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Imaging the translocations of CLIC4 and Epac1

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Imaging the translocations of CLIC4 and Epac1

Bas Ponsioen

Imaging the translocations of CLIC4 and Epac1

Proefschrift

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Bas Ponsioen
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Introduction

1 Prelude

This thesis comprises a set of explorations into various intracellular and intercellular signaling pathways. Throughout these explorations, 'translocations' have been a constant theme. Scattered across various cell biological topics, this thesis reflects the discovery of three translocations and the experimental work aimed to unravel their molecular mechanisms and cell biological relevances. At first glance, the word 'translocation' is self-explanatory; moving from one place ('locus') to the other ('trans' is across). Circulating red blood cells can be considered to undergo continuous translocation, but the work presented here focuses on molecular movements within the micrometer-sized dimensions of the cell.

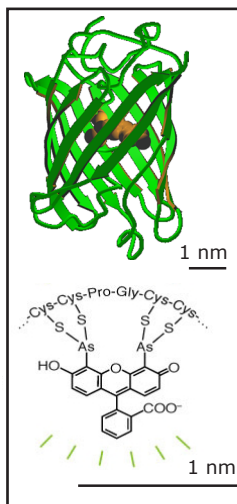
The interior of a cell is a tangle of continuous activity. On the to-do-list: thousands of simultaneous interactions and enzymatic conversions that cooperate to maintain healthy cellular physiology and, in addition, to accomplish cell type-specific functions. The immense ensemble of molecules that constitute a living cell requires strict regulation of all possible interactions and enzymatic activities. We can formulate two basic principles that are prerequisites to control such 'organized complexity'. First, interactions among constituents, be it proteins, lipids, ions or small molecules, are to be **specific**. It is not surprising that cell biology has described a vast amount of conserved recognition domains that confer specificity on molecular interactions. The specificity of a recognition domain is accomplished by the unique combination of its three-dimensional structure and charge distribution, fitting to that of its binding partner following the principle of key-and-lock. Second, most enzymatic conversions are **regulated**. In other words, they can be 'switched on and off'. For example, many enzymes undergo conformational changes that either silence or release their catalytic activities.

Regulation of specific interactions often results in the redistribution of molecules within the cell (see section 3). Such 'translocations' can result in strong enrichment at intracellular compartments where their action is required. Therefore, translocations are intrinsic to signaling cascades.

Translocations studied in this thesis are based on passive diffusion. Passive transport is distinct from active transport by its use of energy. Passive transport is thermodynamically 'downhill', whereas active transport requires input of energy to go 'up the hill'. The latter can, for instance, be provided by coupling the transport process to the dissipating energy of adenosine triphosphate (ATP) hydrolysis.

Active mechanisms enable transport of large cargo, such as that of vesicles along the microtubule network. Also transport against the chemical gradient is ATP-consuming, e.g. the strong enrichment of calcium in the endoplasmic reticulum (10^5 over cytosolic concentrations). Other examples of active transport are the transfer of newly synthesized polypeptides across the ER membrane or import/export through the nuclear pore complex. However, active transport is relatively slow. In contrast, the 'downhill' diffusional redistributions are generally rapid processes.

Obviously, fluorescence microscopy is the method of choice to reveal and study rapid translocations in living cells. It allows visualization with high spatial and temporal resolution. Furthermore, it enables comparison of pre- and post-stimulus conditions and cell-to-cell differences can be appreciated without disturbing averaging effects. Not surprisingly, the use of fluorescence microscopy has been a common denominator throughout the studies presented in this thesis. Thus, before advancing to mechanisms (section 3) and examples (section 4) of translocations in cell signaling, the employed microscopic techniques will be briefly introduced.



Box 1 Potential pitfalls of fluorescent tagging

A potential pitfall in fluorescence microscopy is the use of fusion proteins. Most fluorophores have molecular weights of ~ 30 kD. Thus, in the case of CLIC4 (29kD), fusion with a fluorophore doubles the molecular weight. Such bulky tags may sterically hinder protein-protein interactions. In line with this, the choice to tag either the N- or C-terminus could influence subcellular localization of the fusion construct. We have observed the latter for both Epac1 and CLIC4 and used the tagged version whose localization was most comparable to that of the endogenous counterparts. In the case of gap junctional protein connexin43, C-terminal fusion of GFP even disrupted the formation of proper gap junctions. Therefore, a major challenge in fluorescence microscopy is to identify a genetically encoded fluorescent tag of minimal size. The lab of Roger Tsien (Noble Prize, 2008) seemed to catch a glimpse of the holy grail when they introduced the tetracycline-system [1]. The concept was to insert six residues (Cys-Cys-Pro-Gly-Cys-Cys) into a protein of interest and subsequently label this motif specifically with a fluorescent dye (for comparison: GFP consists of ~ 240 amino acids). Unfortunately, the labeling conditions await further optimization, since the labeling step seriously compromises cellular viability.

2 Measurement of translocations in living cells using fluorescence microscopy

To monitor a protein of interest by fluorescence microscopy, it is genetically fused to a fluorescent protein moiety. The first, green fluorescent protein (GFP), was derived from the jellyfish *Aequorea victoria* and multiple others have been cloned from other sea organisms. Subsequent mutational modifications have led to optimizations in terms of spectral properties, brightness and stability (reviewed in [2]). The color palette spans the spectral range from blue (BFP) to red (mRFP). As described in **Box 1**, caution is demanded when tagging a protein of interest with a fluorescent moiety.

Four different techniques have been employed in this thesis:

- 1) Confocal laser scanning microscopy (CLSM), which yields high spatial resolution by selective rejection of out-of-focus emission from the detectors.
- 2) Fluorescence Resonance Energy Transfer (FRET), which reports protein-protein interactions.
- 3) Fluorescence Lifetime Imaging (FLIM), a technique to determine FRET.
- 4) Fluorescence Recovery After Photobleaching (FRAP), which reveals mobility of fluorescent molecules.

2.1 Fluorescence Resonance Energy Transfer (FRET)

FRET is the radiationless transfer of energy from an excited donor to an acceptor fluorophore. It occurs under stringent conditions. First, the donor emission spectrum must overlap with the absorption spectrum of the acceptor. Second, donor and acceptor fluorophores must be in close proximity, i.e. < 8 nm when using fluorophores in the visible spectrum. A third determinant is formed by the relative orientations of the fluorophore dipoles. FRET is evident from quenched donor fluorescence and gained acceptor fluorescence. Thus, (a change in) FRET can be registered as (a shift in) relative donor and acceptor emission intensities.

The distance constraint is most important for the application of FRET in cell biology. When using common fluorophore combinations (e.g. CFP/YFP or GFP/mRFP), FRET is strictly limited to distances of less than 8 nm. Thus, FRET registers intermolecular distances comparable to the sizes of the studied proteins themselves (5-15 nm) and has therefore been termed a 'molecular ruler' (to put this in perspective: confocal microscopy is limited to a resolution of ~200 nm). FRET can be measured by several experimental approaches, each with its own advantages and disadvantages. As outlined in **Box 2**, the technique of choice depends on the question to be addressed.

Besides its use in revealing interactions between proteins, FRET is also employed in a growing num-

Box 2 Three approaches to measure FRET

FRET between CFP and YFP can be measured in several ways. The three major ones are:

- 1) Monitoring of YFP/CFP ratio. Excite CFP and detect total CFP and total YFP emission from a single cell. The pooling increases signal-to-noise ratio (S/N), allowing sub-second sampling.
- 2) Sensitized YFP emission. Excite CFP and record CFP- and YFP-images. Post-acquisition analysis calculates YFP emission resulting from FRET by subtraction of false signals.
- 3) Measure donor lifetime (FLIM). Excite donor and follow kinetics of fluorescence decay (fluorescence lifetime, τ). FRET accelerates fluorescence decay and can thus be registered as a decrease in τ .

Technique	Advantages	Disadvantages
Monitor YFP/CFP	* good signal-to-noise * rapid sampling * experiments are easy and fast * very low exposure (bleaching, toxicity)	* no spatial information * not quantitative
Imaging sensitized YFP emission	* spatial information with confocal resolution * quantitative	* best for high fluorophore expression * high exposure * labor intensive
FLIM	* spatial information * quantitative	* slow image acquisition

ber of gene-encoded sensors that report intracellular signaling events. These are typically small molecules that can not be made fluorescent directly, such as the second messengers calcium, $\text{PI}(4,5)\text{P}_2$, Reactive Oxygen Species (ROS), cAMP or cGMP [3,4]. Other FRET sensors have been designed to report kinase/phosphatase activities towards specific consensus domains (for e.g. PKA [5] or PKC [6]) or to monitor activation of GTPases (e.g. RhoA [7] or Rap [8]). Because FRET sensors have been extensively applied in this thesis, the most important ones are here briefly introduced.

2.2 FRET-based sensors for calcium, $\text{PI}(4,5)\text{P}_2$ and cAMP

One of the first genetically encoded FRET-based sensors was the Yellow Cameleon calcium sensor [11]. Its core is composed of the C-terminus of Calmodulin (CaM) and the CaM-binding domain of MLC-kinase M13. This core is flanked by CFP and YFP. The increased interaction between CaM and M13 upon calcium binding shortens the distance between the fluorophores and results in enhanced FRET. In this manner, the Yellow Cameleon sensor can register calcium changes with high spatial and temporal resolution.

Other FRET assays for signaling molecules require the co-expression of two separate constructs. For instance, a CFP- and a YFP-tagged version of the Pleckstrin Homology (PH) domain of PLCd1 (CFP- and YFP-PH) can be combined to measure $\text{PI}(4,5)\text{P}_2$ [12]. In resting cells, the PH-domains rapidly shuttle between

cytosol and PM because $\text{PI}(4,5)\text{P}_2$ binding is characterized by fast on/off rates. Their partial presence at the PM suffices to induce FRET between the fluorescent moieties. $\text{PI}(4,5)\text{P}_2$ hydrolysis results in translocation of the PH domains into the cytosol and dilution of the fluorophores is detected as a loss of FRET. This assay has been important to define the role of $\text{PI}(4,5)\text{P}_2$ in the ligand-induced gating of connexin43-based gap junctions (chapter 7).

The first FRET-based sensors designed to measure the second messenger cAMP were based on the tetrameric Protein Kinase A (PKA) [9,13,14]. The concept was to register the cAMP-dependent dissociation of the two catalytic subunits (YFP-PKA-Cat) from the two regulatory subunits (CFP-PKA-Reg). This sensor has been useful in the demonstration of cAMP oscillations [15,16]. However, this first-generation sensor displays some serious disadvantages.

The recent discovery of Epac [17] provided an interesting alternative for the generation of a FRET-sensor for cAMP, since binding of cAMP has a dramatic effect on Epac's conformation. The concomitantly increased distance between the two termini can be measured as a loss of FRET between terminally fused fluorophores. In this manner, Epac-based FRET sensors have been developed in our lab (chapter 5) and, independently, by the groups of Lohse [18] and Zhang [10]. Epac-based cAMP sensors have several advantages over the earlier PKA-based sensor (chapter 5).

Below: catch a **glimpse** at chapters 5 and 8

Glimpse at ...

Chapter 5 & 8 : CFP-Epac-YFP: an improved sensor for cAMP

Several aspects of the PKA-based cAMP sensor [9] are suboptimal. First, the sensor is easily saturated due to its high-affinity cAMP recognition domain. Second, it has a narrow dynamic range of measurable cAMP concentrations due to non-linearity of the dissociation process. Third, variable expression levels of the two sensor halves hamper quantitative analyses.

Advantages of the Epac-based sensor over the PKA-sensor are (1) lower affinity for cAMP, (2) more or less linear dose-response relationship, (3) larger changes in FRET (resulting in improved signal/noise (S/N) ratio) and (4) the 1:1 ratio of donor and acceptor fluorophore. The first two advantages add to the increased dynamic range of measurable cAMP concentrations, while the constant fluorophore ratio greatly facilitates FRET analyses and enables straightforward cell-to-cell comparison. In addition, the single polypeptide sensor can be easily extended with subcellular targeting motifs to measure cAMP in specific compartments of the cell [10].

CFP-Epac-YFP was further optimized by removal of its PM-targeting DEP domain (ΔDEP) and rupture of the catalytic activity by double point mutagenesis in the GEF domain (T781A/F782A). The resultant CFP-Epac(ΔDEP -C.D.)-YFP is cytosolic, catalytically dead (C.D.) and therefore our cAMP sensor of choice.

To further improve the Epac-based cAMP sensor, its fluorescent moieties have been optimized. For this, a series of sensor variants containing variable donor and acceptor fluorophores (in various combinations) has been generated and compared in their 'cAMP-sensorship' (chapter 8). The lessons learned from these comparative analyses may be useful for the generation of other gene-encoded FRET sensors in the future.

3 Diffusion-based translocations

Translocation events are common to signaling cascades. Several examples will be discussed in section 4 and this thesis describes the translocation of signaling molecules CLIC4 (chapter 2) and Epac1 (chapters 3 and 4). We will here describe the functional significance of translocations in signaling cascades and explain how they can result from diffusion.

3.1 Plasma membrane translocation leads to strong enrichment of signaling proteins

Translocations of signaling molecules add a dimension of regulation to signaling cascades: translocation to the PM may bring signaling protein **P** in the vicinity of PM-localized effectors, whereas it is physically separated from other binding partners. Importantly, the density of **P**-molecules increases dramatically when moving from the relatively voluminous cytosol (m^3) to the limited surface (m^2) of the PM. As briefly explained in **Box 3**, the increase in molecular density may amount to >2 orders of magnitude. Obviously, such enrichment has a huge impact on the reaction rates of signaling processes at the PM. Therefore,

translocations are a major determinant of the switch-like activation patterns of signaling proteins.

The impact of enrichment can be illustrated by the targeting technology that makes use of the 'switchable' interaction between domains of the FK506-binding protein (FKBP) and the FKBP-rapamycin binding (FRB) protein (see **Fig. 1**). The interaction is triggered by the membrane-permeable drug rapamycin. Rapamycin binds FKBP with high affinity and the subsequent FKBP-rapamycin tandem has dramatically increased affinity for FRB. Thus, the drug links the two domains together. For the inducible hydrolysis of the phospho-inositide $\text{PI}(4,5)\text{P}_2$, a 5-phosphatase has been fused to FKBP (FKBP-5-phosphatase) and coexpressed with a PM-anchored FRB domain (FRB-CAAX) [19]. Thus, addition of rapamycin triggers translocation of the cytosolic FKBP-5-phosphatase to the PM, resulting in complete depletion of membrane $\text{PI}(4,5)\text{P}_2$. Key to the success is the enrichment of the phosphatase enzymes. In the absence of rapamycin, $\text{PI}(4,5)\text{P}_2$ levels remain unaffected, because the phosphatases remain diluted in the cytosol. Rapamycin-induced translocation 'turns the switch'. For this thesis, this assay has been used to establish the central role of $\text{PI}(4,5)\text{P}_2$ in gating of connexin43-based gap junctions (chapter 7).

Box 3 "Concentration by translocation"

A simple calculation suffices to estimate the increase in density of a particle that translocates from the cytosol to the PM. Two conditions are compared:

- 1) all molecules locate to the cytosol, or
- 2) all molecules locate to the PM.

For both conditions we determine the particle concentration that allows an average intermolecular distance of 8 nm, which approximates the distance at which protein-protein interactions and FRET occur. For the dimensions of a cell we estimate that:

* its volume is 1 picoliter (10^{-12} liter = 10^{-15} m^3)

* its surface is 600 μm^2 ($= 6 \times 10^{-10}$ m^2 ; precisely determined parameter in electrophysiology)

1) All particles locate to the cytosol

We subdivide the cytosolic volume in hypothetical volumes that are occupied by a single molecule:

→ each molecule occupies $(8\text{nm} \times 8\text{nm} \times 8\text{nm}) = (8 \times 10^{-9})^3 \text{ m}^3$

→ the cell (10^{-15} m^3) contains 1.9×10^9 of such cubic sub-volumes, thus 1.9×10^9 molecules

→ these are $(1.9 \times 10^9) / (6 \times 10^{23}) = 3.3 \times 10^{-15}$ moles

→ intracellular concentration: $(3.3 \times 10^{-15}) / (10^{-12}) = 3.3 \times 10^{-3} = \mathbf{3.3 \text{ mM}}$

2) All particles locate to the PM

We subdivide the membrane surface in hypothetical surfaces that are occupied by a single molecule:

→ each molecule occupies $(8\text{nm} \times 8\text{nm}) = (8 \times 10^{-9})^2 \text{ m}^2$

→ the surface ($6 \times 10^{-10} \text{ m}^2$) contains 9.4×10^6 square sub-surfaces, thus 9.4×10^6 molecules

→ these are $(9.4 \times 10^6) / (6 \times 10^{23}) = 1.6 \times 10^{-17}$ moles

If these 1.6×10^{-17} moles of particles translocate back to the cytosol...

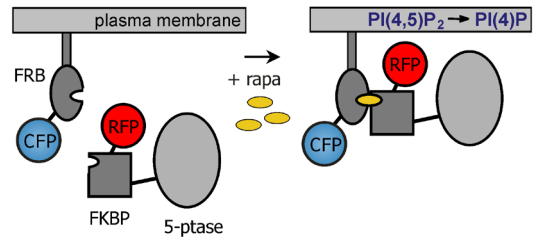
→ intracellular concentration: $(1.6 \times 10^{-17}) / (10^{-12}) = 1.6 \times 10^{-5} = \mathbf{16 \text{ }\mu\text{M}}$

These two concentrations are in a mutual ratio of $\sim 200 \times$.

In other words, translocation to the PM induce a ~ 200 -fold particle enrichment.

Fig 1 *PI(4,5)P₂ depletion by rapamycin-induced translocation of a 5-phosphatase*

A phosphoinositide-specific inositide 5-phosphatase, fused to FKBP and mRFP, resides in the cytosol. Translocation towards the PM is triggered by rapamycin which induces the tight interaction of the FKBP moiety with the FRB of CFP-FRB-CAAX. Fluorophores allow registration of the translocation process. Note that the YFP emission spectrum can be used for readout of PI(4,5)P₂-dependent processes.



3.2 Diffusion-based translocation: a few scenarios

Because free diffusion (also known as Brownian motion) directs signaling molecules to all corners of the cell, signaling molecules encounter their downstream effectors by coincidence. Whether they truly transfer signals depends on binding specificity and on activation status, which is under the regulation of alternative signaling events. To explain translocations of signaling modules, one element must be added: one of the interactors is tethered to a static subcellular compartment. We assume the PM-anchored target **T** and the cytosolic protein **P**. In resting conditions, there is no binding affinity between **P** and **T**. Brownian motion dictates the stochastic movement of **P** in an equilibrium shuttling between cytosol and PM. However, when mutual affinity is 'switched on', the PM-localized target **T** becomes a sink for **P**. Diffusion drives **P** to accumulate, or translocate, to target **T**.

The 'switch' can be turned on at the level of the diffusible protein or that of the anchor at the PM. At

the molecular level, switching can be done in several ways. One of the binding partners can be post-translationally modified. For instance, phosphorylation of protein **P** may define a new recognition domain that triggers translocation to **T**. Conformational changes may also expose affinity domains that are otherwise inaccessible. Such conformational rearrangements may, on their turn, be triggered by phosphorylation but also by binding of (upstream) signaling components. An example of the latter is the cAMP-induced conformational change of Epac1 that triggers PM translocation (chapter 3). An affinity domain may further be released by dissociation from a complex in which it was previously shielded. Conversely, mechanisms where binding capacity is acquired by association into a protein complex can be considered 'coincidence detectors'.

These scenarios are summarized in **Table 1** and are referred to in the next section. It must be stressed that this limited list represents only a fraction of all possible mechanisms in cell signaling.

	What turns the switch?
Modify diffusable protein P	1a) post-translational modification 1b) conformational change 1c) association to protein X 1d) dissociation from protein X ... or combinations thereof
Modify anchor target T	2a) post-translational modification 2b) conformational change 2c) association to protein X 2d) dissociation from protein X ... or combinations thereof

Table 1 *Scenarios of switchable interactions that underly translocation*

4 GPCR signaling is full of translocations

This section surveys a subset of signaling pathways that are under the control of G Protein-Coupled Receptors (GPCRs). Selected are those that have been subject of study in the course of this thesis: via the G protein subunits $G_{\alpha_{13}}$, G_{α_s} and G_{α_q} . Meanwhile, these pathways illustrate the occurrence of translocations in cell signaling. Thus, the focus lies on localizations and translocation mechanisms rather than on the extensive treatment of each and every signaling protein.

4.1 G Protein-Coupled Receptors

GPCRs constitute one of the largest mammalian protein families, with nearly 1000 members in the human genome [20]. They are characterized by seven transmembrane domains and coupling through heterotrimeric G proteins. GPCRs occur in all cells of the body, but expression depends on cell type, function and developmental stage. They can be activated by a wide variety of stimuli, including neurotransmitters, peptide hormones, lipids, ions, and light. GPCR functions vary from transmission of sensory information (e.g. >200 family members expressed in the olfactory organ [21]) to stimulation of proliferation and migration [22].

The heterotrimeric G proteins consist of an alpha subunit (G_{α}) and a dimer beta/gamma subunit ($G_{\beta\gamma}$) [23]. For the many different signaling cascades, the

human GPCR family utilizes a repertoire of at least 20 G_{α} , 5 G_{β} and 12 G_{γ} isotypes [24]. In the absence of receptor stimulation, G_{α} and $G_{\beta\gamma}$ are tightly associated in the tripartite complex [25] and achieve PM localization by prenylation of G_{γ} and palmitoylation of G_{α} [20,25]. The GTPase activity is contained by the larger G_{α} subunit. Like all GTPases, G_{α} cycles between a GDP-bound, inactive state and a GTP-bound, active state (**Fig. 2A**). Guanine Exchange Factors (GEFs) enhance transition to the GTP-bound state, whereas GTPase Activating Proteins (GAPs) enhance GTP hydrolysis. The association with $G_{\beta\gamma}$ stabilizes the inactive state of G_{α} [25].

Ligand binding, however, induces the activated conformation of the GPCR that binds $G_{\alpha}/G_{\beta\gamma}$ via the third intracellular loop [26] and serves as a GEF for the recruited G_{α} subunit. Using a rapid fluorescent readout, Vilardaga et al. showed that $\alpha 2a$ -adrenergic receptors undergo this shape change within 50 milliseconds [27]. Activation of G_{α} is, on its turn, accompanied by a conformational change that induces dissociation from $G_{\beta\gamma}$ [28]. Next, G_{α} redistributes laterally along the PM (it remains palmitoylated) to find its downstream effector(s). Although not fully understood, some reports have claimed that dissociation from $G_{\beta\gamma}$ relieves the protection from depalmitoylation. As a consequence, the G_{α} subunit may rapidly lose its PM localization and the subsequent translocation towards the cytosol may constitute a shut-off mechanism for signaling towards PM-localized effectors [20].

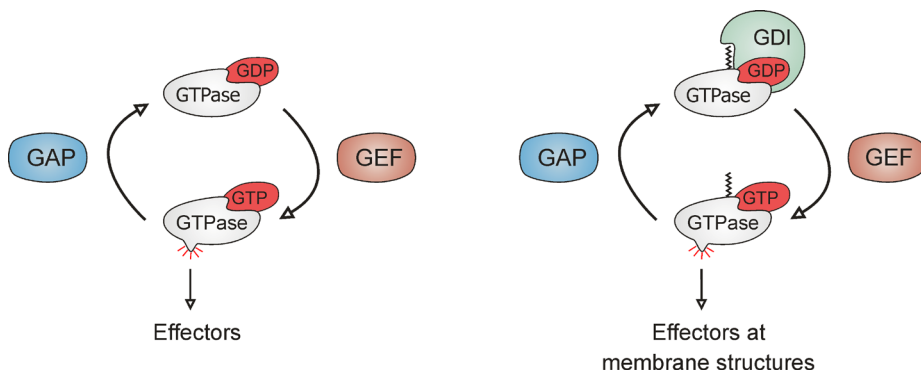


Figure 2 The GTPase cycle

A) Cycling of GTPase proteins between the inactive, GDP-bound and the active, GTP-bound conformation is regulated by guanine exchange factors (GEFs) and GTPase activating proteins (GAP). See text for details
 B) In its inactive conformation, the small GTPase RhoA is kept in the cytosol by RhoGDI, which shields the fatty acid moiety.

4.2 $G\alpha_{13}$ signaling

Activated $G\alpha_{13}$ subunits mainly signal through Guanine Exchange Factors (GEFs) for the small GTPase Rho. These include PDZ-RhoGEF, AKAP-Lbc [35], p115RhoGEF and LARG [25,36]. Together with Ras, Rac, Cdc42 and Rap, RhoA belongs to the family of small Rho-GTPases (21kD) [37], which, similar to the larger $G\alpha$ subunit (43kD), cycle between the GDP- and GTP-bound state (**Fig. 2A**).

RhoA activation has dramatic effects on cellular morphology. It does so mainly via its effector Rho-kinase (or ROCK), which induces actomyosin contraction by phosphorylation of myosin light chain (MLC). Pronounced Rho-dependent morphological changes can best be studied by stimulating the $G\alpha_{13}$ -coupled receptors for lysophosphatidic acid (LPA) in N1E-115 cells (e.g. see chapter 2).

The cycling of RhoA is accompanied by dynamic shuttling between cytosol and PM, contributing to switchable signaling behavior. PM localization is accomplished by prenylation of its very C-terminus. The attached fatty acid tail tends to insert into the inner leaflet of the PM (*scenario 1a*), unless this is prevented by Guanine nucleotide Dissociation Inhibitor (GDI) proteins, which locate to the cytosol (**Fig. 2B**). As long as RhoA is complexed to GDI, which shields the C-terminus and the prenyl modification, it remains in the cytosol. LPA receptor stimulation dissociates this complex and thus triggers translocation of RhoA to the PM (*scenario 1d*). Interestingly, this translocation

is independent of RhoA activation itself, since also the inactive mutant Rho(N19) translocates upon LPA stimulation [38]. Thus, LPA induces translocation of prenylated RhoA towards the PM, where its transition to the active state is catalyzed by $G\alpha_{13}$ -activated Rho-GEFs.

In this thesis, we demonstrate that LPA-induced $G\alpha_{13}$ /RhoA signaling triggers an additional PM translocation: that of chloride intracellular channel 4 (CLIC4). Although the biological function of CLIC4 recruitment to the PM has remained unclear, we could establish that cytosolic CLIC4 is targeted to the activated LPA receptor complex (chapter 2).

Below : catch a **glimpse** at chapter 2

Glimpse at ...

Chapter 2 : CLIC4 translocates to activated, $G\alpha_{13}$ -coupled receptors at the plasma membrane

Chloride intracellular channels (CLICs) are a family of six proteins that are thought to function as chloride channels in intracellular organelles and, also, the PM [29]. Chloride conductances have been reported, but only in *in vitro* experiments using artificial membranes [30]. Altogether, the cellular roles of CLIC proteins are poorly understood.

Using time-lapse microscopy, we showed that LPA stimulation triggers the rapid translocation of CLIC4 from the cytosol to the activated receptor at the PM. CLIC4 recruitment is transient (lasting 5-10 min) and strictly dependent on $G\alpha_{13}$ and RhoA activation. In apparent contradiction to its nomenclature, we find no evidence that CLIC4 can insert into the PM or modulate chloride currents.

Although the biological function of CLIC4 translocation has not been resolved to date, we speculate that CLIC4 translocates to the receptor complex to regulate signal transfer from $G\alpha_{13}$ towards RhoA (activation) at the PM. Interestingly, CLIC4 has been reported to interact with the scaffold protein AKAP350 [31], while a family member of the latter, AKAP-Lbc is a RhoGEF, that directly links $G\alpha_{12}$ to RhoA [32]. Furthermore, CLIC4 binds the adaptor protein 14-3-3 [33], which is an activator of AKAP-Lbc [34]. Therefore, it is tempting to hypothesize that CLIC4 translocates to a protein complex containing the activated receptor, $G\alpha_{13}$ and an AKAP scaffold with GEF activity towards RhoA. In this model, the spatial regulation of CLIC4 will undoubtedly add to the switch-like regulation of RhoA activity.

4.3 G_{α_s} signaling

Activated G_{α_s} subunits stimulate adenylyl cyclases (ACs) to synthesize the second messenger cyclic adenosine monophosphate (cAMP) from ATP. The second messenger cAMP is involved in numerous cellular functions, depending on cell type and activated receptor. For example, cAMP stimulates cell growth in many cell types while inhibiting it in others [41-43]. The diverse effects of cAMP are mediated by protein kinase A (PKA), exchange protein directly activated by cAMP (Epac) and cyclic nucleotide-gated channels (CNGCs). The classical cAMP effector PKA is a tetramer consisting of two regulatory and two catalytic subunits [44]. The catalytic subunits are released when cAMP binding to the regulatory subunits dissociates the complex (*scenario 1d*). The released catalytic domains phosphorylate various substrates on typical PKA-consensus motifs. cAMP-mediated cell differentiation is characterized by the induction of specific genes through the transcription factor CREB (cAMP responsive element binding protein), which translocates to the nucleus upon phosphorylation by PKA [41] (*scenario 1a*).

Given that PKA is involved in numerous parallel signaling cascades, it is a challenge to understand how the kinase is activated in the right place and at the right time. It is generally believed that specificity is achieved, in part, through the compartmentalization of PKA at different subcellular locations through interaction with so-called A Kinase Anchoring Proteins (AKAPs) [35,45-47]. At least 50 different AKAP isoforms target the regulatory subunits of PKA to various intracellular compartments (reviewed in [35]).

To restrict cAMP signaling to these compart-

ments, AKAP anchors also cluster the cAMP-degrading phosphodiesterases (PDEs) [48,49], implying that cAMP levels are reduced in the vicinity of PKA. Interestingly, PKA phosphorylation is known to upregulate PDE activity [50], establishing a feedback-loop that rapidly terminates the cAMP signal. Thus, the AKAP scaffold restricts cAMP turnover to the micrometer scale [51,52]. Indeed, using a FRET reporter for PKA activity, Saucerman et al (2006) demonstrated that the occurrence of local cAMP gradients is enhanced by PDE activity [53]. Another study used an Epac-based cAMP sensor to show that following receptor stimulation, cAMP elevations in the nucleus follow with a lag time and with decreased amplitude as compared to elevations near the PM [10].

cAMP is a small messenger molecule that can pass through gap junctions [54-57]. We have studied this gap junctional communication (GJC) of cAMP and observed that PDE activity determines the degree of cAMP exchange between adjacent cells (chapter 6).

Below : catch a **glimpse** at chapter 6

More recently, the discovery of Epac as a direct effector of cAMP [17] has triggered the elucidation of many cAMP-regulated processes that could not be explained by the classical effector PKA. Epac1 and Epac2 act as GEFs for the small G Proteins Rap1 and Rap2. Unlike RhoA, Rap GTPases are permanently anchored to membrane compartments via its prenylated C-terminus [62], including the Golgi network, vesicular membranes and the plasma membrane [63-66]. It plays roles in diverse processes typical for the PM, including cell adhesion [58,64,67], adherens junction formation

Glimpse at ...

Chapter 6 : Gap junctional communication allows sharing of cAMP

Gap junctions are clusters of transmembrane channels that allow direct cell-to-cell transfer of ions and small molecules [39]. Apart from a few cell types, most cells in normal tissues show gap junctional communication (GJC) [40]. GJC of cAMP is of particular interest because cAMP has numerous effects on cell behavior, including inhibition of proliferation and migration [41]. Using our FRET-based cAMP sensors, we directly monitored intercellular exchange of physiologically raised cAMP in monolayers of Rat-1 fibroblasts, that endogenously express Cx43. Our data show that cAMP translocation from cAMP donor to acceptor cells occurs over the entire range of physiological cAMP concentrations. We further indicate the phosphodiesterases (PDE) as the primary determinants for the relative cAMP levels in acceptor cells as compared to donor cells.

Importantly, these data demonstrate that GJC of cAMP may be relevant to the shared properties of Cx43-expressing tissue cells. Gap junctions are tumour suppressors in cells with affected signaling functions. It has been proposed that 'normal' cells may deliver signaling intermediates through junctional exchange, permitting survival of mutant cells with blocked enzymatic pathways [39]. cAMP may be a concrete example of such intermediate messenger. Furthermore, GJC is an important mechanism in the regulation of embryogenesis [40]. The several connexin isoforms are differentially expressed among embryonic tissue types and therefore, GJC of cAMP may tissue-specifically ensure concerted regulation of cAMP-dependent processes. These examples are particularly interesting from the perspective of compartmentalization. Since cAMP is mainly produced at the PM, its diffusion through GJs may be relatively insensitive to PDE-mediated degradation in the cytosol. As a consequence, intercellular exchange of cAMP may be most relevant to cAMP-regulated processes at the PM.

[59] and insulin secretion [68,69].

Epac GEFs contain a regulatory region with one (Epac1) or two (Epac2) cAMP-binding domains, a Di-shavelled, Egl-10, Pleckstrin (DEP) domain, and a catalytic region for GEF activity [60]. Recently, the structures of both the inactive and the active conformation of Epac2 were solved [70,71]. This revealed that in the inactive conformation the regulatory region occludes the Rap binding site and that this is relieved by a conformational change induced by cAMP binding.

Similar to PKA, also Epac signaling is governed by compartmentalization. Interestingly, in cardiomyocytes Epac1 has been found in the same complex as PKA, anchored by the muscle-specific mAKAP [72]. The complex, which also contains the PDE4D3, is a crossroad of cAMP signaling involved in the regulation of ERK5. Specifically Epac2 is part of the Rim2-piccolo complex involved in cAMP-dependent exocytosis [73]. Both Epac1 and Epac2 are targeted to microtubules via an interaction with the Light Chain of Microtubule

Associated Protein (MAP-LC) [74]. In addition, Epac translocations can be triggered by hormone stimulation. Li et al. (2006) found that Epac2 translocates to the plasma membrane by binding to the small GTPase Ras, when Ras acquires the active conformation [75] (*scenario 2b*).

In this thesis, we uncovered two additional mechanisms of Epac1 targeting in response to extracellular signals. Epac1 translocates to the PM upon binding of cAMP (chapter 3) and upon activation of members of the Ezrin/Radixin/Moesin (ERM) family (chapter 4). Both types of PM recruitment appeared important for Epac1-mediated cell adhesion. This is not surprising, since activation of Rap at the PM is crucial for cell adhesion [64]. Thus, not only is Epac1 compartmentalized, several extracellular cues can regulate Epac1 function via dynamic translocations from one compartment to the other.

Below : catch a **glimpse** at chapters 3 and 4

Glimpse at ...

Chapter 3 : Epac1 translocates to the plasma membrane upon cAMP binding

Epac1 is a GEF for the small G protein Rap and is thereby involved in processes such as integrin-mediated cell adhesion [58] and cell-cell junction formation [59]. In the absence of cAMP, Epac1 localizes to the nuclear envelope, the cytosol and, to a minor extent, the PM. Here, we report that cAMP induces the translocation of Epac1 towards the PM. Combining high-resolution confocal fluorescence microscopy with FRET assays, we observed that Epac1 translocation is a rapid and reversible process. This dynamic redistribution of Epac1 requires both the cAMP-induced conformational change and the DEP domain.

In line with its translocation, Epac1 activation induces Rap activation predominantly at the PM. Furthermore, translocation-deficient Epac1 mutants were impaired in their ability to induce Rap-mediated adhesion of Jurkat T cells. This indicates that the cAMP-induced translocation enhances Rap-mediated cell adhesion.

Interestingly, our data imply that cAMP exerts dual regulation on Epac1. The cAMP-induced conformational change not only elicits GEF activity by Epac1 [60], it also induces its translocation to the compartment where its GEF activity should activate Rap. This dual regulation guarantees that Epac1 acts as a binary switch for Rap activation.

Chapter 4 : Epac1 is recruited to the PM by activated ERM proteins

Besides the cAMP-induced translocation, we revealed an additional translocation mechanism that targets Epac1 to the PM. This redistribution is mediated by direct interaction with members of the Ezrin/Radixin/Moesin (ERM) family. Recruitment to ERM proteins is distinct from the cAMP-induced translocation in several aspects. First, it is not mediated by the DEP domain but by the N-terminal 49 residues that precede the DEP domain. Second, it is independent of Epac1 conformational state. And third, it targets Epac1 to specific, asymmetrical regions of the PM, whereas cAMP-binding induces uniform PM targeting.

We find that the Epac1-ERM interaction is regulated by conformational opening of ERM proteins. In agreement with this, thrombin receptor signaling, which induces the conformational opening of ERM proteins [61], triggers the recruitment of Epac1 to asymmetrical zones of the PM.

Also ERM-mediated recruitment is important for activation of Rap signaling. Epac1(Δ 49), which is deficient in ERM binding, was impaired in its ability to induce adhesion upon Epac1-activating stimuli. Considering that both the cAMP-dependent Epac1 translocation and the interaction with ERM proteins contribute to Rap activation (and hence cell adhesion), we suggest that these two targeting mechanisms synergistically cooperate to effectively couple GEF activity of Epac1 to its effector Rap. Within the context of translocations as enrichment mechanisms for signaling molecules (section 2.4), it is tempting to speculate that the marked asymmetrical PM distribution imposed by ERM proteins may further enrich Epac1 to the appropriate compartment for Rap activation, thereby increasing its efficiency of action.

4.4 $G\alpha_q$ signaling

Activated $G\alpha_q$ subunits trigger the activity of phospholipase C ($PLC\beta$), which hydrolyzes the PM-specific phospho-inositide $PI(4,5)P_2$. The cleavage generates the second messengers inositol-triphosphate (IP_3) and diacylglycerol (DAG). $PI(4,5)P_2$ itself is also a second messenger involved in a vast amount of PM-localized processes, many of which are related to actin polymerization. In this way, $PI(4,5)P_2$ plays roles in processes ranging from actin cytoskeletal remodeling to regulation of ion transporters [76].

In unstimulated cells, $PLC\beta$ is anchored to the PM via electrostatic interactions between its C-terminal domain (CT-domain) and negative charges at the inner leaflet (Singer et al). When activated $G\alpha_q$ interacts with $PLC\beta$ [77], it elicits the $PI(4,5)P_2$ hydrolyzing activity. The loss of negatively charged $PI(4,5)P_2$ induces translocation of $PLC\beta$ to the cytosol [78]. Probably, this cytosolic translocation is further enhanced by GAP activity of the $PLC\beta$ CT-domain [79], which inactivates $G\alpha_q$ and thereby disrupts the interaction. Thus, hydrolysis of its substrate $PI(4,5)P_2$ and inactivation of its upstream signal form the switch to induce $PLC\beta$ translocation to the cytosol (*scenario 1d*; here translocation is away from the PM). Obviously, this negative feedback mechanism may contribute to the transient kinetics of $G\alpha_q$ -mediated $PI(4,5)P_2$ hydrolysis.

$PI(4,5)P_2$ hydrolysis is involved in the gating of several PM-localized channels, such as the KCNQ potassium channel [80] and the transient receptor potential cation channels TRPM7 [81] and TRPM8 [82]. In this thesis, we established the crucial role of $PI(4,5)P_2$ in ligand-induced closure of connexin43-based gap junctions (this thesis, chapter 7).

Below : catch a **glimpse** at chapters 7

Glimpse at chapter 7

Chapter 7 : $PI(4,5)P_2$ regulates communication via connexin43-based gap junctions

Cell-cell communication through connexin43 (Cx43)-based gap junction channels is rapidly inhibited upon activation of GPCRs, that couple through $G\alpha_q$. However, the mechanism has long remained unknown. Here, we show that Cx43-based cell-cell communication is inhibited by depletion of $PI(4,5)P_2$ from the plasma membrane. Knockdown of phospholipase $C\beta 3$ ($PLC\beta 3$) inhibits $PtdIns(4,5)P_2$ hydrolysis and keeps Cx43 channels open after receptor activation. Furthermore, when $PI(4,5)P_2$ is overproduced by overexpressed $PI(4)P$ -5-kinase, Cx43 channel closure is impaired.

Next, we showed that $PI(4,5)P_2$ depletion is not only required but also sufficient for gap junction closure. For this, we used the translocatable FKBP-5-phosphatase described in section 3.1. Rapamycin-induced recruitment of FKBP-5-phosphatase towards PM-anchored FRB-CAAX induced immediate $PI(4,5)P_2$ depletion without generation of second messengers IP_3 and DAG [80]. Thus, the observed reduction in gap junctional communication implies that hydrolysis of $PI(4,5)P_2$ itself is a direct signal for channel closure.

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Chapter 2

Spatiotemporal regulation of CLIC4, a novel player in the $G\alpha_{13}$ -RhoA signaling pathway

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Submitted

Spatiotemporal regulation of CLIC4, a novel player in the $G\alpha_{13}$ -RhoA signaling pathway

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Abstract

CLIC4 ("Chloride Intracellular Channel" 4) is a soluble protein structurally related to omega-type glutathione-S-transferases (GSTs) and implicated in various biological processes, ranging from chloride channel formation to morphogenesis. However, the function of CLIC4 and whether it is regulated remain unclear. Using time-lapse microscopy, we show that G_{13} -coupled receptor agonists, such as lysophosphatidic acid and thrombin, trigger the rapid translocation of CLIC4 from the cytosol to the activated receptor at the plasma membrane. CLIC4 recruitment is transient (lasting 5-10 min) and strictly dependent on $G(\alpha)_{13}$, RhoA activation and F-actin integrity. CLIC4 does not appear to enter the plasma membrane or to modulate chloride currents. CLIC4 translocation requires at least six conserved residues, including reactive Cys35, whose equivalents are critical for substrate binding in omega-GSTs. Our results show that CLIC4 is regulated by G_{13} -linked RhoA pathway to be targeted to G_{13} -coupled receptor complexes at the plasma membrane, and they suggest that CLIC4 binds an as-yet-unknown substrate in a manner analogous to GST-substrate interaction.

Introduction

Chloride Intracellular Channels (CLICs) are a family of six proteins that are thought to function as chloride channels in intracellular organelles and at the plasma membrane. They have been implicated in several cellular functions linked to ionic transport, such as charge compensation required for tubule formation in the excretory cell of *C. elegans* [1,2], tubular morphogenesis in mammalian endothelial cells [3] and acid secretion in bone resorbing osteoclasts [4]. However, many other findings are difficult to reconcile with membrane ion transport, such as involvement of CLIC4 in keratinocyte differentiation [5] and apoptosis [6,7], binding of CLIC4 to the centrosome [8,9] and localization of CLIC1 and CLIC3 to the nuclear matrix [10,11]. In general, the cellular roles of CLIC proteins are poorly understood.

CLIC proteins are structurally distinct from members of the CLC chloride channel family, the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) or known ligand-gated chloride channels [12]. In contrast, the 220 amino acid core region shared by the CLIC proteins shows significant structural homology to the omega-class of Glutathione S-Transferases (GST) [13]. The first CLIC proteins to be isolated were purified using IAA-94, an analogue of the GST-inhibitor ethacrynic acid [14]. To date, the evolu-

tionary relationship with omega-GSTs is not well understood. Although cellular CLICs, like GSTs, are globular, signal peptide-less [14] proteins occurring predominantly in the soluble form, IAA-94-inhibitable chloride conductances have been demonstrated in artificial liposomes containing reconstituted CLIC1 [15], CLIC4 [16] or CLIC5 [17]. Importantly, these chloride conductances emerge in the absence of any accessory protein, and were strictly dependent on oxidative conditions and acidic pH (< 6.0). Importantly, however, the cell interior is a reducing environment with a pH that is mostly >7.2. These observations have led to the membrane-insertion model, wherein reactive oxygen species (ROS) trigger insertion of cytosolic CLIC proteins into lipid bilayer, enhanced by the local, relatively low pH at the inner leaflet of the plasma membrane. In support of this, CLIC1 undergoes a redox-controlled structural transition that is required for *in vitro* channel activity [15]. Moreover, β -amyloid-induced ROS generation has been proposed to cause a CLIC1-mediated chloride conductance in microglia of Alzheimer patients [18]. Finally, Singh and co-workers have postulated that the chloride conductances of CLIC1 and CLIC5 are regulated by cytoskeletal actin filaments, adding a layer of control to the insertion model [19]. However, *in vivo* membrane recruitment of CLIC proteins evoked by physiological signaling cascades has never been demonstrated to date.

We here describe the translocation of CLIC4 towards

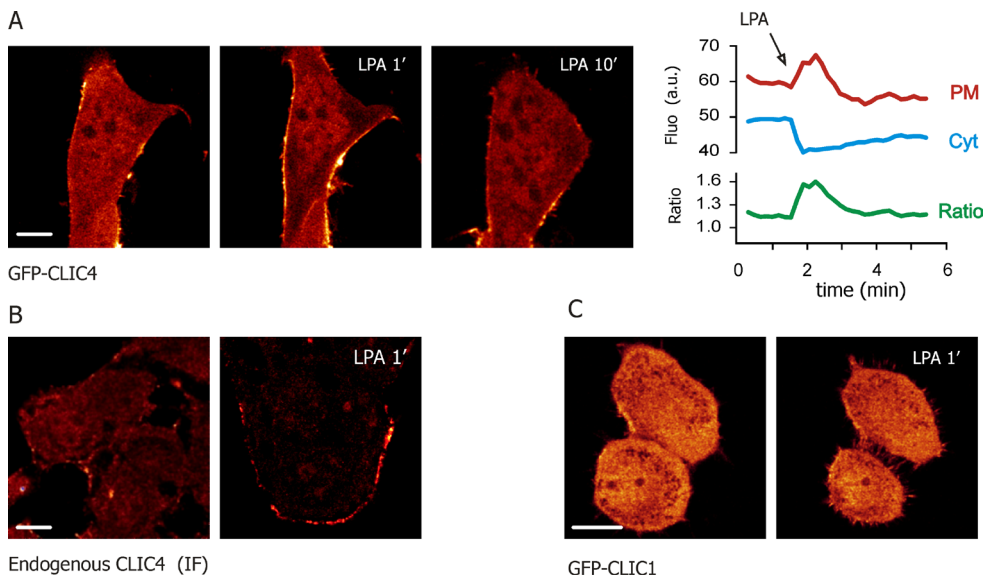


Figure 1 Agonist-induced translocation of CLIC4 towards the plasma membrane.

A) LPA-induced translocation of GFP-CLIC4 in N1E-115 cells visualized by confocal microscopy. In resting cells, GFP-CLIC4 resides mainly in the cytosol with some patch-like accumulation at the cell periphery. LPA (1 μ M) induces a rapid but transient recruitment of GFP-CLIC4 towards the plasma membrane. Translocation can be measured semi-quantitatively by monitoring the accumulation of GFP fluorescence at the plasma membrane (PM, red trace) and the concomitant depletion of fluorescence from the cytosol (Cyt, blue trace). Net translocation is expressed as the ratio PM/Cyt (green trace).

B) Localization of endogenous CLIC4 in resting and LPA-stimulated N1E-115 cells. Plasma membrane accumulation was markedly increased by LPA stimulation (1 min).

C) Failure of GFP-CLIC1 to translocate in LPA-stimulated N1E-115 cells. Scalebars in all shown images: 10 μ m.

Table 1 Signatures of depolarizing chloride efflux and GFP-CLIC4 translocation correlate

Responses to LPA, Thrombin, S1P and Bradykinin were tested in N1E-115 cells. Isoproterenol, the agonist of adrenergic receptors, was added to A431 cells.

G Proteins: bradykinin stimulation in N1E-115 cells results in strong and specific activation of Gαq-PLC pathway [37]. Isoproterenol stimulation evokes strong and specific Gαs-mediated cAMP production in A431 cells due to high expression of adrenergic receptors. Inhibition of Gαi by PTX (200ng/ml, 16hr) did not affect chloride efflux or CLIC4 translocation. Thus, both chloride efflux and CLIC4 translocation involve Gα₁₃ and do not require Gαq (calcium release) or Gαs (cAMP synthesis) or Gαi (PI3K activation).

Rho and downstream effectors: involvement of the Gα₁₃ effector RhoA in chloride efflux and CLIC4 translocation was shown by RNAi-RhoA and treatment with C3 toxin (30μg/ml, 16hr). All tested RhoA effectors were excluded from involvement (see Fig 2A,C).

Agonist/treatment	CLIC4 translocation	Cl channel activation (depolarization)	Cytoskeletal contraction (RhoA activation)
Serum (5 %)	+	+	+
LPA (1 μM)	+	+	+
S1P (1 μM)	+	+	+
Thrombin (TRP)	+	+	+
Bradykinin (Ca ²⁺ rise)	-	-	-
Isoproterenol (cAMP)	-	-	-
LPA/TRP			
+ Pertussis toxin	+	+	+
+ Gα ₁₃ /RhoA RNAi	-	-	-
+ C3 exo-enzyme	-	-	-
+ Y-27632	+	+	-
+ dn-mDia1	+	+	+
+ Wortmannin	+	+	+

the plasma membrane of N1E-115 neuroblastoma cells following stimulation of lysophosphatidic acid (LPA) receptors. CLIC4 translocation is immediate and transient, and depends on activation of the G(α)₁₃-RhoA pathway as well as on actin polymerization. Interestingly, LPA stimulation is known to evoke a transmembrane chloride efflux in N1E-115 cells, but the nature of the putative channel has remained elusive [20,21]. Therefore, we thoroughly tested the involvement of CLIC4 in LPA-induced chloride efflux, but found no evidence. In contradiction with the membrane-insertion model, our efforts to show CLIC4 membrane insertion or inducible channel function *in vivo* were unsuccessful. Instead, we find that the LPA-induced translocation targets CLIC4 towards the upstream, activated LPA receptor complex. We further show that the conserved residues that confer GST-homology to CLIC proteins, including cysteine35, are essential for CLIC4 translocation. As such, this is the first study to attribute a function to the GST-like structural features of CLIC4. Despite the crucial importance of reactive Cys35 [22] we find that CLIC4 translocation is not redox-regulated. Our data suggest that the GST-like structural fold harbors a recognition domain for an as-yet unknown binding partner that is essential for translocation. We propose that transient recruitment of CLIC4 to the activated LPA receptor plays a role in coupling ligand-activated GPCRs to downstream G(α)₁₃-RhoA signaling.

Results

We set out to examine if CLIC4 may function in one or more receptor-linked signaling pathways, particularly those involving Cl channel activation and cytoskeletal remodeling. To this end, we used N1E-115 neuronal cells, because receptor signaling, ion channel activation and cytoskeletal regulation have been extensively examined in these cells. Most, if not all, cell types express multiple CLIC family members. PCR analysis revealed that N1E-115 cells express CLIC4, CLIC1 and CLIC6 (data not shown).

Translocation of CLIC4 towards the plasma membrane mediated by the G(α)₁₃-RhoA pathway

To monitor CLIC4 localization and trafficking, we generated constructs of CLIC4 fused to various fluorophores. When expressed in N1E-115 cells, GFP-tagged CLIC4 was distributed homogeneously through the cytosol (Fig. 1A). Upon addition of LPA, we observed a rapid translocation of GFP-CLIC4 towards discrete domains of the plasma membrane, concomitant with depletion of CLIC4 from the cytosol (Fig. 1A). GFP-CLIC4 accumulation was maximal after ~1 min. and had disappeared after about ~10 min of LPA addition. A similar translocation was observed with C-terminally tagged CLIC4-YFP (data not shown). Importantly, this translocation pattern was also observed for endogenous CLIC4 (Fig. 1B), indicating that the GFP tag has little or no effect on CLIC4 targeting. In marked contrast, CLIC1 (GFP-CLIC1 and CLIC1-YFP) did not undergo detectable translocation upon LPA stimulation (Fig. 1C). CLIC4 depletion from the cytosol occurred in a homogeneous manner (Fig. 1A and supplementary movie 1), strongly suggesting that cytosolic CLIC4 is freely diffusible. Indeed, photobleaching (FRAP) experiments showed that cytosolic GFP-tagged CLIC4 is as mobile as free GFP (data not shown).

What signaling events underlie the rapid recruitment of CLIC4? Translocation was insensitive to pertussis toxin (PTX), ruling out the involvement of G_i-linked signaling pathways. Instead, translocation was only observed with GPCR agonists that activate the G_{12/13}-linked RhoA pathway leading to cytoskeletal contraction in N1E-115 cells, notably LPA, thrombin receptor-activating peptide (TRP) and sphingosine-1-phosphate (S1P). In contrast, G_q-coupled receptor agonists such as bradykinin failed to induce CLIC4 translocation, as summarized in Table 1. As a direct test for involvement of the G_{12/13}-RhoA pathway we used RNAi constructs to knockdown G(α)₁₃ and RhoA. This abolished agonist-induced CLIC4 translocation, as did pre-treatment of the cells with the Rho-inactivating C3 exo-enzyme. That C3 and RNAi treatment were effective was evidenced by loss of agonist-induced cell rounding (Fig.

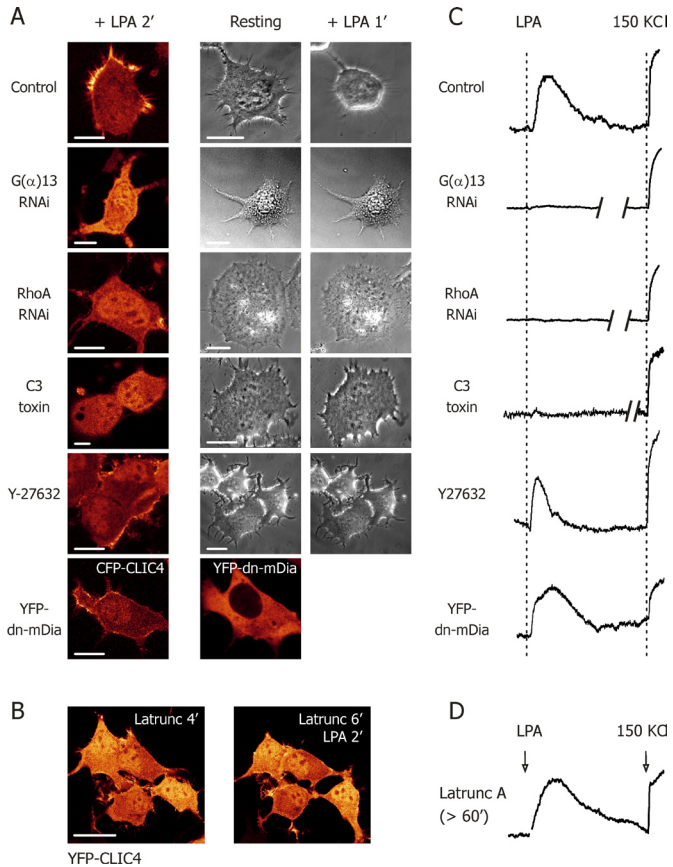
Figure 2 LPA-induced CLIC4 translocation and chloride efflux are both in the $G_{\alpha_{13}}$ -Rho pathway but can be dissociated

A) GFP-CLIC4 translocation and cell morphology controls under various conditions affecting $G_{\alpha_{13}}$ -RhoA signaling in N1E-115 cells. RNAi-encoding vectors were transfected for 48 hr together with GFP-CLIC4 (or transfection marker H2B-mRFP in the cell morphology controls; not shown in the images). C3 toxin (30 μ g/ml) was incubated for 16 hr and Y-27632 (30 μ M) for 30 min. Cells expressing dominant-negative mDia1 (YFP-mDia1-FHZA Δ N) were selected for maximal YFP signal; imaging of co-expressed CFP-CLIC4 showed LPA-induced translocation (lower panels).

B) Incubation with Latrunculin A (1 μ M, 4 min.) abolished LPA-induced translocation of GFP-CLIC4. Scalebars in (A,B) are 10 μ m.

C) Membrane potential measurements in LPA-stimulated N1E-115 cells using the voltage-sensitive dye (see M&M). Indicated RNAi or drug treatments corresponds with those shown in panel A ($n > 100$). (transfection marker H2B-CFP was used to avoid leak-through in the voltage-dye detection channel). These experiments revealed a marked correlation with CLIC4 translocation. All measurements were calibrated by using 150 mM KCl to eliminate the transmembrane potential.

D) Latrunculin A (up to 1 hour pre-incubation) does not affect LPA-induced membrane depolarization, whereas it abolishes CLIC4 translocation (panel B). Trace representative for $n = 8$.



2A) and induction of neurite outgrowth (data not shown). Agonist-induced CLIC4 translocation was not unique for N1E-115 cells, as it was also observed in Rat-1 fibroblasts, HEK293 cells, Hela and A431 carcinoma cells stimulated with either LPA or TRP (data not shown). It thus appears that CLIC4 recruitment at the plasma membrane is a common cellular response to activation of the $G_{12/13}$ -RhoA pathway.

RhoA is a key regulator of actomyosin-based contractility and its major downstream effectors are Rho kinase (ROCK), mDia1 and PIP5-Kinase. However, incubation with the ROCK inhibitor Y-27632, at doses that abolished agonist-induced cell rounding (30 μ M), did not affect CLIC4 translocation, nor did expression of dominant-negative mDia1 (FHZA Δ N) or incubation with the PIP5K inhibitor Wortmannin. However, agonist-induced CLIC4 translocation was inhibited by briefly pretreating the cells with latrunculin A (1 μ M; 4 min.), a toxin that prevents F-actin polymerization and thereby disrupts the cytoskeleton (Fig. 2B). These results indicate that CLIC4 recruitment depends on F-actin integrity rather than actomyosin contractility.

CLIC4 recruitment can be dissociated from chloride channel activation

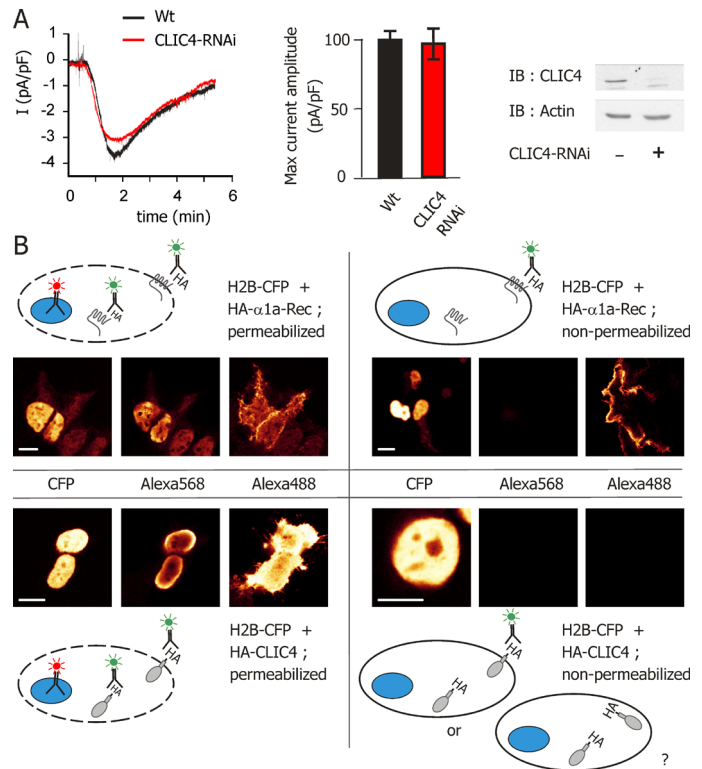
Ion conductance studies using artificial lipid bilayers have suggested that CLICs may auto-insert into membranes to form anion channels. However, the channel hypothesis has remained controversial to date. We note that the transient kinetics of CLIC4 translocation are strikingly similar to those of the $G_{\alpha_{13}}$ -mediated chloride current in LPA-stimulated N1E-115 cells. This current manifests itself as a transient membrane depolarization (from -60 mV to -15 mV), which can be monitored by using a voltage-sensitive dye (Fig. 2C). In common with CLIC4 translocation, agonist-induced membrane depolarization was abolished after knockdown of $G_{\alpha_{13}}$ and RhoA as well as by C3 treatment, but not by Y-27632 (Fig. 2C). However, membrane depolarization was insensitive to latrunculin A at doses that blocked CLIC4 translocation (even when incubated for >1hr) (Fig. 2D). We therefore conclude that CLIC4 recruitment to the plasma membrane does not underlie chloride channel activation.

Yet, it cannot be ruled out that CLIC4 has channel-forming ability or is a channel regulator in our system. We therefore performed patch-clamp experiments to measure LPA-induced chloride currents in N1E-115 cells. As shown

Figure 3 *CLIC4 is not required for LPA-induced chloride efflux and does not pass the plasma membrane*

A) Representative voltage-clamp recordings of inward chloride currents in LPA-stimulated N1E-115 cells. Black, control cells; red, cells expressing RNAi-CLIC4 (3 targeting constructs). The bar diagram shows mean peak amplitudes $\pm$ s.e.m. Transmembrane currents are corrected for membrane conductance, which is a measure for total membrane surface (pA/pF). Western blot shows CLIC4 levels in untransfected and knockdown N1E-115 cells.

B) Extracellular detection of HA-epitope in non-permeabilized HEK293 cells. Cells were transfected with transfection marker H2B-CFP plus HA-tagged (α 1a)adrenergic receptor (HA- α 1a-R) (upper panels) or HA-CLIC4 (lower panels). HA-CLIC4-expressing cells were stimulated with LPA (2 min.) before fixation. In non-permeabilized cells, HA- α 1a-R was detected selectively at the cell surface (upper right), in contrast to the intracellular signal in permeabilized cells (upper left). Expression and detection of HA-CLIC4 were verified in permeabilized HEK293 cells (lower left). Under non-permeabilizing conditions, the HA-tag of CLIC4 was not detected (lower right). Scalebars: 10 μ m.



in Fig. 3A, knockdown ($n=20$) of CLIC4 did not significantly modulate the kinetics or amplitude of the LPA-induced inward Cl current. Although CLIC4 knockdown was toxic to N1E-115 cells and the survivors showed approx. 20% residual CLIC4 levels, this result strongly suggests that membrane-targeted CLIC4 does not induce or modulate transmembrane chloride currents in our cell system.

When recruited to the cell periphery, could CLIC4 conceivably bind to or even enter the plasma membrane? To address this question, we examined the fluorescence resonance energy transfer (FRET) between CFP-CLIC4 and membrane-anchored YFP-CAAX, a reporter that inserts into the plasma membrane through its prenylated K-Ras-derived CAAX motif. Following LPA stimulation, there was no detectable increase in the YFP/CFP ratios relative to basal level ($n=4$, $(\Delta F)/F = 1.01 \pm 0.05$). Similar results were obtained with YFP-CLIC4 and CFP-CAAX (data not shown). The apparent lack of FRET strongly suggests that CLIC4 does not approach the plasma membrane closer than about 10 nm. Consistent with this, immunofluorescence studies on N1E-115 and HEK293 cells overexpressing HA-CLIC4 failed to show externalization of the N-terminal HA-tag, whereas the N-terminal HA-tag of the α 1a-adrenergic receptor, which externalizes a similar number of residues as expected for membrane insertion of CLIC4 (34 amino acids, [16]), was readily detected extracellularly (Fig. 3B). In conclusion, cortical CLIC4 accumulation at the plasma membrane does not lead to detectable externalization of the N-terminus.

CLIC4 translocates specifically towards activated G_{13} -coupled receptors

When comparing CLIC4 translocation induced by LPA to that induced by TRP, we noticed striking differences in the patterns of CLIC4 accumulation. LPA stimulation generally resulted in an asymmetric distribution of CLIC4 at the plasma membrane, whereas TRP-stimulated cells showed a more homogeneous distribution of CLIC4 along the plasma membrane. This is best exemplified by the experiment of Fig. 4A, in which a cell was first stimulated with TRP and thereafter (after 10 min.) with LPA. It is clear that the resulting localizations of GFP-CLIC4 are markedly different. Such differential distribution was also observed for endogenous CLIC4 in cells stimulated with LPA or TRP (Fig. 4A). We hypothesized that this differential CLIC4 localization may reflect different membrane localizations of the respective GPCRs.

Indeed, in HEK293 cells expressing HA-LPA2 and GFP-CLIC4 we could co-precipitate GFP-CLIC4 with HA-LPA2 (Fig 4B). This interaction was observed in stimulated as well as non-stimulated cells, likely due to constitutive activation of Rho signaling by the overexpressed HA-LPA2. We tested this hypothesis further by three different approaches. In the first approach, we took advantage of the finding that N1E-115 cells express LPA2 receptors (but not LPA1 or LPA3), which form a macromolecular complex with the scaffold protein NHERF2 (sodium-hydrogen exchanger regulatory factor 2) through a PDZ-domain interaction [23]. (In epithelial cells, NHERF scaffolds localize

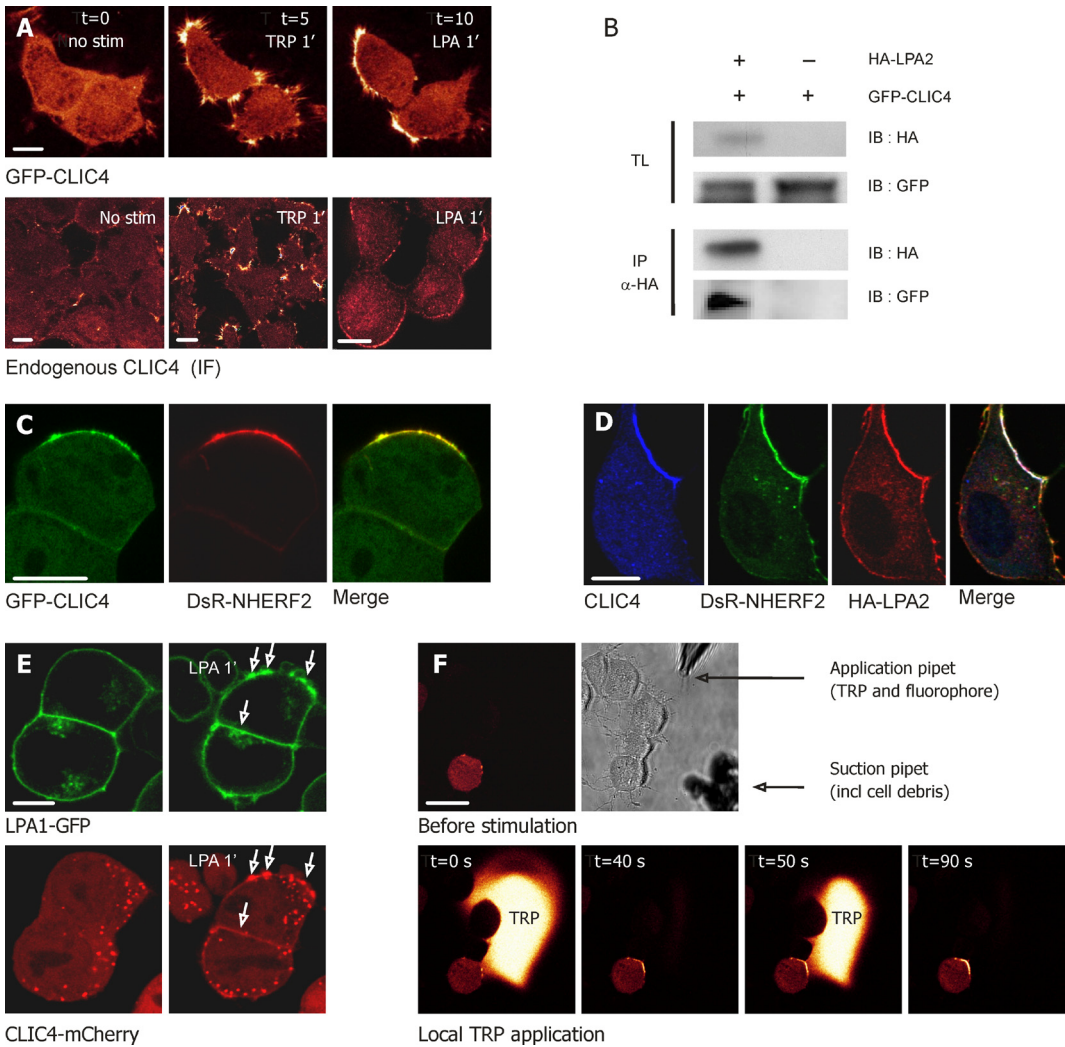


Figure 4 CLIC4 translocation towards activated GPCRs and NHERF2

A) Top panels: differential distribution of translocated CLIC4 in the same N1E-115 cell. The cell was first stimulated with TRP and thereafter with LPA. Lower panels: endogenous CLIC4 in N1E-115 cells stimulated with either TRP or LPA (immunofluorescence). Scalebars: 10 μ m.

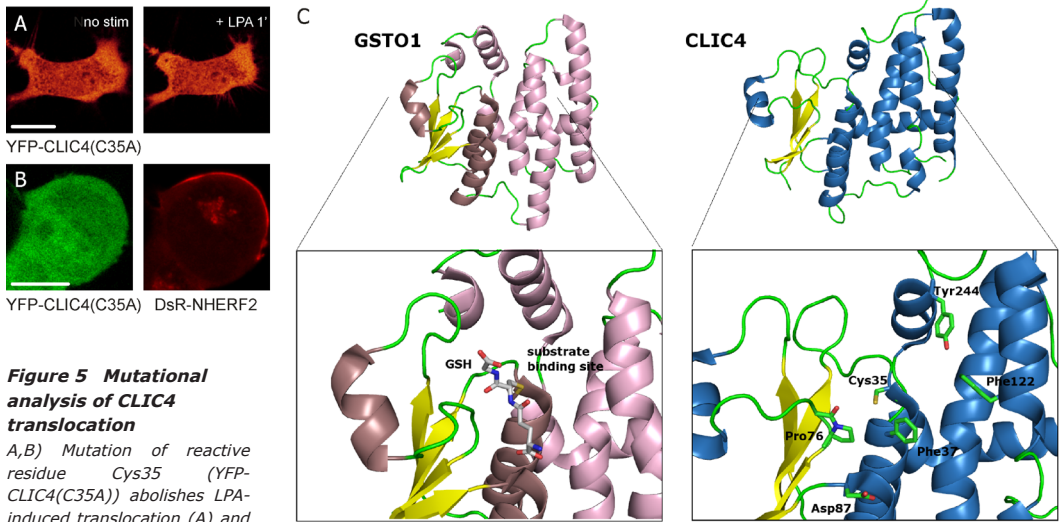
B) HEK293 cells expressing HA-LPA2 and GFP-CLIC4 were lysed and HA-LPA2 was immunoprecipitated. Left lane : GFP-CLIC4 co-precipitated with HA-LPA2, as detected with GFP antibody. Right lane : in the absence of HA-LPA2, co-precipitation of GFP-CLIC4 is not observed.

C) Colocalization of GFP-CLIC4 and DsRed-NHERF2 at polarized regions of the plasma membrane in N1E-115 cells.

D) Triple colocalization of endogenous CLIC4 (Alexa488-conjugated secondary antibody; shown in blue), DsRed-NHERF2 (shown in green) and HA-LPA2 (Alexa633-conjugated secondary antibody; shown in red).

E) CLIC4 translocates to LPA1 receptors. Pictures taken from time-lapse recordings showing LPA1-GFP and CLIC4-mCherry in HEK293 cells before and after LPA stimulation. Arrows indicate plasma membrane regions where LPA1-GFP and CLIC4-mCherry transiently colocalize after LPA administration. Scalebars in (C,D,E) are 10 μ m.

F) CLIC4 accumulates in those membrane domains that are exposed to ligand (TRP). TRP was locally applied by combining an application and a suction pipet. Besides TRP (~ 3 mM), the application pipet contained Calcium Orange (~ 0.2 mM) for continuous monitoring of the flux between both pipets. After verifying the gradient steepness of the TRP/dye mix, a non-stimulated cell was positioned near the pipets and GFP-CLIC4 was imaged during local TRP application. Emissions of GFP and the pipet dye were detected in the same channel (500-555 nm). Top : fluorescence and transmission images before ligand stimulation; arrows indicate pipets (and cell debris attracted by the suction pipet). Bottom: t=0 s, temporary TRP flux touching the "north-east" flank of the cell; t=40 s, GFP-CLIC4 accumulates selectively at the TRP-exposed area of the plasma membrane; t=50 s, a second TRP flux; t=90 s, further accumulation of GFP-CLIC4. This experiment is representative for n=4. Scalebars: 20 μ m.



D	Mutated Residue	YFP-CLIC4	Translocation upon LPA	NHERF2 colocalization
		wt	+	+
Involved in GSH binding	C35A	-	-	-
	F37D	-	-	-
	D87A	-	-	-
	P76A	-	-	-
Involved in substrate binding	F122R	-	-	-
	Y244A	-	-	-

to the apical plasma membrane, where they assemble G protein-coupled receptors, transmembrane ion channels and transporters, [24]). We found that, in N1E-115 cells, GFP-CLIC4 and DsRed-NHERF2 showed perfect colocalization in a polarized, restricted zone along the plasma membrane (Fig. 4B); colocalization was also observed for endogenous CLIC4 and DsRed-NHERF2. In addition, we observed triple colocalization of endogenous CLIC4 with the overexpressed LPA2 receptor (HA-tagged) and DsRed-NHERF2 (Fig. 4C). These results indicate that CLIC4 is targeted specifically to the LPA2-NHERF2 complex.

In the second approach, we co-expressed the GFP-tagged LPA1 receptor and CLIC4-mCherry in HEK293 cells and monitored their spatiotemporal regulation. As shown in Fig. 4D, LPA stimulation caused a rapid accumulation of CLIC4-mCherry at LPA1-GFP-containing subdomains at the plasma membrane, indicating that CLIC4 is being targeted to the activated LPA1 receptor (see also supplementary movie 2). Finally, in yet another approach, we applied a precisely confined stream of TRP (generated through an application and a suction micropipette) to single GFP-CLIC4-expressing N1E115 cells. This induced an immediate translocation of GFP-CLIC4 only to the agonist-exposed area of the plasma membrane (Fig. 4E and Suppl. Movie 3), which is in contrast with the non-polarized membrane targeting observed after bath application of TRP (Fig 4A; $n > 50$). Taken together, these results indicate that CLIC4 is targeted specifically to activated $G(\alpha)_{13}$ -coupled receptor complexes.

Mutational analysis of CLIC4 translocation

We next examined which residues are necessary for CLIC4 translocation, taking into account the structural similarity of CLIC4 to the omega-class of glutathione S-transferase (GST) family [25,26]. The GST-fold itself has an N-terminal thioredoxin-like domain that binds glutathione, and an all-helical C-terminal domain with a binding site for the substrates to which glutathione is conjoined. The omega-GSTs typically contain a reactive cysteine that activates glutathione by forming a mixed-disulfide [27]. This single reactive cysteine has been conserved in the CLIC proteins (Cys35 in CLIC4), but it is unclear why. Under reducing conditions, as normally found in the cytosol, CLICs exhibit very low affinity for glutathione and no substrates are known to which they display GST activity. Instead it has been proposed that the CLICs are structurally dynamic and possess both a soluble GST-fold and a transmembrane state [15,28].

Strikingly, when Cys35 was mutated into an alanine, the YFP-CLIC4(C35A) mutant failed to translocate to the plasma membrane upon agonist stimulation (Fig. 5A). Likewise, the plasma membrane localization observed with DsRed-NHERF2 was lost (Fig. 5B). In contrast, mutation of two other conserved cysteines, Cys189 and Cys234, did not affect CLIC4 translocation (Table in Fig. 5D; primary data not shown). So, if CLIC4 maintains an as yet undetected enzymatic activity, homology to the omega-GSTs would suggest that Cys35 is essential for catalysis.

Cys35 reactivity [22] could imply that reactive oxygen species (ROS) affect the resting state of CLIC4 and/or its translocation behavior. However, application of an oxidative burst (1 mM H₂O₂) did not trigger CLIC4 translocation, nor did it detectably affect the agonist-induced translocation. Conversely, CLIC4 translocation was not prevented by the anti-oxidant N-acetylcysteine (NaC, 5 mM). These results argue against a role for ROS in recruiting CLIC4 to the plasma membrane.

Next, we mutated residues whose equivalents make up the glutathione-binding site in omega-GSTs. Superposition of the CLIC4 and omega-GST structures revealed that the accommodation of glutathione involves three additional conserved residues, namely Phe37, Pro76 and Asp87 (Fig. 5C). Mutating Phe37 into an aspartate (F37D) adds a repulsive interaction to the carboxyl group of glutathione's gamma-glutamate. Mutating Pro76 will disrupt the glutathione binding site in GST, while the D87A mutation removes a residue that interacts with the amide of the glutathione gamma-glutamyl group in omega-GST. Mutation of any of these three residues abolished agonist-induced translocation of YFP-CLIC4 as well as its colocalization with DsRed-NHERF2 (Fig. 5D). These results suggest that CLIC4 binds a substrate in a manner similar to GST-glutathione binding, and that this binding is required for CLIC4 to respond to GPCR stimulation.

GSTs also bind a second substrate, namely the xenobiotic compound to which glutathione is conjugated. Because this substrate must lie next to glutathione for conjugation, distance constraints reduce the number of residues that need to be considered. Phe122 and Tyr244 are strongly conserved among the CLIC proteins and appear at positions equivalent to the secondary substrate binding site of the GSTs (Fig. 5C). We therefore generated mutants YFP-CLIC4(F122R) and YFP-CLIC4(Y244A). When expressed in N1E-115 or HEK293 cells, both mutants failed to undergo agonist-induced translocation, neither did they colocalize with co-expressed DsRed-NHERF2 (Fig. 5D).

In conclusion, CLIC4 translocation requires residues Cys35, Phe37, Pro76 and Asp87 (equivalents of the GSH-binding residues in GSTs) as well as Phe122 and Tyr244 (equivalents of the secondary substrate-binding residues in GSTs). While the identity of the prospective CLIC4-binding partner remains to be identified, our data strongly suggest that the substrate-binding features of the omega-GSTs have been conserved in CLIC4 along with the fold itself, and that substrate binding is a prerequisite for CLIC4 translocation.

Discussion

In this study we analyze the transient translocation of CLIC4 towards the plasma membrane upon stimulation of G(α)₁₃-coupled GPCRs (Fig. 1). CLIC4 translocation is strictly dependent on G(α)₁₃-mediated activation of RhoA (Fig. 2). Further, we find that the activated GPCR (complex) itself forms the anchor for CLIC4 translocation, explaining the rapid agonist-induced recruitment and receptor-specific distributions along the plasma membrane

(Fig. 4). Whether translocated CLIC4 interacts directly or indirectly with the activated receptor awaits further studies. We identified six conserved residues essential for translocation, whose equivalents in the omega-GST enzymes serve substrate recognition (Fig. 5), putting the GST-derived features of the CLICs in a new perspective.

Although it is tempting to hypothesize that plasma membrane translocation of CLIC4 (chloride intracellular channel) underlies the LPA-induced chloride efflux described previously [20,21], we failed to prove a causal relationship. Neither membrane insertion of HA-CLIC4 (Fig. 3B) nor modulation of LPA-induced currents by altering CLIC4 expression levels (Fig. 3A) could be observed. In addition, the translocation was eliminated upon disruption of the actin cytoskeleton using Latrunculin A, further dissociating CLIC4 translocation from the Latrunculin A-insensitive chloride channel activity (Fig. 2D). Similar results have been described for CLIC5 overexpressed in JEG-3 placental cells, although chloride channel activity was detectable in vitro [17]. It must be emphasized, however, that the present study was restricted to GPCR signaling in N1E-115 cells, hence the membrane-insertion model cannot be excluded for other cell systems or other subcellular organelles. This is underlined by the increased anion conductances demonstrated by electrophysiological characterization of CLIC4 overexpressed in HEK293 cells [29]. However, in these cells CLIC4 levels are unphysiologically high (unpublished observation), possibly driving the spontaneous occurrence of artificial membrane insertion. Furthermore, membrane insertion triggered by endogenous signaling cascades has never been reported. Finally, the observed effects on electrical membrane properties [29] might be explained by CLIC4 regulating alternative chloride channels. Tonini and coworkers [30] combined patch-clamp analyses with intracellular application of antibodies via the recording pipette and found that antibody binding to the FLAG-epitope of CLIC1 fusion constructs prevented the conductance increase only when the FLAG-tag was fused to the N-terminus. These experiments attribute a role to the N-terminus of (over-expressed) CLIC1 in the transmembrane chloride conductance, but do not (as the authors claim) provide evidence for actual membrane insertion, since a regulatory role for CLIC4 cannot be excluded. Thus, the physiologically regulated membrane insertion of endogenous CLIC proteins still awaits direct proof.

Another conceptual problem of N-terminal membrane insertion is the incompatibility with the high structural similarity of the N-terminus with the cytosolic GST proteins. This has necessitated a model wherein the CLICs dynamically switch from a soluble GST-fold to a transmembrane state [15,28]. We found that six conserved residues, whose equivalents in GST proteins interact with substrates of glutathione-conjugation, are indispensable for CLIC4 translocation (Fig. 5). This underscores the importance of the GST-fold for substrate recognition in the anchoring receptor complex (Fig 4) rather than for switching to a membrane insertion domain (Fig. 3). Thus we ascribe an essential role to the enigmatic structural GST-homology of CLIC4. Despite the facts that GST functions are tightly

associated with redox-regulated conjugation processes and that *in vitro* channel properties of CLIC4 are redox-sensitive, we find that CLIC4 translocation is insensitive to redox potential. Therefore, it remains uncertain whether CLIC4 translocation involves a GST-like enzyme activity or only the GST-derived substrate binding features. An interesting possibility is that the GST-homology domain is specialized to recognize a post-translationally modified binding partner. Such post-translational regulation could explain the transient nature of CLIC4 translocation. Further structural understanding of the interaction will likely follow from identifying the direct binding partner in the activated GPCR complex.

As for the translocation of CLIC4, we have not yet determined whether the interaction with the activated GPCR is direct or indirect. In this respect, it is interesting that CLIC4 binds directly to the C-terminal tail of the histamine-receptor H3R [31], strengthening the link between CLIC4 and GPCRs. Likely, however, this interaction is functionally distinct from the $G(\alpha)_{13}$ -induced receptor recruitment described here, since H3R does not couple to $G(\alpha)_{13}$. Furthermore, colocalisation with H3R appears to occur intracellularly and the authors did not show increased interaction upon ligand stimulation. Our demonstration that translocating CLIC4 selectively migrates to a subpopulation of locally activated receptors (Fig. 4F) indicates that the initiation of the translocation process is regulated at the level of the receptor complex rather than at the level of cytosolic CLIC4. Activated GPCRs are transiently decorated with a multitude of proteins, ranging from G protein subunits for downstream signaling to endocytosis machinery components for eventual receptor internalisation. Conceivably, one of these recruited proteins forms the temporary attractant in the dynamic process of CLIC4 translocation. In this respect, it is interesting to note that Dynamin1 (Dnm1), a key player in receptor internalisation, has been found to directly interact with CLIC4 [32]. Still, this is a puzzling option, since we consider it unlikely that CLIC4 translocation is functionally linked to internalisation *per se*, because GPCRs that couple to other G proteins (e.g. $G(\alpha)_q$, $G(\alpha)_s$ or $G(\alpha)_i$; see Table 1) undergo dynamin-assisted internalization without recruiting CLIC4. Furthermore, our preliminary experiments showed that downregulation of CLIC4 does not prolong the kinetics of the LPA-induced MAP kinase response, suggesting that receptor internalisation was unchanged (data not shown).

We speculate that CLIC4 recruitment to activated receptor complexes may play a role in the signal transfer from $G(\alpha)_{13}$ to RhoA. First, this is expected to occur selectively on platforms of activated receptors, where we find translocated CLIC4. Second, we find that CLIC4 translocation is unique for $G(\alpha)_{13}$ -coupled receptors (Table 1). Third, CLIC4 translocation requires RhoA in the activated, GTP-bound state (Fig. 2), possibly implying that its (activity-dependent) translocation to the plasma membrane [33] is linked with that of CLIC4 (Fig. 2). Fourth, this hypothesis would explain why CLIC4 translocation is not affected by inhibition of any of the downstream Rho-effectors ROCK, mDia, PKN (data not shown) and PIP(5) Kinase (Table 1). We have interesting preliminary data

showing that overexpressed mCherry-CLIC4(C35A) has a dominant negative effect on the LPA-induced translocation of co-expressed GFP-CLIC4. Importantly, these confocal timelapse recordings also reveal a loss of the LPA-induced contractile response (data not shown), suggestive of affected RhoA signaling. This observation may indicate that translocatable CLIC4 is a prerequisite for signal transfer towards RhoA. In this context, it is interesting that $G(\alpha)_{12}$, a close family member of $G(\alpha)_{13}$, is directly linked to RhoA by the scaffold protein AKAP-Lbc [34]. Upon LPA stimulation, $G(\alpha)_{12}$ activates AKAP-Lbc, which itself is a RhoA-specific GEF [34]. Several other AKAP scaffolds directly interact with GPCRs [35], but for AKAP-Lbc this remains to be determined. Interestingly, the scaffold protein 14-3-3, which regulates the activation of RhoA by AKAP-Lbc [36], is a direct interactor of CLIC4 [32]. Furthermore, CLIC4 interacts directly with the isoform AKAP350 [8,9]. Thus, it is tempting to speculate that CLIC4 translocates to a protein complex containing the activated receptor, $G(\alpha)_{13}$ and an AKAP scaffold with GEF activity for RhoA. Further studies are required to test this hypothesis wherein CLIC4 plays a central role in GPCR- $G(\alpha)_{13}$ -RhoA signaling.

Materials and Methods

Reagents and antibodies

LPA, S1P, Y-27632, Wortmannin, Latrunculin A, sodium nitroprusside, N-acetyl-cysteine (NaC) and mouse monoclonal antibody were from Sigma Chemical Co. (St. Louis, MO). H2O2 was from MERCK chemicals. PTX was from List Biol. Laboratories (Campbell, California). Thrombin Receptor-activating Peptide (TRP, amino acid sequence SFLRRN) was synthesized in our institute. C3-toxin was received from Shuh Narumiya (Kyoto University). CLIC4 rabbit polyclonal was generated in the institute of MB; HA rat monoclonal (3F10) was from Roche (Inc.); GFP rabbit polyclonal was generated in our institute (WHM); HRP-conjugated secondary antibodies were from DAKO.

DNA constructs

The following expression vectors were described elsewhere: CFP-CAAX and YFP-CAAX [37], H2B-CFP [38]. Vector containing HA-tagged α_{1a} -adrenergic receptor was purchased from www.cdna.org. For generation of GFP-CLIC4, CLIC4 cDNA was isolated from human placenta (clone 11-1a) using a forward primer with a BamHI adaptor immediately preceding the endogenous start codon (CGCG/GATCC ATG GCG TTG TCG ATG CCG C) and a reverse primer with a HindIII adaptor immediately following the endogenous stop codon (GCA/AGCTT TTA CTT GGT GAG TCT TTT GGC). PCR product was subcloned into PCR2.1 Topo vector and from this a Bam-HI insert was ligated into pGFP-C1 vector and verified for proper orientation by sequencing. CFP-, YFP- and HA-CLIC4 were made by substituting GFP of GFP-CLIC4 with fluorophores from pCFP-C1, pYFP-C1 (NheI/KpnI) or oligonucleotides containing coding sequence for HA flanked by restriction

CLIC4	# 1 F	gatcccc GATGTCGCCAAGAGACTTA ttcaagaga TAAGTCTCTTGGCGACATC ttttgga
	# 1 R	agcttttcaaaaa GATGTCGCCAAGAGACTTA tctcttga TAAGTCTCTTGGCGACATC ggg
	# 2 F	gatcccc GGGACCCACCGCATTATA ttcaagaga TAAATGGCGGGTGGTCCC ttttgga
	# 2 R	agcttttcaaaaa GGGACCCACCGCATTATA tctcttga TAAATGGCGGGTGGTCCC ggg
	# 3 F	gatcccc CGCCATTATAACCTTCAA ttcaagaga TTGAAGTTATAATGGCG tt tttgga
	# 3 R	agcttttcaaaaa CGCCATTATAACCTTCAA tctcttga TTGAAGTTATAATGGCG ggg
RhoA	# 1 F	gatcccc TGGACTCCAGAAGTCAAGC ttcaagaga GCTTGACTCTGGAGTCCA ttttgga
	# 1 R	agcttttcaaaaa TGGACTCCAGAAGTCAAGC tctcttga GCTTGACTCTGGAGTCCA ggg
G _{α13}	# 1 F	gatcccc AGTGAATTTGACCAAGTGC ttcaagaga GCACCTGGTCAAATTCAC T ttttgga
	# 1 R	agcttttcaaaaa AGTGAATTTGACCAAGTGC tctcttga GCACCTGGTCAAATTCAC T ggg
	# 2 F	gatcccc AGTGAATTTGACCAAGTGC ttcaagaga GCACCTGGTCAAATTCAC T ttttgga
	# 2 R	agcttttcaaaaa AGTGAATTTGACCAAGTGC tctcttga GCACCTGGTCAAATTCAC T ggg
	# 3 F	gatcccc AGTGAATTTGACCAAGTGC ttcaagaga GCACCTGGTCAAATTCAC T ttttgga
	# 3 R	agcttttcaaaaa AGTGAATTTGACCAAGTGC tctcttga GCACCTGGTCAAATTCAC T ggg

Table of the used RNAi targeting sequences

sites NheI/KpnI. For fluorophore-tagging at the C-terminus, CLIC4 insert was isolated from template GFP-CLIC4 by PCR (forward primer AGCTGATATCTGATGGCGTTGTCGAT; reverse primer AAAAGACTCTACCAAGGCTAGCAGCT) and ligated (EcoRV/NheI) into pcDNA3.1 vectors containing YFP or mCherry C-terminal to a multiple cloning site (MCS). Point mutations in YFP-CLIC4 were generated using Phusion Site-Directed Mutagenesis Kit (Finnzymes Inc.) and verified by sequencing analysis.

Generation of GFP-CLIC1 and CLIC1-YFP was identical to the approach followed for CLIC4 constructs. RNAi constructs were made by insertion (BgIII/HindIII) of ordered oligonucleotides into empty pSuper-vector (Brummelkamp et al, 2002) and verified by sequencing analysis. Target sequences are listed below.

YFP-mDia1(FH2AN) was a kind gift from Art Alberts, DsRed-NHERF2 was a kind gift from Th. Gadella, pSuper-RNAi-RhoA was a kind gift from J. Collard.

Cell Culture, transfections and live cell experiments

N1E-115 neuroblastoma cells and HEK293, Rat-1, MDCK, HeLa and A431 cells were cultured in DMEM. Cells were seeded and cultured on glass coverslips and constructs were transiently transfected using Fugene 6 Transfection Reagent (Roche Inc.). Experiments were performed in a culture chamber mounted on an inverted microscope in bicarbonate-buffered saline (containing, in mM, 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 glucose, 23 NaHCO₃, with 10 mM HEPES added), pH 7.2, kept under 5% CO₂, at 37°C. Agonists and inhibitors were added from concentrated stocks.

Live-cell confocal imaging and image analysis

Coverslips with cells expressing various constructs were mounted in a culture chamber and imaged using an inverted TCS-SP5 confocal microscope equipped with 63x immersion oil lense (N.A. 1.4) (Leica, Mannheim, Germany). Imaging conditions were: CFP, excitation at 442 nm, emission at 465-500 nm; GFP, exc. at 488 nm, em. at 510-560 nm; YFP, exc. at 514 nm, em. at 522-570 nm; mCherry, exc. at 561 nm, em. at 580-630 nm.

For translocation studies, series of confocal images were taken at 5 or 10 s intervals. To quantitatively express the translocation of GFP- or YFP-CLIC4, the ratio of PM to cytosolic fluorescence was calculated by post-acquisition, automated assignment of regions of interest (ROI) using Leica Qwin software (see also van der Wal et al. [37]).

Immunofluorescence stainings

Cells were fixed in paraformaldehyde (PFA) or (for endogenous CLIC4) methanol, permeabilized using 0.1% Triton X100, blocked in 2%

BSA, incubated with primary antibody and subsequently with Alexa-conjugated secondary antibodies. Mounted slides were examined on Leica TCS-SP5 confocal microscope (63x lense, N.A. 1.4). Immunofluorescence experiments attempting to detect externalized HA-epitope of overexpressed HA-CLIC4 (Fig 3) were performed under strictly non-permeabilizing conditions, i.e. without Triton X100-treatment and with PFA as a fixative. However, because even optimized conditions cannot exclude plasma membrane perforation on a subcellular scale (e.g. by shear stress, salt precipitation or cell death), we verified plasma membrane integrity for every studied cell by co-staining of co-transfected H2B-CFP; this internal control showed immunostained H2B-CFP in ~2% of studied cells, underlining the potential for false positive results. Parallel experiments on cells transfected with the α1a adrenergic receptor (HA-α1a-R), which externalizes the N-terminal HA-tag with approx. the same number of externalized residues as the postulated CLIC4 externalization (~34, [15]), confirmed high effectiveness and signal strength of the HA immunostaining procedure.

Membrane potential recordings in N1E-115 cells

N1E -115 cells were loaded with fluorescent dye from the FLIPR Membrane Potential Assay Kit (Molecular Devices Inc., R8128) for 5-10 minutes and mounted on an inverted Zeiss microscope (40x lense, N.A. 1.2). Excitation was at 515 nm from a monochromator Hg-lamp, fluorescence (longpass filtered >540 nm) was detected with a photonmultiplier tube (PMT) and excitation intensity was adapted to yield a standardized baseline output signal. Diaphragms were used to collect emission selectively from transfected cells (discriminated by H2B-CFP transfection marker using 425 nm excitation light).

Dynamic monitoring of YFP/CFP FRET was performed as described in van der Wal et al., [37].

Patch clamp recordings

Electrophysiological recordings were collected using the HEKA EPC9 system. Current recordings were digitized at 100 kHz or 10 Hz (steady-state whole-cell currents).

Borosilicate glass pipettes were fire-polished to 2–4 M Ω . After establishment of the G Ω seal, the patched membrane was ruptured by gentle suction to obtain whole-cell configuration. Solutions were (in mM) 120 whole-cell pipette potassiumglutamate, 30 KCl, 1 MgCl₂, 0.2 CaCl₂, 1 EGTA, 10 HEPES, pH 7.2, and 1 MgATP; 140 external solution NaCl, 5 KCl, 0–1 MgCl₂, 0–10 CaCl₂, 10 HEPES, and 10 glucose adjusted to pH 7.3 with NaOH.

Co-immunoprecipitation, SDS-PAGE and immunoblotting

Cells were harvested in Laemmli sample buffer (LSB), boiled for 10 min. and subjected to immunoblot analysis according to standard procedures. Filters were blocked in TBST/5% milk, incubated with primary and secondary antibodies, and visualized by enhanced chemoluminescence (Amersham Pharmacia). For immunoprecipitation of HA-LPAR₂, cells were harvested in lysis buffer containing 1% NP-40, 0.25% sodium desoxycholate, supplemented with protease inhibitor cocktail (Roche). Lysates were spun down and the supernatants were subjected to immunoprecipitation using protein A beads-conjugated antibodies for 4 hrs at 4°C. Beads were dissolved in LSB, proteins were eluted by sonication and analyzed by SDS page and immunoblotting according to standard protocols.

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Chapter 3

Direct spatial control of Epac1 by cAMP

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Direct spatial control of Epac1 by cAMP

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Abstract

Epac1 is a GEF for the small G-protein Rap and is directly activated by cAMP. Upon cAMP binding, Epac1 undergoes a conformational change that allows interaction of its GEF domain with Rap, resulting in Rap activation and subsequent downstream effects, including integrin-mediated cell adhesion and cell-cell junction formation. Here, we report that cAMP also induces the translocation of Epac1 towards the plasma membrane. Combining high-resolution confocal fluorescence microscopy with TIRF and FRET assays, we observed that Epac1 translocation is a rapid and reversible process. This dynamic redistribution of Epac1 requires both the cAMP-induced conformational change as well as the DEP domain. In line with its translocation, Epac1 activation induces Rap activation predominantly at the plasma membrane. We further show that the translocation of Epac1 enhances its ability to induce Rap-mediated cell adhesion. Thus, regulation of Epac1-Rap signaling by cAMP includes both the release of Epac1 from auto-inhibition and its recruitment to the plasma membrane.

Introduction

cAMP is an important second messenger that mediates many cellular hormone responses. It has become more and more appreciated that along with the cAMP effector protein kinase A (PKA), Epac proteins also play pivotal roles in many cAMP-controlled processes, including insulin secretion [1,2], cell adhesion [3-7], neurotransmitter release [8-10], heart function [11-13] and circadian rhythm [14]. **Epac1 and Epac2 are cAMP-dependent guanine nucleotide exchange factors (GEFs) for the small G-proteins Rap1 and Rap2 [15,16].** They contain a regulatory region with one (Epac1) or two (Epac2) cAMP-binding domains, a Dishevelled, Egl-10, Pleckstrin (DEP) domain, and a catalytic region for GEF activity [17]. Binding of cAMP is a prerequisite for catalytic activity *in vitro* and *in vivo* [17]. Recently, the structure of both the inactive and active conformation of Epac2 was solved [18,19]. This revealed that in the inactive conformation the regulatory region occludes the Rap binding site, which is relieved by a conformational change induced by cAMP binding.

Like all G-proteins of the Ras superfamily, Rap cycles between an inactive GDP-bound and active GTP-bound state in an equilibrium that is tightly regulated by specific GEFs and GTPase-activating proteins (GAPs). GEF-induced dissociation of GDP results in the binding of the cellular abundant GTP, whereas GAPs enhance the intrinsic GTPase activity of the G-protein, thereby inducing the inactive GDP-bound state. Besides Epac, several other GEFs for Rap have been identified, including C3G, PDZ-GEF and RasGRP, and these act downstream of different signaling pathways [20]. Since Rap localizes to several membrane compartments, including the Golgi network, vesicular membranes and the plasma membrane [21-26], spatial regulation of its activity is expected to be established by the differential distributions of its upstream GEFs, each activating distinct pools of Rap on specific intracellular locations.

Similar to Rap, Epac1 is also observed at many locations in the cell, including the cytosol, the nucleus and the nuclear envelope, endomembranes and the plasma membrane [17,27,28-31]. These various locations may reflect the many different functions assigned to Epac1, such as regulation of cell adhesion, cell junction formation, secretion, regulation of DNA-PK by nuclear Epac1, and regulation of the Na⁺/H⁺ exchanger NHE3 at the brush borders of kidney epithelium [29,32,33]. Apparently, specific anchors are responsible for this spatial regulation of Epac1. Indeed, Epac1 was found to associate with phosphodiesterase 4 (PDE4) in a complex with mAKAP in cardiomyocytes [11], with MAP-LC bound to microtubules [34] and with Ezrin at the brush borders of polarized cells (M. Gloerich, J. Zhao and J.L. Bos, in preparation).

In this study, we report the unexpected observation that, in addition to the temporal control of Epac1 activity, cAMP also induces translocation of Epac1 towards the plasma membrane. Using confocal fluorescence microscopy, Total Internal Reflection Fluorescence (TIRF) microscopy and Fluorescence Resonance Energy Transfer (FRET)-based assays for high spatial and temporal resolution, we observed that translocation of Epac1 is immedi-

ate and that Epac1 approaches the plasma membrane to within ~7 nm. In line with this, Epac1-induced Rap activation was registered predominantly on this compartment. Epac1 translocation results directly from the cAMP-induced conformational change and depends on the integrity of its DEP domain. We further show that Epac1 translocation is a prerequisite for cAMP-induced Rap activation at the plasma membrane and enhances Rap-mediated cell adhesion. Thus, cAMP exerts dual regulation on Epac1 for the activation of Rap, controlling both its GEF activity and targeting to the plasma membrane.

Results

cAMP induces translocation of Epac1 towards the plasma membrane

To study the subcellular localization of Epac1 during activation by cAMP, we monitored GFP-Epac1 using time lapse confocal imaging in HEK293 cells. In accordance with previous reports [17,27,28,30,31], GFP-Epac1 is observed at the nuclear envelope, at the plasma membrane (PM), at endo-membranes and in the cytosol. Upon addition of forskolin, which activates adenylate cyclase to produce cAMP, we observed a pronounced redistribution of GFP-Epac1 towards the periphery of the cell (Fig. 1A and Suppl Movie). Automated image analysis [35] showed that the GFP-Epac1 redistribution is manifest both as a decrease in cytosolic fluorescence and an increase in PM-localized fluorescence, occurring within 2 minutes after stimulation ($\tau_{1/2}$ ~40 s; Fig. 1A). In contrast, fluorescence in the nucleus was constant throughout the experiment (Fig. 1A). We also employed Total Internal Reflection Fluorescence (TIRF) microscopy to monitor GFP-Epac1 selectively at the basal membrane. We observed forskolin-induced accumulation of GFP-Epac1 as an increase of 60 +/- 4 % (mean +/- SEM; n=17) relative to pre-stimulus levels (Fig 1B), suggesting that Epac1 translocation represents a large-scale recruitment to the PM.

Similarly, isoproterenol stimulation, which induces physiological cAMP increases via activation of β -adrenergic receptors, induced rapid Epac1 translocation in A431 cells (Fig. 1C). Epac1 translocation was observed in all cell types tested (HEK293, A431, OVCAR-3, ACHN, RCC10, MDCK, N1E-115, HeLa, Rat-1, GE11, H1299) and over the full range of expression levels (data not shown). Importantly, staining of OVCAR-3 cells with a monoclonal Epac1 antibody showed an increased presence of endogenous Epac1 at the PM after treatment with forskolin, reflecting the PM-translocation of endogenous Epac1 (Fig. 1D). Similar results were obtained with a polyclonal Epac1 antibody (data not shown).

Due to the limited resolution of conventional confocal and TIRF microscopy (~300 nm and ~90 nm, respectively) it is difficult to judge whether GFP-Epac1 indeed translocates to the PM or, alternatively, merely to the cortical actin cytoskeleton just below it. Therefore, we monitored Fluorescence Resonance Energy Transfer (FRET) between YFP-Epac1 and CFP-CAAX, which is membrane-anchored by its prenylated K-Ras CAAX-motif and in these cells con-

Figure 1. Epac1 translocates towards the PM upon elevation of cAMP levels

A) In HEK293 cells stimulation with forskolin (25 μ M) induces translocation of GFP-Epac1 towards the cell periphery. Inset: fluorescence intensity along the red line, showing sharp demarcation of the plasma membrane (width at half-maximum $\sim$ 300 nm).

Right: fluorescence intensity at the plasma membrane (red, PM) and in the cytosol (blue, Cyt) as well as the ratio PM/Cyt (green) during the response to forskolin; fluorescence levels in the nucleus (grey, Nucl) were constant. Traces are representative for $n > 15$.

B) Representative TIRF experiment showing the forskolin-induced accumulation of GFP-Epac1 at the basal membrane of HEK293 cells. Mean increase relative to pre-stimulus levels was $60 \pm 4\%$ (mean $\pm$ SEM; $n = 17$).

C) A431 cells expressing GFP-Epac1 were imaged during isoproterenol (1 nM) stimulation. Translocation of GFP-Epac1 was observed within 1 minute ($n = 8$).

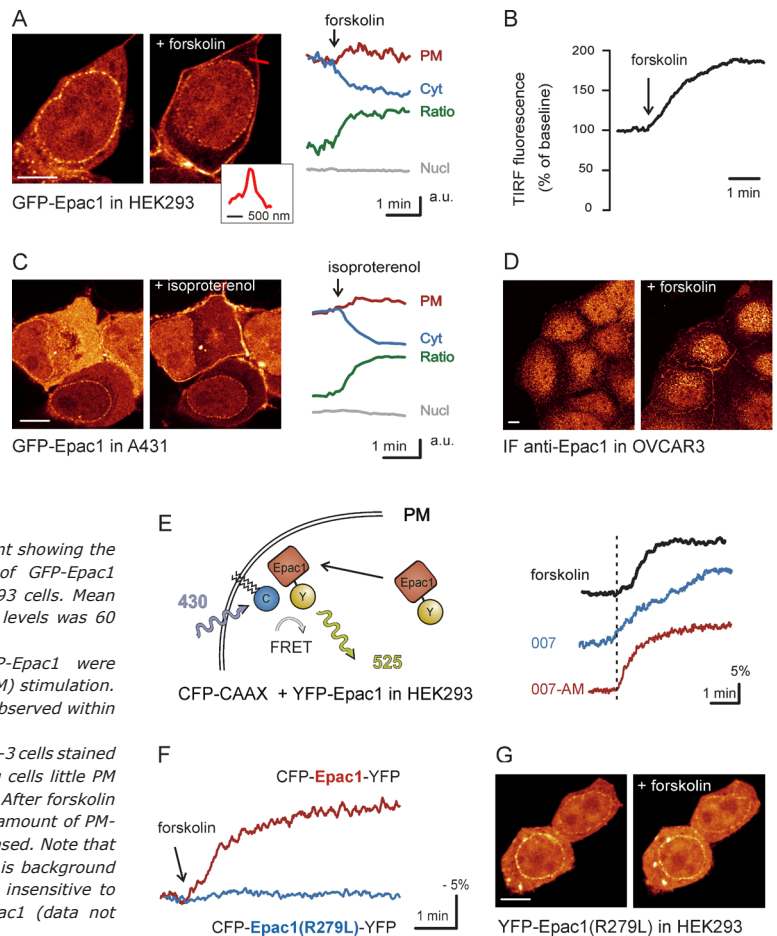
D) Immunofluorescence of OVCAR-3 cells stained for endogenous Epac1. In resting cells little PM localization of Epac1 is observed. After forskolin stimulation (25 μ M, 10 min) the amount of PM-localized Epac1 is markedly increased. Note that the nuclear immunofluorescence is background staining rather than Epac1, as it is insensitive to siRNA-mediated silencing of Epac1 (data not shown).

E) Measurement of FRET between the PM-marker

CFP-CAAX (see M&M) and translocating YFP-tagged Epac1 (traces are representative for $n > 5$). FRET, expressed as YFP/CFP ratio, increases upon addition of forskolin (25 μ M, black). FRET increases were also induced by the Epac-specific cAMP analogue 8-pCPT-2'-O-Me-cAMP (007, 100 μ M; blue) or the more membrane-permeable analogue 8-pCPT-2'-O-Me-cAMP-AM (007-AM, 1 μ M; red).

F) Forskolin (25 μ M) treatment of HEK293 cells transfected with the FRET sensor CFP-Epac1-YFP. The sensor reports the cAMP-induced conformational change as a loss of intramolecular FRET. In contrast to the wild-type sensor (red), the mutant FRET construct CFP-Epac1(R279L)-YFP (blue) lacks the ability to change conformation upon changing cAMP concentrations.

G) Mutagenesis of Arginine279 to Leucine eliminated the ability of YFP-Epac1(R279L) to translocate upon forskolin stimulation (25 μ M). Scale bars: 10 μ m.

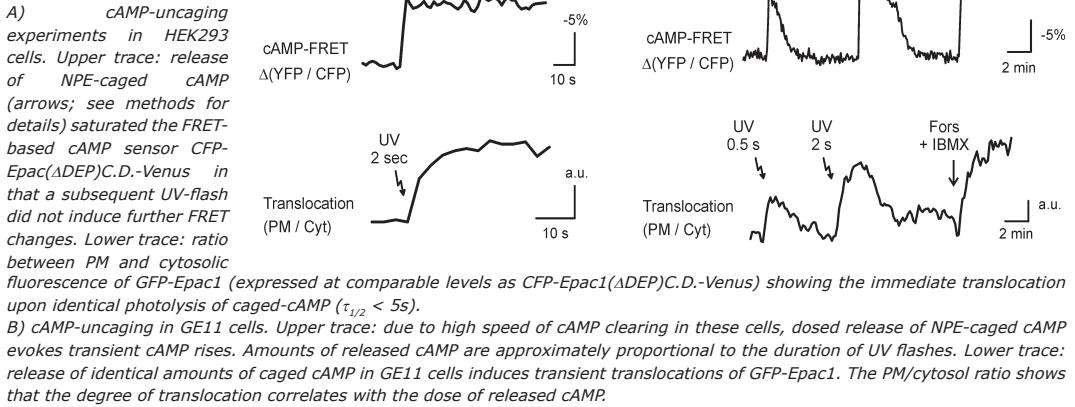


stitutes a marker of the PM (see M&M). Forskolin induced immediate increases in the YFP/CFP ratio (10-15%, Fig. 1E), indicating that translocated GFP-Epac1 approaches the PM to within approximately 7 nm. Similar results were obtained using a C-terminally tagged Epac1 (data not shown).

The translocation of Epac1 can also be induced by the Epac-specific cAMP analogues 8-pCPT-2'-O-Me-cAMP (007) [36] and the more cell-permeable 8-pCPT-2'-O-Me-cAMP-AM (007-AM) [37; chapter 9 of this thesis] (Fig. 1E), suggesting that cAMP induces Epac1 translocation through

activation of Epac1 itself rather than via parallel pathways such as PKA. Indeed, Epac1(R279L), which is mutated in the cAMP binding domain and thereby locked in the auto-inhibited, inactive conformation ([31]; see also FRET analysis on CFP-Epac1(R279L)-YFP and the wildtype sensor in Fig. 1F), lacked the ability to translocate (Fig. 1G), indicating that Epac1 must be in its open conformation to translocate. In addition, forskolin-induced translocation of GFP-Epac1 was not affected by inhibition of PKA with H89 (data not shown). Thus, we show that Epac1 translocates towards the PM when it is bound by cAMP.

Figure 2. Epac1 translocation is a highly dynamic and reversible event



Epac1 translocation dynamically follows cAMP levels

High-resolution confocal imaging shows that translocating GFP-Epac1 is recruited from a homogeneous cytosolic pool. Indeed, fast FRAP experiments [35] confirmed that the mobility of GFP-Epac1 approaches that of free GFP (data not shown). Conversely, active transport appears not to be involved, since Epac1 translocation is insensitive to disruption of the actin cytoskeleton (Cytochalasin D, 1 μ g/ml, or Latrunculin A, 1 μ M) or the microtubule network (Nocodazol, 25 ng/ml) (data not shown). Thus, upon cAMP-binding Epac1 finds the PM by passive diffusion.

To examine the dynamics of Epac1 translocation, we loaded HEK293 cells with NPE-caged cAMP and transiently released cAMP by UV-induced photolysis of the NPE-cage. Using CFP-Epac(Δ DEP-CD)-Venus [38], an improved variant of the previously published cAMP sensor [39], we first established experimental conditions to instantly saturate Epac1 with cAMP (Fig. 2A). When applying identical UV pulses to cells expressing comparable levels of GFP-Epac1, we observed very rapid translocation that was halfway within 5 seconds and near-complete in approximately 20 seconds (Fig. 2A).

To investigate whether PM recruitment of Epac1 is a reversible event, we photoreleased NPE-caged cAMP in GE11 cells, which rapidly clear cAMP, likely due to high PDE activity [39,40]. This allowed cAMP transients to be evoked repetitively, as detected by the FRET-based cAMP sensor (Fig. 2B). When similar amounts of cAMP were released in GE11 cells expressing GFP-Epac1, we observed rapid translocations to the PM followed by relocation to the cytosol (Fig. 2B). Analyses of the PM/cytosol ratio show that the translocation kinetics closely resemble the dynamic course of the cAMP levels. These experiments indicate that Epac1 translocation is a rapidly reversible event and that the momentaneous cAMP levels dictate the degree of PM localization.

Epac1 conformational change rather than downstream signaling is required for its translocation

As opening up of Epac1, which is essential for cAMP-induced translocation (Fig. 1G), also releases its catalytic activity, we examined whether downstream signals may be required for its recruitment to the PM. As shown in Fig. 3A, overexpression of RapGAP1 inhibits cAMP-induced Rap activation by keeping both Rap1 and Rap2 in the GDP-bound, inactive state. RapGAP1 overexpression did not affect the translocation of GFP-Epac1 (Fig. 3A,B), suggesting that Rap activity is not required. In line with this, co-expression of constitutively active Rap1A(G12V) did not affect GFP-Epac1 localization in unstimulated cells (Fig. 3A), nor did it affect the magnitude or kinetics of the 007-AM-induced translocation (Fig. 3A,B). Furthermore, in OVCAR-3 cells, the cAMP-binding mutant Epac1(R279L) does not translocate when Rap is transiently activated through activation of endogenous Epac1 (data not shown). Taken together, these data indicate that Epac1 translocation results from its conformational change rather than downstream signaling via Rap. This was further supported by using the CFP-Epac1-YFP probe, that allows the simultaneous visualization of localization as well as conformational state via intramolecular FRET. These experiments showed that the kinetics of Epac1 translocation closely follow those of its conformational state (Fig. 3C).

The DEP domain is essential but not sufficient for Epac1 translocation

To determine which domains of Epac1 are involved in the translocation, a series of deletion mutants were analyzed (Fig. 4A). Removal of the DEP domain, which is essential for proper intracellular targeting and functioning of DEP domain-containing proteins such as Dishevelled [39] and numerous RGS proteins [27,41-45], completely abolished Epac1 translocation (Fig. 4B). Mutation of Arginine 82

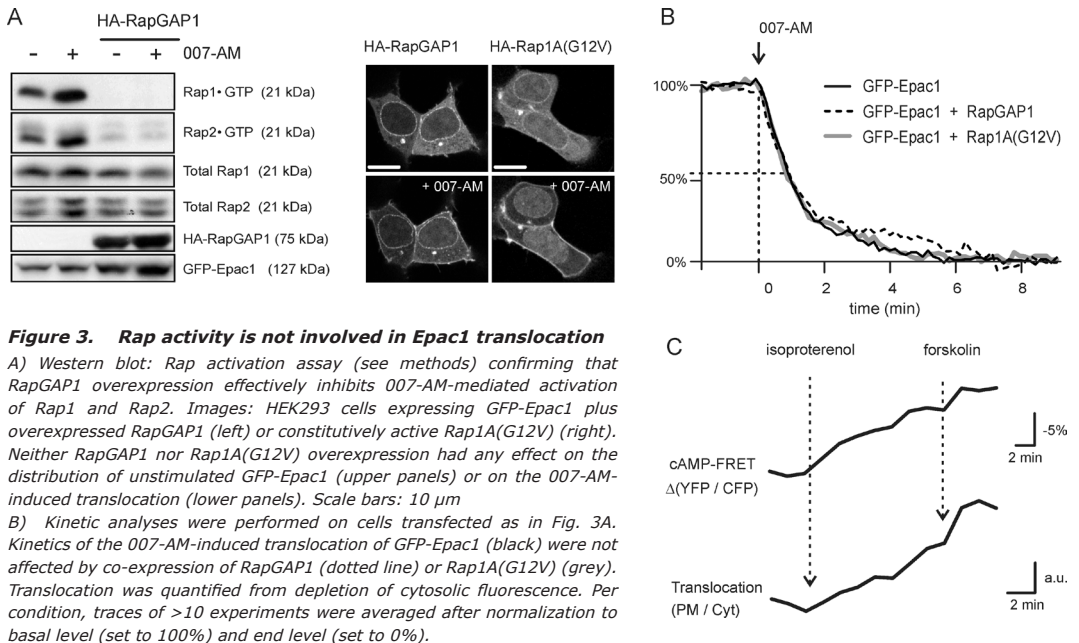


Figure 3. Rap activity is not involved in Epac1 translocation

A) Western blot: Rap activation assay (see methods) confirming that RapGAP1 overexpression effectively inhibits 007-AM-mediated activation of Rap1 and Rap2. Images: HEK293 cells expressing GFP-Epac1 plus overexpressed RapGAP1 (left) or constitutively active Rap1A(G12V) (right). Neither RapGAP1 nor Rap1A(G12V) overexpression had any effect on the distribution of unstimulated GFP-Epac1 (upper panels) or on the 007-AM-induced translocation (lower panels). Scale bars: 10 μ m

B) Kinetic analyses were performed on cells transfected as in Fig. 3A. Kinetics of the 007-AM-induced translocation of GFP-Epac1 (black) were not affected by co-expression of RapGAP1 (dotted line) or Rap1A(G12V) (grey). Translocation was quantified from depletion of cytosolic fluorescence. Per condition, traces of >10 experiments were averaged after normalization to basal level (set to 100%) and end level (set to 0%).

C) A431 cells expressing CFP-Epac1-YFP were imaged by simultaneous detection of CFP- and YFP emission (see Materials and Methods) allowing the analysis of both Epac1 activation state (FRET, Ratio YFP/CFP, upper trace) and its translocation to the PM (Ratio PM/Cyt, lower trace). Submaximal stimulation of A431 cells with 0.5 nM isoproterenol evokes slow cAMP accumulation and thereby induces gradual activation of Epac1; subsequently, forskolin (25 μ M) was added to saturate CFP-Epac1-YFP. The experiment illustrates that the kinetics of the construct's PM-translocation strongly resemble its gradual activation (representative for $n=6$).

within the DEP domain of Epac1, which localizes within a proposed interaction surface crucial for the DEP-mediated targeting of Dishevelled-1 and Ste2 [46-49], also abolished the cAMP-induced translocation, further illustrating the critical role of the DEP domain in mediating the cAMP-induced translocation. Nonetheless, GFP-DEP (comprising amino acids 50-148) was not observed at the PM (data not shown). Similarly, the YFP-tagged regulatory region of Epac1 (YFP-Epac1-Reg), comprising the DEP domain and the cAMP binding domain, did not localize at the PM nor did it translocate to the PM upon cAMP elevations (Fig. 4A,B). However, application of 007-AM to cells transfected with both YFP-Epac1-Reg and the CFP-tagged complementary catalytic region of Epac1 (CFP-Epac1-Cat), which are able to reconstitute the structure of the full length protein (data not shown), induced the combined translocation of both fragments (Fig. 4C). This demonstrates that the DEP domain is required for the translocation of Epac1 to the PM, but that it can function only in conjunction with the catalytic region.

Epac1 translocation enhances Rap-dependent cell adhesion

As the main pool of Epac1 redistributes to the PM after its activation, we explored whether the translocation of Epac1 is a prerequisite for activation of Rap at this compartment. For this, HEK293 cells were transfected with the YFP-tagged Ras-binding domain (RBD) of RalGDS

(YFP-RBD(RalGDS)), which recognizes Rap1 specifically in its GTP-bound, activated state [23]. When cells were co-transfected with HA-Epac1, addition of 007-AM resulted in the rapid accumulation of YFP-RBD(RalGDS) at the PM, as visualized both by TIRF (Fig 5A, left panel) and confocal microscopy (right panel). Interestingly, such accumulation was not observed on other subcellular compartments, suggesting that in HEK293 cells cAMP signaling via Epac1 activates Rap predominantly at the PM (Fig 5A). To test for the role of Epac1 translocation in Rap activation at the PM, we compared YFP-RBD(RalGDS) membrane recruitment in cells expressing either CFP-Epac1 or CFP-Epac1(Δ DEP) by TIRF microscopy. 007-AM induced recruitment of the probe to the basal membrane in cells expressing CFP-Epac1 (12 of 14 cells), whereas this was almost absent in cells co-expressing CFP-Epac1(Δ DEP) (1 of 9 cells; $p < 0.01$; Fig. 5B). Thus, translocation of Epac1 is required for Rap activation at the PM.

Epac1-Rap signaling is involved in integrin-mediated cell adhesion by regulating both the affinity and avidity of actin-associated integrin molecules [50]. For Jurkat T-cells, it has been shown that this requires the presence of active Rap at the PM [23]. To study the role of Epac1 translocation in its ability to mediate integrin regulation, we measured adhesion of Jurkat T-cells in response to 007. For this, the cells were transfected with luciferase together with either wild-type Epac1 or the non-translocating Epac1 variants mutated in their DEP domain, and

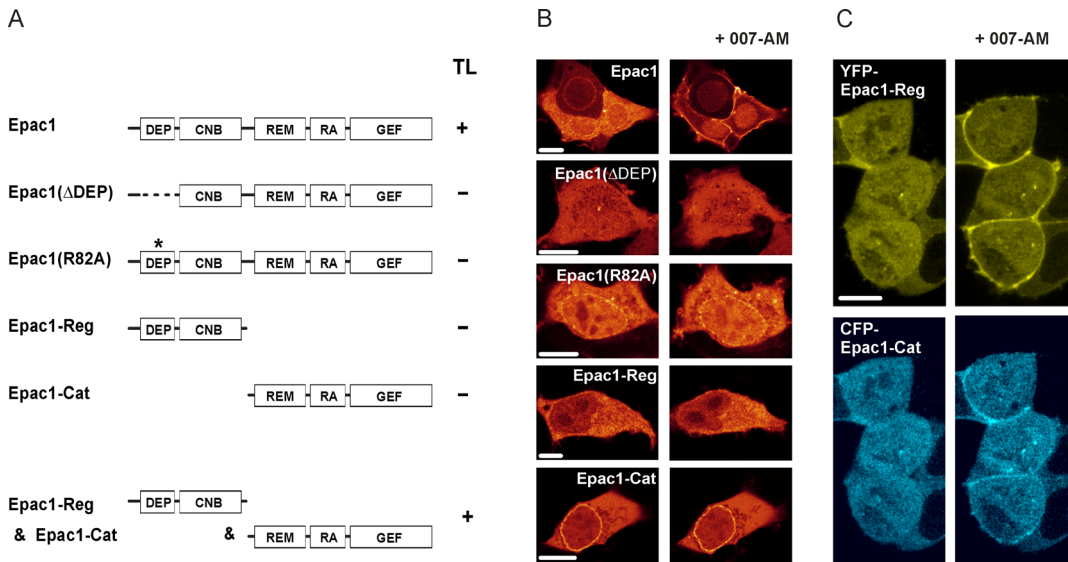


Figure 4. The DEP domain is essential but not sufficient for Epac1 translocation

A,B) Overview of Epac1-mutants and their abilities to translocate (TL) upon addition of 007-AM (1 μ M). Full-length Epac1 consists of a DEP domain (amino acid 50-148), the cyclic nucleotide binding domain (CNB), the Ras exchange motive (REM), a putative Ras association (RA) domain and the catalytic CDC25 homology GEF domain. Removal of the DEP domain (amino acid 50-148) or disruption of the interaction surface within the DEP domain (R82A) abolishes the translocation of Epac1 in response to 007-AM. The separate regulatory region (YFP-Epac1-Reg, amino acid 1-328), containing both the DEP domain and cAMP binding domain (CNB), did not translocate after 007-AM stimulation, indicating that the DEP domain cannot mediate the localization of Epac1 at the PM in the absence of the catalytic region. In accordance with the crucial role of the DEP domain, the complete catalytic region (CFP-Epac1-Cat, amino acid 330-881) was not present at the PM either.

C) In contrast to the separately expressed regulatory and catalytic region, coexpression of YFP-Epac1-Reg (upper panels) + CFP-Epac1-Cat (lower panels) restored the 007-AM-induced translocation, resulting in colocalization of both constructs at the PM. Scale bars: 10 μ m

adhesion was quantified by detection of luciferase emission. Indeed, 007 induced strong adhesion to fibronectin of wildtype Epac1-transfected cells (Fig. 5C). In contrast, this cAMP-induced effect on cell adhesion was impaired when the translocation-deficient mutants Epac1(Δ DEP) or Epac1(R82A) were expressed (Fig. 5C). Rap1-GTP pull-down experiments in Jurkat T-cells show that expression of these mutants indeed result in less Rap1 activation compared to wildtype Epac1 (Fig. 5D), implying that a significant fraction of Rap1 resides at the PM in these cells. These data indicate that the translocation of Epac1 significantly enhances the signaling cascade towards Rap-mediated cell adhesion.

Discussion

In the current study we have shown that cAMP induces the translocation of cytosolic Epac1 towards the PM. Epac1 translocation is a generally occurring, physiological event, as it was observed in a wide array of cell types, both with overexpressed and endogenous Epac1 (Fig. 1). The translocation depends solely on the cAMP-induced conformational state of Epac1, since it could be induced by the

Epac-selective analogue 007(-AM) and was prevented by a mutation in the cAMP binding pocket (R279L, Fig. 1F,G). Furthermore, since neither activation nor inhibition of Rap could affect Epac1 translocation, involvement of Rap-mediated signaling could be excluded (Fig. 3). Finally, the degree of Epac1 translocation closely followed the levels of free cAMP within the cells and showed similar kinetics as the cAMP-induced conformational change (Fig. 2 and Fig 3C). Thus, in addition to releasing Epac1 from auto-inhibition, direct binding of cAMP also regulates the translocation of Epac1 towards the PM.

Epac1 translocation is based on passive diffusion, since fluorescence distribution in the cytosol is homogeneous throughout the translocation without discernible discrete moving structures that would indicate active transport. In line with the diffusion model, Epac1 translocation shows rapid and reversible kinetics after cAMP-uncaging (Fig. 2) and is not affected by disruption of the actin cytoskeleton or the microtubule network (data not shown). These data imply that upon transition to its opened conformation, Epac1 acquires an affinity for an anchoring factor at the PM, to which it is subsequently targeted via passive diffusion.

Deletion and point mutations have indicated the DEP domain as an essential determinant of translocation. This

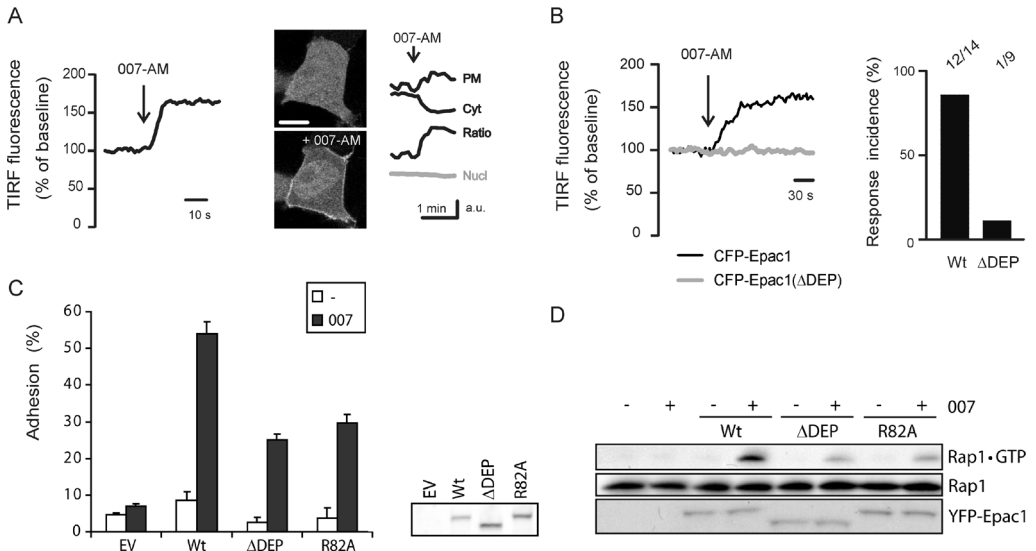


Figure 5. The translocation of Epac1 enhances Rap activation and Rap-mediated adhesion of Jurkat T-cells

A) Left panel, *in vivo* TIRF imaging of Rap1 activation in HEK293 cells using YFP-RalGDS(RBD). When co-expressed with HA-Epac1, YFP-RalGDS(RBD) translocates to the basal membrane within seconds after 007-AM stimulation (1 μ M). The 007-AM-induced increase was 46 $\pm$ 6 % (mean $\pm$ SEM) relative to pre-stimulus levels ($n=9$). Right panel, confocal imaging of YFP-RalGDS(RBD) translocation showing accumulation at the plasma membrane (PM) as well as the simultaneous depletion of cytosol (Cyt); fluorescence in the nucleus was constant (Nucl). Accumulation of YFP-RalGDS(RBD) was not observed on intracellular membranes. Scale bar: 10 μ m.

B) HEK293 cells were transfected with CFP-Epac1 or CFP-Epac1(Δ DEP) and recruitment of co-transfected YFP-RBD(RalGDS) to the basal membrane was measured by TIRF microscopy. Cells expressing low levels of CFP- and YFP-tagged fusion proteins were selected. Traces are representative experiments. Bar graph shows relative occurrence of 007-AM-induced membrane accumulation of YFP-RBD(RalGDS): CFP-Epac1, 12 out of 14 cells; CFP-Epac1(Δ DEP), 1 out of 9 cells.

C) Jurkat T-cells were transfected with either wildtype Epac1 or the non-translocating Epac1 mutants (Epac1(Δ DEP) and Epac1(R82A)) together with a luciferase reporter. Transfected cells were allowed to adhere to fibronectin-coated surface for 45 minutes and adhesion was subsequently detected as luciferase emission. Wildtype-Epac1, when activated by 007 (100 μ M, black bars) greatly enhanced the adhesion as compared to unstimulated conditions (white bars). This effect was impaired when the translocation-deficient mutants Epac1(Δ DEP) or Epac1(R82A) were transfected and it was absent in empty vector-transfected cells (EV). Shown are data from a representative experiment performed in triplo ($n=4$). Total luciferase levels were comparable in all transfections. Right: western blot labeled with the Epac1 antibody (5D3) showing expression levels of the transfected wildtype- and mutant Epac1 used in the adhesion assay.

D) Jurkat T-cells were transfected with similar amounts of either wildtype Epac1 or the translocation-deficient mutants Epac1(Δ DEP) and Epac1(R82A) and GTP-bound Rap1 was pulled down from lysates of cells after stimulation with 007 (100 μ M, 10 min).

is analogous to the targeting function of DEP domains in other proteins such as Dishevelled and RGS [1, 8, 27, 31, 40]. However, crystal structure studies on Epac2 [52] suggest that the DEP domain is solvent exposed regardless of cAMP binding. Therefore, a model wherein the cAMP-induced conformational change renders the DEP domain accessible is unlikely. Indeed, the separate DEP domain or regulatory region of Epac1 did not localize to the PM (Fig. 4). Thus, the DEP domain can only fulfill its function in the context of the structure of the full length protein. The interaction surface for PM anchoring is thus established by combined structural features of the DEP domain and a determinant in the catalytic region, which are dependent on the cAMP-bound conformation. The identification of the membrane anchor would likely help to define the underlying structural mechanism.

Many downstream effects of Epac1 occur at the PM: cell adhesion, cell-cell junction formation and the regulation of NHE3. These effects may require localized activation of Epac1 and, thereby, localized activation of Rap. Indeed, we showed that the translocation of Epac1 is a prerequisite for Rap activation (Fig 5B). Furthermore, translocation strongly enhances Rap-mediated cell adhesion, as the translocation-deficient mutants Epac1(Δ DEP) and Epac1(R82A) were impaired in their ability to induce Rap-dependent adhesion of Jurkat T-cells to fibronectin. It is important to note here, that purified Epac1(Δ 1-148) mediates GDP-dissociation from Rap1 *in vitro* equally well as full-length Epac1 does [51], indicating that the DEP domain is not required for the catalytic activity. The residual effect of the Epac1 mutants on cell adhesion may be due to the relatively high expression of Epac1 in this

system, allowing a fraction of the mutant Epac1 to locate near the PM regardless of the DEP-dependent translocation, thereby activating Rap. Indeed, TIRF experiments showed membrane recruitment of YFP-RBD(RalGDS) in cells expressing CFP-Epac1(Δ DEP) at relatively high levels (data not shown), but not at lower levels (Fig 5B).

Recently, the DEP domain of Epac1 has been shown to be essential for the ability of the regulatory region to disrupt TSH-mediated mitogenesis, further supporting the notion that proper localization of Epac1 via its DEP domain is required for its function [52]. Different anchors may target Epac1 to other cellular compartments and thereby regulate alternative functions of Epac1 that are not linked to (processes at) the PM. Indeed, in cardiomyocytes Epac1 was found in a complex with muscle specific mAKAP, phosphodiesterase 4A and PKA to regulate ERK5 activity [11]. Epac1 is also present at the nuclear membrane (see Fig. 1A) and this binding is maintained during the early time points of cAMP stimulation, indicating that only a subfraction of Epac1 translocates to the PM. Thus, in this respect Epac1 resembles protein kinase A, which is targeted to distinct subcellular compartments through the binding to AKAPs.

PM-localization has also been reported to be essential for signaling via Epac2, which is mediated by binding of its RA domain to activated Ras [30,53]. Originally this was proposed to be regulated by cAMP [30]. However, binding of Epac2 to Ras does not require the open conformation of Epac2 and is not affected by cAMP [53] and Supplemental Fig 1). In addition, expression of an active Ras mutant suffices for targeting Epac2 to the PM [30,53]. Conversely, deletion of the putative RA domain within Epac1 does not affect its cAMP-induced translocation as demonstrated by increased FRET between CFP-CAAX and an YFP-Epac1 mutant lacking the RA domain (see Supplemental Fig 2). These data exclude the possibility that the RA domain is the missing determinant within the catalytic region of Epac1. The different mechanisms of PM targeting distinguish the roles of Epac1 and Epac2, which may add to the understanding of their specific biological functions.

Based on our data, we propose a model where binding of cAMP regulates Epac1 in two manners: it targets Epac1 towards the PM and simultaneously releases the activity of its GEF domain. Such dual regulation imposes signal specificity by guaranteeing that cAMP predominantly affects PM-localized Rap molecules (Fig. 5A), whereas, for example, growth factors such as EGF activate a perinuclear pool of Rap [54]. Analogously, negative regulation of Rap by GAPs may also be spatially confined, as it has been reported that a PM pool of RapGAP restricts Rap activation in COS1 cells [55]. Thus, it appears that the localization of GEFs and GAPs rather than that of the G-protein itself determines the intracellular location of G-protein activity.

We can only speculate why the activation of Epac1 is dynamically regulated by the simultaneous cAMP-dependent translocation rather than by more static confinement via stable association to the PM. The translocation mechanism may serve to dynamically regulate the availability of Epac1 throughout the cell. In addition, the separation between Epac1 and Rap in resting conditions may be an ultimate guarantee against stimulation of Rap by residual

Epac1 activity. Adding a spatial component to the cAMP-mediated regulation of Epac1 undoubtedly renders the transition from unstimulated to stimulated Rap at the PM more pronounced.

Materials and Methods

Reagents and antibodies

Forskolin, IBMX and H89 were from Calbiochem-Novabiochem Corp. (La Jolla, CA); Isoproterenol, EGF, Cytochalasin D, Latrunculin A and Nocodazol from Sigma Chemical Co. (St. Louis, MO); 1-(2-nitrophenyl)ethyl adenosine-3',5'-cyclic monophosphate (NPE-caged cAMP) from Jena Bioscience GmbH (Jena, Germany); 8-pCPT-2'-O-Me-cAMP (007) and 8-pCPT-2'-O-Me-cAMP-AM (007-AM) from Biolog Life Sciences (Bremen, Germany); Fura-Red-AM and BAPTA-AM from Molecular Probes Inc. (Eugene, OR); the Rap1 antibody from SantaCruz Biotechnology (SC-65) and the Rap2 antibody from BD Transduction laboratories (610216). The Epac1 antibody (5D3) has been described [56]. Fibronectin was purified as described [57].

DNA constructs

The following expression vectors were described elsewhere: pcDNA3 CFP-Epac1-YFP and pcDNA3 CFP-Epac1- Δ DEP-C.D.-YFP [39], pcDNA3 CFP-Epac1(Δ DEP-C.D.)-Venus (55), pMT2-SM-HA Rap1A(G12V) and pMT2-SM-HA RapGAP1 [58], pMT2-SM-HA Epac1 [17], pcDNA3 CFP-CAAX [35]. Epac1 (RapGEF3, homo sapiens, GI: 3978530) was cloned C-terminal to YFP in a pcDNA3 vector. Mutations were introduced by site-directed mutagenesis. The separate regulatory (amino acid 1-328) and catalytic (amino acids 330-881) region of Epac1 were cloned into pcDNA3 with an N-terminal CFP and YFP tag, respectively. GFP-RBD(RalGDS) was a kind gift from Mark Philips.

Cell culture

HEK293 (Human Embryonal Kidney) cells and A431 human carcinoma cells were cultured in DMEM; OVCAR-3 and the Jurkat T-cell line JHM1 2.2 were grown in RPMI medium, all supplemented with 10% serum and antibiotics.

Live cell experiments

Cells were seeded in 6-well plates on 25-mm glass cover slips and cultured in 3 ml medium. Constructs were transiently transfected using Eugene 6 Transfection Reagent (Roche Inc.). Experiments were performed in a culture chamber mounted on an inverted microscope in bicarbonate-buffered saline (containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, 23 mM NaHCO₃, with 10 mM HEPES added), pH 7.2, kept under 5% CO₂, at 37°C. Agonists and inhibitors were added from concentrated stocks.

Dynamic monitoring of YFP/CFP FRET

Cells on coverslips were placed on an inverted NIKON microscope equipped with 63x lens (N.A. 1.30) and excited

at 425 nm. Emission of CFP and YFP was detected simultaneously by two photon multiplier tubes (PMTs) through 470 +/- 20 nm and 530 +/- 25 nm bandpass filters, respectively. Data were digitized by Picolog acquisition software (Picotech) and FRET was expressed as the normalized ratio of YFP to CFP signals. The ratio was adjusted to 1 at the onset of the experiment and changes are expressed as percent deviation from this initial value. For some of these experiments, a CFP-tagged version of K-Ras-CAAX was used as PM marker. Whereas in hippocampal neurons K-Ras-CAAX may translocate to endomembranes under certain conditions [59], under our conditions CFP-CAAX localizes to the plasma membrane as we have extensively documented [35,57]. For the experiment described in Fig. 3C the YFP/CFP FRET ratio was determined in imaging mode, by detecting CFP- and YFP images simultaneously on a Leica fluorescence SP2 microscope equipped with a dual-view attachment and a Coolsnap-HQ CCD-camera (Roper Scientific), using ASDMW acquisition software.

Confocal microscopy

Coverslips with cells expressing various constructs were mounted in a culture chamber and imaged at 37 °C using an inverted TCS-SP5 confocal microscope equipped with 63x immersion oil lens (N.A. 1.4; Leica, Mannheim, Germany). Imaging settings were: CFP, excitation at 442 nm, emission at 465-500 nm; GFP, exc. at 488 nm, em. at 510-560 nm; YFP, exc. at 514 nm, em. at 522-570 nm.

For detection of endogenous Epac1 in OVCAR-3 cells, cells were grown on 12 mm glass cover slips for 72 h and after 10 min of stimulation with 25 μ M Forskolin, fixed with 3.8% formaldehyde, permeabilized using 0.1% Triton X100 and blocked in 2% BSA. Cells were incubated with the Epac1 antibody (5D3) and subsequently with Alexa-conjugated secondary antibodies (Invitrogen). Mounted slides were examined using a Axioskop2 CLSM microscope (Zeiss) (63x magnification lenses, N.A. 1.4).

Digital image analysis

For translocation studies, series of confocal images were taken from a medial plane at 5 or 10 s intervals. To quantitate the translocation of constructs, the ratio of cytosolic to PM fluorescence was calculated by post-acquisition automated assignment of regions of interest (ROIs) using Leica Qwin software (see [35] and <http://research.nki.nl/jalinklab/Homepage%20%Phys&ImgGrp%200.htm>). Note that in the individual traces gain of fluorescence at the PM appears small compared to the loss in the cytosol because of the under-representation of membrane area in medial sections; the majority of membrane is present in the basal membrane and in the strongly curved apical parts of the cell. Post-acquisition brightness and contrast adjustments were performed with ImageJ software (NIH).

TIRF microscopy

Cells expressing GFP-Epac1 or YFP-RBD(RalGDS) were mounted in a culture chamber and imaged on a Leica TIRF setup, equipped with 488 argon excitation laser and Hamamatsu EM-CCD detector. A 63x, 1.45 NA objective was used and evanescent field penetration depth was set

to 90 nm. TIRF imaging was at ambient temperature and analysis was with LAS-AF software.

Loading and flash photolysis of NPE-caged cAMP

Cells were loaded by incubation with 100 μ M NPE-caged cAMP for 15 min. Uncaging was done with brief pulses of UV light (340-410 nm) from a 100 W HBO lamp using a shutter. To define exposure times and UV-light intensities for desired cAMP-release, cAMP was monitored radiometrically (CFP/YFP) using the Epac-based sensor CFP-Epac(Δ DEP-C.D.)-Venus [38]. Translocation of GFP-Epac1 was monitored in parallel experiments.

Rap activation assay

Rap activity was assayed as described previously [61]. Briefly, HEK293 cells grown in 9 cm plates were lysed in buffer containing 1% NP40, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 10% glycerol, 2 mM MgCl₂ and protease and phosphatase inhibitors. Lysates were cleared by centrifugation and active Rap was precipitated with a GST fusion protein of the Ras-binding domain of RalGDS precoupled to glutathione-sepharose beads.

Adhesion assay

The adhesion of Jurkat T-cells to fibronectin was measured as described previously [62]. In brief, 96-Well Nunc Maxisorp plates were coated with 5 μ g/ml fibronectin and blocked with 1% bovine serum albumin. 1.2×10^7 Jurkat cells were transiently transfected by electroporation (950 μ F, 250 V) with 5 μ g CMV-luciferase plasmid and pcDNA3 YFP-Epac1 plasmid adjusted to get equal expression levels, supplemented with pcDNA3 empty vector to a total of 40 μ g plasmid DNA, using the Gene Pulser II (Bio-rad). Cells were harvested two days after transfection and resuspended in TSM buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl₂, 2 mM MgCl₂). 2.5×10^4 cells for each well were allowed to adhere for 45 min and non-adherent cells were removed with 0.5% BSA in TSM buffer. Adherent cells were lysed and subjected to a luciferase assay as described previously [63]. For Rap1 activity measurements in Jurkat T-cells, transfected cells were subjected to the Rap activation assay after resuspension in TSM buffer and stimulation with 007 (100 μ M, 10 min).

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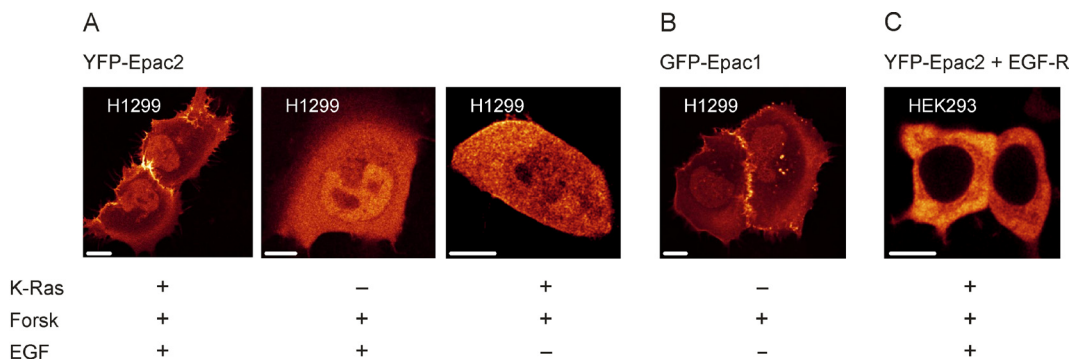
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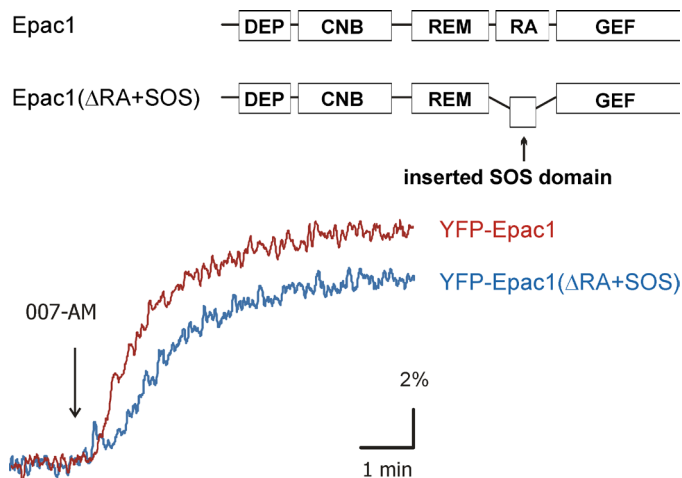
Supplemental Figures



Suppl Fig 1
The mechanism of Epac1 translocation is distinct from that of Epac2 translocation
A) In accordance with Li et al. [30], YFP-Epac2 (RapGEF4, *mus musculus*, GI:9790086, cloned C-terminal to YFP in a pcDNA3 vector) translocates to the plasma membrane in H1299 cells after both forskolin (25 μ M) stimulation and EGF (25 ng/ml)-mediated activation of overexpressed K-Ras (left), while this translocation was not observed when omitting Ras overexpression (middle) or EGF stimulation (right).
B) In contrast, forskolin alone is sufficient for the translocation of Epac1 in H1299 cells.
C) In any of the other cell types positive for Epac1 translocation, such as HEK293 cells, we could not reproduce the translocation of Epac2, even under conditions of EGF-mediated activation of co-expressed Ras. Scalebars: 10 μ m

Suppl Fig 2
The putative RA domain is not required for translocation of Epac1

As the catalytic region of Epac1 is required for PM translocation, we explored the requirement of the putative RA domain within this region (amino acids 519-658). Since deletion of the entire domain abolishes proper folding of the protein (data not shown), we replaced it with the homologous region of the RasGEF Sos1 (GI: 169234770, amino acids 741-776), which lacks an RA domain. The resultant YFP-Epac1(Δ RA+SOS) was expressed in HEK293 cells together with CFP-CAAX and stimulated with 007-AM (1 μ M). The observed FRET increase reports PM translocation of YFP-Epac1(Δ RA+SOS) similarly to wildtype YFP-Epac1, indicating that the RA domain is not essential for Epac1 translocation.



Chapter 4

ERM proteins recruit Epac1 to the plasma membrane and facilitate Epac1-induced cell adhesion

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Manuscript in preparation

ERM proteins recruit Epac1 to the plasma membrane and facilitate Epac1-induced cell adhesion

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Abstract

Epac1 is a GEF for the small G-protein Rap and is directly activated by cAMP. Epac1-Rap signaling is involved in plasma membrane-localized processes such as integrin-mediated cell adhesion and cell-cell junction formation. We previously showed that cAMP induces the translocation of Epac1 to the plasma membrane, thereby enhancing Rap-mediated cell adhesion. We here report an additional mechanism of Epac1 recruitment to the plasma membrane via an interaction with members of the Ezrin/Radixin/Moesin (ERM) family. In contrast to cAMP-dependent Epac1 translocation, this recruitment depends neither on the DEP domain nor on the conformational state of Epac1. Instead, it is regulated by conformational opening of the ERM proteins. Furthermore, whereas cAMP binding targets Epac1 uniformly along the plasma membrane, ERM proteins recruit Epac1 to polarized subcompartments. Finally, we show that the ERM-interaction contributes to Epac1-mediated cell adhesion. Taken together, our data suggest that ERM proteins spatially confine Epac1 to subcompartments of the plasma membrane, thereby contributing to the efficiency of localized Rap signaling.

Introduction

cAMP is a second messenger in a wide variety of hormone responses. cAMP is produced at the plasma membrane by adenylate cyclases, and subsequently becomes compartmentalized due to degradation by spatially restricted phosphodiesterases (PDEs) [1]. Further compartmentalization of cAMP signaling is established by the confined targeting of cAMP effector proteins. For the classical cAMP target Protein Kinase A (PKA), more than 50 A-kinase anchoring proteins (AKAPs) have been identified. AKAPs differentially target PKA to subcellular compartments and are thereby implicated in distinct biological functions of PKA [2].

The discovery of Epac as a direct effector of cAMP [3,4] has triggered the elucidation of many cAMP-regulated processes that could not be explained by the known effectors PKA and cyclic nucleotide-regulated ion channels. Epac1 and Epac2 act as guanine nucleotide exchange factors (GEFs) for the small G proteins Rap1 and Rap2, and thereby function in processes such as cell adhesion [5-9], insulin secretion [10,11] and cell polarity [12,13]. Their catalytic region contains the enzymatically active CDC25 domain, a Ras Association (RA) domain and the Ras Exchange Motif (REM). In auto-inhibited Epac, the catalytic site is sterically covered by the regulatory region, which harbors the cAMP-binding domain(s) and the DEP domain [14]. cAMP binding releases Epac from auto-inhibition by inducing a conformational change, as demonstrated by the crystal structures of active and inactive Epac2 [15,16].

Similar to the compartmentalization of PKA, cAMP-Epac signaling is spatially regulated by several anchoring proteins. For instance, both Epac1 and Epac2 are targeted to microtubules by interacting with Microtubule Associated Protein-Light Chain (MAP-LC) [17]. Other anchors bind specifically to either Epac1 or Epac2, thereby contributing to their distinct functions. For instance, only Epac1 is targeted to the mAKAP complex at the nuclear envelope [18] and, conversely, Epac2 is part of the Rim2-Piccolo complex involved in cAMP-dependent exocytosis [19,20]. In addition, more dynamic targeting mechanisms allow the regulation by intracellular signalling events. For example, Epac2 is recruited to the plasma membrane by the activated GTPase Ras [21] and this was shown to be crucial for its involvement in neurite outgrowth [22]. Recently, we reported that Epac1 translocates to the plasma membrane upon binding of cAMP and we showed that this translocation enhances Rap-mediated cell adhesion [23]. Although the anchor at the plasma membrane remains elusive, it has become clear that cAMP-dependent Epac1 translocation involves the DEP domain (residues 50-148) and depends on the cAMP-induced conformational opening.

In an attempt to identify new binding partners for Epac1, we performed a yeast-two-hybrid screen, which revealed interactions with members of the Ezrin-Radixin-Moesin (ERM) family. ERM proteins show high sequence similarity and function as scaffolding proteins that link the actin cytoskeleton to the plasma membrane [24]. Inactive ERM proteins reside in the cytoplasm in an auto-inhibited state maintained by an intramolecular interaction between

the N-terminal FERM domain and the C-terminal Actin Binding Domain (ABD). For their transition to scaffolds at the plasma membrane, ERM proteins require PI(4,5)P₂ binding and acquisition of the open conformation [25]. The latter requires phosphorylation of the ABD, for which several kinases have been implicated, including PKC α and the Rho-effector Rock [26-30]. ERM proteins directly link the actin cytoskeleton to the PM and recruit of multiple signalling proteins. In this manner, ERM proteins function in numerous processes that involve actin dynamics, such as the formation of microvilli [31], adherens junctions stabilization [24] and leukocyte polarization [32].

In the present study, we show that ERM proteins serve as membrane anchors for Epac1. The underlying interaction is mediated by the Epac1 N-terminus (residues 1-49) and is independent of its conformational state. These properties imply that ERM proteins do not constitute the anchor for the previously described cAMP-induced translocation, which depends on the DEP domain and conformational opening [23]. Instead, the interaction is regulated on the level of the ERM proteins, which selectively bind Epac1 when they are in their active, open conformation. In line with this, ERM activation via thrombin stimulation triggers the immediate recruitment of Epac1 to cortical ERM proteins. This recruitment targets Epac1 to polarized compartments along the plasma membrane, adding a component of spatial regulation to Epac1 functioning. Importantly, we find that the ERM-interaction enhances the ability of Epac1 to induce Rap-mediated adhesion of Jurkat T-cells. Taken together, our data suggest that spatial regulation by ERM proteins regulates the coupling of cAMP-dependent Epac1 activity towards Rap.

Results

Epac1 interacts via its N-terminus to proteins of the ERM family

In an attempt to identify new binding partners for Epac1, a yeast two-hybrid screen was performed using a human placenta cDNA library and full length Epac1 as bait. Positive clones were isolated encoding partial cDNA for the membrane-associated proteins Ezrin and Radixin. Together with Moesin, these proteins belong to the Ezrin/Radixin/Moesin (ERM) family and function as adaptor proteins that link the actin cytoskeleton to the plasma membrane [33].

To confirm the interaction between Epac1 and the ERM family proteins in mammalian cells, HEK293 cells were transfected with HA-Epac1 and Flag-tagged variants of Ezrin, Radixin or Moesin. Indeed, all ERM proteins co-precipitated with Epac1 (Fig 1A). As the ERM proteins share high sequence similarity and bind Epac1 to a similar extent, Radixin was used as a representative family member to further explore the interaction between Epac and ERM proteins. Epac1 and Epac2 are similar in domain architecture, except that Epac2 contains an additional cAMP binding domain [14]. However, Flag-Radixin was not able to co-immunoprecipitate with Epac2 (Fig 1B). Thus, binding to ERM proteins is specific for Epac1, suggesting that the interaction is mediated by a region that

is not conserved in Epac2. Mutational analyses revealed that the interaction with Radixin requires the N-terminal 49 amino acids of Epac1 (Fig 1C), which are indeed absent in Epac2. To further establish this, we tested binding of bacterially purified Ezrin to a series of 20-mer peptides from the Epac1 N-terminus. This assay demonstrated that the Epac1-ERM interaction is direct and further revealed that the interaction surface resides between amino acid 19 and 48 of Epac1 (Supplementary Fig S1). Thus, the Epac1 N-terminal N49 is not only essential but also sufficient to bind ERM proteins.

Importantly, these data exclude the possibility that the interaction with ERM proteins underlies the recently described cAMP-induced PM translocation of Epac1 [23], which is mediated by its DEP domain. In line with this, deletion of the DEP domain (residues 50-148) did not affect the interaction with Radixin (Fig 1C). Thus, our data indicate that Epac1 binds ERM proteins via its N-terminal 49 residues, providing a targeting mechanism that is distinct from the cAMP-induced PM translocation.

Epac1 selectively binds to ERM proteins that are in the open conformation

In resting ERM proteins, binding to the plasma membrane and the actin cytoskeleton is prevented by the intramolecular interaction between its N-terminal FERM domain and C-terminal actin binding domain (ABD) (see scheme in Fig 1A). This auto-inhibition can be relieved by threonine phosphorylation within the ABD (in Radixin: Thr564) [26,34]. Co-immunoprecipitation experiments using either the truncation mutant containing the N-terminal FERM domain and α -helical region (residues 1-492) or the separate C-terminal ABD (residues 493-584) showed that Epac1 binding is mediated by the N-terminal half of Radixin (Fig 2B). Since this region is partially buried in the

globular, inactive ERM conformation, we tested whether Epac1 specifically binds ERM proteins that are in the open conformation. For this, two Radixin mutants were tested (see residues in Fig 2A). The first, Radixin(T564D), contains a phospho-mimicking mutation at the threonine position [35]. The second, Radixin(I577D/F580D) is a novel mutant based on the crystal structure of Moesin [36]. In this mutant, residues of the ABD that form a hydrophobic interaction with the FERM domain were replaced by negatively charged asparagines. Both mutations prevented the interaction between the N- and C-terminal truncation constructs of Radixin (data not shown), and thus result in the constitutively open conformation of full length Radixin. Indeed, both Radixin mutants showed dramatically increased interaction with Epac1 compared to wildtype Radixin (Fig 2C), indicating that Epac1 displays an increased affinity for the open conformation of the ERM binding partners.

To test this further, we stimulated HEK293 cells with thrombin, which induces threonine phosphorylation and thus conformational opening of ERM proteins ([37] and Fig 2D). As shown in Fig 2D, the interaction between Epac1 and wild-type Radixin was enhanced upon thrombin treatment. This could also be measured *in vivo* by measurement of Fluorescence Resonance Energy Transfer (FRET) between YFP-Radixin and Epac1-TdTom. Addition of Trombin Receptor-activating Peptide (TRP) induced an increase in FRET (Fig 2E; Δ TdTom/YFP = 6.0 $\pm$ 1.08 % (average $\pm$ st-dev, n=3), reflecting the instant interaction between Epac1 and Radixin. These data confirm that Epac1 preferably binds to the open conformation of ERM proteins.

In a converse experiment, we examined whether the open conformation of Radixin is truly required for binding to Epac1. For this, the separate C-terminal ABD of Radixin was overexpressed to bind the N-terminus of full-length

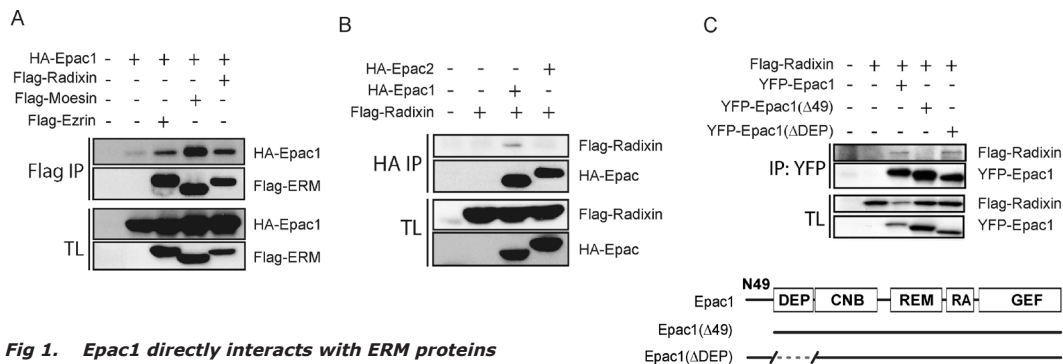


Fig 1. Epac1 directly interacts with ERM proteins

A) Co-immunoprecipitation of HA-Epac1 with Flag-Ezrin, Flag-Radixin, and Flag-Moesin in HEK293 cells.

B) Co-immunoprecipitation of Flag-Radixin with HA-tagged version of Epac1 or Epac2 in HEK293 cells. In contrast to HA-Epac1, HA-Epac2 is unable to co-immunoprecipitate Flag-Radixin. Note that upon co-immunoprecipitation of Radixin with Epac1 is less efficient than the inverse experiment in 1A.

C) Co-immunoprecipitation of Flag-Radixin with HA-tagged wildtype-Epac1, (ΔDEP)-Epac1, Δ49-Epac1 and Epac1(N49) in HEK293 cells. The 49 N-terminal amino acids of Epac1 are both required and sufficient for the interaction with Radixin, whereas the DEP domain is dispensable. Scheme represents domain structure of Epac1, showing the regulatory region with the N-terminal N49, the DEP domain and the cyclic nucleotide-binding domain (CNB), and the catalytic region which contains the REM, RA and the catalytic CDC25 domain.

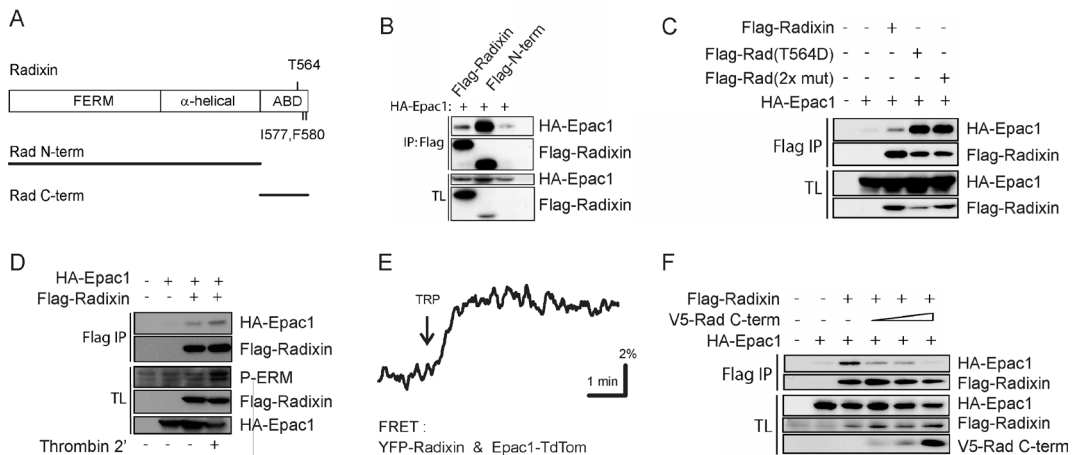


Fig 2. ERM proteins require open conformation to bind Epac1

A) Diagram of Radixin domain structure and the mutants used in experiments described in B) to E). Shown are the membrane binding FERM (four-point one, ezrin, radixin, moesin) domain, the region predicted to form an α -helical coiled-coil and the actin-binding domain (ABD).

B) Co-immunoprecipitation in HEK293 cells of HA-Epac1 with either the N-terminal truncation construct containing the FERM domain and α -helical region of Radixin (residues 1-492) or its C-terminal Actin Binding Domain (ABD, residues 492-584). Epac1 specifically co-immunoprecipitates with the N-terminal construct.

C) Co-immunoprecipitation of HA-Epac1 with wildtype Flag-Radixin and the constitutively opened mutants Flag-Radixin(T564D) and Flag-Radixin(1577D/F580D) in HEK293 cells. The amount of co-immunoprecipitated HA-Epac1 is significantly higher with the conformationally open Radixin mutants, suggesting that Epac1 favors binding to Radixin in the open conformation.

D) Co-immunoprecipitation of HA-Epac1 with Flag-Radixin in HEK293 cells stimulated with thrombin (0.2 mU/ μ l, 2 min). As shown by their phosphorylation-state (Ezrin-T567, Radixin-T564 and Moesin-T558), this induces release of ERM auto-inhibition. Thrombin stimulation results in increased interaction of HA-Epac1 with Flag-Radixin, further supporting that Epac1 favors binding to Radixin in the open conformation.

E) Measurement of FRET between YFP-Radixin and Epac1-TdTom in HEK293 cells during stimulation with TRP. FRET, expressed as TdTom/YFP ratio, increased upon TRP addition. Trace is representative for n=3. Average ratio increase was 6.0 $\pm$ 1.08 % (st. dev.).

F) Co-immunoprecipitation of HA-Epac1 with Flag-Radixin in the presence of increasing amounts of the V5-tagged C-terminal ABD of Radixin in HEK293 cells. Overexpression of V5-ABD dose-dependently decreases the interaction of HA-Epac1 with Flag-Radixin, indicating that HA-Epac1 binds Radixin in a region that is shielded during auto-inhibition of Radixin.

Radixin. This mimics the intramolecular interaction and thus the closed conformation of FL-Radixin. Indeed, co-expression of the Radixin C-terminus resulted in a dose-dependent decrease in binding between Radixin and Epac1 (Fig 1F). All in all, our data show that the binding of Epac1 to ERM proteins is regulated on the level of ERM conformation.

Activated ERM proteins rapidly recruit Epac1 to the plasma membrane

Epac proteins are spatially regulated by interactions with diverse anchoring proteins, and the inducible Epac1-ERM interaction may also represent such a regulatory mechanism. In the inactive, closed conformation, ERM proteins reside in the cytosol [34,38], whereas activated ERM proteins localize to the plasma membrane. To study the potential role of ERM proteins in regulating the subcellular localization of Epac1, we used the constitutively open and thus PM-localized Ezrin(T567D). Whereas Epac1-YFP, when expressed alone, localized mainly to the cytosol and

the nuclear envelope, it showed marked accumulation at the PM when Ezrin(T567D) was overexpressed. This was not observed with CFP-Epac1(Δ 1-49), while the isolated N-terminus of Epac1 (GFP-N49) was sufficient for PM targeting (Fig 3A), indicating that the PM accumulation of Epac1 is indeed mediated by the interaction with ERM proteins.

Strikingly, Ezrin(T567D) expression similarly induced PM accumulation of Epac1(R279L)-YFP, which is mutated in its cAMP binding domain and thereby locked in the auto-inhibited conformation [23,39] (Fig 3A). The latter implies that recruitment by ERM proteins is independent of Epac1 conformational state.

Activated ERM proteins are tethered to the PM via their FERM domains [40], implying that they may recruit Epac1 closely to the PM. To test this, we drove Epac1-YFP to the constitutively open mutant Ezrin(T567D) in cells co-expressing CFP-CAAX, which is membrane-anchored by its prenylated K-Ras CAAX-motif. We observed a prominent loss of FRET upon addition of Ionomycin (Fig 3B; average $\pm$ s.e.m. 23 $\pm$ 3% decrease in ratio YFP/CFP), which

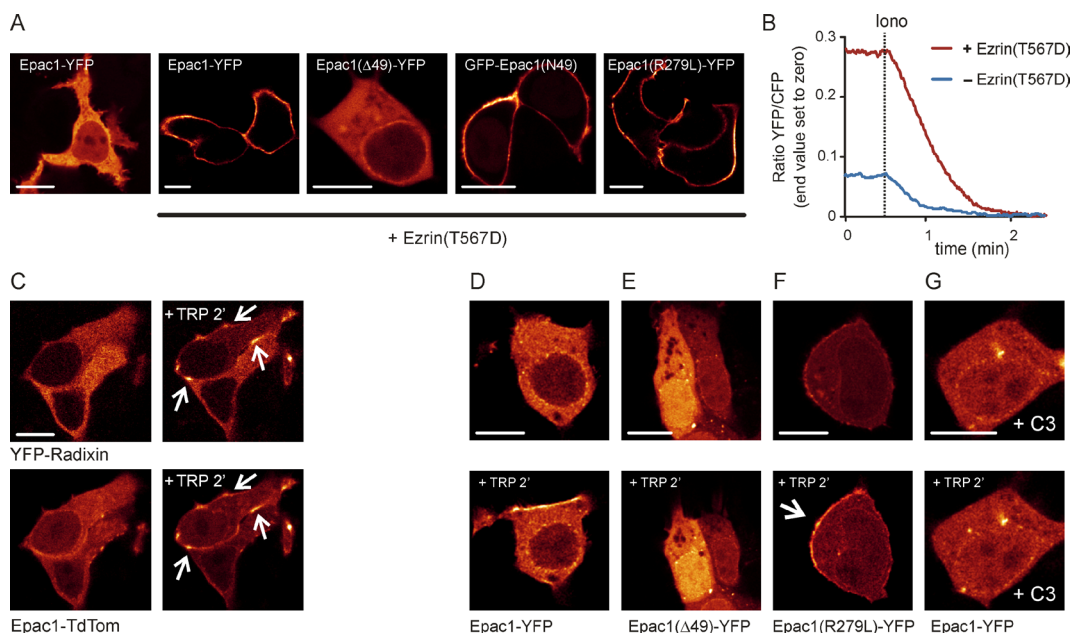


Fig 3. Activated ERM proteins recruit Epac1 to the plasma membrane

A) Subcellular localisation of Epac1-YFP in HEK293 cells. From left to right : Epac1-YFP alone; with coexpressed Ezrin(T567D); Ezrin(T567D) coexpression with deletion mutant Epac1(Δ 1-49)-YFP, with GFP-N49 and with Epac1(R279L)-YFP.

B) Measurement of FRET between the PM-marker CFP-CAAX (see [23]) and Epac1-YFP in the presence of constitutively active mutant Ezrin(T567D) (red trace). Disruption of FRET following Ionomycin-induced CFP-CAAX depalmitoylation revealed high degree of initial FRET (23 $\pm$ 3% decrease in ratio relative to baseline value 1.0 (average $\pm$ s.e.m.; n=4)), which was significantly less ($p < 0.01$) in the absence of Ezrin(T567D) (5 $\pm$ 1%; n=4, blue trace). Shown are representative traces.

C) Stills from time lapse confocal imaging of HEK293 cells expressing YFP-Radixin and Epac1-TdTom. In resting cells Radixin and Epac1 resided in the cytosol. In response to TRP (50 μ M) both constructs redistributed to restricted domains at the PM.

D) TRP stimulation induces PM recruitment of Epac1-YFP in the absence of overexpressed Radixin.

E) Epac1(Δ 49)-YFP is not recruited to the PM upon TRP stimulation. Note the cell contraction triggered by thrombin receptor signaling.

F) TRP induces PM recruitment of the cAMP-binding mutant Epac(R279L)-YFP

G) Epac1 recruitment is prevented when cells are incubated with the exo-enzyme C3, the inhibitor of RhoA (16 hr, 30 μ g/ml). Scalebars in all images: 10 μ m.

removes CFP-CAAX from the PM (unpublished observation). This implies, that ERM-bound Epac1-YFP resides within 10 nm from the PM. This was not observed when Epac1(Δ 49)-YFP was used (average $\pm$ sem 0.5 $\pm$ 0.1 %, data not shown). Without overexpressed Ezrin(T567D), Ionomycin-induced loss of FRET was significantly smaller (average $\pm$ s.e.m. 5 $\pm$ 1%; Fig 3B). The residual effect suggests membrane-targeting of Epac1 by endogenous ERM proteins.

Next, we used confocal microscopy to simultaneously monitor the subcellular localization of YFP-Radixin and Epac1-TdTom during TRP stimulation. Thrombin receptor activation drives ERM proteins into their open conformation [37] and induces their interaction with Epac1 (Fig 2D). We observed PM accumulation of YFP-Radixin and the simultaneous recruitment of Epac1-TdTom to these areas of accumulated YFP-Radixin (Fig 3C; movie can be on the web-

site), in agreement with the TRP-induced FRET increase shown in Fig 2E. Also without Radixin overexpression, TRP elicited the recruitment of Epac-YFP to the PM (Fig 3D). This was disabled by deletion of N49 (Epac1(Δ 49)-YFP, Fig 3E), implying that ERM proteins, when activated by thrombin receptor signaling, recruit Epac1 to the PM. TRP-induced recruitment was also observed for the auto-inhibited Epac1(R279L)-YFP (Fig 3F) and GFP-N49 (data not shown). Furthermore, it was sensitive to the RhoA-inhibitor C3 toxin (Fig 3G), establishing that TRP induces ERM proteins activation through Rho signaling [28,29]. Thus, from our data we conclude that Rho-dependent activation of ERM proteins causes their translocation to the PM and releases their ability to recruit Epac1.

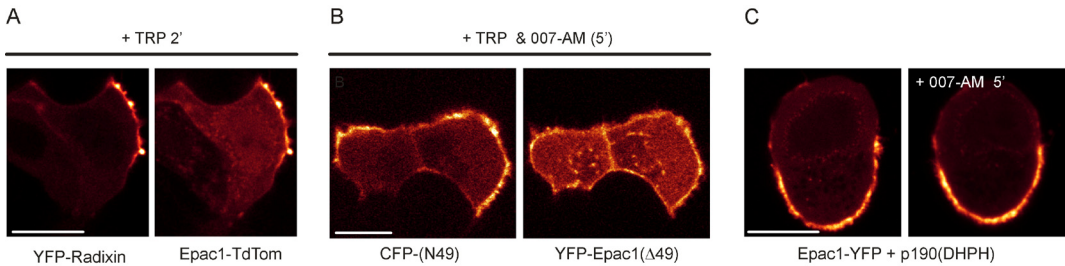


Fig 4. Activated ERM proteins recruit Epac1 to polarized areas of the PM

A) Example of TRP-stimulated HEK293 cell showing polarized localisation of YFP-Radixin and Epac1-TdTom.

B) HEK293 cells were transfected with CFP-N49 and Epac1(Δ 49)-YFP and stimulated with TRP (50 μ M) and 007-AM (1 μ M). Both constructs accumulate at the PM, but show different subcellular distributions.

C) Epac1-YFP shows strongly polarized localisation when p190-RhoGEF(DHPH) is co-transfected. This pattern remains unaltered upon addition of 007-AM (1 μ M).

Scalebars in all images: 10 μ m.

Activated ERM proteins recruit Epac1 to polarized areas of the PM

Interestingly, the distribution patterns induced by ERM-activating differ notably from those described for cAMP-induced translocation of Epac1 [23]. Activated ERM proteins, which localize asymmetrically themselves (Fig 4A, left panel), recruit Epac1 to polarized areas of the PM, whereas cAMP-bound Epac1 distributes more uniformly along the PM (Fig 4A right panel; see also Fig 3C,D,F). This becomes even more apparent when Rho signaling is promoted by co-expression of a the separate DH-PH domain of p190-RhoGEF (p190(DHPH)) [41], resulting in heavily polarized distributions of Epac1-YFP (Fig 4C) and Epac1(R279L)-YFP (data not shown). Colocalization in polarized PM areas was also observed with endogenous radixin, as visualized by immunofluorescence stainings in HEK293 cells stimulated with TRP or transfected with p190(DHPH) (Supplemental Fig 2).

Thus, cAMP-induced translocation and ERM-mediated recruitment induce differential localization patterns of Epac1. To demonstrate this more directly, we transfected HEK293 cells with CFP-N49 and YFP-Epac1(Δ 49) and stimulated these cells with both TRP and 007-AM. PM targeting was observed for both constructs, however, leading to different localizations at the PM. YFP-Epac1(Δ 49), which can translocate upon binding of 007-AM but lacks the ERM interaction domain, distributed uniformly along the PM. On the other hand, CFP-N49, which can interact with ERM proteins but is insensitive to 007-AM, accumulated asymmetrically along the PM (Fig 4B). Thus, in contrast to cAMP-induced Epac1 translocation, activated ERM proteins have the potential to recruit Epac1 to polarized areas of the PM.

Epac1 recruitment by ERM proteins is independent of Epac1 activation state (Fig 3A, 3F), in marked contrast with its cAMP-dependent translocation. Thus, in the absence of cAMP-raising stimuli, ERM proteins recruit auto-inhibited, inactive Epac1 to polarized areas of the PM. We wondered whether this spatial confinement is affected when cAMP binding subsequently elicits the DEP-dependent affinity of Epac1 for the PM. Therefore, we added

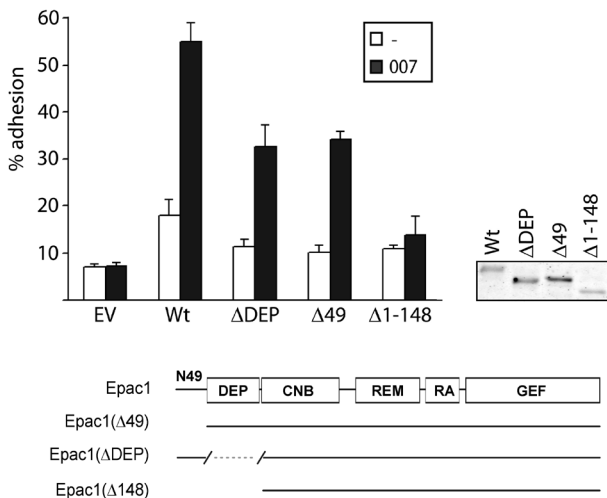
007-AM to cells expressing Epac1-YFP and p190-(DHPH). As shown in Fig 4C, 007-AM did not induce lateral redistribution of the asymmetrically localizing Epac1. Thus, although cAMP induces homogeneous PM translocation in the absence of ERM interactions (YFP-Epac1(Δ 49); Fig 4B and data not shown), active ERM proteins can restrict the localisation of cAMP-activated Epac1 to polarized areas along the PM. Taken together, our data imply that ERM proteins can target the cAMP-dependent GEF activity of Epac1 to specialized areas of the PM.

The Epac1-ERM interaction facilitates 007-induced cell adhesion

Since active ERM proteins are capable of restricting activated Epac1 to a subcompartment of the PM, we wondered whether this interaction facilitates Epac1-mediated Rap signalling. In Jurkat T-cells, activation of Epac1-Rap signaling induces adhesion by increasing the affinity of integrins for their extracellular matrix substrates [42]. To assess whether the interaction of Epac1 with ERM proteins contributes to its ability to induce adhesion, we transfected Jurkat T-cells with wildtype Epac1 or deletion mutants lacking the ERM interaction region (Δ 1-49), the DEP domain (Δ 50-148) or both domains (Δ 1-148). In all cases, a luciferase construct was cotransfected so that adhesion to fibronectin could be quantified by measurement of luciferase activity. As previously described [23], the 007-induced increase in adhesion was greatly impaired, albeit not completely abolished, upon deletion of the DEP domain (Fig 5). Interestingly, a similar intermediate effect was observed upon deletion of N49 (Fig 5). This implies that besides the cAMP-induced, DEP-dependent translocation, also the ERM interaction is involved in Rap signaling by Epac1. Indeed, in Jurkat T-cells expressing an Epac1 mutant deficient in both recruitment mechanisms (Epac1(Δ 1-148)), the 007-induced adhesion was entirely lost. These data strongly suggest that the DEP and the N49 domains cooperate to convey Epac1 GEF activity to PM-localized Rap, thereby inducing cell adhesion.

Fig 5. The Epac1-ERM interaction facilitates 007-induced cell adhesion

Jurkat T cells were transfected with luciferase together with a series of Epac1 constructs: wildtype Epac1, Epac1(Δ 50-148), Epac1(Δ 49) or Epac1(Δ 1-148). Transfected cells were allowed to adhere to fibronectin-coated surface for 45 minutes and thoroughly washed. Adhesion was subsequently detected as luciferase activity. Wildtype-Epac1, when activated by 007 (100 μ M, black bars) greatly enhanced the adhesion as compared to empty vector-transfected cells (EV). This effect was reduced when cells were transfected with Epac1(Δ 50-148), which does not translocate in response to cAMP, or with Epac1(Δ 1-49), which is deficient in binding to ERM proteins. Only in cells transfected with Epac1(Δ 1-148), the 007-induced increase in adhesion was entirely lost. Shown are data from a representative experiment performed in triplo ($n=3$). Total luciferase levels were comparable in all transfections. Inset: western blot labeled with the Epac1 antibody (5D3) showing expression levels of transfected wildtype- and mutant Epac1 used in the adhesion assay.



Discussion

In the current study we reveal the direct interaction between Epac1 and members of the Ezrin/Radixin/Moesin (ERM) family (Fig 1). We determined that the Epac1 N-terminus (residues 1-49, or N49) interact with the N-terminal half of ERM proteins, which also harbors the FERM domain (Fig 2). Importantly, the Epac1-ERM interaction is independent of the conformational state of Epac1, since it also applies to the cAMP binding mutant Epac1(R279L), which is locked in the inactive, auto-inhibited conformation (Fig 3). Instead, we show that the interaction is regulated on the level of the ERM proteins. To bind Epac1, ERM proteins require the open, activated conformation that is triggered by threonine phosphorylation in the ABD (Thr564 in Radixin) (Fig 2).

Recently, we reported that conformational opening upon cAMP binding also triggers the translocation of Epac1 to the PM. To date, the identity of the involved membrane anchor has remained unknown. Although the ERM proteins, which can directly bind the PM, appear interesting candidates, their role as anchors in the cAMP-dependent translocation is excluded by their ability to recruit the auto-inhibited Epac1(R279L). Furthermore, cAMP-dependent translocation is mediated by the DEP domain (residues 50-148) and does not require the N-terminal N49 (Fig 4B), which is the determinant for ERM-interaction.

Instead, we show that ERM proteins are anchors for an alternative mechanism of Epac1 recruitment. To localize to the PM, ERM proteins require the activated, open conformation [26], which is also a prerequisite for Epac1 binding (Fig 2). Taken together, recruitment of Epac1 to the PM directly follows from targeting of ERM proteins themselves. This was clearly illustrated by the simultaneous PM recruitment of YFP-Radixin and Epac1-TdTom (Fig 3C) when we activated ERM proteins via thrombin receptor stimulation [37]. Using FRET sensors for cAMP [43]

and for PKA-activity [44] we further established that cAMP levels are not elevated upon TRP stimulation (data not shown), supporting the notion that ERM-mediated Epac1 recruitment is independent of Epac1 activation state.

The recruitment to ERM proteins contributes to the ability of Epac1 to induce Rap-dependent [8] adhesion of Jurkat T-cells. The 007-induced adhesion of cells expressing Epac1(Δ 1-49) was significantly reduced as compared to cells expressing wildtype Epac1 (Fig 5). Interestingly, we previously reported that also the DEP-dependent translocation of cAMP-bound Epac1 enhances Jurkat cell adhesion [23]. Indeed, while deletions of DEP or N49 alone resulted in partial decreases in adhesion, their combined deletion (Epac1(Δ 1-148)) could suppress 007-induced adhesion completely. These data suggest that the two targeting mechanisms involving DEP and N49 cooperate to establish Rap activation. This implies that the interaction with ERM proteins facilitates the coupling between cAMP-activated Epac1 and its effector Rap.

In our current model, ERM proteins contribute to the Epac1-mediated activation of Rap by spatial confinement of the RapGEF. First, they position Epac1 in the vicinity of the PM (<10 nm), as demonstrated by FRET experiments using the PM-marker CFP-CAAX (Fig 3). Second, Epac1 accumulates at subdomains of the PM due to the asymmetrical distribution of ERM proteins themselves (Fig 4). Although the ERM proteins provide PM targeting of Epac1, Rap-mediated cell adhesion is still dependent on Epac1 activation by 007. The observation that Epac1 activation does not affect the ERM-induced spatial confinement (Fig 4) implies that ERM proteins can spatially regulate the GEF activity of Epac1.

The observation that spatial regulation of Epac1 activity by ERM proteins facilitates Rap-dependent adhesion may be explained in multiple ways. First, the combination of ERM- and cAMP-dependent targeting is expected

to increase the avidity for the PM, thereby increasing the frequency of interaction events between Epac1 and Rap molecules. Second, the asymmetrical localizations, to which Epac1 is targeted, may reflect signaling platforms specialized for the coupling between Epac1 and Rap. In support of this, Rap has been shown to display polarized PM distributions in several cell types [12,45-47]. Third, the inhomogeneous targeting may result in the activation of a specific pool of Rap and, thereby, of specific Rap effectors. For example, ERM proteins may confine Rap activity to areas where integrin-mediated adhesion can occur. Finally, ERM-mediated Epac1 targeting may serve a combination of these functions.

Signaling via RhoA has been reported to induce ERM activation [37]. Using the RhoA-inhibitor C3, we established that TRP induces Epac1 recruitment via RhoA activation (Fig 3G). Thereby, the Epac1-ERM interaction further connects the actions of RhoA and Rap. Both GTPases play central roles in actin remodeling and share involvement in processes such as cell morphology, regulation of focal adhesions [48] and migration [49]. Interestingly, crosstalk between Rap and RhoA at several levels has been reported. Two Rho-GAPs, ARAP3 [50] and RA-RhoGAP [51] have been identified as effectors of Rap1. Conversely, RhoA can activate the atypical PLC ζ [52], which acts as a GEF for Rap1 [53]. In addition, Schmidt and co-workers found that PLC ζ can be activated downstream of Rap2B activity [54]. Our current data add to this interconnectivity by showing that RhoA signaling confers spatial regulation to Epac1-mediated Rap activation. Besides RhoA signaling, ERM proteins can be activated by several other signaling pathways, including phosphorylation by PKC α [27], PKC θ [55] and NIK [56]. This implies that such pathways also cross-talk to Rap via spatial regulation of Epac1.

Epac1 targeting by ERM proteins may also be of major importance in epithelial cells, where ERM proteins strongly accumulate to the microvilli at the apical membrane. ERM proteins are involved in the dynamic regulation of actin assembly, which underlies the highly curved architecture of the microvilli [57]. Indeed, along with ERM proteins, Epac1 strongly accumulates in the microvilli of epithelial cell types (e.g. MDCK, HeLa, OVCAR; data not shown) and we find that this depends on ERM binding (data not shown). Epac1 has been reported to inhibit the apically localized sodium-proton (H⁺) exchanger 3 (NHE3) in the proximal tube of the kidney [58] and in the intestinal epithelia [59]. Since NHE3 functions in signaling complexes that also contain Ezrin [60,61], it is likely that ERM-mediated Epac1 targeting is of great importance for this function.

Spatial confinement of Epac1 by ERM proteins is another example of compartmentalized Epac1 signaling. The ensemble of Epac anchors, also containing MAP-LC [17], mAKAP [18] and the Rim2-piccolo complex [19], allows the involvement of Epac in several functions at different subcellular locations. In addition, our recruitment studies indicate that the anchoring function of ERM proteins are subject to dynamic regulation. Analogously, signaling of cAMP effector PKA is extensively compartmentalized by >50 AKAPs. It is interesting to note that Ezrin also serves as an AKAP [59,62,63]. Thereby, Ezrin may form a cross-

road for two cAMP-dependent signaling routes.

Our current data show that ERM-mediated PM recruitment facilitates Rap-dependent cell adhesion. This strongly suggests that the ERM proteins define a compartment at the PM where Epac1-Rap signaling is efficiently coupled to downstream adhesive events.

Materials and Methods

Reagents and antibodies

8-pCPT-2'-O-Me-cAMP (007) was obtained from Biolog Life Sciences (Bremen, Germany), human Thrombin from Sigma-Aldrich (T7572). Thrombin Receptor-activating Peptide (TRP, residues SFLRRN) was synthesized in our institute. The mouse monoclonal GFP antibody was obtained from Roche, the FlagM2 antibody from Sigma-Aldrich, the phosphoERM (Ezrin T567, Radixin T564 and Moesin T558) antibody from Cell Signaling Technology and the monoclonal anti-HA antibody from Covance (HA11). Ezrin recombinant protein was purified as described previously.

DNA constructs

Ezrin (EZR, homo sapiens, GI: 161702985), Radixin (RDX, homo sapiens, GI: 62244047), Moesin (MSN, homo sapiens, GI: 53729335), Epac1 (RapGEF3, homo sapiens, GI: 3978530) and Epac2 (RapGEF4, mus musculus, GI:9790086) were cloned C-terminal to either a Citrine, TomatoRed or Flag-His tag in a pCDNA3 vector or an HA tag in a PMT2-SM vector, using the Gateway system (Invitrogen). cDNA for Ezrin, Radixin and Moesin was obtained from RZPD (Berlin, Germany). Δ DEP-Epac1 (amino acid 50-148 deleted), Δ 49-Epac1 (N-terminal 49 amino acids deleted), N-terminal Radixin (amino acid 1-492), C-terminal Radixin (amino acid 492-584) and the indicated point mutants were generated by site-directed mutagenesis.

Yeast two-hybrid screening

Human full length Epac1 (RapGEF3, homo sapiens, GI: 3978530) cloned in a pB27 vector was screened with a randomly primed human placenta library by Hybrigenics S.A. (Paris, France), as previously described (ref).

Cell culture

HEK293 (Human Embryonal Kidney) cells were cultured in DMEM, supplemented with 10% serum and antibiotics.

Immunoprecipitation

HEK293 cells cultured in 5cm dishes were transfected with Eugene 6 Transfection Reagent (Roche Inc.) with the indicated constructs. For experiments in which Epac1 was immunoprecipitated, cells were lysed in a buffer containing 50mM Tris pH 7.5, 200mM NaCl, 20mM MgCl₂, 1% NP40, 10% glycerol and protease and phosphatase inhibitors. For the reverse experiments in which ERM proteins were immunoprecipitated, a buffer containing 1% Triton X100, 0.5% DOC, 50mM Tris pH 7.5, 150mM NaCl, 2mM EDTA pH 8.0 and protease and phosphatase inhibitors was used. Cell pellets were spun down by centrifugation and lysates were incubated with sepharose A beads (Pharmacia) cou-

pled to the appropriate antibody. After extensive washing with lysis buffer, bound proteins were eluted in Laemmli buffer and analyzed by SDS-Page.

Live cell experiments

Cells were seeded in 6-well plates on 25-mm glass cover slips and cultured in 3 ml medium. Constructs were transiently transfected using Fugene 6 Transfection Reagent (Roche Inc.). Experiments were performed in a culture chamber mounted on an inverted microscope in bicarbonate-buffered saline (containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 23 mM $NaHCO_3$, with 10 mM HEPES added), pH 7.2, kept under 5% CO_2 , at 37°C. Agonists and inhibitors were added from concentrated stocks.

Dynamic monitoring of TomatoRed/YFP FRET

Cells on coverslips were placed on an inverted NIKON microscope equipped with 63x lense (N.A. 1.30) and excited at 490 nm. Emission of YFP and TomatoRed was detected simultaneously by two photon multiplier tubes (PMT) through 555 +/- 20 nm and 610 +/- 25 nm bandpass filters, respectively. Data were digitized by Picolog acquisition software (Picotech) and FRET was expressed as the normalized ratio of TomatoRed to YFP signals. Changes are expressed as percent deviation from this initial value.

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Chapter 5

Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator

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Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator

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Running title: Epac as a FRET-based cAMP sensor

Abstract

Epac1 is a guanine nucleotide exchange factor for Rap1 that is activated by direct binding of cAMP. *In vitro* studies suggest that cAMP relieves the interaction between the regulatory and catalytic domains of Epac. Here we monitor Epac1 activation *in vivo* by using a CFP-Epac-YFP fusion construct. When expressed in mammalian cells, CFP-Epac-YFP shows significant fluorescence resonance energy transfer (FRET). FRET rapidly decreases in response to the cAMP-raising agents, whereas it fully recovers after addition of cAMP-lowering agonists. Thus, by undergoing a cAMP-induced conformational change, CFP-Epac-YFP serves as a highly sensitive cAMP indicator *in vivo*. When compared to a protein kinase A (PKA)-based sensor, Epac-based cAMP probes display an extended dynamic range and a better signal-to-noise ratio; furthermore, as a single polypeptide, CFP-Epac-YFP does not suffer from the technical problems encountered with multi-subunit PKA-based sensors. These properties make Epac-based FRET probes the preferred indicators for monitoring cAMP levels *in vivo*.

Introduction

cAMP is a common second messenger that activates protein kinase A (PKA), cyclic nucleotide-regulated ion channels and Epac (for exchange proteins directly activated by cAMP). Epacs are guanine nucleotide exchange factors (GEFs) for Rap1 and Rap2 [1]. Rap GTPases cycle between an inactive GDP-bound and an active GTP-bound state, with GEFs mediating the exchange of GDP for GTP. Rap proteins are involved in a number of biological processes, most notably the regulation of cell adhesion through integrins and cadherins [2]. The GEF Epac1 consists of a C-terminal catalytic domain characteristic for exchange factors for Ras family GTPases and an N-terminal regulatory domain. The latter domain contains a cAMP-binding site similar to those of protein kinase A (PKA) and, in addition, a DEP domain that mediates membrane attachment [3,4].

In vitro studies have shown that cAMP is absolutely required for the activation of Epac [5]. It has been hypothesized that the regulatory domain of Epac functions as an auto-inhibitory domain, which is relieved from inhibition by cAMP, but direct proof for this notion is lacking. In this model, Epac is folded in an inactive conformation at low cAMP levels, thereby preventing Rap binding due to steric hindrance. cAMP binding unfolds the protein, allowing Rap to bind. This is somewhat analogous to the mechanism of PKA regulation by cAMP; in its inactive conformation, two regulatory subunits are bound to two catalytic subunits. Upon binding of cAMP this complex falls apart, resulting in the release of active enzymes.

In the present study, we set out to measure Epac activation *in vivo* by sandwiching Epac between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) and then measure fluorescence resonance energy transfer (FRET) between the two fluorescent moieties. FRET, the radiationless transfer of energy from a fluorescent donor to a suitable acceptor fluorophore, depends on fluorophore orientation and on donor-acceptor distance at a

molecular scale. We show that in mammalian cells, CFP-Epac-YFP displays significant energy transfer which rapidly diminishes following a rise in intracellular cAMP and increases again in response to a fall in cAMP. This indicates that cAMP causes a significant conformational change *in vivo* and supports the unfolding model for Epac activation. Taking advantage of this property, we characterized CFP-Epac-YFP as a FRET sensor for cAMP and generated cytosolic, catalytically dead mutants. We show that the Epac-based cAMP indicators outperform the previously reported PKA-based cAMP sensor [6-8] in several aspects.

Results and Discussion

cAMP induces a conformational change in Epac

To monitor cAMP-induced conformational changes in Epac, we generated a construct in which Epac1 was fused N-terminally to CFP and C-terminally to YFP, as shown in Fig. 1A. Using a GST-RaIGDS assay (see supplementary information), it was checked that this construct was able to activate Rap1. CFP-Epac-YFP was transiently expressed in human A431 cells, where it localized to membranes and the cytosol (see further). Fluorescence spectra of these cells revealed significant FRET (Fig. 1B, red line), indicating that CFP and YFP are in close proximity (~ 3 -4 nm). Stimulation with forskolin, a direct activator of adenylyl cyclase, significantly decreased FRET (green line). Similar responses were observed in other cell types, including HEK293, N1E-115 and MCF-7 cells. Thus, cAMP induces a significant conformational change in Epac, in support of the unfolding model (Fig. 1A).

We next analyzed the kinetics of cAMP-induced FRET changes by ratiometric recording of CFP and YFP emission using a dual-photometer setup (see Methods). Within seconds after addition of forskolin, FRET started to decrease, usually dropping to a minimum level within 2-3 minutes (Fig. 1C). In the presence of the phosphodiesterase inhibitor IBMX (100 μ M), forskolin evoked an average decrease

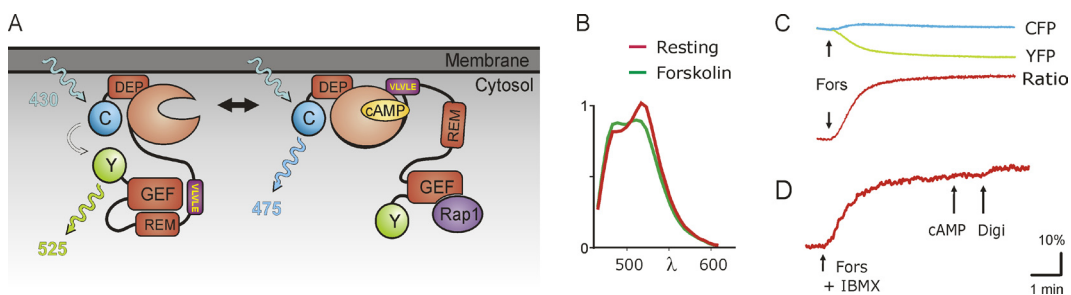


Figure 1. A cAMP-induced conformational change in Epac detected by FRET

A) Model for the conformational change upon binding of cAMP to the regulatory domain of Epac (adapted from Bos, 2003). Upon cAMP binding, the VLVLE sequence can interact with the regulatory domain, releasing the inhibition of the GEF domain by the REM domain. FRET between the CFP- and YFP-tags allows detection of this conformational change. B) Emission spectra of CFP-Epac-YFP, excited at 430 nm. Red line, resting level; green line, 3 min after forskolin treatment (25 μ M). C) Time course of cAMP-induced CFP-Epac-YFP activation, monitored in A431 cells by FRET. Increases in the ratio CFP/YFP reflect unfolding of Epac. Arrow, addition of forskolin (Fors, 25 μ M). D) Cells were treated with forskolin (25 μ M) and IBMX (100 μ M) and subsequently permeabilized using digitonin (Digi, 10 μ g/ml) in the presence of 2 mM cAMP.

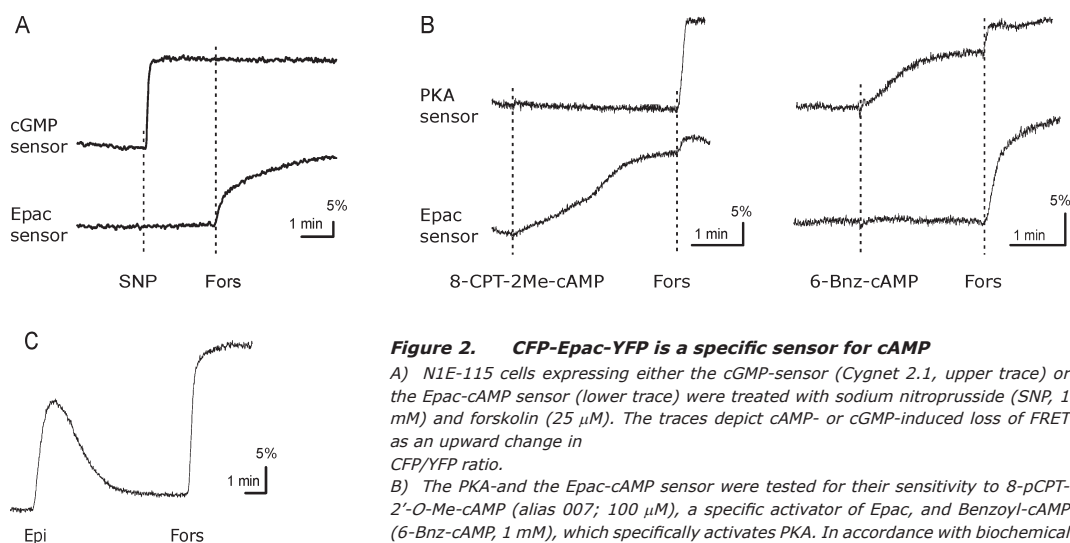


Figure 2. CFP-Epac-YFP is a specific sensor for cAMP

A) N1E-115 cells expressing either the cGMP-sensor (Cygnets 2.1, upper trace) or the Epac-cAMP sensor (lower trace) were treated with sodium nitroprusside (SNP, 1 mM) and forskolin (25 μ M). The traces depict cAMP- or cGMP-induced loss of FRET as an upward change in CFP/YFP ratio.

B) The PKA and the Epac-cAMP sensor were tested for their sensitivity to 8-pCPT-2'-O-Me-cAMP (alias 007; 100 μ M), a specific activator of Epac, and Benzoyl-cAMP (6-Bnz-cAMP, 1 mM), which specifically activates PKA. In accordance with biochemical data (not shown), the slow and incomplete increases in CFP/YFP ratio in the upper

right and lower left panels are caused by limited diffusion of these compounds over the plasma membrane.

C) Typical example of an agonist-induced cAMP response recorded with CFP-Epac-YFP in a Rat-1 fibroblast. Epi, epinephrine (250 nM); forskolin (25 μ M) was added to calibrate the response.

of 30 $\pm$ 3 % in CFP/YFP emission ratio. This reflects near-complete saturation of cAMP binding to Epac as deduced from experiments where cells were subsequently permeabilized with digitonin (10 μ g/ml) in the presence of 2 mM extracellular cAMP (Fig. 1D). This caused at most a moderate (on average 3%) further drop in FRET.

Epac activation is independent of subcellular localization

CFP-Epac-YFP localized to the cytosol and to membranes, in particular to the nuclear envelope and to perinuclear compartments. We confirmed proper targeting of CFP-Epac-YFP by comparing its distribution with that of immunolabeled endogenous Epac in OVCAR3 cells. Identical localization patterns were observed (Zhao *et al.*, in preparation), in agreement with a previous report [9]. Thus, CFP-Epac-YFP can be used as a FRET probe to image Epac activation. As activation of its downstream target Rap1 is membrane-delimited [10,11], we set out to visualize Epac activation throughout the cell by two different imaging FRET techniques (see supplementary information). The results reveal that, at least in these cells, agonists induce homogeneous FRET changes throughout the cell. Thus, Epac activation is not confined to membranes, indicating that cAMP binding is the main determinant of Epac activation.

CFP-Epac-YFP as a novel fluorescent cAMP indicator

Having shown that FRET changes in CFP-Epac-YFP reflect cAMP binding, we next investigated how well the Epac construct performs as an *in vivo* sensor for cAMP. We first tested whether CFP-Epac-YFP is insensitive to cGMP, given

that cGMP binds to Epac with an affinity similar to that of cAMP, but fails to activate the enzyme [12]. In N1E-115 neuroblastoma cells, which express soluble guanylyl cyclase, a massive increase in intracellular cGMP levels ensued upon stimulation with the NO-donor sodium nitroprusside, as recorded by the cGMP-sensitive FRET sensor Cygnets-1 [13]. In contrast, the Epac FRET signal was not affected by nitroprusside treatment (Fig. 2A). We conclude that cGMP does not detectably affect the conformation of Epac.

We next tested two cAMP analogues that are specific for either Epac or PKA. As shown in Fig. 2B, the Epac-specific compound 8-p-CPT-2'-O-Me-cAMP [14] reduced FRET in the Epac-, but not in the PKA-cAMP sensor. Conversely, the PKA-specific compound 6-Bnz-cAMP [15] specifically diminished the FRET signal only in cells expressing the PKA-based sensor (Fig. 2B). Thus, the Epac-cAMP sensor preserves its specificity for cAMP analogues.

We further tested the Epac-FRET construct in various cell types, including Rat-1 and NIH3T3 fibroblasts, mouse GE11 epithelial cells, mouse N1E-115 neuroblastoma and human MCF7 breast carcinoma cells. Addition of various cAMP-raising agents and receptor agonists, including forskolin, epinephrine, prostaglandin E1 and neurokinin A caused robust FRET decreases in all cases. In general, forskolin induced a sustained decrease in FRET, whereas in most cell types receptor agonists such as PGE1 and epinephrine elicited transient signals lasting for 10-15 minutes (Fig. 2C and data not shown). The transient nature of the epinephrine-induced signal is due to homologous receptor desensitization, since a second but distinct stimulus is still capable of decreasing FRET. We conclude that CFP-Epac-YFP is a specific, highly sensitive and reliable indicator of both transient and sustained changes in intracellular cAMP levels.

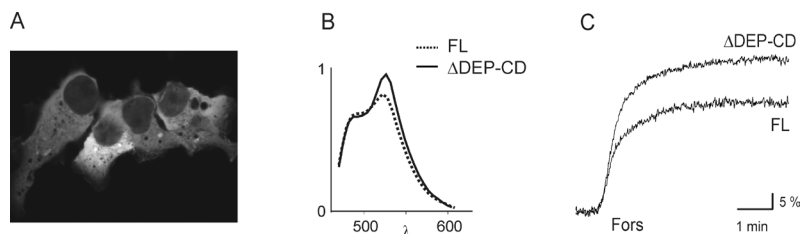


Figure 3. CFP-Epac(DDEP-CD)-YFP is cytosolic, catalytically inactive, and has improved signal-to-noise ratio

A) Confocal micrograph of HEK293 cells expressing CFP-Epac(DDEP-CD)-YFP shows absence of membrane labeling.

B) Emission spectra of CFP-Epac-YFP (dashed line) and CFP-Epac(DDEP-CD)-YFP (solid line), excited at 430 nm.

C) Comparison of forskolin-induced change in CFP/YFP ratio in cells expressing CFP-Epac-YFP (FL) and CFP-Epac(DDEP-CD)-YFP. Representative traces from seven experiments each.

A catalytically inactive, cytosolic mutant displays increased FRET responses

To generate a cytosolic variant, we next deleted the DEP domain (aa 1-148), which is the main determinant of membrane DEP-YFP, located almost exclusively to the cytosol (Fig. 3A) in HEK293 localization [16,17]. Indeed, this chimera, CFP-Epac(and other cells. This mutation also diminished Epacs ability to activate Rap1 significantly (see supplementary information). We further introduced mutations (T781A, F782A) to render the indicator catalytically dead. These residues were predicted to affect Rap1-binding based on the crystal structure of SOS, a closely related GEF [18]. The resulting construct, CFP-Epac(DDEP-CD)-YFP displayed no detectable Rap1 activation (see supplementary information).

Spectral analysis revealed that the basal FRET level in the cytosolic variants was significantly above that of the full-length chimera (Fig. 3B). FRET in CFP-Epac(DDEP-CD)-YFP expressing cells reliably decreased upon stimulation with cAMP-raising agonists. Importantly, maximal changes in CFP/YFP ratio outperformed that of the full-length chimera by approximately 50% in magnitude ($\sim 45\%$ versus $\sim 30\%$), significantly increasing the signal-to-noise ratio (Fig 3C). Because selectivity remained unaltered as compared to CFP-Epac-YFP (not shown), the cytosolic localization, catalytic inactivity and improved signal-to-noise ratio make CFP-Epac(DDEP-CD)-YFP the indicator of choice for monitoring cytosolic cAMP levels.

Epac-based cAMP sensors display an extended dynamic range

Previously described PKA-based cAMP sensors are tetramers consisting of two catalytic and two regulatory domains. These probes contain four cAMP binding sites and have submicromolar (~ 300 nM) affinity *in vivo* [19]. cAMP binding in PKA displays cooperativity with an apparent Hill coefficient of 1.6 [20]. As a consequence, this probe has a steep dose-response relationship that rapidly reaches saturation. In contrast, *in vitro* studies have shown that the affinity of the single cAMP binding site in Epac is at least

an order of magnitude lower [21]. We determined the affinities of the different fluorescent Epac constructs for cAMP *in vitro* by fluorescence ratiometry (see supplementary information). The results revealed affinities of ~ 50 , ~ 35 and ~ 14 mM for CFP-Epac-YFP, CFP-Epac(DDEP-CD)-YFP, respectively. Thus, the Epac-cAMP sensors should display right-shifted and extended dynamic range of DEP-YFP and CFP-Epac(ranges.

To test this notion *in vivo*, cells expressing either CFP-Epac-YFP or the PKA-cAMP sensor were cocultured on coverslips and neighboring cells expressing comparable amounts of Epac and PKA, respectively, were analyzed for FRET changes. Dosed photorelease of NPE-cAMP, a membrane-permeable caged cAMP analogue, was used to evoke identical incremental changes in intracellular cAMP in the two neighbor cells (Fig. 4A). Sequential increases in cAMP caused a rapid decrease in FRET and subsequent apparent saturation of the response in the PKA sensor, whereas the Epac sensor showed a much larger dynamic range. In line with these observations, the responses to forskolin-induced robust cAMP increases (Fig 4B) were rapid and saturating for the PKA-based sensor, whereas FRET in the Epac-based sensor changed more gradually and often did not saturate completely (Fig. 1D).

The shifted and extended dynamic range of Epac for cAMP has important consequences for measuring physiological cAMP levels. As shown in Fig. 4C, in GE11 cells isoproterenol triggers a rapid and rather sustained FRET change ($\sim 30\%$). In isoproterenol-pretreated cells, addition of lysophosphatidic acid (LPA) resulted in a rapid recovery of the FRET signal, as one would expect for a Gi-coupled receptor agonist that lowers cAMP levels [22]. Of note, the PKA-based sensor failed to record this rapid effect of LPA, apparently due to saturation of the probe, but rather reported a substantial lag period (up to several minutes; Fig. 4C, middle trace). That it fails to record the true kinetics of the LPA-induced cAMP response becomes evident when the Epac-based sensor is used. As is shown in Fig. 4C, CFP-Epac-YFP detects the initial fall in cAMP levels within seconds after LPA addition.

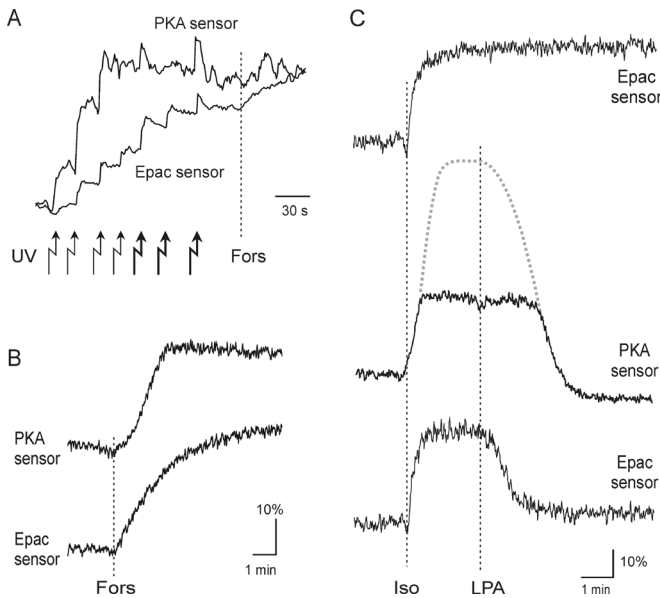


Figure 4. The Epac-cAMP sensor exhibits an extended dynamic range as compared to the PKA-cAMP sensor
A) Flash photolysis (thin arrows, 1/15 sec; thick arrows, 1/4 sec) of NPE-caged cAMP in neighboring A431 cells expressing either PKA-cAMP sensor or Epac-cAMP sensor (as recognized by partial decoration of membranes). Forskolin (50 μ M) was added to further increase cAMP levels. Traces are normalized for comparison. B) Typical responses to forskolin (50 μ M), recorded with the PKA- and the Epac-cAMP sensor in A431 cells. 10-90% response rise times differed significantly (34 ± 5 s for PKA, $n=9$; 248 ± 38 s for Epac, $n=9$; $p<0.005$). Note the sharp transition from the dynamic response range to the saturated plateau phase in the PKA sensor trace. C) Upper trace, sustained cAMP elevation evoked by isoproterenol (10 μ M) in a GE11 epithelial cell. Middle and lower traces, registration of cAMP decreases induced by subsequent addition of LPA (5 μ M), visualized with the PKA probe and the Epac probe, respectively. Note that Epac reveals the immediate LPA effect, whereas it is obscured by saturation of the PKA-cAMP sensor.

Conclusions

Our results support a model in which cAMP binding to the regulatory domain of Epac releases an inhibitory conformation that prevents binding to Rap1 [23]. Importantly, the FRET signal not only reflects binding of cAMP but also activation of Epac because cGMP, which binds with a similar affinity but fails to activate Epac [24] is without effect. We employed this property to show that the local, membrane-delimited activation of Rap-1 [25,26] is not due to local activation of Epac. The here observed uniform Epac activation contrasts with the findings of Zaccolo and Pozzan (2000) who detected subcellular cAMP gradients in cardiac myocytes with the PKA-based cAMP sensor. This is likely explained by cell-type specific differences in activity and intracellular distribution of the phosphodiesterases that shape such cAMP gradients, because we failed to detect gradients of cAMP using the PKA probe in our cells.

Of note, our *in vivo* data based on photolysis of NPE-caged cAMP (Fig. 4A) strongly support the notion that cAMP differentially regulates its effectors, i.e. low cAMP concentrations signal mainly via PKA, while at higher doses cAMP exerts additional effects via Epac activation [27].

This study further shows that Epac-based FRET constructs are ideally suited as cAMP sensors in that they display improved characteristics compared to the commonly used PKA-based sensors. First, the moderate affinities of our Epac constructs (14 to 50 μ M) result in a right-shifted dose-response relationship that matches physiological cAMP levels (Fig. 4). During review of this manuscript, a K_d of 2.3 μ M was reported for a FRET sensor based on

Epacs isolated cAMP-binding domain [28]. Thus, Epac-based sensors provide a wide range of affinities which allows matching the sensors to the anticipated cAMP levels. Second, the PKA regulatory subunits contain two cAMP binding sites each that exhibit cooperative binding (Hill coefficient of 1.6) resulting in a very steep response. In contrast, the single cAMP binding domain of Epac1 results in an extended dynamic range. Third, Epac needs only a single cAMP molecule for a $\sim 30\%$ FRET change while four molecules of cAMP are needed to cause a comparable change in two donor-acceptor pairs in PKA. Together with the lower affinity of Epac, this results in reduced buffering of cytosolic cAMP. This is not trivial since expression levels of cytosolic FRET probes commonly are in the micromolar range (0.1-5 μ M [29]), i.e. at cAMP levels found in the cytosol upon receptor stimulation. Fourth, the Epac-cAMP sensor is a single polypeptide, eliminating expression- and stoichiometry-related problems encountered with the PKA-based versions. For instance, imbalanced expression levels of regulatory and catalytic subunits of PKA hampers quantitative analyses of FRET changes. Furthermore, a single cDNA construct allows easy generation of stably transfected cell lines, which is often a problem with the PKA-based sensor (unpublished observations). Fifth, monomeric Epac sensors show faster activation kinetics than the slowly dissociating PKA-based sensors [30]. In addition, the cytosolic CFP-Epac(δ DEP-CD)-YFP construct exhibits even larger cAMP-induced FRET changes, resulting in a superior signal-to-noise ratio. Together, these properties make Epac-based FRET probes the preferred fluorescent indicators for monitoring elevated cAMP levels in living cells.

Methods

Materials

Isoproterenol, 1-oleoyl-LPA, prostaglandin E1, epinephrine and sodium nitroprusside were from Sigma Chemical Co. (St. Louis, MO); IBMX, forskolin, and neurokinin A were from Calbiochem-Novabiochem Corp. (La Jolla, CA); 1-(2-nitrophenyl)ethyl adenosine-3',5'-cyclic monophosphate (NPE-caged cAMP) was from Molecular Probes Inc. (Eugene, OR); 8-p-CPT-2'-O-Me-cAMP and N⁶-Benzoyladenosine-3',5'-cyclic monophosphate were kindly provided by Hans Gottfried Genieser (Biolog Life Sciences (Bremen, Germany)).

DNA Constructs

eCFP ("non-sticky", A206K [1]), a multiple cloning site with BglII/EcoRV/NheI/SacI restriction sites, and eYFP (A206K) were cloned in-frame, and inserted in pCDNA3 (Invitrogen) using HindIII/XbaI. Full-length Epac1 was generated by PCR using human Epac1 (#AF103905) and cloned in-frame into the restriction sites EcoRV/NheI of the MCS using the primers 5'-TTGATATCTGATGGTGTGAGAAG-GATGCACC-3' and 5'-GGGGCTAGCTGGCTCCAGCTCTCGGG-3'. The resultant construct contained the linker SGLRSRYL, separating eCFP from Epac1, and ASEL, separating Epac1 from eYFP.

CFP-Epac1(δ DEP)-YFP was generated using the upstream primer 5'-TTGATATCAGCCCGTGGGAATCATG-3' instead, deleting aa 1-148. The latter construct was rendered catalytically dead (CFP-Epac1(δ DEP-CD)-YFP) by pointmutating T781A and F782A in the GEF domain. The chosen residues were predicted to affect Rap1-binding based on the crystal structure of the Son of Sevenless (SOS) protein, a GEF for H-Ras and a close family member of Epac[2].

The PKA-based cAMP sensor, consisting of two expression vectors encoding the YFP-tagged catalytic and CFP-tagged regulatory domain of PKA, was as published [3,4]. The FRET-sensor for cGMP, termed Cygnet-2.1 for cyclic GMP indicator using energy transfer, consists of a truncated form of the cGMP-dependent protein kinase sandwiched between CFP and YFP and was used as published [5].

Cell Culture, transfections and live cell experiments

Cells were seeded on glass coverslips, cultured, and transfected with constructs as described [31]. Experiments were performed in a culture chamber mounted on an inverted microscope in bicarbonate-buffered saline (containing, in mM, 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 glucose, 23 NaHCO₃, with 10 mM HEPES added), pH 7.2, kept under 5% CO₂, at 37°C. Agonists and inhibitors were added from concentrated stocks. Expression levels of fluorescent probes were estimated as described [32].

Dynamic FRET monitoring

Cells on coverslips were placed on an inverted NIKON Microscope and excited at 425 nm. Emission of CFP and YFP

was detected simultaneously through 470 $\pm$ 20 and 530 $\pm$ 25 nm bandpass filters. Data were digitized and FRET was expressed as ratio of CFP to YFP signals, the value of which was set to 1.0 at the onset of the experiments. Changes are expressed as percent deviation from this initial value of 1.0.

Loading And Flash Photolysis of NPE-caged cAMP

Cells were loaded by incubation with 100 μ M NPE-caged cAMP for 15 min. Uncaging was with brief pulses of UV light (340-410 nm) from a 100 W HBO lamp using a shutter. For comparison, traces were normalized with respect to baseline and final FRET values.

Confocal FRET imaging

We recently described FRET imaging by sensitized emission on a Leica TCS-SP2 confocal microscope (Mannheim, Germany) in detail [6]. Briefly, reference cells expressing only CFP or YFP were seeded together with the CFP-Epac-YFP expressing cells and simultaneously imaged in the same field of view. Three images were collected: the donor image (CFP, excited at 430 nm and detected from 460-510 nm), sensitized emission image (YFP, excited at 430nm and detected from 528-603 nm) and the acceptor image (YFP, excited at 514 nm and detected from 528-603 nm). All images were shading-corrected. Donor leakthrough in the sensitized emission channel and false acceptor excitation that occurred at 430nm were corrected using correction factors derived from the reference cells as described [7]. FRET efficiency was expressed by dividing the sensitized emission image with the donor image.

Fluorescence spectra were recorded with the λ -scan functionality of the Leica confocal microscope from living cells, excited at 430 nm. Spectra are the mean of 10 scans from different cells.

Fluorescence Lifetime Imaging.

FLIM experiments were performed on a Leica inverted DM-IRE2 microscope equipped with Lambert Instruments (Leutingewolde, the Netherlands) frequency domain lifetime attachment, controlled by the vendors EZflim software. CFP was excited with $\sim$ 4 mW of 430 nm light from a LED modulated at 40 MHz and emission was collected at 450-490 nm using an intensified CCD camera. Calculated CFP lifetimes were referenced to a 1 μ M solution of Rhodamine-G6 in medium, set at 4.11 ns lifetime. CFP-Epac-YFP expressing cells were cocultured with reference cells that expressed CFP only.

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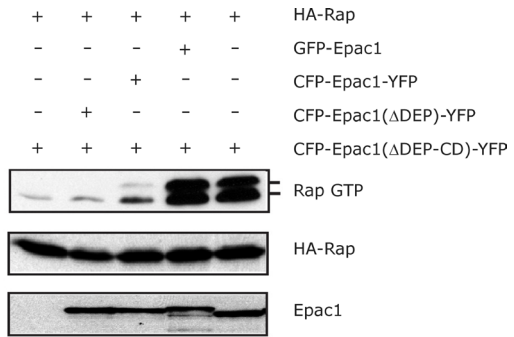
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Supplemental Figures

Suppl Fig 1. *In vivo* guanine nucleotide exchange (GEF) activity of Epac-based FRET probes

Different fluorescently tagged Epac constructs were tested for their guanine exchange activity towards Rap1. Indicated constructs were transfected in NIH3T3 cells, which do not express detectable amounts of endogenous Epac1. After 48 hours, cells were stimulated with 100 μ M 8-p-CPT-2'-O-Me-cAMP for 15 min. Cells were lysed and assayed for GTP-bound Rap1 using GST-RalGDS as an activation-specific probe [1]. Upper panel, pull-down samples were probed with an antibody against Rap1 (Santa Cruz, SC-65). The upper band is HA-tagged Rap1, the lower band is endogenous Rap1. Middle panel, expression of HA-Rap1 as detected with an anti-HA monoclonal antibody (12CA5). Lower panel, expression of Epac1 constructs was verified using an Epac1 specific mouse monoclonal antibody (5D3). Note that, in line with the reported dependence of Epac on correct subcellular localization, loss of the DEP domain significantly interferes with Rap activation. Residual activity is completely lost in CFP-Epac1(Δ DEP-CD)-YFP, the mutant that lacks Rap1 binding.

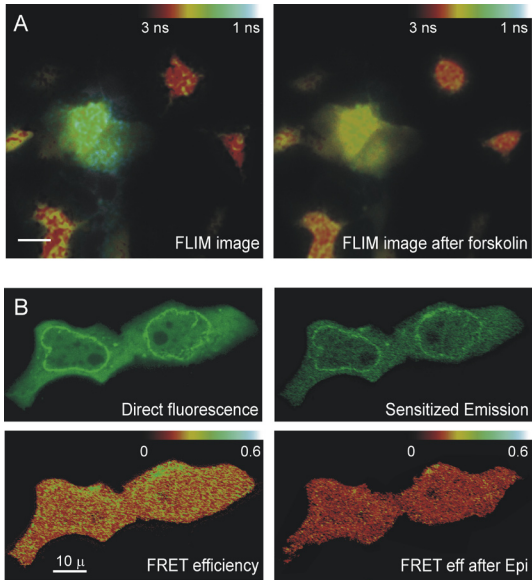


Suppl fig 2. *Epac activation is independent of subcellular localization*

Activation of the downstream target of Epac1, Rap1, reportedly is membrane-delimited, but conflicting views exist on whether this predominantly occurs at endomembranes or at the plasma membrane [2,3]. We therefore set out to visualize Epac activation throughout the cell by two different FRET techniques. Initially, we confirmed that tagging of Epac with GFPs does not interfere with its proper localization by comparing the cellular distribution of CFP-Epac-YFP to that of immunolabeled endogenous Epac in OVCAR3 cells. In good agreement with published data for untagged Epac [4], CFP-Epac-YFP localized in the cytosol as well as to membranes (the nuclear envelope, perinuclear membranes, and to a lesser extent the plasma membrane).

Widefield Fluorescence Lifetime IMaging (FLIM; see Methods) reports FRET quantitatively as a decrease in the excited-state lifetime of the fluorescent donor molecule. For reference, A431 cells expressing CFP-Epac-YFP were imaged along with HEK293 control cells expressing cytosolic CFP. In resting cells, our FLIM analysis failed to reveal spatial differences in FRET efficiency (Fig. 2A, left panel). Furthermore, activation of Epac with cAMP-raising agonists caused a similar FRET decrease throughout the cells (Fig. 2A, right panel).

To better resolve subcellular details, we employed a recently developed, highly corrected confocal laser scanning FRET microscopy approach [5] that allows discrimination of CFP-Epac-YFP activation in the cytosol and at membranes. In the cell types studied, Epac activation state as deduced from FRET did not depend on membrane localization (lower left panel). cAMP-raising agonists such as epinephrine (250 nM) caused similar FRET changes at membranes and in the cytosol (lower right panel). The homogeneous FRET values determined for CFP-Epac-YFP throughout the cells are likely due to the rapid diffusion of cAMP in the cytosol. Taken together, our data demonstrate that Epac1 activation is not localized to membranes and further indicate that binding to cAMP is the main determinant of Epac activation.



Suppl Figure 2. *Spatial distribution of Epac activity as detected by fluorescence resonance.*

A) (left panel) FRET in CFP-Epac-YFP expressing A431 cells as detected by FLIM. The homogeneous lifetime of $\sim$ 1.7 ns throughout the cell indicates $\sim$ 30% FRET efficiency. For reference, CFP in control cells displays a lifetime of $\sim$ 2.4 ns. (right panel) Stimulation with forskolin (1 μ M) decreases FRET, causing the lifetime to increase to $\sim$ 2.2 ns.

B) Confocal images of an A431 cell expressing CFP-Epac-YFP. Upper left, YFP fluorescence; upper right, sensitized emission, i.e. calculated YFP emission resulting from FRET; lower left, calculated FRET efficiency in resting cell; lower right, FRET efficiency after epinephrine treatment (250 nM).

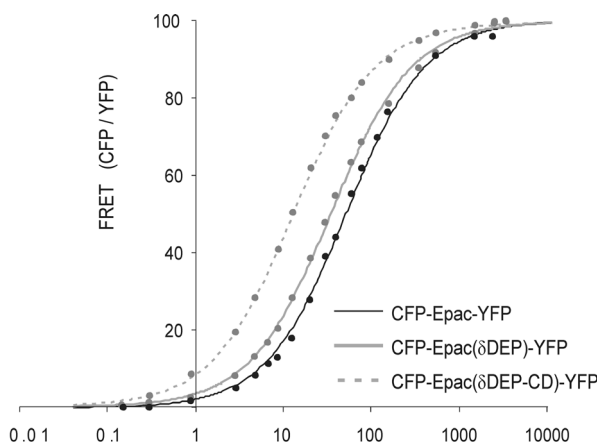
Suppl fig 3. Fluorescently tagged Epac constructs bind cAMP with micromolar affinities

To determine dissociation constants (K_d) towards cAMP, five 15-cm petridishes of HEK293 cells were transfected for each of the constructs. Cells were harvested 24h post transfection, washed in PBS and homogenized in hypotonic medium (PBS:H₂O, 1:2) with a Downs piston. The homogenate was cleared by high-speed centrifugation for 10 minutes and subsequently ionic concentrations were corrected towards intracellular levels (in mM: 140 KCl, 5 NaCl, 1 MgCl₂ and 10 HEPES for pH 7.2).

FRET changes caused by consecutive additions of cAMP were recorded in the stirred cuvet of a PTI Quantmaster dual channel spectrofluorimeter (Lawrenceville, NJ). FRET was expressed as the ratio of the YFP channel (530 +/- 10 nm) and the CFP channel (490 +/- 10 nm), when excited at 420 +/- 3 nm. For analysis, we used the Hill-function:

$$\text{FRET}([\text{cAMP}]) =$$

$$\text{FRET}_{\text{max}} * ([\text{cAMP}]^n / (K_d^n + [\text{cAMP}]^n))$$

**Suppl Figure 3.**

Dose-response relationship for cAMP-induced FRET changes for CFP-Epac-YFP (red), CFP-Epac(Δ DEP)-YFP (green), and CFP-Epac(Δ DEP-CD)-YFP (blue) *in vitro*. Apparent dissociation constants were $50 \pm 3 \mu\text{M}$, $35 \pm 3 \mu\text{M}$, and $14 \pm 2 \mu\text{M}$, respectively ($N=3$). Hill coefficients did not differ significantly from 1 (0.97, 0.95 and 0.94, respectively). Shown are data and fitted curve of a representative example.

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Chapter 6

Direct measurement of cyclic AMP diffusion and signaling through connexin43 gap junctional channels

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Direct measurement of cyclic AMP diffusion and signaling through connexin43 gap junctional channels

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Abstract

Gap junctions (GJ) are clusters of transmembrane channels that allow direct cell-to-cell transfer of ions and small molecules. The GJ-permeant signaling molecule cAMP is of particular interest because of its numerous cellular effects. However, to assess the biological relevance of GJ-mediated cAMP transfer, quantitative aspects must be determined. Here we employed cAMP indicators based on fluorescence resonance energy transfer (FRET) to study propagation of cAMP signals to neighbor cells through connexin43 (Cx43)-based gap junctions in Rat-1 cells quantitatively. Intracellular cAMP levels were selectively raised in single cells by either photorelease of caged cAMP or stimulation of G(s)-coupled receptors. cAMP elevations spread to adjacent cells within seconds in a Cx43-dependent manner. We determined that Rat-1 cells follow cAMP rises in surrounding monolayer cells to approx. 40% in amplitude. This degree of cAMP transfer sufficed to evoke a well-characterized response to cAMP in neighbour cells, i.e. the PKA-mediated phosphorylation of the ER transcription factor in A431 carcinoma cells. We conclude that contacting cells can cooperatively regulate cAMP-sensitive processes via gap junctional diffusion of cAMP.

Introduction

The second messenger cAMP signals a wide variety of cellular activities including changes in metabolism, gene transcription and secretion. Furthermore, in many cell types cAMP is a negative regulator of cell proliferation and migration. Basal cytosolic cAMP levels are in the low μM range [1], tightly controlled by the counteracting activities of adenylyl cyclases (AC) and phosphodiesterases (PDEs). ACs are activated by $G(\alpha)_s$ subunits and inhibited by $G(\alpha)_i$. Activated ACs can evoke transient cAMP elevations, while PDEs mediate subsequent degradation and are the main determinants of basal cAMP levels. Members of both AC and PDE enzyme families differ in their regulatory and pharmacological characteristics. The cell type-dependent expression of AC and PDE isoforms therefore determines the balance in cAMP turnover.

Gap junctions (GJs) are transmembrane channels that are formed by docking of two connexons, each contributed by one of the adjoining cells and each consisting of 6 connexin molecules. GJs allow the intercellular exchange of small molecules (<1-2 kD), including ions,

small peptides [2] and second messengers such as IP3 and cyclic nucleotides [3,4]. The gap junctional communication (GJC) of cAMP is of special interest because of cAMP's involvement in multiple signaling cascades. It remains unclear, however, whether intercellular cAMP diffusion has signaling functions between adjacent cells. This may critically depend on GJ permeability for cAMP, tissue geometry and PDE activity in receiving cells. Quantitative studies in a relevant cell system are required to assess the importance of cAMP-GJC.

Thus far, studies on cAMP-GJC employed indirect, non-quantitative readouts [5-9], while direct, quantitative readouts such as radiolabeled cAMP have been applied in artificial systems of gap junctions reconstituted in liposomes [10,11]. Here, we use recently developed fluorescent cAMP sensors as direct readouts for GJC of cAMP in Rat-1 cells. The used FRET-reporters are the fluorescent equivalents of PKA [12] and the exchange factor Epac1 [13] and therefore allow direct readout of cAMP. The tetrameric PKA sensor is most suitable for the detection of subtle cAMP changes due to its high affinity (~ 300 nM). However, the relative expression levels of its subunits in-

Figure 1 Flash photolysis of NPE-caged cAMP demonstrates GJ diffusion of cAMP

A) Rat-1 fibroblasts were seeded at low density and transfected with Epac-based FRET sensor. Left: after incubation with NPE-caged cAMP (5 min, 100 μM), cAMP was photo-released with UV light (0.5 sec from a 10-fold attenuated Hg arc lamp) while FRET was recorded ratiometrically by confocal microscopy (ratio changes in %). Right: 4 sec exposure induced saturation of the Epac sensor as judged from response kinetics. Essentially identical results were obtained with the PKA-based sensor.

B) Left: flash-photolysis of caged cAMP in one cell of a Rat-1 doublet (see inset; red, illuminated cell; blue, neighbor cell). 0.5 sec UV flashes caused non-saturating rises in cAMP that were followed by increases in the neighbor cells with 5 to 15 sec delay in $\sim 55\%$ of all experiments. If cAMP transfer was detected, 50 μM 2-APB was added to close GJs. Subsequent UV flashes caused cAMP increases that were restricted to the illuminated cell (9 out of 10). Essentially identical results were obtained with the PKA sensor (not shown, $n=6$). Right: typical FRAP experiment (see methods) showing that 50 μM of 2-APB effectively abolishes transfer of the widely used GJC tracer calcein ($n=9$).

C) Left: experimental setup similar to B), except that here the high-affinity PKA sensor was used. A 0.5 sec UV flash caused [cAMP] to increase in both the illuminated cell and its neighbor. The GPCR agonist TRP (12.5 μM), which blocks GJC in a physiological manner, abolished the neighbor response after a subsequent flash (2 sec; 4 out of 6 exp.). Right: calcein-FRAP experiments confirmed GJ closure by TRP (6 out of 8 exp.).

D) Left: similar uncaging experiment in a doublet of Rat-RNAi-Cx43 cells. No increases in neighbor cells were observed ($n=8$). Calibration was performed by UV-illumination of both cells. Right trace: calcein FRAP experiments confirming the lack gap junctional communication in Rat-RNAi-Cx43 cells ($n=12$).

Inset: Western blot analysis with anti-Cx43 antibody to show downregulation of Cx43 protein.

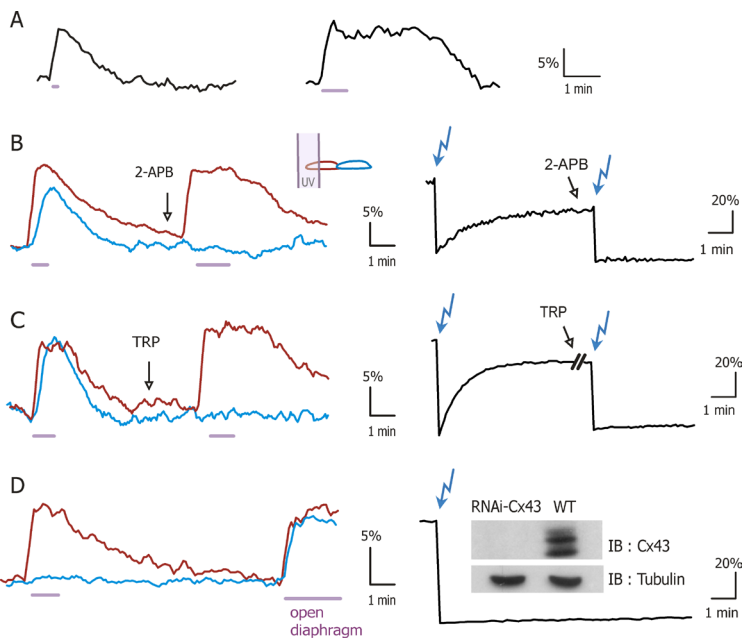
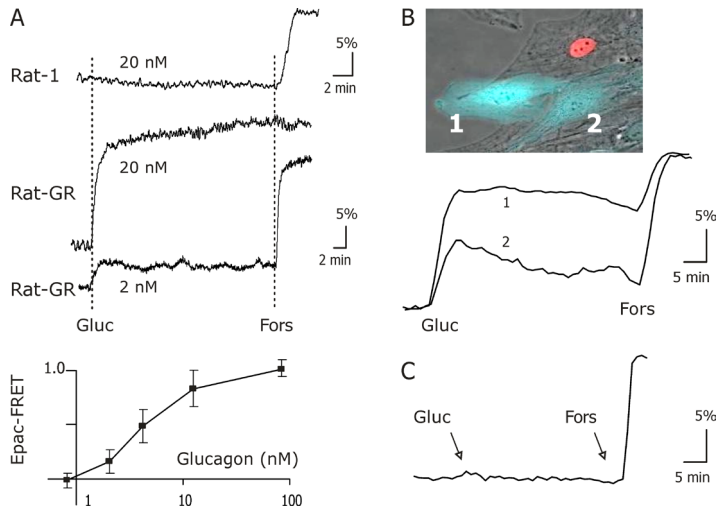


Figure 2 cAMP-GJC measurements using the Rat-GR Donor cell line

A) Upper trace: native Rat-1 cell is unresponsive to glucagon (20 nM), as monitored by the highly sensitive PKA sensor. Middle and lower trace: responses of Rat-GR cells to saturating (20 nM) and submaximal (2 nM) glucagon doses, respectively, measured with the Epac sensor. All experiments were calibrated by addition of forskolin (50 μ M) and IBMX (100 μ M). Below: dose-response relationship characteristic for the Rat-GR cell line determined with Epac-FRET; amplitudes as % of maximal FRET changes induced by forskolin and IBMX. Each data point stems from multiple cells ($n=10$ to 15) simultaneously recorded in multiple-position mode experiments (mean $\pm$ s.e.m.).

B) Two Acceptor cells adenovirally transduced with the Epac sensor bordered on a single Rat-GR Donor cell expressing the nuclearly localizing H2B-mRFP for recognition. Following addition of glucagon (20 nM) Epac-FRET detects cAMP increased in both neighbor cells (observed 17 out of 23). The difference in amplitude may be explained by differing degrees of GJC. Consistent with this, the left cell showed a faster rise time than the right one. Traces were normalized to maximal FRET changes induced by forskolin (50 μ M).

C) Epac-measurement in a Rat-RNAi-Cx43 cell adjacent to a Rat-GR Donor. No cAMP elevations were detected after administration of glucagon ($n=14$).



evitably vary from cell to cell and this hampers quantitative analyses. The single-polypeptide Epac sensor, that was previously developed in our lab, provides a more quantitative readout and thus enables to determine the exact relationship between source cells and receiving cells. Using this fluorescent toolbox we determined that Rat-1 cells follow cAMP rises in surrounding monolayer cells to approx. 40% in amplitude. Furthermore, we present evidence that cAMP diffusion can mediate structural changes in transcription factors in adjacent A431 carcinoma cells. Finally, we show that PDE activity is a major limiting factor in the degree of cAMP spreading in a monolayer.

Results and Discussion

Direct measurement of gap junctional diffusion of cAMP using flash photolysis

We first examined gap junctional diffusion of cAMP using the cAMP sensor based on Epac [13]. We used Rat-1 cells as a model system since these cells are well coupled by connexin43(Cx43)-based gap junctions. Fig. 1A shows the dosed release of cAMP by flash-photolysis of NPE-caged cAMP in a single, isolated Rat-1 cell as detected by the Epac-based sensor. It can be seen that this sensor accurately monitors the rapid increase in cAMP induced by a 0.5 s UV flash (details in Methods) and its subsequent clearance, which presumably represents breakdown by intracellular phosphodiesterases (PDEs; left trace). Prolonged UV exposure induced a marked plateau reflecting saturation of the Epac sensor (right trace).

Next, we examined the intercellular diffusion of cAMP in contacting Rat-1 cells. When caged cAMP was photo-released in one of two neighboring Rat-1 cells (using a directed UV beam; Fig. 1B), a substantial rise in cAMP levels was observed in the neighboring cell in 55% of all experiments. The ~ 10 s response delay reflects gap junctional diffusion. Occasional lack of cAMP transfer most likely reflects poor GJC between these subconfluent cells because a similar success rate was observed in FRAP experiments with the GJ-permeable tracer calcein (hundreds of experiments over the years); see right panels in Fig. 1B,C for example traces). To confirm that this effect was mediated by gap junctional diffusion rather than an alternative (paracrine) mechanism, we used the (non-specific) GJC blocker 2-APB [16]. As shown in Fig. 1B, 2-APB completely blocked the cAMP elevation in the neighboring cell (9 out of 10 experiments). Identical results were obtained with the PKA-based FRET sensor ($n=6$; data not shown). The PKA-FRET experiments confirm that 2-APB blocked GJC completely, as the high affinity of this sensor is expected to pick up even minor changes in [cAMP]. Similarly, when GJC was disrupted by addition of GPCR agonists, such as Thrombin Receptor activating Peptide (TRP, SFLRRN) or endothelin (ET) [17], the rise in cAMP was strictly confined to the UV-flashed cell (Fig. 1C). 2-APB- and agonist-induced inhibition of GJC was confirmed by calcein FRAP experiments (Fig. 1B,C, right panels).

Rat-1 cells express Cx43 as the only GJ protein [17] as confirmed by RNAi-mediated knockdown of Cx43. Cx43 knockdown cells were completely communication-deficient (Rat-RNAi-Cx43; Fig. 1D, right panel and data not shown).

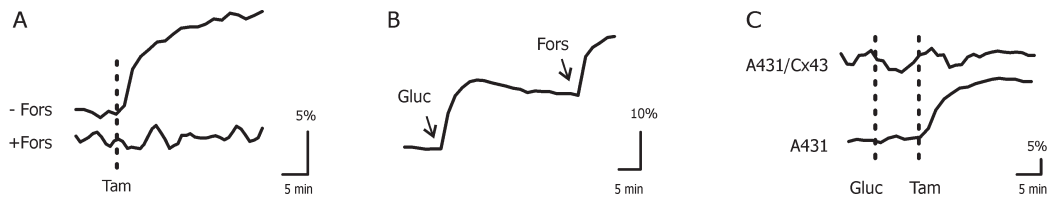


Figure 3 *cAMP-GJC mediates PKA-dependent structural changes in estrogen receptor (ER) in adjacent cells.*

A) YFP-ER-CFP FRET in A431/Cx43. Upper trace: the anti-estrogen tamoxifen forces the ER reporter into the inactive conformation as reported by FRET in CFP-ER-YFP ($n=20$). Lower trace: after pretreatment with 25 μ M forskolin (10 min) the tamoxifen-effect is abolished (11 of 13 exp.).

B) Following addition of glucagon (20 nM), Epac-FRET measures cAMP increases in A431/Cx43 cells that are adjacent to Rat-GR Donors (9 of 10 exp.). A431/Cx43 cells themselves are unresponsive to glucagon (data not shown).

C) Upper trace: glucagon-stimulation of Rat-GR Donor cells prevents the response of YFP-ER-CFP to tamoxifen in neighboring A431/Cx43 cells (representative trace, $n=7$). Lower trace: the preventive action of Rat-GR stimulation is not observed when cocultured with native A431 cells expressing YFP-ER-CFP ($n=8$).

When cAMP was photoreleased in one of two neighboring Cx43-deficient Rat-1 cells, the resulting rise in cAMP was not mirrored in the neighboring cell. In conclusion, Cx43-based gap junctions allow rapid and efficient exchange of cAMP between neighboring cells.

Gap junctional diffusion of cAMP following receptor stimulation

We next raised cAMP in the "Donor" cell in a more physiological manner, namely by stimulating a prototypic Gs-coupled hormone receptor. Rat-1 cells retrovirally transduced with the $G(\alpha)_s$ -coupled glucagon receptor (Rat-GR) were stimulated with glucagon and the Gs-mediated rise in cAMP was monitored over time using the Epac sensor. Glucagon-induced cAMP elevations were sustained for up to 1 hour. Whereas high glucagon concentrations saturated the Epac sensor (Fig. 2A, middle trace), lower doses of the hormone evoked intermediate responses (lower trace). The dose-response curve, determined with the quantitative Epac sensor, indicates an EC_{50} value of ~ 4 nM, in agreement with the literature [18]. In co-culture, Rat-GR cells serve as cAMP Donors for wildtype Rat-1 cells, which lack functional glucagon receptors (Fig. 2A, upper trace).

We co-cultured Rat-GR Donor cells (expressing H2B-mRFP as a transfection marker) with wild-type Rat-1 cells expressing the Epac sensor (1:50 ratio) to ensure that Acceptors contact single Donors only. Addition of 20 nM glucagon, which elicits near-maximal responses in the Donors, caused substantial cAMP rises in adjacent Acceptors (17 out of 23 experiments). In the representative experiment in Fig. 2B, the two Acceptors showed $\sim 75\%$ and 50% cAMP responses compared to maximal activation induced by forskolin.

When Rat-GR Donors were mixed 1:50 with Cx43-knockdown cells expressing the Epac sensor, no cAMP signal was observed in the neighboring cells (Fig. 2C, $n=14$), consistent with a requirement for gap junctions to transfer cAMP. Thus, the specific sensitivity to glucagon and the

ability to tune its responses (Fig. 2A) make the Rat-GR cell line a convenient tool to investigate gap junctional diffusion of cAMP.

cAMP diffusion mediates transcription factor phosphorylation in adjacent cells

To show that the intercellular exchange of cAMP can serve a signaling function, we monitored the cAMP-sensitive modification of the estrogen receptor (ER). The ER, a cytosolic transcription factor, plays a key role in breast cancer development; anti-estrogens such as tamoxifen find wide clinical application. Using a FRET-based ER construct (YFP-ER-CFP), Michalides and coworkers showed that tamoxifen induces a conformational change in the ER that inactivates this transcription factor. Strikingly, a rise in cAMP abrogates the tamoxifen-induced shape change [14]. These authors further showed that cAMP acts via PKA-mediated phosphorylation of the ER at Ser305 [14], thereby rendering the cells resistant to tamoxifen.

We used this system to study whether intercellular diffusion of cAMP may confer tamoxifen-insensitivity to neighboring cells. To this end, we used Cx43-expressing human A431 carcinoma cells (A431/Cx43) as Acceptors. When co-cultured with glucagon-sensitive Rat-1 cells, A431/Cx43 cells form functional heterotypic gap junctions with the Rat-1 cells (data not shown). As expected, A431 cells expressing the YFP-ER-CFP reporter became resistant to tamoxifen after addition of forskolin (Fig. 3A). When these cells were co-cultured with glucagon-sensitive Rat-1 cells, addition of glucagon caused a significant rise in cAMP in adjacent A431/Cx43 Acceptor cells (Fig. 3B). As shown in Fig. 3C (upper trace), glucagon completely prevented the tamoxifen-induced ER shape change in A431 cells. Again, this effect was not observed in wildtype A431 cells lacking gap junctions (Fig. 3C, lower trace). From these results we conclude that gap junctional diffusion of cAMP from normal fibroblasts to adjacent carcinoma cells can influence the conformation state of the ER transcription factor in the cancer cell.

Figure 4 Quantification of cAMP-GJC shows relevance over entire physiological range

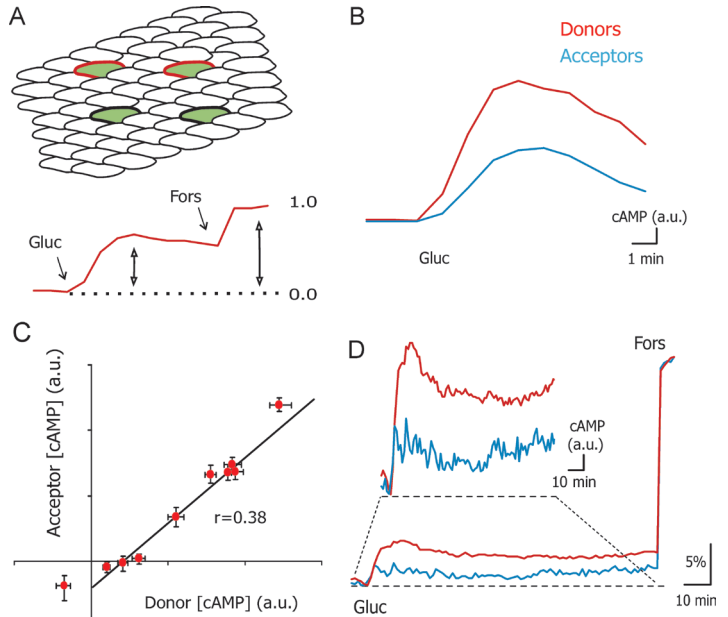
A) Coculture graphic: Donors and Acceptors, both expressing the Epac sensor (green), were jointly seeded on a sub-confluent monolayer of non-fluorescent Rat-GR Donors. Donors were discriminated by selective labeling with Cell Tracker Orange (red). Glucagon responses in several Donors and several Acceptors were simultaneously measured in multiple-position acquisition mode (15 min, measurement interval 1 min).

B) Average responses of 15 Donors (red) and 15 Acceptors (blue) from a representative multiple-position experiment. The kinetics of Acceptor cAMP levels closely follow the Donor responses. Shown are intracellular cAMP concentrations calculated from the FRET data as follows: glucagon-induced FRET changes were expressed as percentages of the forskolin + IBMX response (see example in Fig. 4A) and subsequently converted to [cAMP] according to previously determined sigmoidal relationship between [cAMP] and FRET.

Note that [cAMP] is given in arbitrary units because the *in vitro* K_d (14 μ M, Ponsioen et al.[13]) may not necessarily reflect the *in vivo* affinity; however, the shape of the converted curves is independent of K_d.

C) Double-log plot of Acceptor versus Donor responses, where Acceptors are singular cells while Donor responses represent the entire Donor monolayer. Each point in the plot combines Donor and Acceptor responses recorded in the same experiment (mean $\pm$ s.e.m., $15 < n < 20$ for each cell type). Plotted are [cAMP] (a.u.), calculated as described for B). A linear regression fit yields a slope of 0.38 ($p < 0.001$). Note that the slope of the regression line does not depend on absolute calibration of [cAMP].

D) Similar experimental setting as in B) and C), but stimulated with 2 nM glucagon and monitored for 2 hours. $N=15$ for both Donors and Acceptors. Although glucagon induced modest responses in the Donor monolayer, the Acceptor cAMP levels followed proportionally. FRET curves are normalized to saturating forskolin responses. Inset: same responses converted to [cAMP] as described in B). Dashed lines are zero.



Single cells proportionally follow the cAMP levels of surrounding cells

We next set out to quantify to which degree single cells follow the cAMP signal in neighboring monolayer cells. The Epac sensor was transduced into Donor and Acceptor Rat-1 cells and both were seeded sparsely on a monolayer of non-fluorescent Rat-GR Donors (Fig. 4A). cAMP levels in the Donor monolayer were increased with various doses of glucagon and responses in both Epac sensor expressing Donor and Acceptor cells were monitored in multiple-position acquisition mode. As can be seen from the representative experiment in Fig. 4B, cAMP levels in Acceptor cells closely follow the kinetics of the Donor responses with a lag time of less than one minute. The Donor and Acceptor response amplitudes, plotted in Fig. 4C, show a striking proportionality (regression coefficient 0.38, $p < 0.001$) indicating that Acceptors, although surrounded by Donors, maintain (or 'clamp') their cAMP levels down to 38% of the Donor levels, by counterbalancing the cAMP influx via clearance mechanisms (e.g. PDE-mediated degradation). This proportionality is observed for all cAMP concentrations in the Donor monolayer down to micromolar con-

centrations (see legend for considerations regarding the relationship between FRET and [cAMP]), suggesting that cAMP diffusion through gap junctions is relevant for cAMP levels in the entire physiological range.

When monitored over a period of 2 hours, the Donor response was continuously mirrored in the Acceptor cells, even when imposing relatively minor cAMP elevations in the Donor monolayer (Fig. 4D). More precisely, the integrated response amplitude of the Acceptors ($n=15$) in Fig. 4D was 37% of that of the Donors, in excellent agreement with a regression coefficient of 0.38 (Fig. 4A).

Thus, as a result of GJC, the cAMP levels in individual cells proportionally follow those of the surrounding cells over the entire range of physiological cAMP levels.

PDE activity is a major determinant of cAMP spreading

Penetration of cAMP from source cells into surrounding Acceptor cells depends both on the diffusional resistance of the GJ and clearing by the Acceptor cells. As shown in Fig. 4C, cAMP levels in Acceptor cells reach an equilibrium

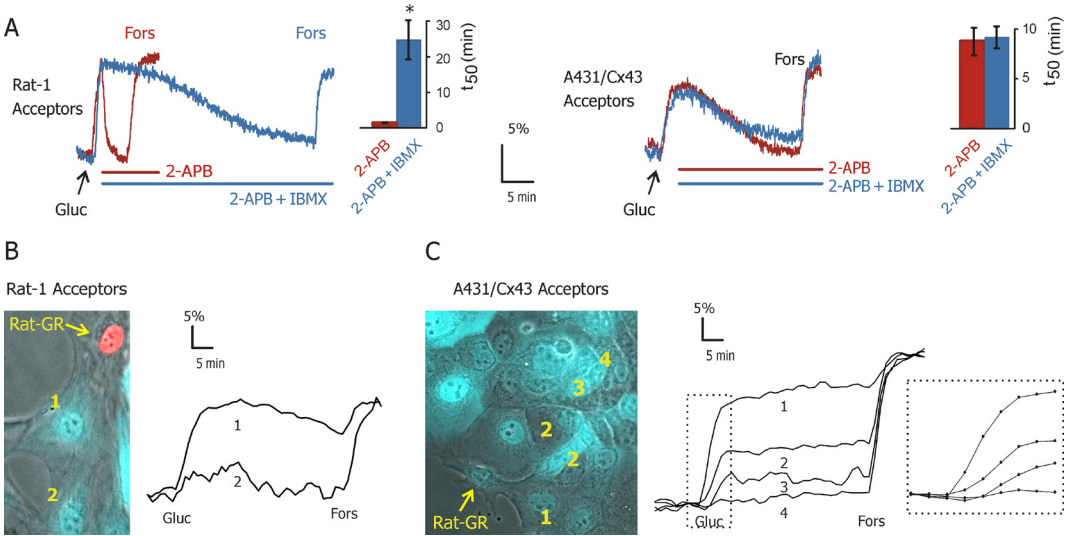


Figure 5 PDE degradation rate determines spreading of cAMP over multiple cell layers

A) Acceptor cells expressing the Epac sensor (left traces, Rat-1; right traces, A431/Cx43) were seeded on monolayers of Rat-GR Donors. The supply of cAMP from glucagon-stimulated Donors was instantly blocked by addition of 2-APB (50 μ M) in the absence (red traces) or presence (blue traces) of IBMX (200 μ M). Bar graphs: 50% decay time (t_{50} ; mean $\pm$ s.e.m.; Rat-1 Acceptors, -IBMX $n=5$, +IBMX $n=3$, * $p<0.001$; A431+Cx43 Acceptors, -IBMX $n=4$, +IBMX $n=4$).

B) A single Rat-GR Donor cell (expressing H2B-mRFP) feeds into a number of surrounding Rat-1 Acceptors expressing the Epac sensor. Traces represent cAMP kinetics in the first-order and second-order neighbor cell (representative for $n=4$). Measurements were done in sub-confluent cultures allowing easy definition of successive neighbors.

C) Same experiment as in B), but the Rat-GR Donor now feeds into an island of Epac sensor expressing A431/Cx43 Acceptors. Rat-GR Donors were discriminated by morphology. Figures exemplify the assigned neighbor orders. In A431/Cx43 Acceptors, cAMP increases were observed up to the 3rd order neighbors (representative for $n=4$). Right panel: rising phases display the propagation of cAMP through the consecutive Acceptor layers.

state (~40% of Donor levels) within minutes, showing that at this stage clearance compensates for the continued influx. What are the main determinants of cAMP clearing? We set out to compare Rat-1 cells with A431/Cx43 cells, since the latter exhibit slower cAMP decay. This is evident from the experiments shown in Fig. 5A (red traces), in which 2-APB is used to instantly block cAMP supply from glucagon-stimulated Donors to the Acceptors. Whereas Rat-1 Acceptors cleared cAMP with a half time (t_{50}) of approx. 1 min (Fig. 5A, left panel, red trace), the t_{50} in A431/Cx43 Acceptors was approx. 9 min (Fig. 5A, right panel, red trace). To distinguish PDE-mediated cAMP breakdown from other possible clearing mechanisms we conducted similar experiments in the presence of the broad-spectrum PDE inhibitor IBMX. When added simultaneously with 2-APB, IBMX caused a marked increase in the decay t_{50} in Rat-1 Acceptors (Fig. 5A, left panel, blue line; for quantification see bar graph), indicating that in these cells PDE activity is a major determinant of cAMP clearance. In contrast, in A431/Cx43 cells clearance of cAMP was only

marginally slower in the presence of IBMX, indicating that in these cells PDE-mediated breakdown plays a minor role (Fig. 5A, right panel).

We next tested penetration of cAMP from single Rat-GR Donors into monolayers of either of the two Acceptor cell types (seeded 1:50). In Rat-1 Acceptor monolayers, stimulation of single Rat-GR Donors induced cAMP increases in direct neighbors and, with much lower amplitudes, in second-order neighbors (Fig. 5B), but not in third-order neighbor cells. In contrast, in monolayers of A431/Cx43 Acceptor cells, cAMP often penetrated well into third-order neighbors (Fig. 5C). Importantly, control calcein-transfer experiments showed lower coupling in A431/Cx43 cells than in Rat-1 cells (data not shown), excluding the possibility that the observed differences are explained by differing degrees of GJ-coupling. Therefore, our data indicate that, depending on the cell type, the PDE activity can be a major factor determining cAMP penetration into surrounding Acceptor cells.

Concluding Remarks

In this study, we have used FRET-based sensors to study gap junction-mediated transfer of cAMP in normal fibroblasts. Both the Epac-based sensor and the high-affinity PKA sensor revealed that subtle cAMP rises rapidly spread to neighbor cells and that the kinetics of the rise and fall are nearly synchronous in Donor and Acceptor cells (Fig. 1). Using the more quantitative Epac sensor, we determined that cAMP levels in single Rat-1 cells follow the collective cAMP changes in surrounding monolayer cells to ~40% (Fig. 4). This proportional relationship was observed over the entire range of physiological cAMP concentrations, including (sub-)micromolar deviations (Fig. 4A). These data suggest that GJC can establish concerted regulation of cAMP-sensitive processes among tissue cells.

The observed Acceptor-Donor proportion of ~40% is determined by the cAMP permeability of Cx43 as well as by PDE degradation rates in the Acceptor cells. In a recent study, Bedner and coworkers reported the order of cAMP permeabilities for a number of connexin family members: Cx43 > Cx26 > Cx45 = Cx32 > Cx47 > Cx36 [8]. How these different permeabilities affect the Acceptor-Donor proportion of cAMP levels in cell systems as described here, remains to be determined. The same is true for the extrapolation of our findings to the 3D architecture of real tissues. It is expected that in 3D tissue the number of gap junctional connections per cell exceeds those encountered in a 2D monolayer, which would only further increase synchronization of cAMP levels between contacting cells. Our data further indicate that, depending on the cell type, PDE activity in receiving cells can critically determine the extent of cAMP-GJC: A431/Cx43 Acceptors, which exhibit relatively low PDE activity, follow Donor cAMP levels more efficiently than Rat-1 cells (Fig. 5) despite their poorer coupling when compared to Rat-1 cells. Physiological rises in cAMP were sufficient to confer tamoxifen-resistance to neighboring A431/Cx43 carcinoma cells (Fig. 3), illustrating how cell behaviour is influenced by GJ diffusion of cAMP. Among the many processes regulated by cAMP, its anti-proliferative effects on most cell types [19-21] is of special interest. Although speculative, it remains tempting to consider cAMP-GJC as a mechanism for the growth inhibitory effects of Cx43-based gap junctions [5,6,22].

Materials and methods

Chemicals

Glucagon, endothelin and 4-hydroxytamoxifen were from Sigma, 1-(2-nitrophenyl)ethyl adenosine-3',5'-cyclic monophosphate (NPE-caged cAMP) and Cell Tracker Orange were from Molecular Probes Inc. (Eugene, OR), (2-aminoethoxy)diphenylborane (2-APB) was from Cayman Chemical (Ann Arbor, MI), Calcein-AM, forskolin and IBMX were from Calbiochem-Novabiochem Corp. (La Jolla, CA), and Anti-Cx43 polyclonal mouse antibody was from Sigma. All other chemicals were of analytical grade.

DNA Constructs

pcDNA expression vectors with the following inserts were described elsewhere: CFP-Epac-DDEP-C.D.-YFP [13]; the PKA-based sensor consisting of RII-CFP and Cat-YFP [12] and YFP-ER-CFP [14]. CFP-Epac-DDEP-C.D.-YFP was cloned into the adenoviral vector from Invitrogen's Virapower Adenoviral Expression System via its pENTR intermediate vector (NotI/NotI into NotI-site of a pENTR variant whose ccdB gene was removed from the MCS). The human Glucagon Receptor (hGR) was purchased from UMR cDNA Resource Center (Rolla, USA) and cloned directly into a retroviral LZRS-vector (EcoRI, XhoI).

Cell lines

Rat-RNAi-Cx43; Cx43 was knocked down in Rat-1 cells by stable expression of retroviral pSuper containing the RNAi target sequence (GGTGTGGTGTCAGTGCTC) (pRS Cx43). To generate virus, Phoenix-Eco package cells were transfected with pRS Cx43 and the supernatant containing viral particles was harvested after 72 hrs. Rat-1 cells were infected with 1 ml of viral supernatant supplemented with 10 μ l Dotap (Roche; 1 mg/ml) and plated in selection medium 48 hrs later (puromycin, 2 μ g/ml, Sigma). After two weeks of selection, colonies were picked and tested for Cx43 expression and GJIC. The monoclonal Rat-RNAi-Cx43 cell strain was cultured from the colony that expressed lowest Cx43 and, as a result, was deficient in communication.

Rat-GR Donor cells were generated as follows: Phoenix-Eco package cells were transfected with LZRS-hGR and Rat-1 cells were transduced following procedures as described above. In this case, however, a polyclonal population was cultured by continuous puromycin selection (2 μ g/ml). Test assays with Epac-FRET indicated that the resultant population was 100% glucagon responsive. Human A431 carcinoma cells stably transfected with Cx43 cDNA were kindly provided by B. Giepmans [15].

Cell culture, transfection and live cell imaging

Rat-1 fibroblasts and derivatives (Rat-GR and Rat-RNAi-Cx43) and A431/Cx43 cells were seeded in 6-well plates on 25-mm glass cover slips and cultured in 3 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% serum and antibiotics. Constructs were either transiently transfected using Eugene 6 Transfection Reagent (Roche Inc.) or transduced via the Virapower Adenoviral Expression System (Invitrogen). For co-cultures containing cAMP Donor and Acceptor cells (see text), cells were separately transfected or transduced with sensor (YFP-ER-CFP, Epac sensor) or transfection marker (H2B-mRFP); after 24 hrs donors and acceptors were jointly seeded for the experiment one day later (48 hrs). Depending on the experiment, donors were stained with Cell Tracker Orange before mixing with the Acceptors. Live cell imaging was performed in a culture chamber mounted on an inverted microscope in bicarbonate-buffered saline (containing (in mM) 140 NaCl, 5KCl, 1MgCl₂, 1CaCl₂, 10 glucose, 23 NaHCO₃, with 10 mM HEPES added), pH 7.2, kept under 5% CO₂, at 37°C.

Confocal FRET measurements and photolysis of caged cAMP

Rat-1 fibroblasts were loaded with NPE-caged cAMP by 5' incubation in a concentration 100 μ M. Uncaging of cAMP was with brief pulses of UV light (340-410 nm) from a 100 W HBO lamp using a shutter. For spatially resolved imaging of cAMP we measured FRET of the Epac- or PKA-based sensor ratiometrically on a Leica TCS-SP2 confocal microscope (Mannheim, Germany).

GJC assays using calcein-FRAP

Monolayers of cells were loaded with the GJ-permeable dye calcein-AM (10 min, 5 μ M) and subsequently washed with DMEM to de-esterify the AM-moiety for 15'. Cytosolic calcein was bleached by high-intensity laser illumination directed at a single cell ($\sim$ 3 s, 50-fold scanning power) and the subsequent GJ exchange of calcein was monitored by confocal time-lapse imaging and normalized to calcein signal from remote, non-bleached cells.

FRET monitoring with high temporal resolution

Cells on coverslips were placed on an inverted NIKON Microscope and excited at 425 nm. Emission of CFP and YFP was detected simultaneously through 470 $\pm$ 20 and 530 $\pm$ 25 nm bandpass filters. Data were digitized with a 500 ms interval and FRET was expressed as ratio of CFP to YFP signals, the value of which was set to 1.0 at the onset of the experiments. Changes are expressed as percent deviation from this initial value of 1.0. Agonists and inhibitors were added from concentrated stocks.

FRET monitoring with spatio-temporal resolution in multi-position mode

FRET was monitored on a Leica TCS-SP2 coupled to a Coolsnap CCD camera. Using Leica's ASMDW acquisition software, time-lapse series were recorded simultaneously in multiple cells on predefined locations on the cover slip. CFP and YFP images were simultaneously detected by projection on two halves of the CCD chip, respectively, via a Dual View Filter Cube comprised of a dichroic filter and mirrors (Leica, Mannheim). Ratios of background-corrected CFP and YFP were calculated from the signal in manually defined Regions Of Interest (ROIs) in cells and background areas.

SDS-PAGE and immunoblotting

Cells were harvested in Laemmli sample buffer (LSB, 50mM TrisHCl pH 6.8; 2%SDS; 10% glycerol; 5% β -mercaptoethanol; 0.1% bromophenol blue). Samples in LSB were boiled for 10 minutes, subjected to SDS-page and transferred to nitrocellulose filters. Filters were blocked in 5% milk in TBST and incubated with rabbit polyclonal anti-Cx43 antibody and subsequent HRP-conjugated swine anti-rabbit secondary antibody (DAKO). Loading control was with mouse anti- α -tubulin and HRP-conjugated rabbit anti-mouse (Sigma).

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Chapter 7

Regulation of connexin43 gap junctional communication by phosphatidylinositol 4,5-bisphosphate

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Regulation of connexin43 gap junctional communication by phosphatidylinositol 4,5-bisphosphate

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Abstract

Cell-cell communication through connexin43 (Cx43)-based gap junction channels is rapidly inhibited upon activation of various G protein-coupled receptors; however, the mechanism is unknown. Here, we show that Cx43-based cell-cell communication is inhibited by depletion of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) from the plasma membrane. Knockdown of phospholipase C β 3 (PLC β 3) inhibits PI(4,5)P₂ hydrolysis and keeps Cx43 channels open after receptor activation. Using a translocatable 5-phosphatase, we show that PI(4,5)P₂ depletion is sufficient to close Cx43 channels. When PI(4,5)P₂ is overproduced by PtdIns(4)P 5-kinase, Cx43 channel closure is impaired. We find that the Cx43-binding partner ZO-1 interacts with PLC β 3 via its third PDZ domain. ZO-1 is essential for PI(4,5)P₂-hydrolyzing receptors to inhibit cell-cell communication, but not for receptor-PLC coupling. Our results show that PI(4,5)P₂ is a key regulator of Cx43 channel function, with no role for other second messengers, and suggest that ZO-1 assembles PLC β 3 and Cx43 into a signalling complex to allow regulation of cell-cell communication by localized changes in PI(4,5)P₂.

Introduction

Communication between adjacent cells through gap junctions occurs in nearly every tissue and is fundamental to coordinated cell behaviour. Gap junctions are composed of connexins, consisting of an intracellular N-terminus, four transmembrane domains and a cytosolic C-terminal tail. Six connexins oligomerize into a pore-forming connexon, and alignment of two connexons in apposing cell membranes forms a gap junction channel. These channels allow direct cell-to-cell diffusion of ions and small molecules (<1-2 kDa), including nutrients, metabolites, second messengers and peptides, without transit through the extracellular space [1-3]. Gap junctions play important roles in normal tissue function and organ development [4-6] and have been implicated in a great diversity of biological processes, notably electrical synchronization of excitable cells, energy metabolism, growth control, wound repair, tumour cell invasion and antigen cross-presentation [7-13]. The importance of gap junctions is highlighted by the discovery that mutations in connexins underlie a variety of genetic diseases, including peripheral neuropathy, skin disorders and deafness [5,14].

Connexin43 (Cx43) is the most abundant and best studied mammalian connexin. Cx43-based gap junctional communication is of a particular interest since it is regulated by both physiological and pathophysiological stimuli. In particular, Cx43-based cell-cell coupling is rapidly disrupted following stimulation of certain G protein-coupled receptors (GPCRs), such as those for endothelin, thrombin, nucleotides and bioactive lipids [15-21]. Disruption is transient as communication is restored after about 20-60 min., depending on the GPCR involved [22]. GPCR-mediated inhibition of intercellular communication will have broad consequences for long-

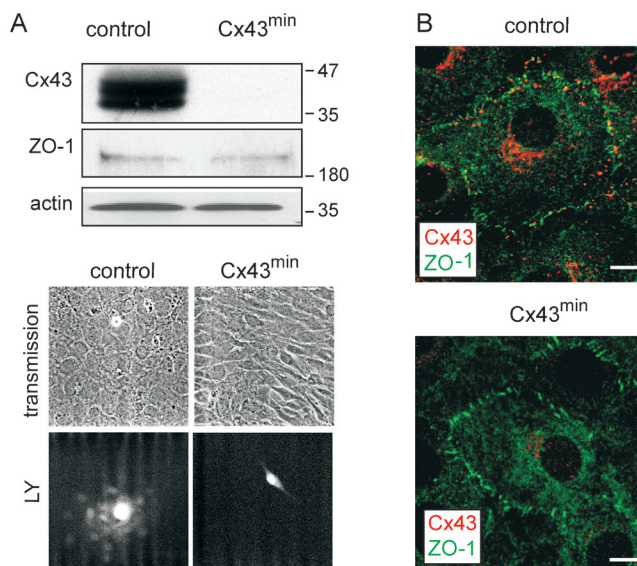
range signalling in cells and tissues where Cx43 is vital, such as dermal fibroblasts, glial cells and heart. However, the link between receptor stimulation and Cx43 channel closure has remained elusive to date. Numerous studies on the 'gating' of Cx43 channels have focused on a possible role for phosphorylation of Cx43 by various protein kinases, in particular protein kinase C (PKC), mitogen-activated protein (MAP) kinase and c-Src, but the results remain ambiguous [23-25]. One of the difficulties with unravelling the regulation of Cx43 channel function is that Cx43 functions in a multiprotein complex that is currently ill understood [26]. One established component of this assembly is the scaffold protein ZO-1, which binds directly to the C-terminus of Cx43 via its second PDZ domain [27,28]. ZO-1 has been suggested to participate in the assembly and proper distribution of gap junctions, but its precise role in the Cx43 complex remains unclear [29,30].

In the present study, we sought to identify the signalling pathway that leads to inhibition of Cx43 gap junctional communication in fibroblasts. Using a variety of experimental approaches, we show that the levels of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) at the plasma membrane dictate the inhibition (and restoration) of Cx43 gap junctional communication in response to GPCR stimulation, with no role for PI(4,5)P₂-derived second messengers. We further show that ZO-1, via its third PDZ domain, interacts with phospholipase C β 3 (PLC β 3) and is essential for G_q/PLC-coupled receptors to abrogate Cx43-based cell-cell communication. Our results suggest a model in which ZO-1 serves to organize Cx43 and PLC β 3 into a complex to allow exquisite regulation of Cx43 channel function by localized changes in PI(4,5)P₂.

Figure 1. Cx43 is the only functional connexin in Rat-1 cells

A) Rat-1 cells were transduced with Cx43 shRNA (Cx43^{min}) or with a non-functional shRNA (control). Top panel: Immunoblot showing that stable expression of Cx43 shRNA leads to disappearance of Cx43 (Cx43^{min} cells), while leaving ZO-1 expression unaltered. Actin served as loading control. Bottom panel: wide-field images of control and Cx43^{min} Rat-1 cells. Cx43^{min} cells lack cell-cell communication as determined by Lucifer Yellow (LY) diffusion.

B) Confocal images of control and Cx43^{min} cells immunostained for Cx43 (red) and ZO-1 (green) (scale bars, 5 μ m).



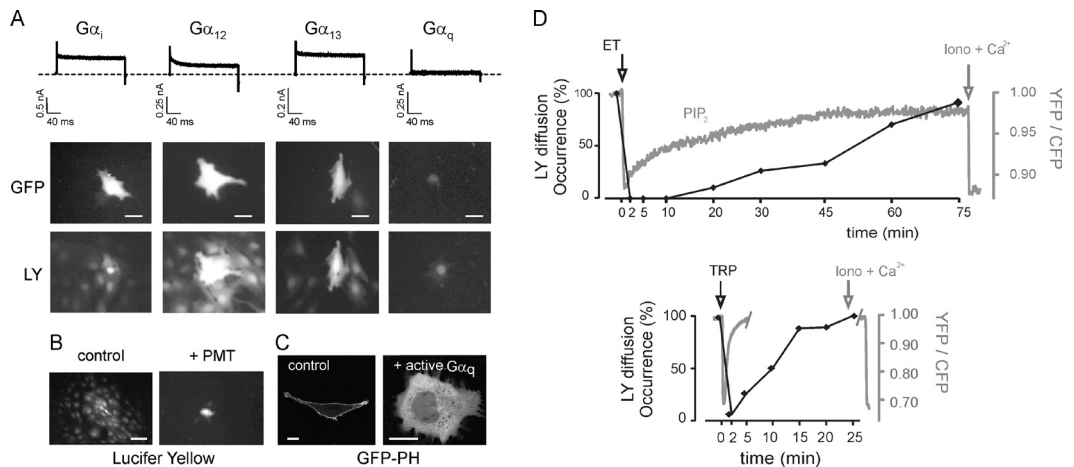


Figure 2. Activated Gα_q disrupts Cx43-based gap junctional communication: correlation with PI(4,5)P₂ depletion.

A) Intercellular communication in Rat-1 cells transfected with various activated (GTPase-deficient) Gα subunits. Upper panel: Electrical cell-cell coupling measured by a single patch-clamp electrode [18]. Whole-cell current responses to 10-mV voltage pulses (duration 100 ms; holding potential -70 mV) were recorded from confluent Rat-1 cells. Note dramatic increase in cellular input resistance (i.e. a decrease in conductance) by activated Gα_q but not other Gα subunits. Middle and bottom panels: Rat-1 cells cotransfected with active Gα subunits and GFP (10:1 ratio). GFP-positive cells were microinjected with Lucifer Yellow (LY) and dye diffusion from the microinjected cells was monitored. Wide field pictures of GFP and LY diffusion as indicated (scale bars, 10 μm). B) Disruption of gap junctional communication by *Pasteurella multocida* toxin (PMT; 1 μg/ml; 3 hrs preincubation), an activator of Gα_q as measured by LY diffusion (scale bars, 20 μm). C) Depletion of PI(4,5)P₂ from the plasma membrane by activated Gα_q. HEK293T cells were transfected with the PI(4,5)P₂ sensor GFP-PH, alone or together with active Gα_q (1:10 ratio). GFP-PH localizes to the plasma membrane where it binds PI(4,5)P₂ (left panel). Co-expression with activated Gα_q causes GFP-PH to relocate to the cytosol (right panel). Scale bars: 10 μm. D) Monitoring PI(4,5)P₂ levels (red trace) and intercellular communication (black; n ≥ 20 per time point) in Rat-1 cells following addition of endothelin (50 nM) (upper panel) or TRP (50 μM) (lower panel). Data points show the percentage of injected cells that spread LY to their neighbors. Temporal changes in plasma membrane-bound PI(4,5)P₂ were measured by changes in FRET between CFP-PH and YFP-PH. Ionomycin, which evokes an immediate and complete depletion of PI(4,5)P₂ from the plasma membrane when applied at high doses (5 μM) together with 5 mM Ca²⁺ [34] was used for calibration.

Results

Regulation of Cx43 gap junctional communication by the Gα_q-PLCβ-PI(4,5)P₂ hydrolysis pathway

Rat-1 fibroblasts are ideally suited for studying Cx43 channel function since they express Cx43 as the only functional connexin [31,32]. Stable knockdown of Cx43 expression (using pSuper shRNA) resulted in a complete loss of intercellular communication, consistent with Cx43 being the only functional gap junction protein in Rat-1 cells (Fig. 1A). Fig. 1B shows that the Cx43-binding partner ZO-1 retains its submembranous localization in Cx43 knockdown cells.

To assess which G protein(s) mediate(s) inhibition of gap junctional communication, we introduced active versions of Gα_i, Gα₁₂ and Gα₁₃ subunits into Rat-1 cells and examined their impact on cell-cell coupling. Expression of active Gα_q resulted in complete inhibition of intercellular communication, whereas active Gα_i, Gα₁₂ and Gα₁₃ left cell-cell coupling unaltered, as evidenced by Lucifer Yellow (LY) diffusion and electrophysiological assays (Fig.

2A). Disruption of gap junctional communication induced by active Gα_q was persistent, as opposed to the transient inhibition observed after GPCR stimulation [33]. Similarly, treatment of Rat-1 cells with *Pasteurella multocida* toxin, a direct activator of Gα_q [34], caused persistent abrogation of cell-cell coupling (Fig. 2B). Gα_q couples to PLCβ to trigger PI(4,5)P₂ hydrolysis leading to production of the second messengers inositol-(1,4,5)-trisphosphate (IP₃) and diacylglycerol (DAG) [35]. We monitored PI(4,5)P₂ in living cells by using a GFP fusion protein of the PH domain of PLCδ1 (GFP-PH) as a probe [36-38]. In control cells, the PI(4,5)P₂ probe was concentrated at the plasma membrane. In cells expressing active Gα_q, however, the probe was spread diffusely throughout the cytosol, indicative of PI(4,5)P₂ depletion from the plasma membrane (Fig. 2C). While these results are consistent with Gα_q mediating agonist-induced inhibition of intercellular communication, they should be interpreted with caution since constitutive depletion of PI(4,5)P₂ from the plasma membrane promotes apoptosis [39,40].

To monitor the kinetics of PI(4,5)P₂ hydrolysis and

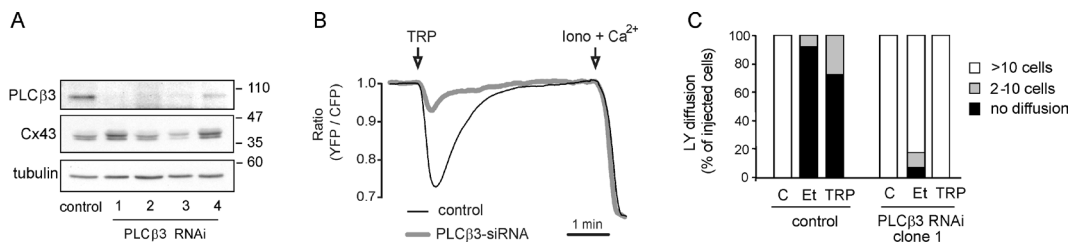


Figure 3. Targeted knockdown of PLCβ3 prevents receptor-mediated PI(4,5)P₂ hydrolysis and inhibition of junctional communication.

A) PLCβ3 knockdown in Rat-1 cells as detected by immunoblotting. Expression of PLCβ3 in Rat-1 cells expressing a non-functional shRNA (control) and in four subclones (1-4) stably expressing different PLCβ3 shRNA constructs. Total cell lysates were immunoblotted for PLCβ3, Cx43 and α-tubulin as indicated.

B) Temporal changes in plasma-membrane PI(4,5)P₂ levels following thrombin receptor stimulation of normal (red trace) and PLCβ3-deficient Rat-1 cells (blue trace). TRP, 50 μM; Ionomycin, 5 μM.

C) Bar graphs showing the percentage of communicating cells in control and PLCβ3 knockdown cells (clone1) treated with either endothelin (Et, 50 nM) or TRP (50 μM), as indicated ($n \geq 25$ for each dataset). LY injections were done at 2 min. after addition of agonist.

resynthesis with high temporal resolution, we made use of the fluorescence resonance energy transfer (FRET) between the PH domains of PLCδ1 fused to CFP and YFP, respectively [41]. When bound to plasma membrane PI(4,5)P₂, CFP-PH and YFP-PH are in close proximity and show FRET. Following PI(4,5)P₂ breakdown, the probes dilute out into the cytosol and FRET ceases. The prototypic G_q-coupled receptor agonist endothelin, acting through endogenous ET(A) receptors, induced an acute and substantial decrease in PI(4,5)P₂, reaching a maximum after 30-60 sec.; thereafter, PI(4,5)P₂ slowly recovered to near basal levels over a period lasting as long as 45-60 min. (Fig. 2D, upper panel; red trace). Sustained PI(4,5)P₂ hydrolysis by ET(A) receptors has been reported previously [42] and may be explained by the fact that activated ET(A) receptors follow a recycling pathway back to the cell surface rather than the lysosomal degradation route [43]. The kinetics of endothelin-induced inhibition and recovery of cell-cell communication followed those of PI(4,5)P₂ hydrolysis and resynthesis, respectively, with communication being restored after about 75 min. (Fig. 2D, upper panel; black trace).

More transient PI(4,5)P₂ depletion and recovery kinetics were observed with a thrombin receptor (PAR-1) activating peptide (TRP), which correlated with a more short-lived inhibition of gap junctional communication (Fig. 2D, lower panel). Furthermore, a desensitization-defective mutant NK2 receptor (for neurokinin A) that mediates prolonged PI(4,5)P₂ hydrolysis [44] inhibits gap junctional communication for prolonged periods of time when compared to the wild-type NK2 receptor [45]. While these results reveal a close correlation between the duration of PI(4,5)P₂ depletion and that of communication shutoff, we note that the restoration of cell-cell communication consistently lagged behind the recovery of PI(4,5)P₂ levels. Nevertheless, our findings strongly suggest that the G_q/PLCβ-mediated hydrolysis and subsequent resynthesis of PI(4,5)P₂ dictate the inhibition and restoration of Cx43

gap junctional communication, respectively.

Knockdown of PLCβ3 prevents cell-cell uncoupling

The Gα_q-activated PLCβ enzymes comprise four members (β1-4) [35]. PLCβ1 and β3 are ubiquitously expressed, whereas PLCβ2 and β4 expression is restricted to hematopoietic cells and neurons, respectively. Rat-1 cells express PLCβ3, but no detectable PLCβ1 (Fig. 3A and results not shown). We stably suppressed PLCβ3 expression using the pSuper short hairpin RNA (shRNA) expression vector [46]. Four different target sequences were selected to correct for clonal variation and off-target effects. Immunoblot analysis shows a marked reduction in PLCβ3 expression in different clones (Fig. 3A). When comparing PI(4,5)P₂ dynamics in PLCβ3 knockdown versus control cells, agonist-induced PI(4,5)P₂ breakdown was strongly reduced in the PLCβ3-deficient cells (Fig. 3B). PLCβ3 knockdown cells showed normal basal cell-cell communication but failed to shut off cell-cell communication following GPCR stimulation (Fig. 3C). These results indicate that PLCβ3 is a key player in the control of intercellular communication, supporting the view that GPCRs inhibit gap junctional communication through the G(α)_q/PLCβ3-PI(4,5)P₂ hydrolysis pathway.

PLC-mediated PI(4,5)P₂ hydrolysis generates the second messengers IP₃ and DAG, leading to Ca²⁺ mobilization and protein kinase C (PKC) activation, respectively. Previous pharmacological studies already suggested that neither Ca²⁺ nor PKC have a critical role in GPCR-mediated inhibition of cell-cell coupling [47], a notion supported by additional experiments using 'caged' IP₃, the cell-permeable Ca²⁺ chelator BAPTA-AM and a PKC-activating bacterial PLC [48] (data summarized in Supplemental Table 1). Whether PI(4,5)P₂-derived second messengers are dispensable for Cx43 channel closure upon GPCR activation remains debatable, however, since the supporting pharmacological evidence is indirect.

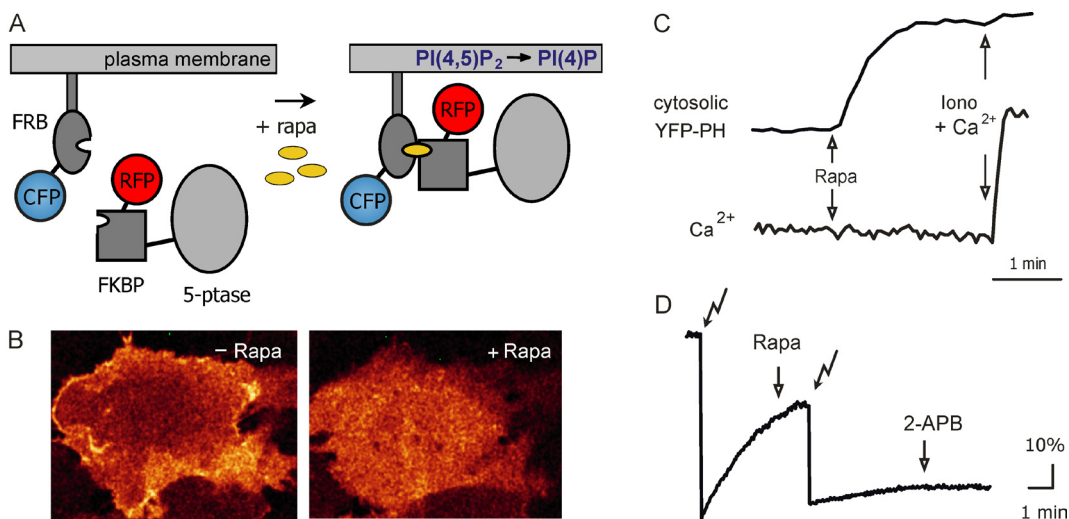


Figure 4. PI(4,5)P₂ depletion by 5-phosphatase inhibits gap junctional communication.

A) Schematic representation of rapamycin-induced PI(4,5)P₂ degradation at the plasma membrane. Rapamycin induces dimerization of FKBP domains to FRB domains. Rapamycin recruits the phosphoinositide-5-phosphatase-FKBP fusion protein (mRFP-FKBP-5-ptase) to FRB tethered to the plasma membrane (PM-FRB-CFP), resulting in the rapid conversion of PI(4,5)P₂ into PI(4)P.

B) Confocal images of YFP-PH in Rat-1 cells before (left) and after (right) addition of rapamycin (100nM). In addition to YFP-PH, PM-FRB-CFP and mRFP-FKBP-5-ptase were also correctly expressed (images not shown). Note that the translocation of YFP-PH into the cytoplasm is complete, indicative of massive PI(4,5)P₂ hydrolysis.

C) Representative responses to rapamycin and ionomycin. Top, cytosolic levels of YFP-PH; bottom, Ca²⁺ dye Oregon Green. Ionomycin treatment could not induce further translocation of YFP-PH, indicating that rapamycin-induced PI(4,5)P₂ degradation was complete (n=10). Rises in cytosolic Ca²⁺ were never observed (n=4), confirming that PI(4,5)P₂ hydrolysis did not generate second messengers.

D) Gap junctional communication in Rat-1 cells transfected with PM-FRB-CFP and mRFP-FKBP-5-ptase, assayed by fluorescence recovery after photobleaching (FRAP) of calcein. While cells showed efficient communication before rapamycin-treatment, gap junctional exchange was significantly decreased at 2 min after addition of rapamycin (0.25 x recovery rate before rapamycin, n=15, p<0.005). The gap junction blocker 2-APB was added at 50 μM.

Conversion of PI(4,5)P₂ into PI(4)P by phosphoinositide 5-phosphatase is sufficient to inhibit cell-cell communication

To examine whether the depletion of PI(4,5)P₂ suffices to inhibit Cx43 gap junctional communication, we used a newly developed method to rapidly deplete PI(4,5)P₂ without activating PLC. In this approach, PI(4,5)P₂ at the plasma membrane is converted into PI(4)P and free phosphate by rapamycin-inducible membrane targeting of the human type IV phosphoinositide 5-phosphatase (5-ptase) [49] (see also [50]). The method is based on the rapamycin-induced heterodimerization of FRB (fragment of mammalian target of rapamycin [mTOR] that binds FKBP12) and FKBP12 (FK506-binding protein 12), as schematically illustrated in Fig. 4A. In this approach, a mutant version of 5-ptase with a defective membrane targeting domain (CAAX-box) is fused to FKBP12 and tagged with monomeric red fluorescent protein (mRFP), while its binding partner FRB (fused to CFP) is tethered to the plasma membrane through palmitoylation (construct PM-FRB-CFP) [51]. In the absence of rapamycin, 5-ptase resides in the cytosol and leaves PI(4,5)P₂ levels at the plasma membrane un-

altered (Fig. 4A, left panel). Upon addition of rapamycin (100 nM), FKBP and FRB undergo heterodimerization and the 5-ptase is recruited to the plasma membrane (Fig. 4A, right panel).

We expressed the mRFP-FKBP-5-ptase and PM-FRB-CFP fusion proteins in Rat-1 cells and confirmed their proper intracellular localization by confocal microscopy (not shown). Addition of rapamycin caused a rapid and complete depletion of PI(4,5)P₂, as shown by the disappearance of the PI(4,5)P₂ sensor YFP-PH from the plasma membrane (Fig. 4B and 4C, upper trace n=10). As expected, no Ca²⁺ signal was detected following the 5-ptase-mediated conversion of PI(4,5)P₂ into PI(4)P (n=4; Fig. 4C). To determine how the 5-ptase-induced hydrolysis of PI(4,5)P₂ affects gap junctional communication, we measured the intercellular diffusion of calcein (added as membrane-permeable calcein-AM) using FRAP (Fluorescence Recovery After Photobleaching) [52] (Fig. 4D). Rat-1 cells expressing mRFP-FKBP-5-ptase and PM-FRB-CFP showed efficient intercellular transfer of calcein. At 2 min after rapamycin addition, however,

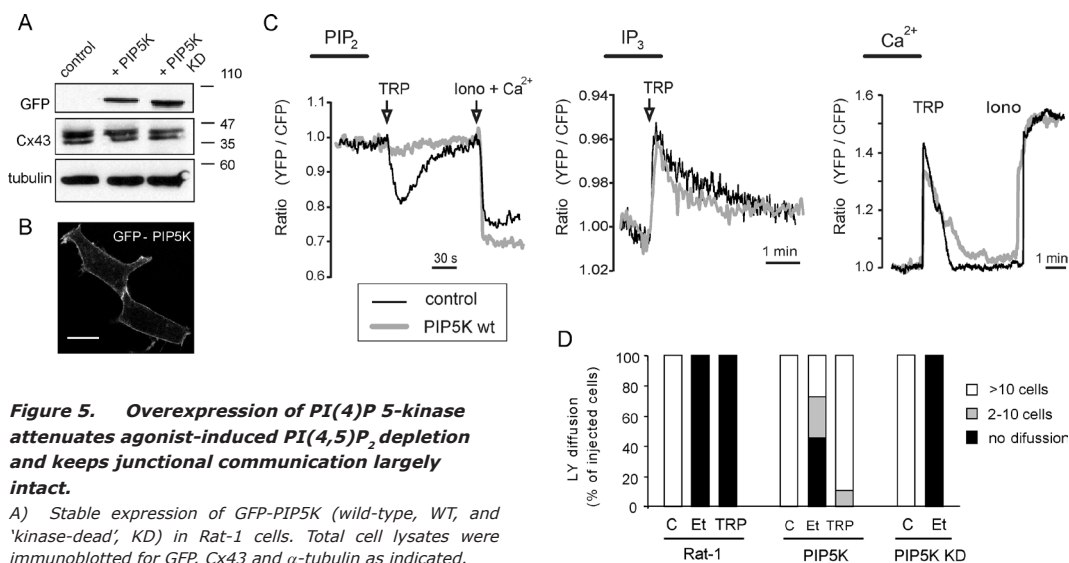


Figure 5. Overexpression of PI(4)P 5-kinase attenuates agonist-induced PI(4,5)P₂ depletion and keeps junctional communication largely intact.

A) Stable expression of GFP-PIP5K (wild-type, WT, and 'kinase-dead', KD) in Rat-1 cells. Total cell lysates were immunoblotted for GFP, Cx43 and α -tubulin as indicated.

B) Localization of GFP-PIP5K in Rat-1 cells (scale bar, 10 μ m).

C) Temporal changes in the levels of PI(4,5)P₂, IP₃ and Ca²⁺ measured by the respective FRET-based sensors, as detailed in the Methods section.

Control and PIP5K-overexpressing Rat-1 cells were stimulated with TRP (50 μ M). In control cells (red trace), PI(4,5)P₂ levels rapidly fall after TRP stimulation, whereas PIP5K overexpression (blue trace) largely prevents the drop in FRET indicating that PI(4,5)P₂ levels remain high (i.e. above FRET threshold). Ionomycin, 5 μ M.

D) Bar graphs showing the percentage of communicating cells (LY diffusion) in control Rat-1 cells and cells expressing either wild-type (wt) or kinase-dead (KD) PIP5 kinase. Cells were left untreated (C) or stimulated with GPCR agonists (endothelin, 50 nM; TRP, 50 μ M) as indicated ($n \geq 20$ for each dataset). Residual response to endothelin is explained by excessive depletion of PI(4,5)P₂ (cf. Fig. 2D). LY injections were done at 2 min. after addition of agonist

intercellular dye diffusion was inhibited as inferred from a strongly reduced fluorescence recovery rate (Fig. 4D; $n=15$, $p<0.005$; about 0.25 $\times$ the recovery rate before rapamycin addition). The recovery of calcein fluorescence could not be decreased any further by addition of the gap junction blocker 2-APB (2-aminoethoxy-diphenylborane; 50 μ M; Fig. 4D) [53]. Rapamycin did not affect cell-cell communication in non-transfected cells (data not shown). Thus, PI(4,5)P₂ depletion by 5-phosphatase activation is sufficient to inhibit Cx43 gap junctional communication, with no need for PI(4,5)P₂-derived second messengers.

Overexpression of PI(4)P 5-kinase prevents inhibition of cell-cell communication

PI(4,5)P₂ at the plasma membrane is generated mainly from PI(4)P by PI(4)P 5-kinase (PIP5K) [54,55]. As a further test of the PI(4,5)P₂ hypothesis, we stably over-expressed PIP5K (type I α , fused to GFP) in Rat-1 cells in an attempt to prevent PI(4,5)P₂ depletion following GPCR stimulation (Fig. 5A). As shown in Fig. 5B, transfected GFP-PIP5K localizes to the plasma membrane. In the PIP5K-overexpressing cells, PI(4,5)P₂ levels remain elevated (i.e. above FRET threshold levels) after agonist addition (Fig. 5C). Nonetheless, GPCR agonists still induced transient rises in IP₃ and Ca²⁺ (Fig. 5C), indicating

that excessive synthesis of PI(4,5)P₂ does not interfere with its hydrolysis. Basal cell-cell communication in PIP5K-overexpressing cells was not significantly different from that in control cells. However, the PIP5K-overexpressing cells failed to close their gap junction channels upon addition of TRP and, to a lesser extent, endothelin (Fig. 5D). That endothelin is still capable of evoking a residual response in PIP5K-overexpressing cells may be explained by the fact that endothelin is by far the strongest inducer of PI(4,5)P₂ depletion (Fig. 2D).

Expression of a 'kinase-dead' version of PIP5K had no effect on either PI(4,5)P₂ hydrolysis or inhibition of cell-cell communication (Fig. 5D). We conclude that Cx43 channel closure is prevented when PI(4,5)P₂ is maintained at adequate levels.

No detectable PI(4,5)P₂ binding to the C-terminal tail of Cx43

PI(4,5)P₂ can modulate the activity of various ion channels and transporters, apparently through direct electrostatic interactions [56,57]. By analogy, regulation of Cx43 channels by PI(4,5)P₂ would imply that basic residues in Cx43 bind directly to the negatively charged PI(4,5)P₂. Indeed, the regulatory cytosolic tail of Cx43 (aa 228-382) contains a membrane-proximal stretch of both

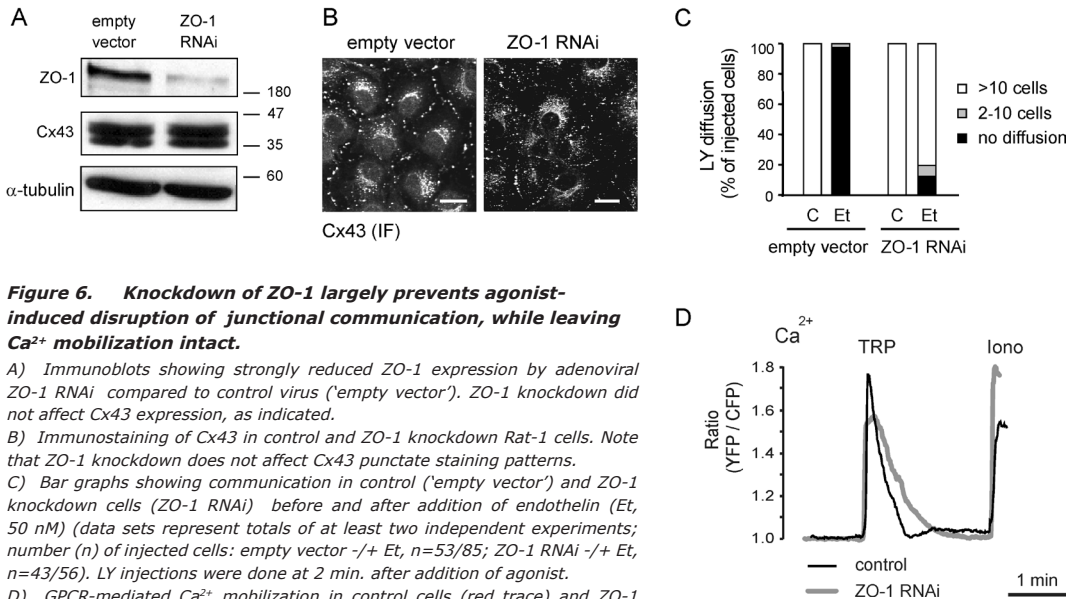


Figure 6. Knockdown of ZO-1 largely prevents agonist-induced disruption of junctional communication, while leaving Ca²⁺ mobilization intact.

A) Immunoblots showing strongly reduced ZO-1 expression by adenoviral ZO-1 RNAi compared to control virus ('empty vector'). ZO-1 knockdown did not affect Cx43 expression, as indicated.

B) Immunostaining of Cx43 in control and ZO-1 knockdown Rat-1 cells. Note that ZO-1 knockdown does not affect Cx43 punctate staining patterns.

C) Bar graphs showing communication in control ('empty vector') and ZO-1 knockdown cells (ZO-1 RNAi) before and after addition of endothelin (Et, 50 nM) (data sets represent totals of at least two independent experiments; number (n) of injected cells: empty vector -/+ Et, n=53/85; ZO-1 RNAi -/+ Et, n=43/56). LY injections were done at 2 min. after addition of agonist.

D) GPCR-mediated Ca²⁺ mobilization in control cells (red trace) and ZO-1 knockdown cells (blue trace). Ca²⁺ was measured using the FRET-based Yellow Cameleon probe. TRP, 50 μM; Ionomycin, 5 μM.

basic and hydrophobic residues (²³¹VFFKGVKDRVKGK/R²⁴³) that could constitute a potential PI(4,5)P₂ binding site. Local depletion of PI(4,5)P₂ might then dissociate the juxtamembrane region of the Cx43 tail from the plasma membrane leading to channel closure. We reasoned that if the Cx43 juxtamembrane domain binds PI(4,5)P₂ *in situ*, mutations within this domain might interfere with PI(4,5)P₂-regulated channel closure. We therefore neutralized the membrane-proximal Arg and Lys residues by mutation to alanine resulting in eight distinct Cx43 mutants, notably K237A,K241A; R239A,R243A; K241A,R243A; R239A,K241A; K237A,R239A; R239A,K241A,R243A; K237A,R239A,K241A and the '4A' mutant, K237A,R239A,K241A,K243A. When expressed in Cx43-deficient cells, however, all these mutants were trapped intracellularly and failed to localize to the plasma membrane (Supplemental Fig. 1A). While this result reveals a previously unknown role for the membrane-proximal Arg/Lys residues in Cx43 trafficking, it precludes a test of the Cx43-PI(4,5)P₂ interaction hypothesis.

We next examined whether PI(4,5)P₂ can specifically bind to either the Cx43 C-terminal tail (Cx43CT; aa 228-382) or a Cx43CT-derived juxtamembrane peptide (Cx43JM; aa 228-263) *in vitro*. We generated a GST-Cx43CT fusion protein and determined its ability to bind phosphoinositides *in vitro* using three distinct protocols. GST-PH(PLCδ1) was used as a positive control. In the first approach, agarose beads coated with either PI(4,5)P₂ or PI(4)P were incubated with GST-Cx43CT or GST-PH and then pulled down by centrifugation. PI(4,5)P₂ beads

readily brought down the GST-PH polypeptide but not GST-Cx43CT (Supplemental Fig. 2A). Second, we incubated GST-Cx43CT with ³²P-labeled PI(4,5)P₂ and examined the ability of excess phosphoinositides to displace bound ³²P-PI(4,5)P₂. While GST-PH showed again strong PI(4,5)P₂ binding that could readily be displaced by excess PI(4,5)P₂, there was no detectable binding of PI(4,5)P₂ to Cx43CT above that observed with GST alone (Supplemental Fig. 2B). Finally, we found that PI(4,5)P₂ (and other phosphoinositides) immobilized on nitrocellulose strips failed to bind either Cx43CT or a 35-aa juxtamembrane domain peptide (Cx43JM; aa 228-263; [55]) (results not shown). Thus, PI(4,5)P₂ does not detectably bind to the juxtamembrane domain of Cx43, nor to the full-length regulatory tail (aa 228-362), at least *in vitro*.

ZO-1 is required for GPCRs to inhibit junctional communication

The very C-terminus of Cx43 binds directly to the second PDZ domain of ZO-1, but the functional significance of the Cx43-ZO-1 interaction is not understood. We asked if ZO-1 has a role in modulating gap junctional communication in response to GPCR stimulation. We already showed that RNAi-mediated depletion of Cx43 does not significantly affect the levels and localization of ZO-1 (Fig. 1B). Conversely, when ZO-1 expression was knocked down by shRNA, Cx43 levels were unaltered (Fig. 6A). ZO-1 knockdown Rat-1 cells retained their fibroblastic morphology

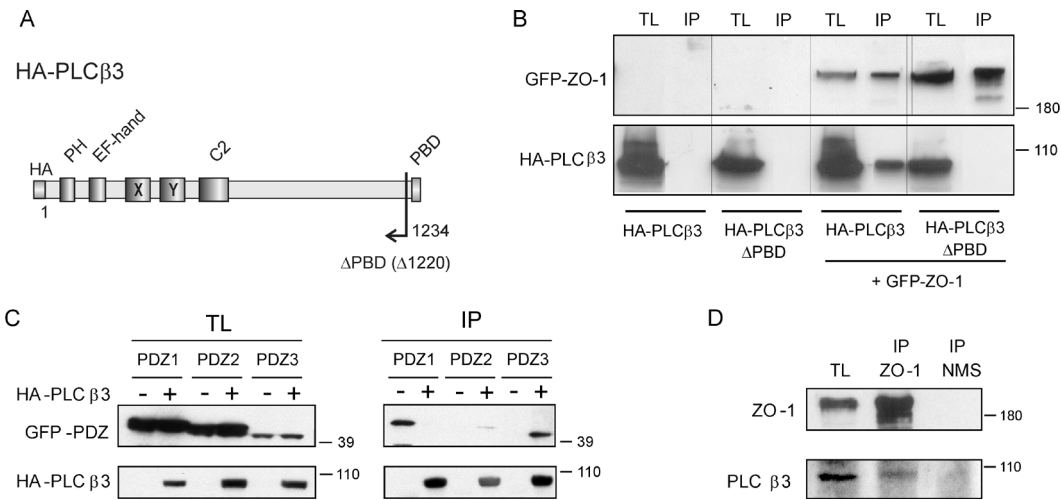


Figure 7. Association of ZO-1 with PLCβ3
A) Schematic representation of HA-PLCβ3. X, Y represent the catalytic domains; the C2 domain interacts with activated G_{α_q} [32]; ΔPBD: mutant PLCβ3 lacking the C-terminal residues 1220-1234 (comprising the PDZ domain binding motif).
B) Co-immunoprecipitation of GFP-ZO-1 and HA-PLCβ3 expressed in HEK293 cells. TL: total cell lysates; IP, denotes immunoprecipitation using anti-GFP antiserum. Samples were immunoblotted for GFP (top) and HA (bottom).
C) Co-immunoprecipitation of HA-PLCβ3 and GFP-tagged individual PDZ domains of ZO-1 expressed in HEK293 cells. TL: total cell lysates; IP, denotes immunoprecipitation using anti-HA antibody. Samples were immunoblotted for GFP (top) and HA (bottom).
D) Endogenous ZO-1 immunoprecipitated (IP) from Rat-1 cells. Total cell lysates (TL) and samples from ZO-1 immunoprecipitates (IP) were blotted for both ZO-1 and PLCβ3 as indicated. NMS: normal mouse serum.

and showed normal Cx43 punctate staining and cell-cell coupling (Fig. 6B,C), showing that ZO-1 is dispensable for the formation of functional gap junctions. When ZO-1 knockdown cells were stimulated with endothelin, however, the inhibition of cell-cell communication was severely impaired (Fig. 6C). Importantly, overall $PI(4,5)P_2$ -dependent Ca^{2+} mobilization was not affected in the ZO-1 knockdown cells (Fig. 6D). We conclude that ZO-1 is essential for the regulation of gap junctional communication by G_q /PLC-coupled receptors, but not for linking those receptors to PLC activation. A plausible explanation for these findings is that ZO-1 serves to bring the $PI(4,5)P_2$ -metabolizing machinery into proximity of Cx43 gap junctions.

Direct interaction between ZO-1 and PLCβ3

As a test of the above hypothesis, we examined if ZO-1 can interact with PLCβ3. PLCβ3 can associate with at least two scaffold proteins, NHERF2 (in epithelial cells) and Shank2 (in brain), via a C-terminal PDZ domain-binding motif [58,59]. We co-expressed HA-PLCβ3 and GFP-ZO-1 in HEK293 cells and performed immunoprecipitations using anti-GFP antibody (Fig. 7A). Cell lysates and immunoprecipitates were blotted for GFP and HA. As shown in Fig. 7B, PLCβ3 and ZO-1 can indeed be coprecipitated. Next, we co-expressed ZO-1 and a PLCβ3 truncation mutant that lacks the C-terminal 14 residues (HA-PLCβ3-ΔPBD;

Fig. 7A), and performed anti-GFP immunoprecipitations. Fig. 7B shows that truncated PLCβ3 fails to interact with ZO-1, indicating that PLCβ3 interacts with ZO-1 through its very C-terminus, containing the PDZ domain-binding motif. Considering that ZO-1 has three distinct PDZ domains, we examined which (if any) PDZ domain binds PLCβ3. We expressed GFP-tagged versions of the three individual PDZ domains in HEK293 cells, either alone or together with HA-PLCβ3. We immunoprecipitated PLCβ3 using anti-HA antibody and blotted total cell lysates and precipitates for both HA and GFP. As shown in Fig. 7C, we find that PLCβ3 binds to PDZ3 but not to PDZ1 or PDZ2.

To verify that the ZO-1-PLCβ3 interaction exists endogenously, we precipitated ZO-1 from Rat-1 cells and blotted for both ZO-1 and PLCβ3. Fig. 7D shows that PLCβ3 co-precipitates with ZO-1. The reverse co-precipitation could not be done, since precipitating antibodies against PLCβ3 are presently not available. Nonetheless, these results suggest that ZO-1, through its respective PDZ2 and PDZ3 domains, assembles Cx43 and PLCβ3 into a signalling complex and thereby facilitates regulation of gap junctional communication by PLC-coupled receptors.

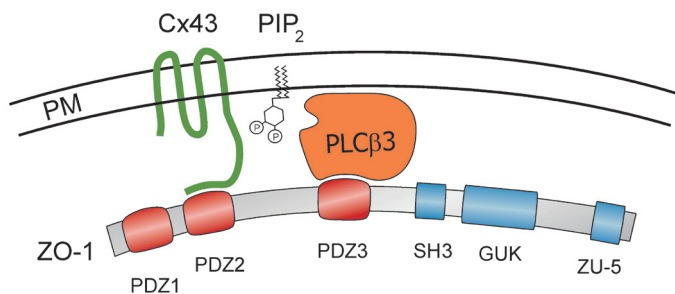


Figure 8. Schematic drawing of the proposed model

ZO-1 is proposed to assemble Cx43 and PLCβ3 into a complex, thereby facilitating regulation of Cx43 channel function by localized changes in PI(4,5)P₂ upon receptor activation. Since we found no evidence for direct binding of PI(4,5)P₂ to Cx43, PI(4,5)P₂ might regulate junctional communication in an indirect manner, for example via a Cx43-associated protein that modifies the Cx43 regulatory tail and thereby shuts off channel function. PM, plasma membrane. See text for details.

Discussion

A critical and long-standing question in gap junction biology is how junctional communication is regulated by physiological and pathophysiological stimuli. Relatively little progress has been made in identifying receptor-induced signalling events that modulate the channel function of Cx43, the best studied and most abundant mammalian connexin. In particular, regulation of Cx43 channel activity via G-protein signalling has not been systematically examined to date. In the present study, we identify the G_q-linked PLCβ-PI(4,5)P₂ hydrolysis pathway as a key regulator of Cx43-based gap junctional communication in normal fibroblasts. We demonstrate that loss of PI(4,5)P₂ from the plasma membrane is necessary and sufficient to close Cx43 channels, without a role for PI(4,5)P₂-derived second messengers. In other words, PI(4,5)P₂ itself is the responsible signalling molecule. A second novel finding is that the Cx43-binding partner ZO-1 binds to PLCβ3 and is essential for PI(4,5)P₂-hydrolyzing receptors to regulate gap junctional communication.

PI(4,5)P₂ as a key regulator

Our conclusion that PI(4,5)P₂ at the plasma membrane regulates Cx43 channel function is based on several lines of evidence. First, active Gα_q (but not Gα_i, Gα₁₂ or Gα₁₃) depletes PI(4,5)P₂ from the plasma membrane and abrogates gap junctional communication. Second, knockdown of PLCβ3 inhibits agonist-induced PI(4,5)P₂ depletion and prevents disruption of cell-cell communication. Third, conversion of PI(4,5)P₂ into PI(4)P by a translocatable 5-phosphatase is sufficient to inhibit intercellular communication. Fourth, maintaining PI(4,5)P₂ at adequate levels by overexpression of PIP5K renders Cx43 channels refractory to GPCR stimulation, although second messenger generation still occurs.

Acting as a signalling molecule in its own right, PI(4,5)P₂ can regulate local cellular activities when its levels rise and fall; in particular, PI(4,5)P₂ can modulate the activity of various ion channels and transporters, presumably through electrostatic interactions [60,61]. Although the existence of such interactions in living cells remains large-

ly inferential and PI(4,5)P₂-binding consensus sequences have not been clearly defined, the common theme is that the negatively charged PI(4,5)P₂ binds to a motif with multiple positive charges interdispersed with hydrophobic residues [62,63]. The Cx43 C-terminal juxtamembrane domain indeed contains such a putative PI(4,5)P₂-binding motif (aa 231-243), although this stretch also meets the criteria of a tubulin-binding domain [64]. Extension of the above model to Cx43 channel gating would then imply that local loss of PI(4,5)P₂ could release the Cx43 regulatory tail from the plasma membrane to render it susceptible to a modification leading to channel closure. However, our investigations to detect specific binding of PI(4,5)P₂ to the Cx43 C-terminal tail or its juxtamembrane domain *in vitro* yielded negative results. Rather, mutational analysis revealed that those basic residues in the juxtamembrane domain have a hitherto unrecognized role in the trafficking of Cx43 to the plasma membrane. These findings do not, of course, rule out the possibility that PI(4,5)P₂ does bind directly to Cx43 *in situ*.

Aside from modulating ion channel activity, PI(4,5)P₂ has been implicated in cytoskeletal remodeling, vesicular trafficking and recruitment of cytosolic proteins to specific membranes [65,66]. Although Cx43 can interact with cytoskeletal proteins, such as tubulin and drebrin [67,68], cytoskeletal reorganization does not play a significant role in regulating Cx43 junctional communication because cytoskeleton-disrupting agents (cytochalasin D, nocodazole, Rho-inactivating C3 toxin) have no detectable effect on GPCR regulation of cell-cell coupling [69] and Supplemental Table 1). Furthermore, we found that GPCR-induced inhibition and recovery of gap junctional communication are insensitive to agents known to interfere with Cx43 trafficking and internalization, including cycloheximide, brefeldin A, monensin, ammonium chloride and hypertonic sucrose (Supplemental Table 1).

Numerous studies have suggested that closure of Cx43 channels in response to divergent stimuli somehow results from Cx43 phosphorylation [70,71]. Several protein kinases, including PKC, MAP kinase, casein kinase-1 and Src, are capable of phosphorylating Cx43 at multiple sites in the C-terminal tail. These phosphorylations

have been implicated not only in Cx43 channel gating but also in Cx43 trafficking, assembly and degradation. The link between Cx43 phosphorylation and altered cell-cell coupling is largely correlative, however, as the functional significance of most of these phosphorylations has not been elucidated. Our previous studies suggested that c-Src-mediated tyrosine phosphorylation of Cx43 underlies disruption of gap junctional communication, as inferred from experiments using both constitutively active and dominant-negative versions of c-Src [72,73]. To date, however, we have been unable to detect GPCR-induced tyrosine phosphorylation of Cx43 in a physiological context. Furthermore, the Src inhibitor PP2 does not prevent GPCR agonists from inhibiting Cx43-based gap junctional communication in either Rat-1 cells or primary astrocytes (Supplemental Table 1; [74]). Therefore, tyrosine phosphorylation of Cx43 leading to loss of cell-cell coupling, as observed with constitutively active c-Src and v-Src [75], may not actually occur under physiological conditions; an issue that warrants further investigation.

Essential role for ZO-1

Another novel finding of the present study concerns the role of ZO-1, an established binding partner of Cx43 [76,77]. Originally identified as a major component of epithelial tight junctions [78], ZO-1 is thought to serve as a platform to scaffold various transmembrane and cytoplasmic proteins. ZO-1 and its close relative ZO-2 have several protein-interaction domains, including three PDZ domains, one SH3 domain and one GUK domain. In epithelial cells, ZO-1 and ZO-2 act redundantly to some extent in the formation of tight junctions [79,80]. In non-epithelial cells lacking tight junctions, ZO-1 has been attributed a role in the assembly and stabilization of Cx43 gap junctions [81-83], but its precise role has remained elusive. Our knockdown studies herein show that ZO-1 is essential for G_q /PLC-coupled receptors to inhibit intercellular communication, but not for coupling those receptors to PLC activation, as inferred from Ca^{2+} mobilization experiments; this result suggests that loss of ZO-1 at Cx43 gap junctions is not compensated for by ZO-2. We find that ZO-1 binds directly to the very C-terminus of PLC β 3 via its third PDZ domain. In the simplest model compatible with our findings, ZO-1 serves to assemble Cx43 and PLC β 3 into a complex to permit regulation of gap junctional communication by localized changes in PI(4,5) P_2 , as schematically illustrated in Fig. 8. Since we found no evidence for direct binding of PI(4,5) P_2 to Cx43 *in vitro*, PI(4,5) P_2 might regulate junctional communication in an indirect manner, for example via a Cx43-associated protein that modifies the Cx43 regulatory tail and thereby shuts off channel function. Precisely how PI(4,5) P_2 regulates the Cx43 multiprotein complex remains a challenge for future studies.

Materials and methods

Reagents

Materials were obtained from the following sources: endothelin, thrombin receptor-activating peptide (TRP; sequence SFLLRN), neurokinin A, Cx43 polyclonal and α -tubulin monoclonal antibodies from Sigma (St. Louis, MO); Pasteurella multocida toxin from Calbiochem-Novabiochem (La Jolla, CA); Cx43 NT monoclonal antibody from Fred Hutchinson Cancer Research Center (Seattle WA); actin monoclonal from Chemicon International (Temecula, CA); polyclonal PLC β 3 antibody from Cell Signalling; ZO-1 monoclonal antibody from Zymed; HRP-conjugated secondary antibodies from DAKO and secondary antibodies for immunofluorescence (goat-anti-mouse, Alexa488 and goat-anti-rabbit, Alexa594) from Molecular Probes. HA, Myc and GST monoclonal antibodies were purified from hybridoma cell lines 12CA5, 9E10 and 2F3, respectively. GFP antiserum was generated in our institute.

cDNA constructs

Constructs encoding active (GTPase-deficient) G_α subunits, eGFP-PH_{PLC β 31}, eCFP-PH_{PLC β 31}, eYFP-PH_{PLC β 31}, eGFP-tagged mouse type-Ia PI(4)P 5-kinase have been described [84-86]. Mouse PLC β 3 cDNA was obtained from MRC gene service, cloned into pcDNA3-HA by PCR (primers listed in Table S2) and ligated into pcDNA3-HA XhoI/NotI sites. HA-PLC β 3- Δ PBD was obtained by restriction of the full-length construct with Eco47III, cleaving off the very C-terminal 14 residues. Human ZO-1 was cloned into XhoI and KpnI sites of pEGFP C2 (Clontech). GFP-based Yellow Cameleon 2.1 has been described [87]. Constructs encoding cytosolic 5-phosphatase fused to FKB12-mRFP and PM-FRB-CFP have been described [88].

Cell culture and cell-cell communication assays

Cells were cultured in DMEM containing 8% fetal calf serum, L-glutamine and antibiotics. For cell-cell communication assays, cells were grown in 3-cm dishes and serum starved for at least 4 hrs prior to experimentation. Monitoring the diffusion of Lucifer Yellow (LY) from single microinjected cells and single-electrode electrophysiological measurements of cell-cell coupling were done as described [89]. Images were acquired on a Zeiss Axiovert 135 inverted microscope, equipped with an Achroplan $\times$ 40 objective (N.A. 0.60) and a Nikon F301 camera.

SDS-PAGE, immunoblotting and immunoprecipitation

Cells were harvested in Laemmli sample buffer (LSB), boiled for 10 min. and subjected to immunoblot analysis according to standard procedures. Filters were blocked in TBST/5% milk, incubated with primary and secondary antibodies, and visualized by enhanced chemoluminescence (Amersham Pharmacia). For immunoprecipitation, cells were harvested in 1% NP-40, 0.25% sodium desoxycholate lysis buffer. Lysates were spun down and the supernatants were subjected to immunoprecipitation using protein A-conjugated antibodies for 4 hrs at 4°C. Proteins were eluted by boiling for 10 min. in LSB and analyzed by immunoblotting.

Immunostaining and fluorescence microscopy

Cells grown on coverslips were fixed in 3.7% formaldehyde in PBS for 15 min. Samples were blocked and permeabilized in PBS containing 1% BSA and 0.1% Triton X-100 for 30 min. Subsequently, samples were incubated with primary and secondary antibodies for 30 min. each in PBS/1% BSA, washed five times with PBS and mounted in MOWIOL (Calbiochem). Confocal fluorescence images were obtained on a Leica TCS NT (Leica Microsystems, Heidelberg, Germany) confocal system, equipped with an Ar/Kr laser. Images were taken using a 63x NA 1.32 oil objective. Standard filter combinations and Kalman averaging were used. Processing of images for presentation was done on a PC using the software package Photoshop (Adobe Systems Incorporated Mountain View, California, USA).

Live-cell imaging

All live imaging and time-lapse experiments were performed in bicarbonate-buffered saline containing (in mM) 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 glucose, 23 NaHCO₃, 10 HEPES (pH 7.2), kept under 5% CO₂, at 37°C. Images of live cells expressing GFP-PH and GFP-PIP5K were recorded on a Leica TCS-SP2 confocal microscope (Mannheim, Germany), using a 63x lens, N.A. 1.4.

PI(4,5)P₂, IP₃ and Ca²⁺ imaging by FRET ratiometry

Temporal changes in PI(4,5)P₂ levels in living cells were assayed by the FRET-based PI(4,5)P₂ sensor, PH-PLCδ1, as described [90]. In brief, Rat-1 cells were transiently transfected with CFP-PH and YFP-PH constructs (1:1 ratio) using Eugene transfection agent and placed on a NIKON inverted microscope equipped with an Achroplan × 63 (oil) objective (N.A. 1.4). Excitation was at 425±5 nm. CFP and YFP emissions were detected simultaneously at 475±15 and 540±20 nm, respectively and recorded with PicoLog Data Acquisition Software (Pico Technology). FRET is expressed as the ratio of acceptor to donor fluorescence. At the onset of the experiment, the ratio was adjusted to 1.0, and FRET changes were expressed as relative deviations from base line. Temporal changes in IP₃ levels were monitored using a FRET-based IP₃ sensor, in which the IP₃-binding domain of the human type-I IP₃ receptor (aa 224 to 605) is fused between CFP and YFP, essentially analogous to the sensor described previously [91]. *In vitro* binding studies showed that it bound IP₃ with an apparent K_d of approx. 5 nM. Intracellular Ca²⁺ mobilization was monitored using the CFP/YFP-based Ca²⁺ sensor Yellow Cameleon 2.1 [35,92,93]. Traces were smoothened in Microsoft Excel using a moving average function ranging from 3 to 6.

PI(4,5)P₂ depletion by rapamycin-induced translocation of phosphoinositide 5-phosphatase

Rat-1 cells were transiently transfected with PM-CFP-FRB and mRFP-tagged FKBP-phosphoinositide 5-phosphatase (mRFP-FKBP-5-ptase) [94]. Cells were selected for experimentation when sufficient protein levels were expressed as judged by CFP and mRFP fluorescence. For PI(4,5)P₂ measurements, the YFP-PH construct

was cotransfected. For Ca²⁺ measurements, cells were loaded with Oregon-Green-AM. To monitor gap junctional communication cells were loaded with calcein-AM and analyzed by Fluorescence Recovery After Photobleaching (FRAP) [95]. These experiments were performed on a Leica TCS-SP2 confocal microscope (Mannheim, Germany), using 63x lens, N.A. 1.4.

Overexpression of PI(4)P 5-kinase

To overexpress PI(4)P 5-kinase (PIP5K; type Iα) [96], virus containing the LZRS-PIP5K constructs was generated as described above. Rat-1 cells were incubated with 1 ml of viral supernatant supplemented with 10 μl Dotap. 48 hrs after infection, cells were plated in selection medium. Transfected cells were selected on zeocin (200 μg/ml, Invitrogen) for 2 weeks and colonies were examined for PIP5K expression.

RNA interference

To generate Cx43-deficient Rat-1 cells, Cx43 was knocked down by stable expression of retroviral pSuper (pRS) [97] containing the RNAi target sequence GGTGTGGCTGT-CAGTGCTC. pRS-Cx43 was transfected into Phoenix-Eco package cells and the supernatant containing viral particles was harvested after 72 hrs. For infection, cells were incubated with 1 ml of viral supernatant supplemented with 10 μl Dotap (Roche; 1 mg/ml). 48 hrs after infection, cells were selected on puromycin (2 μg/ml) for 2 weeks. Single cell-derived colonies were tested for Cx43 expression and communication. PLCβ3 was stably knocked down by retroviral expression of PLCβ3 shRNA. Four different target sequences were selected, namely ACTACGTCT-GCCTGCGAAATT, GATTCGAGAGGTACTGGGC, TTACGTTG AGCCCGTCAAG, CCCTTTGACTTCCCAAGG. Non-functional shRNA was used as a control. ZO-1 was transiently knocked down by adenoviral expression of ZO-1 RNAi. First, ZO-1 RNAi oligos containing the ZO-1 target sequence GGAGGGCCAGCTGAAGGAC were ligated into pSuper after oligo annealing. Next, the oligos together with the H1 RNA promotor were subcloned into pEntr1A (BamHI/XhoI) and recombined into pAd/PL-Dest according to protocol (Virapower Adenoviral Expression System; Invitrogen). Virus was produced in 293A packaging cells according to standard procedures. Supernatant containing virus particles was titrated on Rat-1 cells to determine the amount needed for ZO-1 knockdown.

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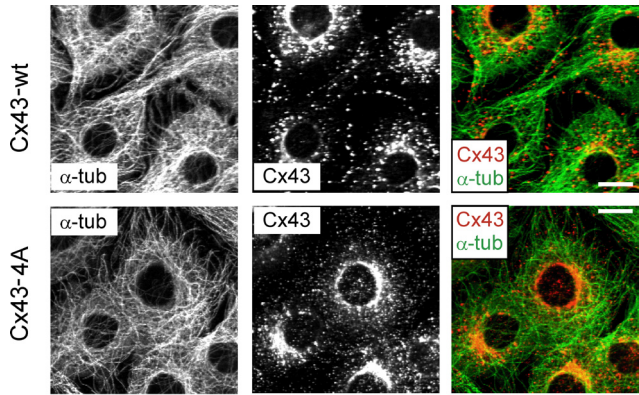
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Supplemental Figures

Suppl Fig 1. Cx43-4A mutant accumulates intracellularly

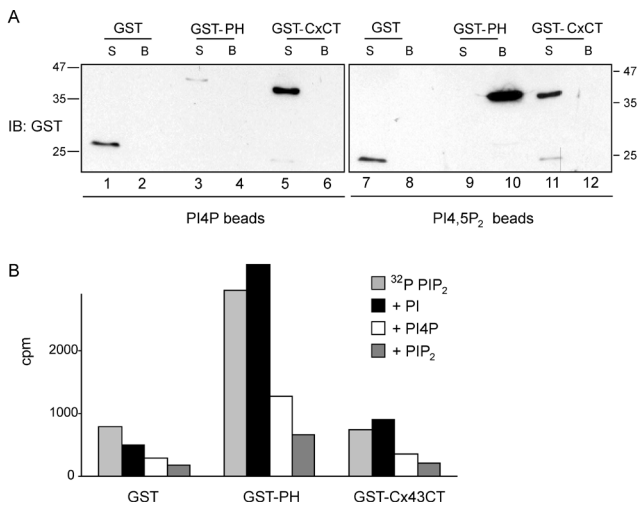
Confocal images of Cx43-deficient Rat-1 cells expressing wild-type Cx43 (wt) and Cx43-4A, in which basic Arg/Lys residues in the C-terminal juxtamembrane domain were neutralized by mutation into Ala. Cells were immunostained for Cx43 (red) and α -tubulin (green). Note intracellular accumulation of Cx43-4A and lack of detectable plasma membrane staining. Similar intracellular accumulation was observed with seven other Cx43(K/R>A) mutants, notably K237A,K241A; R239A,R243A; K241A,R243A; R239A,K241A; K237A,R239A; R239A,K241A,R243A; and K237A,R239A,K241A. Scale bars: 5 μ m.



Suppl Fig 2. No detectable phosphoinositide binding to the Cx43 C-terminal tail (CT)

A) Pull-down of GST alone, GST-PH and GST-Cx43CT (aa 227-382) fusion proteins using PI(4)P- and PI(4,5)P₂-coated agarose beads (Molecular Probes), as indicated. GST fusion proteins were purified from DH5a bacteria following standard procedures. Anti-GST monoclonal antibody was purified from hybridoma cell line 2F3. 10 mg of GST or fusion protein was incubated with 7 ml of beads (~70 pmol) in 300 ml Triton-X100 buffer for 4 hrs at 4°C; beads were spun down by centrifugation. 20 ml of the supernatant (S) was used as input control. Protein was eluted from the beads (B) by boiling for 10 min. in Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting for the presence of GST or GST-fusion protein.

B) Binding of ³²P-labeled PI(4,5)P₂ to GST fusion proteins. GST, GST-PH and GST-Cx43CT were coupled to glutathione beads and incubated with [³²P]-PI(4,5)P₂ alone or together with excess unlabeled PI, PI(4)P or PI(4,5)P₂ for 1 hr at 4°C, as indicated. Beads were washed extensively (after centrifugation) and bound ³²P activity was measured.



Supplemental Tables

Suppl Table 1.

Pharmacological agents showing no effect on either basal or GPCR-regulated cell-cell communication. Cell-cell communication in Rat-1 cells was determined by LY diffusion. See also Postma et al. [18].

Agent	Conc	Remarks
Phorbol ester: TPA (long - term) TPA (acute)	200 nM 100 nM	6- 24 hrs preincubation to downregulate PKC 0-15 min.
PC-PLC (<i>B. cereus</i>)	1 U/ml	Generates diacylglycerol and activates PKC [42]
Pertussis toxin	200 ng/ml	6-16 hrs preincub.
Caged IP ₃ Caged Ca ²⁺		Transient rises in cytosolic Ca ²⁺ upon UV-induced uncaging
BAPTA-AM	5 mM	Ca ²⁺ signaling blunted
Nocodazole Cytochalasin D Rho-inactivating C3 toxin	10 μM 10 μM 100 ng/ml	Cell contraction [55]
Staurosporine K252a PD598059 Ro-31-8220 PP2	100 μM 5 μM 10 μM 10 μM 50 μM	General protein kinase inhibitor (10 min. preincub.) General protein kinase inhibitor MAP kinase inhibitor PKC inhibitor Src family kinase inhibitor (20 min. preincub.)
Cycloheximide Brefeldin A	200 μM 5 μg/ml	60 min. preincub. 30 min. preincub.
Monensin NH ₄ Cl	5 μM 20 mM	
Hyperosmotic sucrose	0.5 M	

Suppl Table 2.

Oligos used for cloning, mutagenesis and RNAi constructs.

F, forward; R, reverse. All sequences 5'→ 3'.

PLCβ3 F	gatacctcagtggttaacgatgagcgaggcgagcccgcg
PLCβ3 R	GATCTCTAGAGCGGCCGCTCAAAGCTGGGTGTTTTCTCTGGCTC
Cx43 RNAi F	GATCCGgtgtgctgtcagtgctcTTCAAGAGagcactgacagccacccTTTTGGAAA
Cx43 RNAi R	AGCTTTTCAAAAAGgtgtggtgtcagtgctcTCTCTTGAAGagcactgacagccacccG GG
PLCβ3 RNAi 1 F	GATCCCCACTACGTCTGCCTGCGAAATTTCAAGAGAATTTTCGAGGCAGAGTAGTGTTTTGGAAA
PLCβ3 RNAi 1 R	AGCTTTTCAAAAACACTACGTCTGCCTGCGAAATTTCTTGAATTTTCGAGGCAGAGTAGTGGGG
PLCβ3 RNAi 2 F	GATCCGgattcgagaggtactgggcTTCAAGAGAgccagctacctctcgaatcTTTTGGAAA
PLCβ3 RNAi 2 R	AGCTTTTCAAAAAGattcgagaggtactgggcTCTCTTGAAGccagctacctctcgaatcGGG
PLCβ3 RNAi 3 F	GATCCCTtactgtgagccgctcaagTTCAAGAGActtgacgggctcaacgtaaTTTTGGAAA
PLCβ3 RNAi 3 R	AGCTTTTCAAAAATtactgtgagcccgctcaagTCTCTTGAActtgacgggctcaacgtaaGGG
PLCβ3 RNAi 4 F	GATCCcctttgacttccccaggTTCAAGAGAccttggggaagtcaaggTTTTGGAAA
PLCβ3 RNAi 4 R	AGCTTTTCAAAAACcctttgacttccccaggTCTCTTGAActtggggaagtcaaggGGG
ZO-1 RNAi F	GATCCGgaggggagcgtgaaggacTTCAAGAGAgctcctcagctggccctcTTTTGGAAA
ZO-1 RNAi R	AGCTTTTCAAAAAGgaggggcca gctgaaggacTCTCTTGAAGctcctcagctggccctccGGG
Cx43 4A F	Acggagaaaaccatcttcatcttcatgtgtgtgtgtccttgggtgtcctcgttgaacatcattgagctcttctac gtcttcttcaaaggcgtgagcgatggtggtggcgaggagcaagcgatccttaccacgcca
Cx43 4A R	Ggcgtgtgaaggatgctgtgctcccgccacggcatccgcaacgccttgagaagacgtagaagagctcaatga tgttcaaaggcagagacaccaaggacaccaccagcatgaagatgatgaagatggttttctc

Chapter 8

A Comparison of Donor-Acceptor Pairs for Genetically Encoded FRET Sensors: Application to the Epac cAMP Sensor as an Example

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A Comparison of Donor-Acceptor Pairs for Genetically Encoded FRET Sensors: Application to the Epac cAMP Sensor as an Example

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Abstract

We recently reported on CFP-Epac-YFP, an Epac-based single polypeptide FRET reporter to resolve cAMP levels in living cells. In this study, we compared and optimized the fluorescent protein donor/acceptor pairs for use in biosensors such as CFP-Epac-YFP. Our strategy was to prepare a wide range of constructs consisting of different donor and acceptor fluorescent proteins separated by a short linker. Constructs were expressed in HEK293 cells and tested for FRET and other relevant properties. The most promising pairs were subsequently used in an attempt to improve the FRET span of the Epac-based cAMP sensor. The results show significant albeit not perfect correlation between performance in the spacer construct and in the Epac sensor. Finally, this strategy enabled us to identify improved sensors both for detection by sensitized emission and by fluorescent lifetime imaging. The present overview should be helpful in guiding development of future FRET sensors.

Introduction

Fluorescence Resonance Energy Transfer (FRET), the radiationless transfer of energy from a donor fluorophore to an acceptor, has become an important tool in cell biology because it allows visualization of protein-protein interactions by light microscopy. FRET is also ideally suited as the read-out for so-called 'sensors', i.e. genetically engineered constructs that report on the conformation or activation state of proteins. We previously reported on CFP-Epac-YFP [1], a sensor construct that consists of part of the cAMP-binding protein Epac1 sandwiched between cyan- and yellow fluorescent proteins. The construct unfolds upon binding of the second messenger cAMP to the Epac moiety and cAMP increases are thus easily followed as a drop in FRET. Whereas the cAMP-induced FRET change in CFP-Epac-YFP is already quite robust (CFP/YFP emission ratio changes by ~30%), the recent introduction of a range of new fluorescent proteins prompted us to further optimize FRET span and other properties of the sensor by systematic variation of the donor- and acceptor fluorescent proteins.

The physical requirements for resonant energy transfer are summarized in 2 equations:

$$E = 1 / (1 + (r/R_0)^6) \quad [2]$$

with

$$R_0 = [Ck^2\eta^{-4}\phi_D\epsilon_A J(\lambda)]^{1/6} \quad [2,3].$$

As can be seen from the inverse 6th power in equation 1, transfer efficiency E depends steeply on the distance (r) between donor and acceptor, relative to the characteristic Förster radius (R_0 , the distance at which transfer is half-maximal for that particular FRET pair). R_0 in turn depends on several factors including κ^2 , which describes the alignment of donor- and acceptor fluorescent dipoles and $J(\lambda)$, which represents the overlap of donor emission spectrum and acceptor excitation spectrum. Furthermore, donor quantum yield (ϕ_D) and acceptor absorption coefficient (ϵ_A) are important determinants of R_0 . Why these considerations are important for FRET constructs will become clear in the following.

FRET changes may be detected by several microscopical techniques [4], the most important of which are sensitized emission (SE) and fluorescence lifetime imaging (FLIM). In SE the donor is excited with light of suitable wavelength, and energy transfer is quantified from the ratio of donor and acceptor emission. Given proper correction for spectral bleedthrough and cross excitation completely quantitative results can be obtained [5]. In contrast, FLIM measurements rely on the donor signal only. In these measurements, the characteristic decay of donor fluorescence upon excitation is followed with sub-nanosecond time resolution. FRET is then apparent as a shortening of the donor decay time, essentially as: $E = 1 - \tau_{D+A} / \tau_D$, where τ_{D+A} and τ_D are the excited-state lifetimes of the donor in the presence and absence of the acceptor, respectively.

Recent years have seen an enormous expansion in the number of available fluorescent proteins (FPs). Almost every month new versions that differ in color, brightness or other characteristics are being added. Many of these have potential for use in FRET pairs. What makes a good

FRET pair for *in vivo* sensors? Keeping with the example of CFP-Epac-YFP, even if we ignore the design considerations for the cAMP-sensing core and focus on just the FPs, the answer is already complicated. Loosely grouped, we can distinguish photophysical-, biological- and detection considerations.

Photophysics

Obviously, for optimal FRET imaging one needs a constellation that maximizes FRET span when cAMP levels change. Thus, Förster radius of the FRET pair, brightness and dipole orientation enter the equation. Other important photophysical properties concern photostability (e.g. bleach rate and photochromism [6]), insensitivity to microenvironmental conditions such as pH or ionic strength [7] and, in case of uncaging experiments, insensitivity to UV light. Finally, FPs must fold efficiently at 37°C and they must rapidly attain their final spectral properties (maturation).

Biology

As sensors must be biologically inert, the fluorescent moieties may not influence cellular function and localization of the tagged protein. For that matter, the size of attached fluorophores must be minimal and their tendency to dimerize or aggregate excluded [8]. In general, longer excitation wavelengths are preferred over near-UV or blue excitation for reasons of phototoxicity [9,10,11], tissue penetration and autofluorescence. Unfortunately, many red FPs start their life as immature green protein which might complicate their use in FRET applications [12]. Obviously, this makes quick maturation an extremely important parameter.

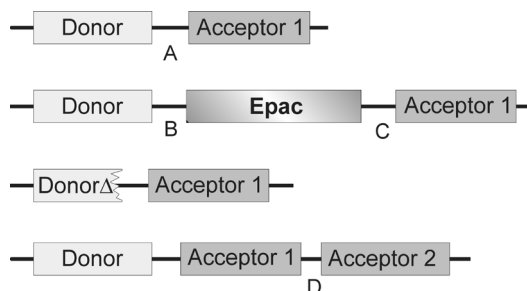
Detection

To complicate things even further, different detection techniques stress different qualities of the FPs. For example, low cross-excitation and high acceptor brightness (i.e., high quantum yield and high absorption) are important for SE determinations, whereas for FLIM detection these factors are less important. In fact, a high acceptor quantum yield may even be unfavorable for FLIM [13]. Conversely, SE is insensitive to multi-exponential decay of the fluorescent donor whereas FLIM analysis is severely hampered when more than one decay constant is present [14]. For another example, the photomultiplier detectors generally used in confocal imaging setups are most sensitive in the blue range of the spectrum, whereas the charge-coupled device (CCD) or avalanche photodiode (APD) detectors found in most FLIM setups favor red colors.

In all, an overwhelming amount of design considerations dazzle the beginner FRET constructionist. In this paper we describe the results of a study aimed at optimizing FRET pairs for use in biosensors like the Epac-based cAMP sensor. Our strategy was to create a wide selection of FP combinations separated by a short spacer, express them in HEK293 cells and test for FRET efficiency and other relevant properties mentioned above. Besides spectral variants, we also included dipole orientation mutants

Figure 1. Schematic overview of the constructs used in this study.

Donor and acceptor fluorophore are connected by a peptide stretch (Linker A: SGLRSRYPFASEL) or by the Epac1(Δ DEP-C.D.) sensor core [1]. Within this stretch, the amino acids PF were replaced by the Epac domain itself, leaving linkers B: SGLRSRY and C: ASEL. For truncated donor constructs (CFP Δ and GFP Δ) GITLGMDELYK was deleted from the donor FPs and SGLRS from the linker. In tandem acceptor constructs the acceptors were separated by a supplementary linker (Linker D: PNFVFLIGAAGILFVSGEL) except for tdHcRed and tdTomato which have distinct linkers, namely NG(GA)₆PVAT) and (GHGTGSTGSGSSGTASSEDNNMA), respectively.



and constructs with duplicate (tandem) acceptors in our study. The most promising pairs were then cloned into the Epac FRET sensor and tested for performance as cAMP indicator in living cells. Based on the above-mentioned design considerations, we identify two of the new sensors, CFP-Epac-cp¹⁷³Venus and GFP Δ -Epac-mRFP, as improved alternatives for SE and FLIM detection, respectively. The results further show overall good correlation between performance of FRET pairs in the linker construct and in the Epac sensor. Therefore, the present overview should be generally helpful in guiding development of new FRET sensors.

Results

Primary considerations and constructs included in the study

Our search for optimal FRET pairs is guided by three primary considerations.

(i) Optimization of FPs in the CFP-YFP part of the spectrum. The original EPAC sensor contains CFP and YFP [15]. These first-generation FPs are extremely popular for FRET, and therefore many laboratories have equipment and filters for detection in this part of the spectrum. Whereas CFP-Epac-YFP competes with the best FRET sensors in displaying robust FRET changes, we observed during extended characterization that this particular acceptor displays some reversible photochromism when excited with UV light (i.e., during cAMP uncaging experiments). Furthermore, the enhanced YFP shows some pH- and Cl⁻-dependence [16,17] Ponsioen, unpublished observations). Therefore, the aim of this part of the study was to optimize CFP-Epac-YFP with respect to FRET span, pH resistance and UV-insensitivity. Constructs included are summarized in Fig. 1-3. For details on constructs and molecular cloning the reader is referred to the Methods section.

(ii) The broad emission spectrum of CFP shows considerable overlap with that of YFP. The often weak sensitized emission signals may thus be obscured in CFP leakthrough of several times its magnitude. For FRET ratiometry this is particularly unfavorable because a rise in FRET will result in opposing signals in the acceptor channel: sensitized emission of the acceptor increases, but this is masked in

part by the concomitant drop in CFP leakthrough. In this part of our study, we therefore aimed to maximize FRET span by minimizing spectral overlap between donor and acceptor emission. We tested a range of red-shifted acceptors for their effectiveness in combination with the CFP donor (Fig. 2-3).

(iii) Finally, we tested a series of constructs that had GFP as fluorescent donor. The rationale is fourfold: first, GFP is almost twice as bright as CFP, and second, its longer optimal excitation wavelength (489 nm versus 432 for CFP) nicely matches 488nm laser lines present in most confocal microscopes. Third, 488-nm excitation is significantly less harsh for the cells. Fourth, GFP is intrinsically better suited for FLIM measurements because its fluorescence decays mono-exponentially, as opposed to the double decay time constants observed for CFP [14,18].

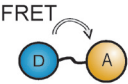
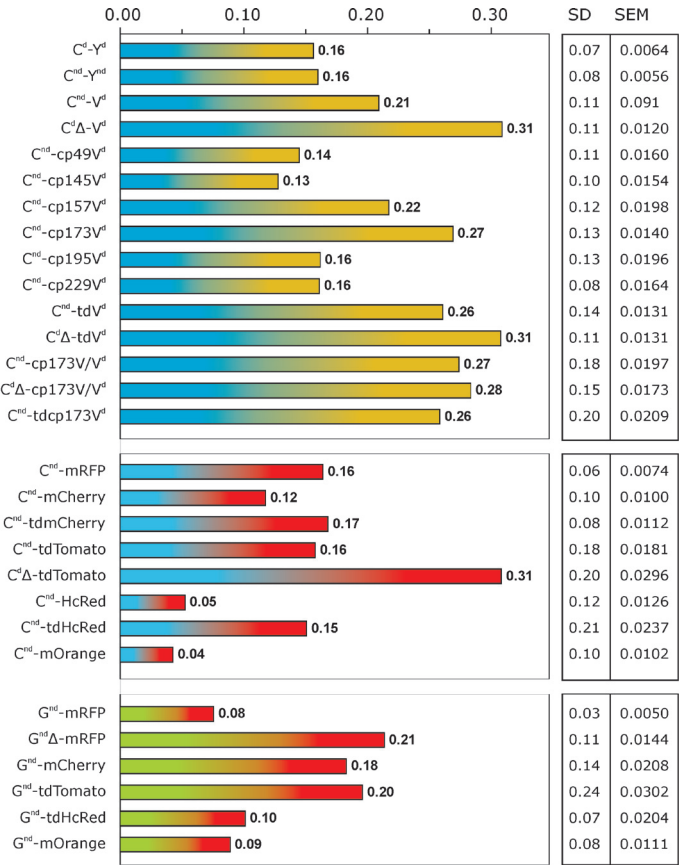
In all cases, initial experiments were performed on constructs consisting of fluorescent donors and acceptors separated by a small flexible spacer (13 amino acids; Fig. 1). FRET efficiency was tested by frequency-domain FLIM on a wide-field microscope (Fig. 2). Selected FP pairs were then inserted in the Epac sensor and tested for performance by FRET ratiometry (Fig. 3), see Methods for details.

CFP-YFP FRET pair analysis

Effect of dimerization

GFP-derivatives such as CFP and YFP have an inherent tendency to form dimers at high concentrations [19] [20]. The presence of two FPs in single-polypeptide FRET constructs multiplies this potential problem. A dimerization-disrupting mutation (A206K, [19]; here denoted as nd for non-dimerizing) was therefore introduced in both FPs in the construct (CFPnd-linker-YFPnd; Fig. 2). Following expression in HEK293 cells, FRET was determined by imaging the lifetime of CFP (see Methods). Both constructs gave essentially similar FRET efficiencies ($E=0.16$). Thus, dimerization does not seem to influence FRET in these constructs although conceivably the short 13-aa linker could restrict freedom of orientation in this construct, thereby masking the possible effect of the mutation.

We next investigated the effects of nd-mutations in the Epac sensor. CFPnd-Epac-YFPnd was prepared by inserting the cAMP-sensitive protein fragment Epac(Δ DEP-C.D.)



FRET Efficiency (E)

Figure 2. FRET in donor-linker-acceptor constructs as detected by frequency-domain FLIM.

The indicated constructs were expressed in HEK293 cells and FRET efficiency *E* was determined as detailed in Material and Methods. Shown are mean (bars), standard deviation (SD) and standard error of the mean (SEM) of 20-400 cells. For further detail, see text.

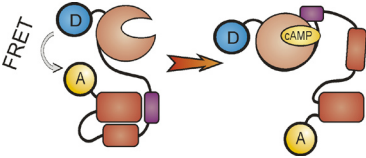
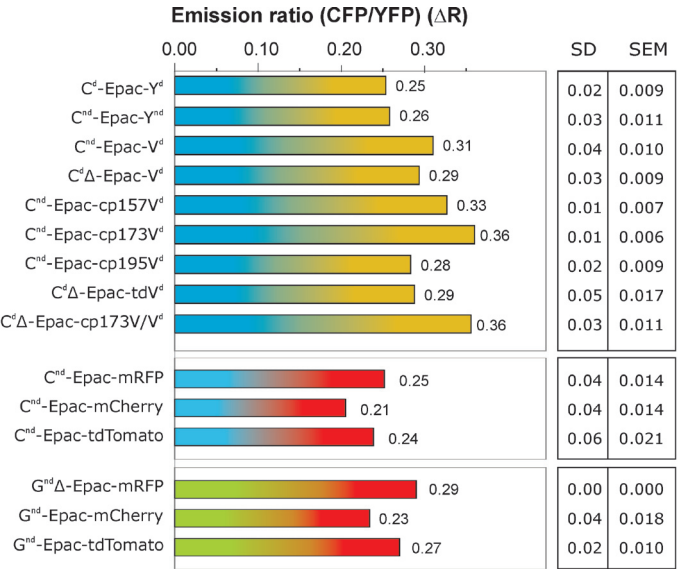


Figure 3. FRET span in cAMP sensors.

The indicated constructs were expressed in HEK-293 cells and assayed for cAMP-induced changes in donor to acceptor ratio on a fluorescence microscope equipped with dual photometers. Donor and acceptor emission were read out simultaneously, and the baseline ratio was set to 1.0 at the onset. FRET span ΔR was determined by calculating the ratio change following addition of IBMX and Forskolin. This raises intracellular cAMP levels maximally and saturates the sensor. For further detail, see the text and Methods.

[1] into the linker region. To directly compare this construct to the parental CFP-Epac(Δ DEP-C.D.)-YFP sensor, both were expressed in HEK293 cells and subjected to ratiometry on a dual-photometer setup (see Methods). Following recording of a baseline, cells were treated with a mix of forskolin (25 μ M) and the phosphodiesterase inhibitor IBMX (100 μ M) to saturate cAMP binding to EPAC [1] (Fig. 4). The FRET span ΔR was then taken to be the relative drop in ratio. Remarkably, the A206K mutations had very little effect on the FRET span ($\Delta R = 0.26$ and 0.25 for A206K (nd) and control (d), respectively; Fig. 3). Thus, in both these configurations dimerization seems to be of little importance, although we anticipate that in other configurations the effects may be significant.

While imaging these cells by confocal microscopy, we noted that in some cell types the constructs displayed a slight tendency to form highly fluorescent aggregates (speckles) in a fraction of the cells (Fig. 5). Surprisingly, the constructs containing nd mutations did not perform better in this respect. We speculate that this may be because although the dimerization constant for individual non-dimerizing FPs is significantly diminished [19], the presence of two FP moieties increases avidity of the constructs, but alternative explanations may also hold true. The insert is also an important factor determining aggregation because in the Epac(Δ DEP-C.D.) containing constructs speckle formation was cured almost completely.

Substitution of YFP for Venus

From studies on YFP-expressing cells we deduced that both pH dependence and UV-induced photochromism in CFP-linker-YFP reside within the YFP moiety. We therefore replaced it by Venus [21], a variant with significantly lower pKa that therefore should be less pH-sensitive. We tested the constructs for UV-induced photochromism by subjecting cells to brief flashes of UV from a Mercury arc lamp, filtered through a Dapi filter cube (Fig. 6). In YFP-containing constructs, this caused an intensity-dependent increase in YFP brightness that amounted to up to 10% in the acceptor channel. In contrast, Venus proved completely re-

sistant to a similar UV treatment. Moreover, Venus also significantly boosted the FRET efficiency of the construct ($E = 0.21$) as compared to YFP ($E = 0.16$; Fig. 2). Note however that Venus lacks the non-dimerizing mutation.

We next tested Venus in the cAMP sensor by preparing CFP-Epac-Venus. Ratiometric determination of cAMP-induced FRET changes (Fig. 3) showed that Venus boosted the FRET span significantly ($\Delta R = 0.31$, compared to $\Delta R = 0.25$ for CFP-Epac-YFP). Thus, both in terms of UV-insensitivity and in FRET span of the sensor Venus proves a much better FRET acceptor.

Varying donor-acceptor distance

Systematic studies into the effect of linker length on FRET have clearly demonstrated the importance of minimizing donor-acceptor distance for FRET efficiency [22,23]. In general, very little can be removed from the N- or C-termini of FPs without adversely influencing their performance [24]. In an attempt to minimize distance, we removed 11 C-terminal aa from CFP, as well as 5 more aa from the linker (CFP Δ ; Fig. 1). Indeed, removal of these 16 aa resulted in significantly improved FRET efficiency ($E = 0.31$, as compared to $E = 0.21$ for the control Venus construct; Fig. 2). Note however that apart from bringing donor and acceptor closer together, this deletion may also diminish rotational freedom.

Whether the CFP Δ deletion is advantageous for any particular FRET sensor construct will of course depend on the tertiary conformation of- and rotational freedom within these chimeras. The equivalent deletion in the Epac-sensor yielded a small drop rather than an increase in FRET span ($\Delta R = 0.29$ versus $\Delta R = 0.31$ for the control construct CFP-Epac-Venus; Fig. 3). Thus, inclusion of the CFP Δ mutation proved of no advantage for the Epac sensor.

Dipole alignment

FRET depends not only on spectral overlap and on donor-acceptor distance but also strongly on fluorescent dipole orientation [3]. We next set out to systematically vary the orientation of Venus to CFP by substituting the YFP

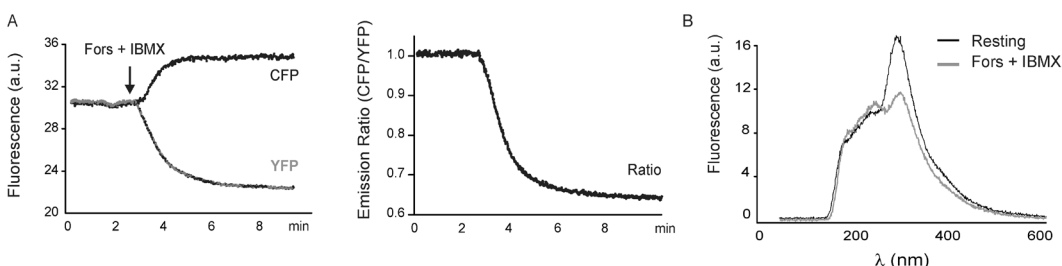


Figure 4. Typical FRET responses in HEK293 cell expressing CFPnd-Epac-cp¹⁷³Venus to stimulation with IBMX/Forskolin.

A) Left : CFP and YFP emission from a single HEK293 cell expressing the improved cAMP sensor were detected at 4 samples per second, following addition of IBMX and Forskolin. Right : the YFP/CFP ratio dropped by almost 35% within minutes. Shown is a typical recording.

B) A single cell spectral fingerprint, obtained before (black) and after (grey) IBMX and Forskolin using a spectrometer. For further detail see Methods.

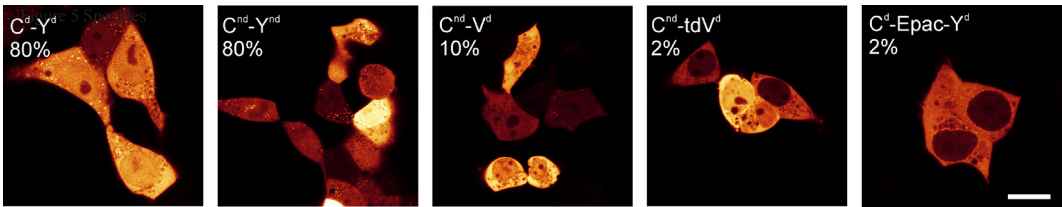


Figure 5. Speckle formation.

HEK293 cells were transfected with indicated constructs and further cultured for 24-36 hours. Shown are confocal sections chosen to maximally visualize any speckles. Note that most speckles go unnoticed by wide-field fluorescence microscopy. Also indicated are estimated percentages of cells showing at least some speckles. Scale bar, 10 μ m.

acceptor with a set of orientation mutants, the so-called circularly permuted (cp) FP versions [25] [26]. In these mutants, tilting of the dipole is achieved by relocating the amino- and carboxyl termini of the acceptor fluorophore to alternative locations on the surface of the FP barrel. All 6 cpVenus versions [27] were inserted in the linker construct. FLIM analysis of cells expressing these constructs showed that only cp¹⁷³Venus insertion resulted in a significant improvement ($E = 0.27$), whereas other permutations gave comparable (cp¹⁵⁷) or even worse (cp^{49/145/195/229}) FRET values than the precursor ($E = 0.21$; Fig. 2).

As dipole orientation in the cAMP sensor may differ from that in the linker construct, a selection of cpVenus mutants were also tested in the Epac construct. To investigate the effect of rotation of the dipole along two axes, we tested cp195, which has its N-terminus on the same side of the barrel as wtVenus, and cp173, the N-terminus of which is on the opposing side of the barrel. FRET spans of cp¹⁹⁵Venus ($\Delta R = 0.33$) and cp¹⁷³Venus ($\Delta R = 0.36$) both exceeded that of the CFP-Epac-Venus construct ($\Delta R = 0.31$; Fig. 3). It was also checked that cp173 retained the favorable UV-insensitivity (Fig. 6). In conclusion, both in the linker construct and in the cAMP sensor inclusion of cp¹⁷³Venus increases performance significantly.

Tandem acceptors

Finally, we studied the effect of presenting the fluorescent donors with duplicate (tandem) acceptors. This may be expected to increase FRET by raising the effective absorption of the acceptor, albeit at the cost of increased construct size. The two fluorophores in a tandem acceptor are

also likely to be oriented differently, thereby easing on the requirement of donor-acceptor dipole alignment. Double acceptors have previously been used to minimize the effects of dimerization caused by FPs [28] [29]. To increase the possibility of presenting acceptors at a favorable angle, three types of tandem acceptors were made: tandem Venus, tandem cp¹⁷³Venus, and a tandem of cp¹⁷³Venus with Venus. As compared to the control construct CFP-linker-Venus ($E = 0.21$), each of the tandem receptors yielded a significantly higher FRET efficiency (range: $E = 0.26$ - 0.27 ; Fig. 2) with CFP-linker-cp¹⁷³Venus-Venus giving the best results. This suggests that the strategy to present the donor with acceptors at different angles may be fruitful to some degree. We therefore also combined the tandems cp¹⁷³Venus-Venus and Venus-Venus with the effective 16aa deletion. The resulting constructs once more showed increased FRET efficiencies ($E = 0.28$ and 0.31 , respectively), although they did not surpass the CFP Δ -linker-Venus construct ($E = 0.31$). In these cases, the increased construct size is thus not balanced by improved FRET efficiency. However, it is worthwhile to note that these tandem acceptor constructs displayed no tendency to form aggregates in cells (Fig. 5).

Finally, we tested a subset of these FRET pairs in the Epac sensor. In these constructs, the 12aa C-terminal deletion of CFP was combined with the tandem acceptors cp¹⁷³Venus-Venus and cp¹⁷³Venus-cp¹⁷³Venus. The resulting cAMP sensors showed cAMP-induced FRET changes of $\Delta R = 0.36$ and $\Delta R = 0.29$, respectively (Fig. 3). Thus, the increased size of the construct is again not compensated by a significant increase in FRET span.

Figure 6. UV-induced photochromism.

Change in ratio of YFP to CFP emission in CFPnd-linker-YFPnd (squares) and CFPnd-linker-Venus^d (triangles) following exposure to UV light for the indicated times. CFPnd-linker-YFPnd as well as free YFP (data not shown) display a dose-dependent increase in emission that maximizes at about 10%, whereas Venus and cp¹⁷³Venus (not shown) are insensitive to UV exposure. See Methods for further detail.

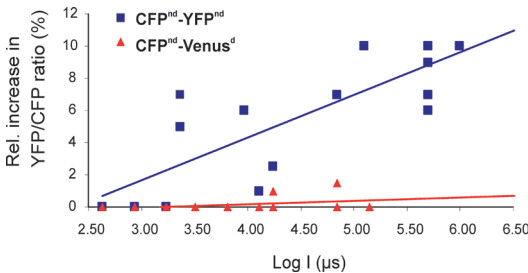
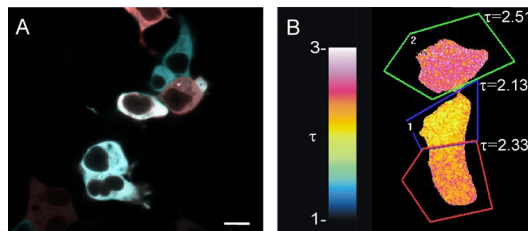


Figure 7. Slow green-to-red maturation of tdTomato and its effects on FRET.

A) Cells expressing CFPnd-EPAC-tdTomato for 24 hr display a spectrum of colors when viewed by eye using an Omega X154 triple-color (CFP-YFP-RFP) cube. For reproduction reasons, the confocal picture shows a mix of green (470-530 nm) and red (570-670) emission to closely match the image visible by eye. In contrast, CFPnd-EPAC-mRFP and CFPnd-EPAC-mCherry show a more homogeneous red color.

B) Cell-to-cell variability in maturation of CFPnd-linker-tdTomato causes significant deviations in the fluorescence decay times detected in the CFP channel, as measured by frequency-domain FLIM. Scale bar, 12 μ m.



Overall, our data show significant, albeit not perfect correlation between performance of FP pairs in the linker construct and in the Epac sensor. In the linker construct, beneficial effects resulted from changing distance (the C-terminal deletion CFP Δ), orientation (cp¹⁷³Venus), as well as from doubling the acceptor.

Importantly, incorporation of Venus in the cAMP sensor cured pH- and UV-sensitivity. Several additional alterations boosted the FRET span, including the cp¹⁷³Venus and the tandem acceptor versions. For future experiments, the former variant (CFP-EPAC-cp¹⁷³Venus) combines a good FRET span with small size. The CFP Δ -Epac-cp¹⁷³Venus-Venus is the favorable candidate if speckle formation is a problem.

CFP-RFP FRET pair analysis

As outlined before, spectral overlap of CFP and YFP complicates quantitative FRET measurements and it diminishes the FRET span of ratiometric sensors. Red-shifted acceptors could cure these drawbacks, but these are also likely to have less spectral overlap with CFP and thus less FRET. Whether this is a curse or a blessing depends on the FRET sensor at hand.

Efficient FRET from CFP to the *Discosoma* RFP (DsRed) [30] has been demonstrated before [31] [32] [33]. However, DsRed is a somewhat cumbersome acceptor because it forms tetramers and shows very slow green-to-red maturation [12]. Therefore we tested some of the newer red FPs, notably mRFP1 [28], mCherry, mOrange [8] and HcRed [34]. Also tested were tandem versions of Tomato ([8]; not tested as monomer), and of HcRed [35] and mCherry.

Monomeric red acceptors

In the linker construct CFP-linker-YFP, substitution of the acceptor moiety by the monomeric red acceptors mRFP1, mCherry and mOrange (Fig. 2) yielded E values of 0.16, 0.12 and 0.04, respectively, as compared to E = 0.16 for the parent construct. The significant FRET in the former two constructs is somewhat surprising because these red-shifted acceptors have much less spectral overlap with the CFP donor than YFP. Note further that the “improved” successors mCherry and mOrange did not outperform their ancestor mRFP1. In fact, of these three red acceptors mOrange is the brightest and spectrally it matches CFP

best [8], but it performs worst as FRET acceptor in the linker constructs. We observed that standard deviations in these experiments were relatively large (Fig. 2 & 3). This may be attributed to the slow green-(em. 430-550)-to-red (em. 550-700) maturation of the red FPs [12]. Immature forms emit in the donor channel thereby biasing the fluorescence lifetimes significantly.

In the Epac sensor, we next replaced YFP with mRFP1 or mCherry, respectively. Both constructs displayed robust cAMP-induced FRET changes ($\Delta R = 0.24$ and 0.21 , respectively; Fig. 3), comparable to the starting material CFP-Epac-YFP ($\Delta R = 0.25$). Whereas donors and acceptors have better spectral separation, this did thus not materialize in improved FRET span in the ratiometric assay, and in fact the constructs were no match for sensors such as CFP-Epac-cp¹⁷³Venus.

Tandem acceptors

We next prepared versions with tandem repeats of the red acceptor FPs. CFP-linker- tdTomato (E = 0.16), CFP-linker- tdmCherry (E = 0.17) and CFP-linker- tdHcRed (E = 0.15) all yielded good FRET efficiency, with the latter two slightly outperforming their respective monomeric acceptor constructs (Fig. 2).

In addition, in these tandem-acceptor constructs the 16 aa deletion in the linker moiety again proved beneficial. Thus, CFP Δ -linker-tdTomato showed a FRET efficiency E = 0.31, as compared to 0.16 for CFP-linker-tdTomato. This constitutes an improvement of 0.15, and it shows that red FPs can function as very efficient acceptors for CFP.

Finally, we tested performance of tdTomato as acceptor for CFP in the Epac sensor. CFP-Epac-tdTomato showed a robust cAMP-induced change in FRET ratio ($\Delta R = 0.24$) but again no performance increase was observed when compared to CFP-Epac-mRFP (Fig. 3). In addition, in this construct the maturation issues of tdTomato were clearly apparent. Simple visual inspection using a CFP/YFP/RFP triple filter cube plainly revealed a wide variety of maturation stages (Fig. 7A). Importantly, the maturation stage of the acceptor also significantly influences E values as determined by FLIM (Fig. 7B).

In conclusion, whereas these experiments show that efficient FRET from CFP to a variety of red FP acceptors is possible, the improved spectral separation did not result in superior ratiometric FRET sensors in our experiments.

GFP-RFP FRET pair analysis

We next set out to assess performance of constructs containing GFP as FRET donor (Fig. 2). As compared to CFP, the better brightness and longer excitation wavelength of GFP (489 nm versus 432 for CFP) should aid in preventing phototoxicity, and its single-exponential decay is well-suited for FLIM measurements.

Initially, we prepared GFPnd-linker-constructs with the red acceptors mRFP and tdHcRed (Fig. 2). FRET efficiencies as detected by FLIM were $E = 0.08$ for GFPnd-linker-mRFP and $E = 0.10$ for GFPnd-linker-tdHcRed. In an attempt to boost these values, we next replicated the C-terminal deletion of the donor that had proved so effective for CFP donors. Indeed, GFPnd Δ -linker-mRFP showed a significantly enhanced E value of 0.21. Thus, the C-terminal deletion appears to be equally effective in GFP.

We further tested the newer variants mCherry, tdTomato and mOrange for performance with the GFP donor. GFPnd-linker-mCherry and GFPnd-linker-tdTomato showed good FRET ($E = 0.18$ and $E = 0.20$, respectively) but the value for GFPnd-linker-mOrange, $E = 0.09$, was disappointing. Thus, again, although theoretically it has a better spectral fit to the donor, mOrange did not live up to its promise in these experiments. On the other hand, the good FRET efficiency observed with mCherry is promising for future development of FLIM sensors, in particular since Time-Correlated Single Photon Counting (TCSPC) experiments confirmed that the fluorescent decay of the GFP donor in this construct is well-described by a single exponent [36].

The most promising of these FRET pairs were subsequently tested in the Epac sensor. GFPnd Δ -Epac-mRFP, GFPnd-Epac-mCherry and GFPnd-Epac-tdTomato all displayed robust cAMP-induced ratio changes in HEK293 cells ($\Delta R = 0.29, 0.23$ and 0.27 , respectively; Fig. 3).

Which of these constructs is recommendable as cAMP sensor in FLIM and ratiometric experiments? GFPnd Δ -Epac-mRFP showed the largest FRET change but mRFP photobleaches more easily than the other two [8]. For FLIM and emission-ratio experiments this should not present a problem, but acceptor bleaching would limit the useful lifespan of this construct in quantitative sensitized emission experiments [5] because for this technique separate acceptor images must be collected. The second-best construct, GFPnd-Epac-tdTomato, shows an almost equally large FRET span and it is very resistant to photobleaching. On the other hand, in addition to its larger size, the tdTomato moiety introduced maturation problems similar to those observed for CFP-Epac-TdTomato and we therefore do not recommend it for future experiments. Consequently, for sensitized emission imaging GFPnd-Epac-mCherry is the best choice because it combines good maturation and bleaching resistance with a decent FRET span. For FLIM and simple ratiometric experiments GFPnd Δ -Epac-mRFP is preferred.

Conclusions and discussion

This study aimed 1) to determine optimal donor – acceptor pairs for FRET and 2) to use this information to improve the performance of our Epac-based cAMP FRET sensor as a model for single-polypeptide type FRET sensors in general. Performances of linker constructs and cAMP sensors were evaluated bearing in mind the different requisites for FLIM and ratiometric detection. Based on our results, we recommend CFPnd-Epac-cp¹⁷³Venus and CFPnd Δ -Epac-cp¹⁷³Venus-Venus (which is somewhat larger, but completely devoid of speckles) as best choices for ratiometric as well as quantitative SE detection. Both show a 44% increase to the already robust FRET span of the originally published sensor [1] and in addition are much more refractory to pH changes and UV illumination. For detection at longer wavelengths and in particular for FLIM measurements, we present GFPnd Δ -Epac-mRFP and GFPnd-Epac-mCherry as preferred choices. For sensitized emission experiments, the latter outperforms the former in having better acceptor photostability at the expense of a somewhat decreased FRET span.

Noting that a wide, but by no means exhaustive range of FPs was included in this study, our further observations should be helpful in guiding future development of FRET sensors. These observations may be summarized as follows.

First, within the linker construct the majority of FP combinations yielded decent to excellent FRET efficiency ($0.1 < E < 0.31$). Spectral match of donors to acceptors varies widely between these constructs and therefore it appears that the amount of spectral overlap is not the prime determinant of FRET efficiency in these constructs. In general, good FRET of a particular FP pair in the linker construct correlated with a good FRET span in the Epac sensor, which should come as no surprise for a sensor that relies on cAMP-induced loss of basal FRET efficiency.

Second, by comparing results from the linker constructs, the general trend is that C-terminal truncation of the donor (the CFP Δ / GFP Δ mutants) has a beneficial effect on FRET efficiency, particularly when introduced in constructs exhibiting moderate basal E values. This improvement is seen irrespective of the acceptor species, but the similar truncation did not result in significant improvement in the Epac sensors, most likely reflecting a different orientation and/or degree of rotational freedom in the latter constructs.

Third, when introduced in constructs with moderate FRET levels, double-acceptor moieties also significantly enhanced E values (compare CFP-linker-Venus to CFP-linker-Venus-Venus and CFP-linker-cp¹⁷³Venus-cp¹⁷³Venus; compare CFP-linker-mCherry to CFP-linker-mCherry-mCherry; compare CFP-linker-HcRed to CFP-linker-HcRed-HcRed). Again, this effect was not seen in constructs that already showed high E values (compare CFP Δ -linker-Venus to CFP Δ -linker-Venus-Venus and CFP Δ -linker-cp¹⁷³Venus-Venus; compare CFP-linker-cp¹⁷³Venus to CFP-linker-cp¹⁷³Venus-cp¹⁷³Venus). In the cAMP sensor, no beneficial effects were observed (Fig. 3). However, irrespective of the effects of this modification on E values, it is worthwhile to include the tandem acceptors in the FP

repertoire because it cured the tendency to form aggregates displayed by some constructs and can substitute for extensive testing of circularly permuted variants.

Fourth, the range of CFP-redFP constructs demonstrates that various red FPs are good acceptors for CFP. These constructs were prepared in order to boost FRET span by having better spectral separation between donors and acceptors, but in our Epac sensor this theoretical advantage did not materialize.

Fifth, we observed clear positive effects of incorporation of circular permutation mutants of Venus in the linker constructs and Epac sensors. This would seem to indicate that dipole alignment is all-important, but putting our observations in the context of published data may raise some doubt on this interpretation. Both in the linker- and the Epac constructs substitution of Venus by cp¹⁷³Venus yielded the largest improvement. This would indicate that in both these constructs N- and C-terminus of the inserts are in comparable locations and that rotational freedom of the FPs is similar. This is unlikely, given the nature of the very different inserts. Even more remarkable is that cp¹⁷³Venus also is the favorite acceptor in other single chain FRET sensors published to date [27,37,38]. Thus, the widespread preference for cp¹⁷³Venus acceptors raises the suspicion that other qualities, rather than the dipole orientation, cause cp¹⁷³Venus to be such a good acceptor.

Throughout this study we have emphasized that besides FRET efficiency or -span other qualities are equally important for FRET sensors. Slow green-to-red maturation terminated some of the otherwise most promising FRET pairs, e.g. those with tdTomato as acceptor. Similarly, the tendency to form bright speckles (aggregates; vesicles) is an undesirable property that was found in some constructs. We found that introduction of non-dimerizing mutations (A206K) in the FPs had little effect on speckle formation. However, the insert was of significant influence, as the Epac constructs generally were less hampered by speckles. Orientation of donors and acceptors within the linker construct conceivably is such that it allows both FPs of one polypeptide to interact with those of another, effectively increasing the avidity. Importantly, introduction of tandem acceptors cured this flaw. We speculate that this is due to internal dimerization of the tandem acceptors but this was not investigated systematically.

As the double exponential fluorescence decay of CFP is inherently poorly suited for FLIM we based our FLIM sensors on GFP. However, the red acceptors for GFP all exhibit some degree of green-to-red maturation. In practice, mRFP and mCherry which mature relatively fast (< 1 hr and <15 min, respectively) performed well in FLIM sensors. For future development, one must also consider another promising approach that was recently reported. Here, the donor is combined with a dark (i.e., non-fluorescent) acceptor termed REACH, for Resonance Energy Accepting Chromoprotein [13]. REACH is a YFP variant that may be combined with GFP in the Epac sensors, allowing lifetime measurements of this single-exponential donor and obviating the need for narrow spectral filtering of GFP emission.

Materials & Methods

Constructs

The previously published construct CFPnd-Epac1(ΔDEP-C.D.)-YFPnd [1] was used to generate all described constructs. Linker constructs were obtained by excising the Epac1(ΔDEP-C.D.) domain using EcoRV/NheI and replacing it with a linker (GATATCCTTTTGCTAGC, restriction sites EcoRV-NheI are underlined), yielding a 13aa linker (SGLRSRYPFASEL) between the two fluorophores. CFP^d and YFP^d were obtained by introducing the pointmutation K206A [19] in CFPnd and YFPnd using QuikChange (Stratagene). Donor fluorophore truncations (GFPΔ/CFPΔ) were generated by PCR and lacked the C-terminal part of the fluorophore (GITLGMDELYK) as well as the N-terminal part of the linker (SGLRS). Using HindIII/EcoRV, these PCR products were used to replace CFP. Venus and cpVenus FPs were generated by PCR using the following templates: Venus [21] and cpVenus (from Ycam 3.2, 3.3, 3.6, 3.7 and 3.9; [27]). Using NheI/EcoRI these PCR products were used to replace YFP. Tandem versions were generated by digesting EcoRI/Xba and inserting an additional linker (AATTTTGTCTTCCTGATCGCGCCGAGGAATACTCTTCGTATCTAGAGTGAATTCCTAA, newly situated restriction sites Xba-EcoRI are underlined). This linker was digested with XbaI/EcoRI and an additional fluorophore was inserted using NheI/EcoRI, resulting in two acceptor fluorophores separated by a 19aa (PNFVFLIGAAGILFVSGEL) linker. For RFP replacements an identical strategy was followed, however, using additional templates mRFP1 [28], mCherry, tdTomato, mOrange [8], HcRed [34] and tdHcRed [35].

Materials

IBMX, Forskolin and Ionomycin were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA).

Cell culture and transfection

HEK293 cells were seeded on 25-mm glass coverslips in six-well plates in DMEM supplemented with 10% FCS and antibiotics. Constructs were transfected using calcium phosphate precipitate, at ~0.8 μg DNA per well.

Microscopy

Cells in bicarbonate-buffered saline (containing, in mM, 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 glucose, 23 NaHCO₃, 10 HEPES), pH 7.2, kept under 5% CO₂, at 37°C were routinely inspected using a Leica DM-6000 inverted microscope with 63x, 1.32 NA oil immersion objective. For the experiments described in Fig. 7A, a triple band fluorescence filter cube (CFP-YFP-RFP), type X154 (Omega) was used. Confocal images were collected using a TCS-SP5 confocal scanhead attached to the DM6000 microscope (Leica, Mannheim, Germany). Agonists and inhibitors were added from concentrated stocks.

For spectral fingerprinting, an Ocean Optics Type USB2000 spectrometer (Dunedin, Florida, USA) was fitted to an inverted microscope. The emission of single transfected cells was captured (integration time, 1-8 s).

Excitation was from a arc lamp equipped with a monochromator.

To assess UV sensitivity (Fig. 6), cells were followed using time-lapse imaging. After establishing a baseline, cells were subjected to a brief (1 s = 1,000,000 μ s) flash of UV illumination using a DAPI filter cube and an EL-6000 light source (Leica) at full power. For lower exposures, the exposure was attenuated by different combinations of shutter times and neutral density filters, and expressed as equivalent shutter time in μ s. Flash-induced change in YFP/CFP ratio was plotted versus equivalent exposure time.

Dynamic FRET monitoring

Cells on coverslips were placed on an inverted NIKON Diaphot microscope and excited at 425 nm (CFP) or 470 nm (GFP). Emission was detected using optical filters as follows: (band-pass filters) CFP, 470 $\pm$ 20nm; GFP, 510 $\pm$ 30 nm; YFP, 530 $\pm$ 25nm; (long pass filter) orange and red FP variants, 590 LP filter. Data from donor and acceptor were collected simultaneously, digitized and FRET was expressed as ratio of donor to acceptor signals. The FRET value of the baseline was set to 1.0 at the onset of the experiments. Cells were then stimulated with 25 μ M Forskolin and 100 μ M IBMX to maximally raise the intracellular cAMP concentration. Changes are expressed as percent deviation from the initial value of 1.0. Data are from 6–20 cells per experiment. Note that for FRET pairs with widely different spectral properties the FRET span can not be quantitatively compared because of inevitable differences in filters and detector sensitivity.

Fluorescence lifetime imaging

FLIM experiments were performed on an inverted Leica DM-IRE2 microscope using a Lambert Instruments (Leutینگewolde, the Netherlands) frequency domain lifetime attachment, controlled by the vendors LI-FLIM software. CFP & GFP were excited with $\sim$ 4mW of light from a 442nm and a 470nm LED, respectively, modulated at 40MHz and emission was collected at 450–490 and 500–540 nm, respectively, using an intensified CCD camera. Lifetimes were referenced to a 1 μ M solution of Rhodamine-G6 in saline that was set at 4.11 ns lifetime. The measured lifetimes (calculated from phase differences) of CFP and GFP in the absence of acceptors were 2.7 ns. FRET efficiency E was calculated as $E = 1 - (\text{measured lifetime of FRET pair}) / (\text{measured lifetime of donor})$. FRET efficiencies are means of 20–400 cells per construct.

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Chapter 9

8-pCPT-2'-O-Me-cAMP-AM: an improved Epac-selective cAMP analogue

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8-pCPT-2'-O-Me-cAMP-AM: an improved Epac-selective cAMP analogue

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Abstract

Epac1 and Epac2 are guanine nucleotide exchange factors for the small G-proteins Rap1 and Rap2. Epac is activated by direct binding of cAMP. The cAMP analogue 8-pCPT-2'-O-Me-cAMP (alias: 007) can activate Epac, but not protein kinase A and is widely used as a selective activator of Epac. To increase membrane permeability and thus to improve bioavailability we have synthesised the AM-ester of 8-pCPT-2'-O-Me-cAMP. The resultant 8-pCPT-2'-O-Me-cAMP-AM (007-AM) activates Epac much more rapidly and at much lower extracellular concentrations than 007, as demonstrated *in vivo* by the use of an Epac1-based FRET sensor. In line with this, we observed efficient activation of Rap1 and efficient induction of Rap1-mediated biological effects under cell culture conditions upon application of 007-AM.

Introduction

Cyclic adenosine monophosphate (cAMP) is a common second messenger involved in the regulation of any different cellular processes through the activation of protein kinase A (PKA), exchange protein directly activated by cAMP (Epac) and cyclicnucleotide-regulated ion channels [1]. Adenylyl cyclases are responsible for catalysing the formation of cAMP from ATP. Levels of cAMP can be raised in cells in response to a large variety of extracellular stimuli, which act via receptors coupled to heterotrimeric G proteins, which stimulate the activity of adenylyl cyclase. In addition, cAMP levels are controlled by phosphodiesterases (PDE), which catalyse the degradation of cAMP to AMP. In cells, cAMP levels can be artificially elevated by forskolin, which activates adenylyl cyclase directly. Furthermore, cAMP levels can be raised by inhibiting PDEs. These approaches are commonly used in tissue culture experiments, but, by generating cAMP, they do not discriminate between the various target proteins that are activated. Alternatively, membrane-permeable cAMP analogues, which selectively interact with particular receptor proteins, can be applied. For example, signalling pathways activated by PKA and Epac can be distinguished by using 6-Bnz-cAMP and 8-pCPT-2'-O-Me-cAMP, respectively [2]. The latter, 8-pCPT-2'-O-Me-cAMP will from here on be referred to by its alias: 007.

Epac is a guanine nucleotide exchange factor for the small G protein Rap. Rap cycles between a signalling-inactive GDPbound state and a signalling-active GTP-bound state. cAMP-activated Epac catalyses the exchange of Rap-bound GDP for GTP. Epac and Rap function in a number of different cellular processes including insulin secretion, inhibition of cell scattering, neurotransmitter release and cAMP-induced barrier function in endothelial cells [3].

Even though 007 has become a widely used tool in Epac-related research, its biological application is limited by its low membrane permeability, caused by the negatively charged phosphate. However, the negatively charged singly bonded oxygen on the phosphate group can be masked by labile esters. Such a precursor is expected to enter the cell efficiently, where the ester is hy-

drolysed either directly by water or by cellular esterases to liberate the active compound [4]. We therefore synthesised 007-AM from 007 by attaching an acetoxymethyl (AM) ester to one of the oxygens of the phosphate. We could demonstrate that this analogue can be used efficiently in cell culture systems and activates Epac at lower doses and with increased efficiency.

Results and Discussion

We synthesised 8-pCPT-2'-O-Me-cAMP-AM (or 007-AM) from 8-pCPT-2'-O-Me-cAMP (007), whereby acetoxymethyl bromide was used as a donor for the AM group. The product that was obtained had a purity exceeding 97% and consisted of a mixture of the equatorial and the axial isomers of the ester (described in Material and Methods, see Fig. 1). Even though the isomers could be resolved by repetitive analytical HPLC runs, efficient separation on a preparative scale was not possible. Orange peel acetylsterase and esterase from porcine liver cleaved the equatorial isomer about five times more efficiently than the axial isomer within minutes (data not shown). The pharmacokinetics of both isomers are thus expected to be similar, justifying the application of a mixture of both isomers to cells. In any case, the isomeric ratio of an individual synthesis can be easily quality controlled by ^{31}P NMR.

To compare the efficiency of 007-AM and 007 in activating Epac1 *in vivo*, an Epac1-based fluorescence resonance energy transfer (FRET) probe was used. In this assay, activation of Epac1 by the binding of cAMP to the Epac1-FRET probe is measured as a reduction in the FRET signal [5]. A431 cells transfected with the FRET probe were stimulated with 007 or 007-AM (Fig. 2). Stimulation of cells with 100 μM 007 resulted in a decrease of the FRET signal that was approximately one order of magnitude slower than the decrease obtained upon stimulation with 1 μM 007-AM. Furthermore, activation of Epac1 following stimulation with 100 mM 007 could be further enhanced by the addition of forskolin, whereas 1 mM 007-AM induced maximal activity of Epac1 under the given

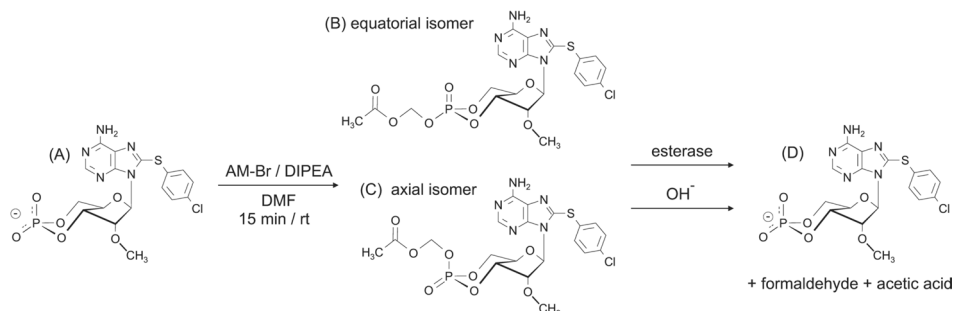


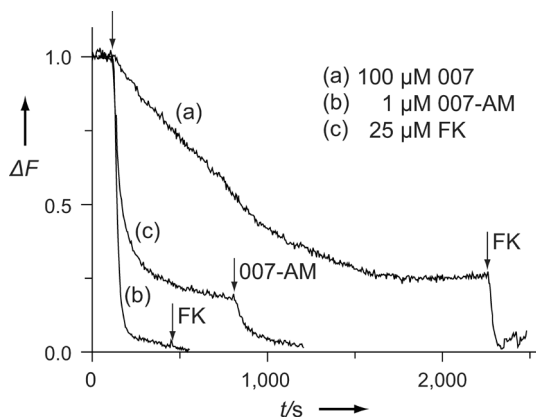
Figure 1. Synthesis and application of 8-pCPT-2'-O-Me-cAMP-AM.

A) 8-pCPT-2'-O-Me-cAMP (007) is converted into 8-pCPT-2'-O-Me-cAMP-AM (007-AM) with acetoxymethyl bromide as a donor of the acetoxymethyl group in the presence of *N,N*-diisopropylethylamine in *N,N*-dimethylformamide. A mixture of the equatorial (B) and axial (C) isomers of the ester is obtained. D) 8-pCPT-2'-O-Me-cAMP-AM is cleaved by esterases to 8-pCPT-2'-O-Me-cAMP and the by-products formaldehyde and acetic acid.

Figure 2.

Epac1 activation in A431 cells.

A431 cells were transfected with the Epac1-FRET sensor and stimulated with 100 μM 8-pCPT-2'-O-Me-cAMP (007) or 1 μM 8-pCPT-2'-O-Me-cAMP-AM (007-AM) followed by 25 μM forskolin (FK) or stimulated by 25 μM forskolin followed by 1 μM 007-AM. The FRET signal was monitored over time and plotted as normalised change in FRET (ΔF); all traces are representative for at least 10 independent experiments.



conditions. The activation of Epac by 007-AM occurs within one minute after application. This is comparable with the kinetics of forskolin-induced Epac activation, and thus 007-AM mimics the "natural" response time upon adenylyl cyclase activation.

The activity of endogenous Epac can be monitored by isolating selectively Rap-GTP from cell lysates. Primary human umbilical vein endothelial cells (HUVEC) were stimulated with different concentrations of 007 and 007-AM (Fig. 3A). Partial activation of Rap was induced by 10 μM 007, and full activation of the G protein was stimulated by 100 μM 007. In contrast, treatment of the cells with just 0.1 μM 007-AM was sufficient to induce full Rap activation.

To determine if 007-AM could efficiently stimulate Rap-dependent processes, biological assays were carried out. In HUVECs, Rap induces a tightening of cell-cell junctions that can be measured as an increase in the electrical

resistance of a cell layer grown on an electrode. 007-AM induced junction tightening at much lower concentrations than 007 (Fig. 3B). Similarly, 007-AM induced adhesion of Jurkat-Epac1 cells to fibronectin more efficiently than 007 (Fig. 3C).

Thus, 007-AM induces Epac1 and Rap1 activation at concentrations that are two to three orders of magnitude lower than those required of the parent compound. In HUVEC cells, sustained Rap1 activation was observed after application of only 0.01 μM 007-AM (Fig. 3A). This concentration is far below the AC_{50} of 007 for Epac1, which was determined to be 1.8 μM *in vitro* [6]. This indicates that 007 accumulates in the cell after the cleavage reaction, which is in accordance with results obtained for other cyclic nucleotide-AM esters [7]. Indeed, 007-AM seems to enter cells much more quickly than 007, as shown by the more rapid activation of the Epac1-FRET sensor by 007-AM in comparison to 007 (Fig. 2).

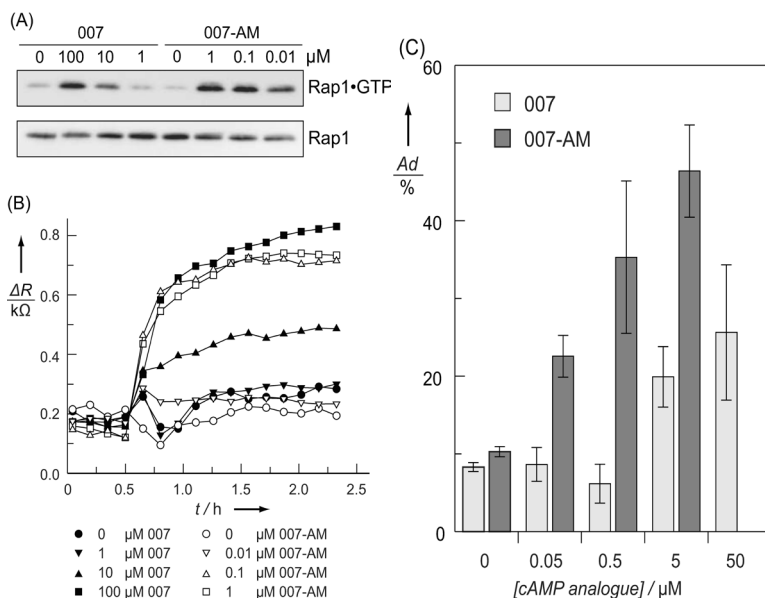
Figure 3.

007-AM acts efficiently on cells.

A) HUVEC cells were stimulated with different concentrations of 8-pCPT-2'-O-Me-cAMP (007) or 8-pCPT-2'-O-Me-cAMP-AM (007-AM), and the levels of Rap1-GTP were measured.

B) HUVECs grown on an electrode were stimulated with different concentrations of 007 or 007-AM as indicated. The change in transendothelial electrical resistance (DR) was measured in real time.

C) Jurkat-Epac1 cells were stimulated with different concentrations of 007-cAMP or 007-AM and seeded on fibronectin coated plates. Adhesion (Ad) was measured after 15 min.



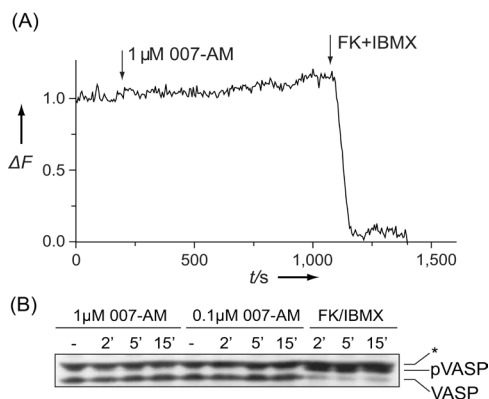


Figure 4.
Selectivity of 007-AM.

A) Ovarc3 cells were transfected with the PKA-FRET probe and stimulated successively with 1 μ M 007-AM and a combination of 25 μ M forskolin (FK) and 100 μ M 3-isobutyl-1-methylxanthine (IBMX). The FRET signal was monitored over time and is plotted here as normalised change in FRET (ΔF). The trace is a representative of three independent experiments.

B) Ovarc3 cells were stimulated with 1 mM or 0.1 μ M of 007-AM or with a combination of 50 μ M forskolin and 500 μ M IBMX (PDE inhibitor), and phosphorylation of VASP was monitored by a phosphorylation-induced mobility shift after the indicated time points. The band marked by asterisk corresponds to unspecific background staining.

The biological selectivity of 007 is probably its greatest benefit for biological research. However, the application of 007-AM causes the accumulation of 007 in cells. To exclude the possibility that a putative high intracellular concentration of 007 caused side effects, 007-AM was applied to cells expressing a PKA-based FRET sensor (Fig. 4A). Upon application of 1 μ M 007-AM, no change in the PKA-FRET signal was observed. In addition, the phosphorylation status of a PKA substrate, vasodilatorstimulated phosphoprotein (VASP), was monitored as a biological measure of the activity of the enzyme. Whereas a clear band shift of VASP is observed after stimulation with forskolin, no effect is observed with 1 μ M 007-AM (Fig. 4B).

To summarise, we have described the synthesis of 007-AM, a precursor that selectively activates Epac and is more efficiently delivered into cells than its parent compound. 007-AM works with high efficiency under biological conditions by stimulating Epac and by activating Rap1-dependent processes, as demonstrated in two model systems. We found that 007-AM is stable for at least two hours in aqueous solution, but in general less stable in sera containing esterases. In addition, it is possible that toxic side effects might be caused by the by-products of the esterase reaction. However, related prodrugs, such as pivampicillin or HesperaTM, both of which release a carboxylic acid and formaldehyde, or EnalaprilTM or acetylsalicylic acid, both of which release acetic acid, are in clinical use arguing for the general safety of AM-ester-based precursors[8]. 007-AM is thus expected to become a powerful tool in Epac- and PKA-related research.

Material & Methods

Synthesis of 8-(4-Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate, acetoxymethyl ester (8-pCPT-2'-O-Me-cAMP-AM)

Synthesis was performed with some minor modifications as described. [9,10] 360 μ mol 8-pCPT-2'-O-Me-cAMP (007), diisopropylethylammonium salt, were suspended in 1000 μ l DMF. After addition of 1800 μ mol (180 μ l; 5 equivalents) acetoxymethyl bromide and 1080 μ mol (185 μ l; 3 equivalents) diisopropylethylamine, the reac-

tion mixture was stirred at ambient temperature for 30 minutes. Progress of AM-ester formation was monitored by analytical HPLC with 40% acetonitrile as eluent. The reaction was stopped by evaporation of all volatile components in a speed-vac centrifuge under reduced pressure with oil pump vacuum, re-dissolved in 0.8 ml DMF, and purified by preparative HPLC using 40% acetonitrile as eluent. The product-containing fractions were collected and evaporated. 110 μ moles of 8-pCPT-2'-O-Me-cAMP-AM (007-AM) were isolated as mixture of axial and equatorial isomers with a purity of > 97% (yield: 30.6%). Formula: C₂₀H₂₁ClN₅O₈PS (MW: 557.9) ESI-MS pos. mode: m/z 558 (M+H)⁺, m/z 486 (M-AM+H)⁺, neg. mode: m/z 556 (M-H)⁻, m/z 484 (M-AM-H)⁻; UV-VIS (pH 7.0) λ max 282 nm (ϵ = 16000). All chromatographic experiments were performed at ambient temperature. The analytical HPLC-system consisted of a L 6200 pump, a L 4000 variable wavelength UV-detector, and a D 2500 GPC integrator (all Merck-Hitachi, Germany). The stationary phase was KromasilTM (Eka Nobel, Sweden) C 8-100, 10 μ m, in a 250 x 4.6 mm stainless steel column. Preparative HPLC was accomplished with KromasilTM C 8-100, 5 μ m material in a 250 x 16 mm stainless steel column. Mass spectra were obtained with an Esquire LC spectrometer (Bruker, Germany) in the ESI-MS mode with 50 % isopropanol / 49.9 % water / 0.1 % formic acid as matrix. UV-spectra were recorded with a Helios β -spectrometer (Spectronic Unicam, United Kingdom) in aqueous phosphate buffer, pH 7.0. All reagents were of analytical grade or the best grade available from commercial suppliers.

Application of 007-AM

Stock solutions of 007-AM were prepared in absolute DMSO at concentrations up to 10 μ M and 007-AM was applied directly to the cell culture dish. When required, 007-AM were pre-diluted in PBS buffer immediate prior to the application. Any exposure of 007-AM to medium, especially to serum, prior to the application was avoided, since esterases, which are present in particular in serum, cause rapid degradation of the AM-ester.

Dynamic FRET monitoring

Dynamic FRET monitoring was done as described

previously[5]. Briefly, cells grown on coverslips and transfected with the FRET probe were placed on an inverted NIKON microscope and excited at 425 nm. Emission of CFP and YFP was detected simultaneously through 470 ± 20 and 530 ± 25 nm band-pass filters. Data were digitized and FRET was expressed as ratio of YFP to CFP signals, the value of which was set to 1.0 at the onset of the experiments. Activation of Epac1 was followed using the FRET probe CFP-Epac1-YFP, and activation of PKA using the PKA regulatory subunit type II fused to CFP and the PKA catalytic subunit fused to YFP [5,11]. Binding of cAMP to both probes induces loss of FRET.

Rap1 activation assay

Rap1 activation assays were performed as previously described [12]. Briefly, equal amounts of cell lysates were incubated with the Ras-binding domain (RBD) of Ral-GDS fused to glutathione S-transferase. This fusion protein was pre-coupled to glutathione-agarose beads to specifically pull down the GTP-bound form of Rap. Samples were analysed by western blotting using Rap1 antibodies (Santa Cruz Biotechnology, USA) and the phosphorylation state of VASP was visualised as band shift upon western blotting with an antibody against VASP (BD Transduction Laboratories, USA).

Transendothelial electrical resistance measurements

Primary human umbilical vein endothelial cells (HUVEC) were seeded at 7×10^4 cells per well (0.8 cm^2) on fibronectin coated electrode arrays and grown to confluency (Applied BioPhysics Inc., Troy, USA). Measurements of transendothelial electrical resistance were performed in real time at 400Hz, 37°C , 5% CO_2 , using an electrical cell-substrate impedance sensing system (ECIS; Applied BioPhysics Inc., Troy, USA). [13] For comparison between different samples, the resistance is plotted as a difference to the basal level of resistance.

Adhesion assay

Jurkat-Epac1 cells transiently transfected with pCMV-luciferase were harvested, resuspended in TSM buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM CaCl_2 , 2 mM MgCl_2 , 0.5% BSA) and gently rotated for 1 hour at 37°C to allow recovery [14]. 96-well Nunc Maxisorp plates (Corning, USA) were coated overnight at 4°C with human serum-purified Fibronectin (5 mg/ml in PBS) and blocked for 1 hour with 1% BSA in TSM. Subsequently, cells were added to the coated wells in the absence or presence of 007 or 007-AM at the indicated concentrations and allowed to adhere for 45 minutes at 37°C . Non-adherent cells were removed with warmed TSM and adherent cells were lysed at 4°C in luciferase lysis buffer (15% glycerol, 25 mM Tris-phosphate pH 7.8, 1% Triton X-100, 8 mM MgCl_2 , 1mM DTT). Luciferase activity was determined using a luminometer (Lumat LB9507). Unseeded cells were lysed separately to determine luciferase counts in the total input. Specific adhesion was determined (counts in cells bound / counts in total input $\times 100$) and plotted directly. Error bars represent standard deviation within each experiment.

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Summary

Samenvatting

Summary

Cells respond to extracellular cues via receptor signaling. In this manner, cellular behavior is under strict control of hormones, growth factors or neurotransmitters. Binding of a ligand to its cognate receptor triggers a cascade of intracellular signaling events. In general, these cascades branch into different series of molecular conversions leading to, for instance, activation of transcription factors, regulation of metabolic processes or changes in cell morphology.

To regulate such 'organized complexity', molecular interactions in the cell are **specific** and **regulated**. Specificity is guaranteed by recognition domains and regulation of interactions or catalytic conversions means that they can be 'switched on or off'. The latter can be realized, for instance, by a conformational change that alters the accessibility of an interaction domain.

Intriguingly, most signaling components function in multiple signaling cascades. For example, the >800 members of the G Protein-Coupled Receptor (GPCR) family trigger a vast variety of cellular responses via a relatively limited set of signaling components. This is achieved by **compartmentalization** of the signaling pathways. For example, GPCRs and multiple downstream signaling components are often clustered into signaling complexes by so-called scaffolding proteins. Such spatial restriction facilitates the coupling of the receptor to its effectors, while it leaves other signaling compartments unaffected. Thus, compartmentalization confers **specificity** to signaling cascades. In addition, such spatial organization can direct signaling activity towards the appropriate subcellular location, depending on the involved process.

Signaling complexes are dynamically regulated, i.e. they can be assembled in response to extracellular signals. The dynamic targeting of a signaling molecule to a specialized compartment becomes manifest as a stimulus-induced **translocation**. In this thesis, we studied the translocations of two proteins: that of chloride intracellular channel 4 (**CLIC4**) and that of exchange protein directly activated by cAMP 1 (**Epac1**). Central in these studies were the microscopical techniques that allowed visualization of these translocations with maximal spatiotemporal resolution, most prominently via confocal imaging and measurement of fluorescence resonance energy transfer (FRET).

Chapter 1 presents a general introduction into the phenomenon of translocations as they occur in cell signaling. The focus lies on signaling cascades activated by GPCRs since these are most relevant to the experimental work described in this thesis. Furthermore, this chapter introduces the microscopical techniques that have been employed to reveal and study 'our' translocations.

In **chapter 2**, we show that CLIC4 translocation is selectively observed in response to $G(\alpha)_{13}$ -activating GPCR agonists and requires active RhoA. Contrasting with the 'membrane insertion model', the proposed activation mechanism for CLIC proteins [1], we show that CLIC4 translocation does not precede channel formation in the plasma membrane. Instead, the translocation anchor is the ligand-activated receptor complex itself. Although we await final proof, our data strongly suggest that recruited CLIC4 plays a regulatory role in the signal transfer from the stimulated GPCR to RhoA activation. In support of this hypothesis, CLIC4 has been reported to interact with the scaffold proteins AKAP350 [2] and 14-3-3 [3]. AKAP-Lbc, a family member of AKAP350, serves both as a GPCR scaffold and as a guanine exchange factor (GEF) coupling $G(\alpha)_{12}$ to RhoA [4] and is, furthermore, under the regulation of 14-3-3 [5]. Therefore, we hypothesize that CLIC4 translocates to a signaling compartment containing the activated receptor, $G(\alpha)_{13}$ and an AKAP scaffold with GEF activity towards RhoA. In this model, the spatial regulation of CLIC4 will undoubtedly add to the switch-like regulation of RhoA activity.

Chapters 3 and 4 describe two distinct plasma membrane translocations of Epac1. The cAMP effector Epac1 is a GEF for the GTPase Rap. Interestingly, Rap activity at the plasma membrane induces cell adhesion. The two types of Epac1 translocation are triggered by cAMP binding and by phosphorylation of Ezrin/Radixin/Moesin (ERM) proteins, respectively. Binding of cAMP induces a conformational change, that not only elicits GEF activity by Epac1 [6], but also releases an affinity for the plasma membrane. As a consequence, Epac1 becomes uniformly distributed along the plasma membrane upon elevated cAMP levels. In contrast, recruitment of Epac1 by ERM proteins is regulated on the level of the ERM proteins. When phosphorylated, they directly bind Epac1, regardless of Epac1's activation state, and thereby recruit the GEF to **asymmetrical** regions of the plasma membrane.

Since both translocation mechanisms could be specifically disabled by mutagenesis, we were able to assess their relative importances for the ability of Epac1 to induce Rap-dependent cell adhesion. Interestingly, we found that the two targeting mechanisms cooperate in this process. We conclude that the targeting mechanisms dynamically compartmentalize cAMP-Epac1-Rap signaling at the plasma membrane, thereby enhancing the efficiency and specificity of action.

Besides Epac1 signaling, also the classical cAMP effector PKA is extensively compartmentalized. To date, more than 50 A kinase anchoring proteins (AKAPs) have been identified. Among the clustered signaling components are PKA, PKA substrates as well as the enzymes that synthesize cAMP (adenylyl cyclases) and degrade cAMP (phosphodiesterases) [7,8]. Clearly, to study compartmentalization of the second

messenger cAMP, measurement with spatiotemporal resolution is required. For this, gene-encoded sensors based on FRET are indispensable. **Chapter 5** describes the development of CFP-Epac-YFP, an Epac-based FRET-sensor for cAMP. The cAMP-induced conformational change is registered as a loss of FRET between the terminally fused fluorophores. This novel cAMP sensor has several advantages over the previously reported PKA-based cAMP sensor. Most importantly, it measures over an increased dynamic range of cAMP concentrations and displays larger changes in FRET. Furthermore, for the study of cAMP compartmentalization, the single polypeptide CFP-Epac-YFP can be targeted to specific subcellular compartments [9], which would be highly complicated (if not impossible) for the two constructs that constitute the PKA-based sensor.

In **chapter 6**, we imaged cAMP with spatiotemporal resolution. Not subcellularly though, but 'intercellularly'. By mixing cells with and without glucagon receptors, we could monitor intercellular transfer of cAMP through gap junctions after glucagon stimulation. From this, we learned that gap junctional exchange of cAMP occurs over the entire range of physiological cAMP concentrations and that phosphodiesterases are the main determinant in controlling the amount of communicated cAMP.

The studies presented in **Chapters 7, 8 and 9** are not directly related to the theme of compartmentalization, but have evolved from the previous chapters. **Chapter 7** shows that the plasma membrane lipid PI(4,5) P_2 directly regulates communication via connexin43-based gap junctions. The trigger for gap junctional closure is the phospholipase C $\beta 3$ (PLC $\beta 3$)-mediated hydrolysis of PI(4,5) P_2 itself rather than the concomitantly produced second messengers diacylglycerol (DAG) or inositoltriphosphate (IP $_3$). **Chapter 8** describes the optimization of the fluorophore pairs used in the Epac-based cAMP sensor. Comparative analyses revealed optimal donor-acceptor pairs depending on the method employed to measure FRET. For the frequently applied ratiometric FRET assays, optimal properties are displayed by the CFP-cp¹⁷³Venus pair. Finally, in **Chapter 9** the Epac1-based FRET sensors were employed to characterize the new Epac1-specific cAMP analogue 8-pCPT-2'-O-Me-cAMP-AM (also known as 007-AM). The hydrophobic acetoxymethyl (AM) ester was attached to render the compound more membrane-permeable than the parent compound 007. Once 007-AM passes the plasma membrane, the AM-group will be cleaved off by intracellular esterases and as a consequence the trapped 007 rapidly accumulates in the cytoplasm. **Indeed, our sensors demonstrated the accelerated kinetics and increased efficiency of Epac1 activation in comparison to 007.**

In conclusion, heaving read this thesis one may appreciate that the use of sophisticated imaging techniques and the development of gene-encoded sensors go hand in hand with expanding insights in the compartmentalization of cell signaling.

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Samenvatting

Cellen zijn eenheden van leven, maar functioneren niet alleen. Binnen een organisme houden ze elkaar voortdurend op de hoogte van wat ze moeten doen: beïnvloeden en beïnvloed worden. Voor het overbrengen van dergelijke boodschappen bestaat een uitgebreid repertoire aan signaalmoleculen, waaronder hormonen, groeifactoren en neurotransmitters. De ontvangers van deze moleculaire vorm van communicatie zijn de receptoren, die door de buitenmembraan van de cel steken. Als een hormoon aan de poorten rammelt, vertaalt de bijbehorende hormoonreceptor het signaal naar het inwendige van de cel, alwaar het stokje wordt doorgegeven door een opeenvolging van verschillende signaalmoleculen. Hun gezamenlijke activiteit mondt uit in een respons die specifiek is voor dat hormoon, bijvoorbeeld het aflezen van bepaalde genen, een vormverandering of een aanpassing van metabolische processen. Een dergelijke lawine van zich vertakkende activatiestappen wordt een signaleringscascade genoemd. Om dit alles in goede banen te leiden, bevatten signaalmoleculen **specifieke** domeinen voor de herkenning van hun interactiepartners. Verder wordt signaaloverdracht nauw **gereguleerd** door weer andere signaleringspaden. Alles bij elkaar vormen de signaleringspaden dus een complex weggennet van chemische omzettingen.

Waar op dit weggennet een signaalmolecuul actief is, is zeer bepalend voor de effecten die het sorteert. Signaleringscascades worden sterk gecompartmentaliseerd binnen de cel: meerdere opeenvolgende schakels van een signaleringsketen worden bijeengebracht in daartoe bestemde complexen. Die maken efficiënte signaaloverdracht mogelijk en garanderen bovendien dat deze overdracht niet 'doorlekt' naar andere compartimenten. Dit zijn dus de knooppunten op het weggennet van de celsignaling. Dergelijke compartimentalisatie verklaart dat signaleringsmoleculen betrokken kunnen zijn bij een verrassend groot aantal verschillende cellulaire functies. Zo effectueren de (ruim) 800 receptoren van de familie der *G protein-coupled receptors* (GPCRs) vele verschillende responsen middels een relatief beperkt arsenaal aan intracellulaire boodschappermoleculen.

Op instigatie van extracellulaire signalen wordt het weggennet regelmatig verlegd. In een mum van tijd kan een nieuw knooppunt zodoende een nieuw type signaaloverdracht mogelijk maken. Dergelijke dynamische herschikkingen van signaleringscomponenten manifesteren zich als **translocaties**. Voortschrijdend inzicht in de ruimtelijke organisatie en de veranderlijkheid van signaleringscascaden vereist microscopische technieken, die deze zichtbaar maken met ruimtelijke en tijdsresolutie. De techniek bij uitstek is fluorescentiemicroscopie, waarbij de gedragingen van een specifiek eiwit nauwgezet kunnen worden gevolgd, nadat

het met behulp van genetische modificatie is voorzien van een fluorescent 'label'. Met gebruikmaking van fluorescentiemicroscopie zijn wij gedurende de totstandkoming van dit proefschrift op translocaties gestuit van twee eiwitten: CLIC4 en Epac1.

Hoofdstuk 1 van dit proefschrift geeft een algemene introductie over translocaties in celsignaling, met speciale aandacht voor cascades die worden geactiveerd door GPCRs. Dit schept tegelijk het kader voor beknopte omschrijvingen van de translocaties die zijn gevonden gedurende deze promotie. Daarnaast behandelt het de microscopische technieken, die deze onderzoeken mogelijk hebben gemaakt.

In **hoofdstuk 2** beschrijven we hoe CLIC4, dat staat voor *chloride intracellular channel 4*, naar de plasma membraan translokeert als de cel wordt gestimuleerd met de GPCR-agonist *lysophosphatidic acid* (LPA). Van de kleine CLIC eiwitten wordt verondersteld dat ze chloridekanalen kunnen vormen als ze worden aangezet tot membraaninsertie. Het is echter belangrijk te onderstrepen dat hierover nog controverse bestaat. Onze interesse werd gewekt door het feit dat LPA zowel een chloridestroom over de plasmamembraan veroorzaakt als de plasma membraan-translocatie van CLIC4. We hebben echter moeten vaststellen dat tussen deze twee gebeurtenissen géén causaal verband bestaat. Wel hebben we kunnen identificeren welke signaleringscomponenten bij CLIC4 translokatie betrokken zijn en naar welke bestemming het voert. CLIC4 translokatie wordt selectief veroorzaakt door stimulatie van $G(\alpha)_{13}$ -gekoppelde receptoren en vereist de actieve vorm van RhoA, een belangrijk signaalmolecuul dat verderop in de $G(\alpha)_{13}$ -signaleringscascade functioneert. Translokerend CLIC4 vervoegt zich bij het signaleringscomplex rondom de zojuist geactiveerde receptor. Al moet definitief bewijs nog geleverd worden, onze experimenten suggereren dat CLIC4 een regulerende rol speelt in de signaaloverdracht van $G(\alpha)_{13}$ naar RhoA. De LPA-geïnduceerde translokatie van CLIC4 naar het geactiveerde LPA-receptor-complex zou kunnen bijdragen aan de markering van het knooppunt waar $G(\alpha)_{13}$ de 'afslag RhoA' kiest. Interessant detail: deze hypothese impliceert dat CLIC4 een functie zou hebben die in het geheel niet aansluit bij de naam van het eiwit.

Hoofdstukken 3 en 4 beschrijven twee verschillende mechanismen waarop Epac1, een *exchange protein directly activated by cyclic AMP*, naar de plasma membraan translokeert. Deze effector van het veel voorkomende boodschappermolecuul cAMP dient als activator van Rap en beïnvloedt daarmee de adhesie van cellen. Het 'ene' type translokatie wordt geïnitieerd door de directe binding van cAMP. De daarmee gepaard gaande conformationele verandering van

Epac1 maakt een affiniteit voor de plasma membraan los en als gevolg hiervan verdeelt Epac1 zich uniform over de plasma membraan. Het 'andere' type translokatie vindt plaats wanneer zogenaamde ERM eiwitten (naar de drie familieleden *ezrin*, *radixin* en *moesin*) van conformatie veranderen. Na openvouwing kunnen ze Epac1 aan zich binden en zodoende recruterend naar het subcompartiment van de plasma membraan waar zij zich verschansen.

De twee translocaties worden dus door verschillende signalen in gang gezet en resulteren in andere ruimtelijke verdelingen van Epac1. Wat ze wél gemeen hebben is dat ze beide een belangrijke bijdrage leveren aan de functie van Epac1 om Rap-afhankelijke celadhesie te stimuleren. We concluderen dan ook, dat de twee translokatiemechanismen samenwerken in het aanleggen van een compartiment, waarin efficiënte signaaloverdracht mogelijk wordt gemaakt van cAMP, via Epac1, naar Rap.

In **hoofdstuk 5** laten we zien dat een cAMP sensor kan worden gemaakt door een blauw en een geel fluorescent eiwit (CFP en YFP) te fuseren aan de twee uiteinden van Epac1. Er vindt dan *fluorescence resonance energy transfer* (FRET) plaats tussen de twee fluorescente uiteinden, maar deze neemt af als cAMP Epac1 van conformatie doet veranderen. Door FRET binnen CFP-Epac1-YFP te meten kan dus worden gevisualiseerd hoe cAMP concentraties in de cel veranderen in tijd en plaats. Op grond van grondige karakterisaties hebben we kunnen aantonen dat deze nieuwe cAMP sensor meerdere voordelen biedt ten opzichte van een eerder gepubliceerde FRET sensor voor cAMP.

Deze FRET-gebaseerde methoden zijn onontbeerlijk in het bestuderen van de ruimtelijke organisatie van cAMP signalering. Immers, net als Epac1 vertoont ook de klassieke cAMP effector *protein kinase A* (PKA) een hoge mate van compartimentalisatie; er zijn meer dan 50 verschillende ankereiwitten bekend, die PKA-signaleringscomplexen bijeenbrengen op specifieke subcellulaire organellen. Kortom, de toekomstige toepassing van cAMP sensors belooft veel bij te dragen aan het begrip van de ruimtelijke aspecten van cAMP signalering.

In **hoofdstuk 6** zetten we de nieuwe cAMP sensor in om uitwisseling van cAMP *tussen* cellen te volgen met ruimtelijke en tijdsresolutie. cAMP uitwisseling vindt plaats via zogenaamde *gap junctions*, kanaaltjes die de inwendige milieus van naburige cellen koppelen. Dat een cAMP molecuul in principe door een gap junction past was al bekend, maar hier laten wij voor het eerst zien dat cAMP-uitwisseling ook werkelijk plaatsvindt over de gehele spanne van fysiologische cAMP niveaus. Daarnaast vinden we dat de activiteit van fosfodiesterases, enzymen die cAMP afbreken, bepalend is voor de hoeveelheid uitgewisseld cAMP.

Hoofdstukken 7, 8 en 9 relateren niet direct aan het thema compartimentalisatie, maar zijn vertakkingen van de onderzoeken in de voorafgaande hoofdstukken. **Hoofdstuk 7** laat zien dat de plasma membraan lipide $PI(4,5)P_2$ een regulerende rol heeft in het open en dicht gaan van gap junctions, die zijn opgebouwd uit connexin43. Sluiting van deze gap junctions is het directe gevolg van $PI(4,5)P_2$ hydrolyse door fosfolipase $\beta 3$ (PLC $\beta 3$), maar staat los van de daaruit voortkomende signaalmoleculen diacylglycerol (DAG) of inositoltrifosfaat (IP $_3$).

In **hoofdstuk 8** optimaliseren we het fluorescente FRET paar van de Epac-gebaseerde cAMP sensor. Na uitgebreid vergelijk hebben we voor verschillende FRET methodieken een optimale combinatie van kleurvarianten kunnen aanwijzen. De veelvuldig toegepaste radiometrische FRET analyse werkt bijvoorbeeld het best met de combinatie CFP-Epac1-cp¹⁷³Venus.

In **hoofdstuk 9** worden de Epac-gebaseerde sensors ingezet ter karakterisatie van de verwante stoffen 8-pCPT-2'-O-Me-cAMP (alias 007) en 8-pCPT-2'-O-Me-cAMP-AM (alias 007-AM). Beiden zijn cAMP analoga, die specifiek Epac activeren, maar PKA ongemoeid laten. Beiden zijn daarom van groot belang voor het ontrafelen van cAMP-afhankelijke celsignalering. De hydrophobe acetoxymethyl (AM-) groep is toegevoegd om 007 makkelijker de plasma membraan te laten passeren. Eenmaal over de membraan wordt de AM-groep verwijderd door intracellulaire enzymen, waarna het overgebleven 007 binnenin de cel gevangen blijft. Inderdaad kon deze versnelde ophoping duidelijk zichtbaar gemaakt worden met gebruik van de fluorescente Epac-gebaseerde sensors.

Over het geheel laat dit proefschrift zien hoe geavanceerde microscopische technieken en de ontwikkeling van genetisch gecodeerde fluorescente sensors de weg bereiden voor toenemend begrip van gecompartmentaliseerde celsignalering.

Curriculum Vitae

List of Publications

Curriculum Vitae

Bas Ponsioen werd geboren op 21 oktober 1977 te Woerden. In 1996 behaalde hij zijn gymnasiumdiploma op het St. Ignatius Gymnasium te Amsterdam en begon hij zijn studie Medische Biologie aan de Universiteit van Amsterdam (UvA). Een eerste wetenschappelijke stage werd verricht op de afdeling Experimentele Dierkunde van de UvA o.l.v. prof.dr. Wytse Wadman. Gedurende deze stage werd Current Source Density analyse toegepast om GABA_A-gemedieerde, inhibitoire chloridestromen te localiseren in het hippocampale netwerk van muizenhersenen. De wens om kennis te maken met fluorescentiemicroscopie voerde naar de groep van dr. K. Jalink op het Nederlands Kanker Instituut (NKI). Gedurende deze tweede stage onderzocht hij de rol van de fosfolipide PI(4,5)P₂

in de nucleus. Na enig oponthoud wegens assistentie van werkcolleges op de biologiefaculteit van de UvA en zitting in het bestuur van het Nederlands Studenten Orkest studeerde hij af in de zomer van 2002. De afstudeerscriptie bij prof.dr. Lopes da Silva (UvA) behandelde de physiologie van trilhaarcellen in het gehoororgaan als basis voor psychofysische fenomenen. Hij was vervolgens kort betrokken bij een samenwerking tussen het instituut voor Atomaire en MOleculaire Physica (AMOLF) en het NKI, met als inzet om beeldvormende massaspectrometrie te realiseren voor biologische toepassingen. Echter, in mei 2003 maakte hij een verse start als promovendus onder begeleiding van prof.dr. W.H. Moolenaar en dr. K. Jalink (NKI), resulterend dit proefschrift.

List of Publications

B.Ponsioen*, J.Zhao*, J.Riedl, F.Zwartkruis, G.van der Krogt, M.Zaccolo, W.H.Moolenaar, J.L.Bos and K.Jalink.
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Spatiotemporal regulation of CLIC4, a novel player in the $G\alpha_{13}$ -RhoA signaling pathway.

Submitted

M.Gloerich*, **B.Ponsioen***, M.Vliem, J.Zhao, Z.Zhang, L.Price, L.Ritsma, H.Rehmann, F.Zwartkruis, K.Jalink and J.L.Bos.

ERM proteins recruit Epac1 to the plasma membrane and facilitate Epac1-induced adhesion.

Manuscript in preparation

L. van Zeijl, **B.Ponsioen**, J.Janssen, K.Jalink and W.H.Moolenaar.

Dual regulation of Cx43 gap junctional communication by GPCRs: a key role for ubiquitin ligase Nedd4.

Manuscript in preparation

B.Ponsioen

Eiwitten voor de goede orde.

De bètacanon. Wat Iedereen moet weten van de natuurwetenschappen (2008) p68-71

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