Close the Gap : a study on the regulation of Connexin43 gap junctional communication

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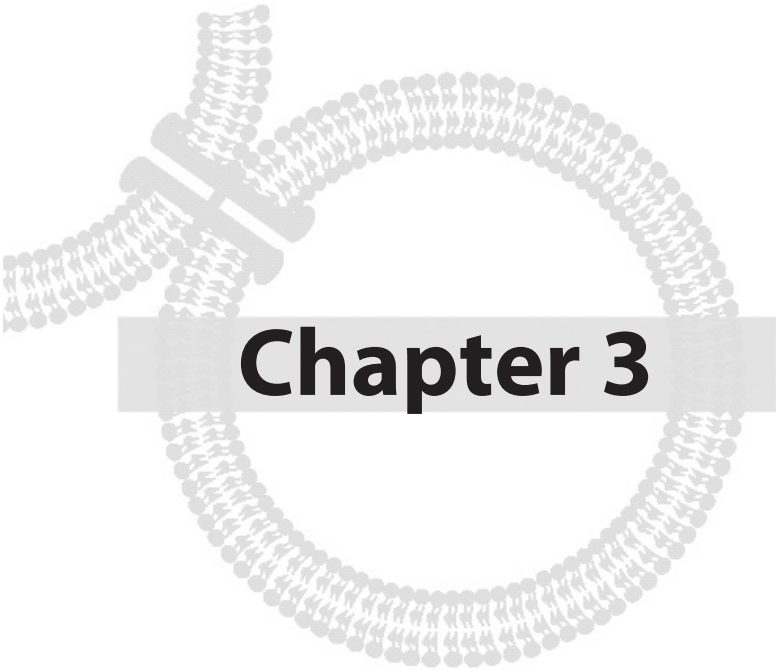

Chapter 3

Inhibition of Connexin43 gap junctional communication by G(q)-coupled receptors: a critical role for residue Tyr265

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Abstract

Cx43-based gap junctional communication (GJC) is rapidly but transiently inhibited by G(q)-coupled receptor agonists, such as endothelin, thrombin and angiotensin. Previously, we showed that inhibition of GJC depends on depletion of phosphatidylinositol 4,5-bisphosphate. However, the molecular details of Cx43 channel regulation remain poorly understood. Previous studies have implicated phosphorylation of the Cx43 C-terminal tail on residues S368, Y247 and Y265, and/or Cx43 internalisation via an Y286-based sorting motif, as key regulatory events. Here, we have analysed the importance of the Cx43 C-terminal domain in channel regulation by studying Cx43 mutants, expressed in connexin-depleted Rat-1 fibroblasts. We find that the Cx43 C-terminal tail (aa 263-382) is essential for channel regulation by GPCR agonists. Furthermore, we find that Cx43 point mutants Y247F, Y265F, Y267F, Y286F and S368A all mediate normal cell-cell communication. After stimulation with GPCR agonist endothelin for 8 minutes, however, the Cx43-Y265F mutant gap junctions are open, in contrast to endogenous Cx43 and the other Cx43 mutants. Although residue Y265 is an established substrate for activated Src, we find that Cx43 tyrosine phosphorylation was not increased in agonist-stimulated cells, while broad-spectrum tyrosine kinase and Src inhibitors had no effect on Cx43 channel closure. Y265 is part of a putative sorting motif, but further mutation of this motif did not affect Cx43 channel closure. Our results indicate that residue Y265 is essential for Cx43 channel disruption by G(q)-coupled receptors, and suggest that Y265 may play a structural role rather than serving as a phosphate acceptor.

Introduction

Gap junctions are groups of intercellular channels that mediate the diffusion of small molecules. The building blocks of gap junctions are proteins called connexins. Six connexins together form a hemichannel, or connexon, that docks with a connexon from the adjacent cell to form a functional channel[1-3]. Gap junctional communication (GJC) is essential for tissue homeostasis and loss or misregulation of GJC is associated with various human pathologies, including cancer[4-14].

In the heart, gap junctional communication between cardiomyocytes ensures efficient electrical coupling and hence the synchronous propagation of action potentials. Connexin43 (Cx43) is the major connexin family member in the myocardium. Misregulation of Cx43 expression, localisation and channel gating may lead to severe cardiac dysfunction[15-19]. In mice, loss of Cx43 in the heart results in lethal ventricular arrhythmias[15].

Previously, our group and others showed that Cx43-based GJC can be inhibited by certain G protein coupled receptor (GPCR) agonists and bioactive lipids[20-27]. Cardiac ischemia may be caused by GPCR agonists angiotensin and endothelins, which are very potent vasoconstrictors[28,29]. Inhibition of gap junctional communication may protect the heart during pathological conditions by limiting the spreading of damage[30,31]. On the downside, however, closure of Cx43 gap junctions may be the cause of arrhythmia and it has been suggested that genetic defects in Cx43 may underlie a predisposition to cardiac arrhythmia[17,19,32,33].

Connexin43 (Cx43) is the most abundant and best studied connexin. Previously, we reported that Cx43 based gap junctional communication can be inhibited by certain G protein coupled receptor (GPCR) agonists[23]. We recently reported that depletion of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) from the plasma membrane is necessary and sufficient for inhibition of communication by GPCRs[20]. However, many details of how GJC is regulated are still unclear.

Various studies suggest that the intracellular C-terminal tail of Cx43 is essential for regulation of Cx43 gap junctions. Many studies have addressed the role of phosphorylation in Cx43 channel regulation; all known phosphorylation sites of Cx43 are located in the C-terminal tail[34-37]. Especially residues Y247 and Y265 as putative targets for tyrosine kinase Src have received their share of attention in the field. In summary, cells expressing v-Src or constitutively active c-Src have been reported to be poor communicators, which is associated with massive tyrosine phosphorylation of Cx43. Mutational analysis has shown that Y265 and, to a lesser extent, Y247 are the Src target residues. Tyrosine phosphorylation may affect GJC by modification of the channel properties and/or by influencing Cx43 turnover and localisation[23,34,38-47]. To date, no other tyrosine kinases that may phosphorylate Cx43 have been identified. It is unclear whether tyrosine phosphorylation of Cx43 plays a role in the inhibition of GJC by GPCRs.

In addition, phosphorylation of residue S368 by protein kinase C (PKC) has been associated with inhibition of communication, possibly by triggering the internalisation and/or promoting the ubiquitination of Cx43[48-54].

Here, we focus on the role of the C-terminal tail of Cx43 in the inhibition of GJC by Gq coupled receptor signalling. We developed a novel system to study Cx43 mutants in their native environment. We knocked down Cx43 in Rat-1 fibroblasts by stable expression of Cx43 shRNA constructs. The Cx43 knockdown cells were used as a reconstitution system for both wildtype and mutant versions of Cx43. Cx43 mutants were compared with wildtype Cx43 for functionality, localisation and response to GPCR agonists.

We show that residue Y265 is essential for regulation of Cx43 based GJC by GPCR signaling. However, tyrosine phosphorylation of Cx43 is not involved in inhibition of GJC. Instead, we propose that Y265 plays a structural role in the multi-protein

complex that regulates Cx43 based gap junctional communication downstream of GPCRs.

Results

Mutational analysis of Cx43 channels in their native cellular context

In our initial attempts to analyze Cx43 mutants, we stably expressed Cx43 wildtype and mutant versions in communication-deficient A431 and HeLa carcinoma cells, and subsequently examined cell-cell communication in response to GPCR stimulation. While stable expression of Cx43 did result in efficient cell-cell coupling, the regulation of Cx43 channel function by receptor agonists was impaired[20,43], suggesting that one or more essential components of the Cx43 gap junction complex, such as for example ZO-1, is lacking or mislocalised.

We therefore sought to analyze Cx43 mutants in their native cellular context, namely Rat-1 fibroblasts, which express Cx43 as the only functional connexin. To this end, we stably knocked down endogenous Cx43 using shRNA, and subsequently introduced shRNA-resistant mutant versions of Cx43 containing two silent mutations in the shRNA target sequence (Fig. 1A). Knockdown of Cx43 reduced its expression by >95% and resulted in a complete loss of intercellular communication[20,56] (Fig. 1B,C), while the Cx43-binding partner ZO-1 retained its submembranous localisation[20]. Expression of shRNA-resistant Cx43 in Cx43 knockdown cells fully restored not only basal cell-cell communication but also its regulation by Gq-coupled receptor agonists, such as endothelin and thrombin (Fig. 1C). It is further seen that reconstituted Cx43 shows an immunostaining pattern similar to endogenous Cx43 (Fig. 1D). Thus, reconstitution of Cx43 in Cx43 knockdown cells provides a suitable system for mutational analysis of Cx43 channel function and regulation.

Critical role for the C-terminal tail of Cx43

The C-terminal tail of Cx43 is thought to act as a regulatory domain. Its integrity is required for disruption of communication induced by non-physiological stimuli such as intracellular acidification and the (v-)Src tyrosine kinase. According to the 'ball-and-chain' model of Cx43 channel regulation, the cytosolic tail of Cx43 is modified to block the channel and thereby inhibit cell-cell communication[40,46]. We expressed a truncated version of Cx43 that lacks the C-terminal 120 residues (aa 263-382; Cx43-Δ263). When expressed in Cx43-knockdown cells, Cx43-Δ263 forms functional channels. However, endothelin stimulation failed to inhibit cell-cell coupling (Fig. 1C), implying that the Cx43 C-terminal tail is essential for GPCR agonists to disrupt cell-cell communication. We also find that this Cx43 mutant is not distributed in the characteristic punctate fashion, but rather forms larger plaques (Fig. 1D), similar to what has been reported for GFP-tagged Cx43[60]. These results

confirm that the Cx43 tail is essential for the regulation of channel function and contributes to the proper formation of junctional plaques.

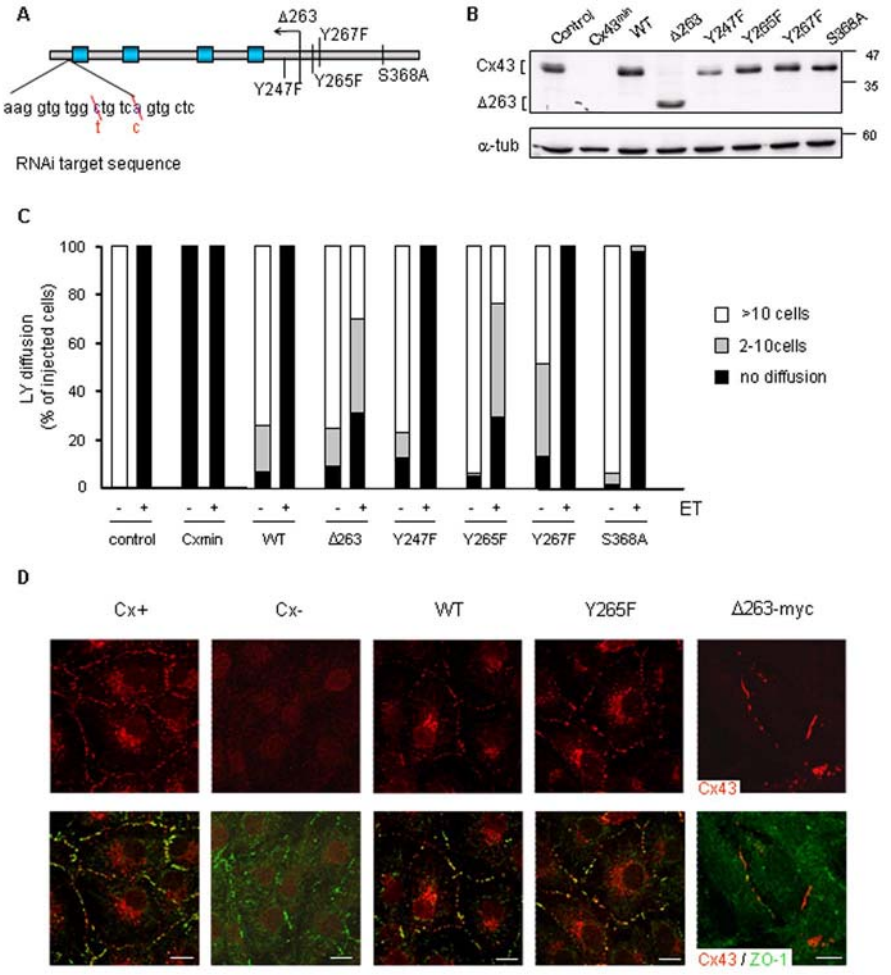

Figure 1: Expression and communication of Cx43 mutants in Cx43 knockdown cells
A: Schematic representation of the Cx43 mutants. Two silent mutations were introduced in the shRNA target sequence to prevent targeting by the Cx43 shRNA construct.
B: Adenoviral expression of Cx43 mutants in Cxmin cells compared to Cx43 expression in control and Cxmin cells. Immunoblot of Cx43 expression (top), α-tubulin is used as loading control (bottom)
C: Bar diagram showing the percentage of cells from which micro-injected Lucifer Yellow spreads to neighbouring cells 8 minutes after endothelin stimulation (n > 80 for each data point).
D: Confocal pictures of Cx43 (red), co-stained with ZO-1 (green) in control cells, Cxmin cells and Cxmin cells reconstituted with Cx43 wild type (WT), Y265F or Δ263-myc. top: Cx43/myc staining, bottom: merge of Cx43/myc and ZO-1 staining. Scale bars: 5 μm.

Residue Ser368 is not involved in inhibition of GJC

Having shown the importance of the Cx43 C-terminal tail, we next created various point mutations in that domain. Phorbol ester-activated PKC phosphorylates Cx43 on residue S368 and thereby decreases gap junctional communication in various cell types[49,51]. We therefore expressed Cx43 mutant S368A in our Cx43 knockdown Rat-1 cells. Cx43(S368A)-based gap junctions show normal intercellular coupling and behave like wild-type junctions in that communication was rapidly and almost completely abolished by endothelin (Fig. 1C). We conclude that PKC target residue S368 is dispensable for the inhibition of communication induced by Gq-coupled receptor agonists. This is consistent with our previous observation that the PKC pathway plays no role in GPCR-induced Cx43 channel inhibition.

Tyrosine265 is essential for GJC inhibition

Earlier studies have shown that Cx43 is tyrosine phosphorylated by oncogenic v-Src, which correlates with permanent inhibition of GJC in v-Src-transformed cells[38,39,44], while activation of c-Src has been implicated in transient inhibition of Cx43-based GJC in normal cells[23,43]. In transfected COS7 cells, activated c-Src phosphorylates Cx43 on residue Y265, but not on Y267. Residue Y247 may serve as a secondary Src target, albeit to a lesser extent than Y265[43,44,47].

We expressed Cx43 mutants Y247F, Y265F and Y267F in the Cx43-depleted Rat-1 cells. All mutants formed functional gap junctions (Fig. 1C), showing an immunostaining pattern similar to that of wt Cx43 (Fig. 1D and data not shown). When the cells were exposed to endothelin, Y247F and Y267F behaved like wildtype Cx43 in that channel function was disrupted upon receptor stimulation. In contrast, in cells expressing mutant Cx43 Y265F GJC was intact when monitored 8 minutes after endothelin stimulation (Fig. 1C). This implicates an essential role for residue Y265.

No tyrosine phosphorylation of Cx43

Since Y265 has been identified as a phosphate acceptor[44], tyrosine phosphorylation of Cx43 in response to GPCR agonists is an obvious explanation for our results. To investigate this, we immunoprecipitated Cx43 from Rat-1 cells after endothelin stimulation, and immunoblotted for phosphotyrosine. As a positive control, we included Cx43 from v-Src-expressing Rat-1 cells, which show massive tyrosine phosphorylation of Cx43. Equal amounts of Cx43 were immunoprecipitated from normal and v-Src-transformed cells. We observed a low level of basal tyrosine phosphorylation of Cx43. However, there was no increase in tyrosine phosphorylated Cx43 in response to endothelin stimulation, while Cx43 from v-Src-transformed cells was strongly phosphorylated (Fig. 2A). As shown in figure 2B, basal tyrosine phosphorylation of Cx43 decreases upon serum starvation. Treatment of the cells with general kinase inhibitor K252a (50 μM) for 30 minutes completely

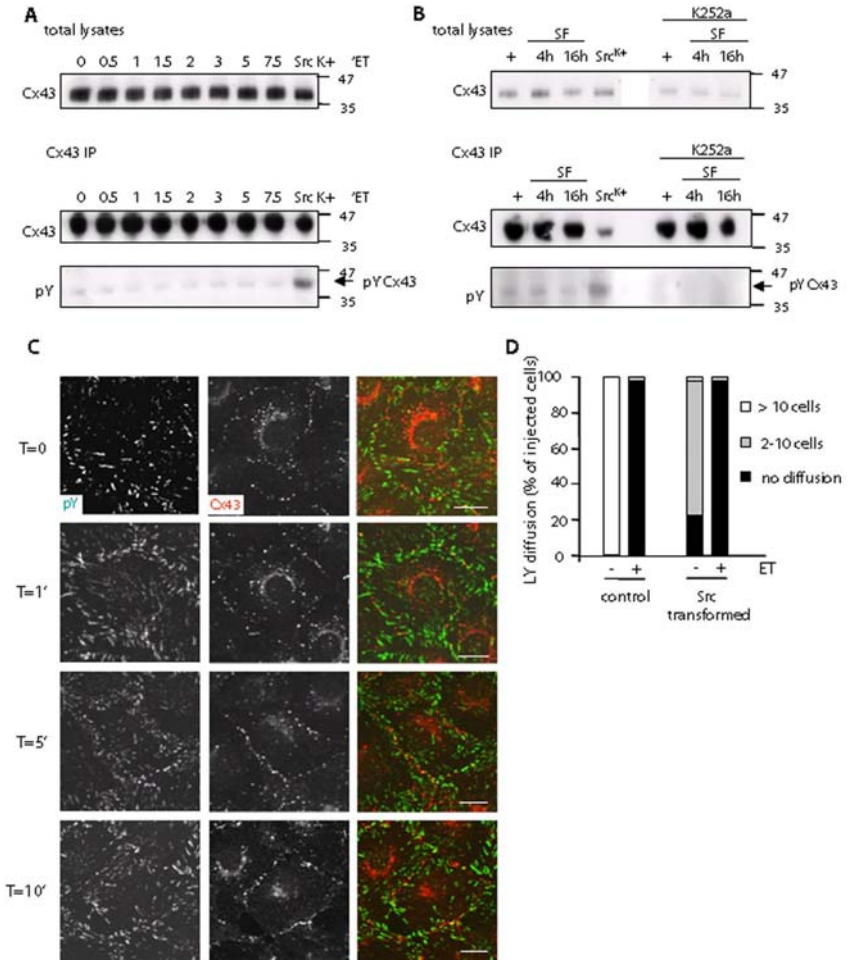

Figure 2: No tyrosine phosphorylation of Cx43 in response to GPCR agonist endothelin

A: Time course of endothelin stimulation, after which Rat-1 cell lysates are subjected to Cx43 immunoprecipitation. Top panel: total lysates immunoblotted for Cx43, Bottom panels: Cx43 immunopricipitates immunoblotted for Cx43 (top) and phosphotyrosine (bottom). V-Src transformed Rat-1 cells were used as a positive control for tyrosine phosphorylation, Cxmin cells were used as a negative control.

B: Left: Rat-1 cells were serum starved for 0, 4 and 16 hours. Cells were lysed and subjected to Cx43 immunoprecipitation. Top panel: Total lysates immunoblotted for Cx43 (top). Cx43 immunopricipitates immunoblotted for Cx43 (top) and phosphotyrosine (bottom) V-Src transformed Rat-1 cells were used as a positive control for tyrosine phosphorylation. Right: Rat-1 cells were serum starved for 0, 4 and 16 hours, and subsequently incubated with K252a (50 μM) for 30 minutes. Cells were lysed and subjected to Cx43 immunoprecipitation. Top panel: Total lysates immunoblotted for Cx43 (top). Cx43 immunoprecipitates, immunoblotted for Cx43 (top) and phosphotyrosine (bottom).

C: Time course of endothelin stimulation, after which Rat-1 cells were fixed and stained for Cx43 (red, middle) and phosphotyrosine (green, left). Scale bars: 5 μm.

D: Bar diagram showing the percentage of cells from which micro-injected Lucifer Yellow spreads to neighbouring cells of control and v-Src transformed cells before and after endothelin stimulation.

inhibited tyrosine phosphorylation of Cx43. Inhibition of GJC by GPCR signalling was not affected by either treatment (Fig. 3A and data not shown). Co-staining the cells for Cx43 and phosphotyrosine revealed no (increase in) co-localization upon agonist stimulation (Fig. 2C).

Additionally, expression of v-Src caused cells to form poor cell-cell contacts, which automatically makes them poor communicators. Cells that do make intercellular contacts showed punctate Cx43 staining at cell-cell contacts and communicated, and communication was inhibited by endothelin (Fig 2D). This suggests that there is no causal relationship between tyrosine phosphorylation of Cx43 and inhibition of communication by GPCR agonists.

Src family kinases do not play a role in inhibition of GJC

When we immunoblotted total lysates of endothelin-stimulated Rat-1 cells with an antibody recognizing Y416 phosphorylated c-Src, we observed a transient increase, indicating activation of c-Src[57] (Fig. 3A). Src activation is at its maximum between 1 and 2 minutes. This is inconsistent with the kinetics of GJC inhibition, which is

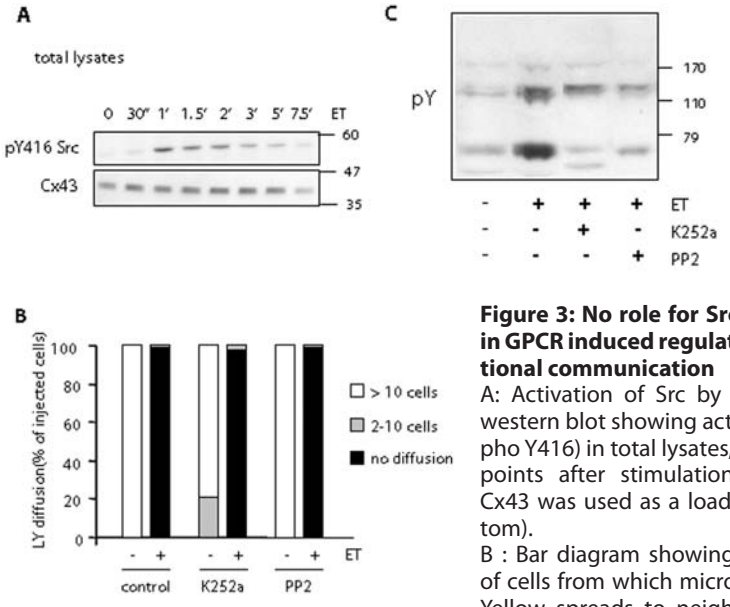

Figure 3: No role for Src family kinases in GPCR induced regulation of gap junctional communication

A: Activation of Src by endothelin. Top: western blot showing activated Src (phospho Y416) in total lysates, at different time points after stimulation of endothelin. Cx43 was used as a loading control (bottom).

B : Bar diagram showing the percentage of cells from which micro-injected Lucifer Yellow spreads to neighbouring cells of control cells and cells preincubated with either Src family kinase inhibitor PP2 (50 μM, 30') or general kinase inhibitor K252a (50 μM, 30') and before and after endothelin (50 nM) stimulation for 8 minutes (n>30 for each data point).

C: Western blot showing tyrosine phosphorylation of total lysates from Rat-1 cells, before and after stimulation with endothelin for 8 minutes (lysates were harvested immediately after microinjection of the cells (Fig. 3B), in control cells and in cells preincubated with either K252a or PP2.

complete within 1 minute after agonist stimulation. Nevertheless, to confirm that phosphorylation of Cx43 by Src is dispensable for GPCR induced inhibition of cell-cell communication, we used PP2, a pharmacological inhibitor of Src family kinases and studied the effect of general kinase inhibitor K252a on GJC regulation. Neither inhibitor had an effect on gap junction closure (Fig. 3B), while showing an almost complete inhibition of general tyrosine phosphorylation (Fig. 3C). Taken together, our results suggest that tyrosine phosphorylation of Cx43 is not required for the GPCR agonist-induced inhibition of cell-cell communication.

Sequence motifs surrounding residue Y265

Aside from being a potential phosphate acceptor, Y265 is part of a YxxΦ motif (where x is any amino acid and Φ is a hydrophobic residue) that may be important for clathrin-mediated endocytosis of Cx43. As described by Piehl *et al.*, a second YxxΦ motif, Y286-F289, is an established Dab2 mediated internalisation

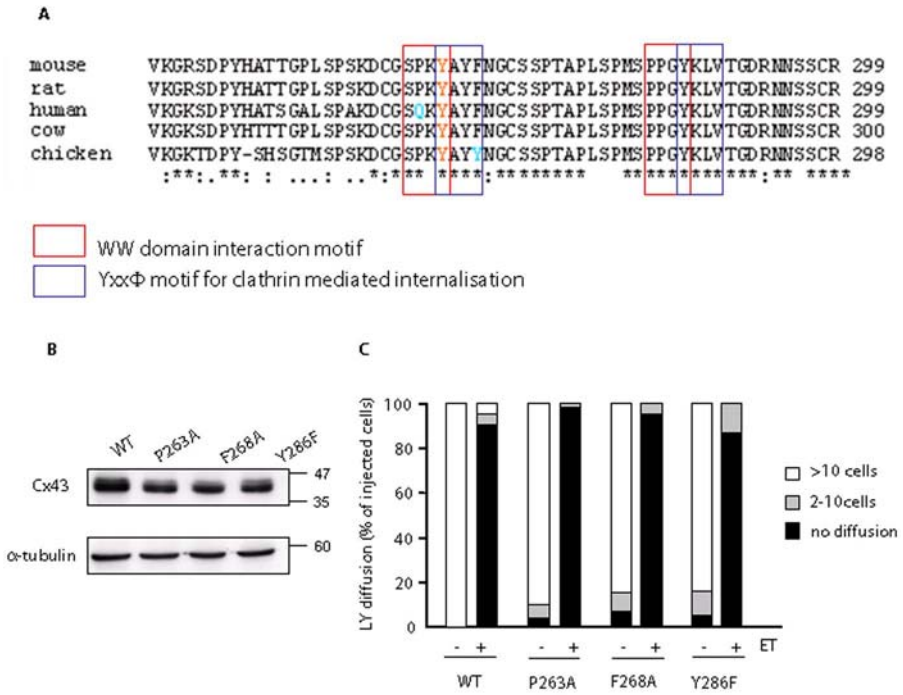

Figure 4: Mutation of putative motifs including Y265

A: Alignment of the residues surrounding Y265 of mouse, rat, human, cow and chicken Cx43. Red and blue rectangles indicate WW domain interaction and YxxΦ motifs, respectively. Y265 is marked in red. Blue residues indicate non-conserved residues that do not fit the indicated motifs.

B: Western blot showing expression of mutants P263A F268A and Y286F (top), α-tubulin is used as loading control (bottom)

C: Bar diagram showing the percentage of cells from which micro-injected Lucifer Yellow spreads to neighbouring cells before and after stimulation of the cells with endothelin (n>30 for each data point).

motif[58] (Fig. 4A). Therefore, we mutated F268 into an alanine to examine the possible involvement of this motif in the GPCR induced block of communication. Mutation of F268 had no effect on inhibition of GJC (Fig. 4B, C), arguing against the hypothesis that Y265 participates in a functional internalisation motif. In addition, we made the Y286F mutation. Also this mutation did not affect inhibition of GJC, excluding the involvement of Y286 and putative surrounding sequence motifs in regulation of GJC by GPCRs.
Finally, we note that rodent, but not human, Y265 is part of a WW binding domain[59] (rodent sequence SPKY versus human: SQKY) (Fig 4A). Mutation of P263 into a glutamine, to resemble human Cx43 or into an alanine had no effect on inhibition of GJC (Fig. 4B,C and data not shown). Thus, known putative sequence motifs surrounding Y265 are seemingly not involved in the regulation of Cx43-based GJC.

Concluding remarks

In this study, we set up a novel system to study Cx43 regulation by expressing shRNA resistant Cx43 mutants in their native cellular environment, Rat-1 Cx43 knockdown cells. This provides Cx43 with an environment in which all components involved in regulation of GJC are present. We found that residue Y265 is essential for inhibition of GJC downstream of GPCR stimulation. Y265 is a known phosphate acceptor and has been reported to be a target for phosphorylation by Src[44]. However, we found no increase in phosphorylation and excluded the involvement of Src family and other kinases. We conclude that tyrosine phosphorylation of Cx43 is not one of the regulating steps in the inhibition of GJC downstream of GPCR's.
Furthermore, we investigated a potential role for Y265 as part of a putative protein motif. The most obvious motifs that include Y265 are a YxxΦ motif and a PxY motif. YxxΦ motifs are involved in clathrin mediated endocytosis[58], and PxY motifs have been reported to mediate binding to WW domains[59]. Notably, Y286 is part of similar motifs (Fig. 4A), and has been implicated in Dab2 mediated, clathrin dependent internalisation, as well as in binding of Cx43 to one of the WW domains of E3 ubiquitin ligase Nedd4. After mutational analysis, we conclude that known protein motifs surrounding Y265 can not explain its importance for regulation of GJC.
We propose that residue Y265 plays an important structural role in the C-terminal tail of Cx43. In spite of the many players that have been identified in the Cx43 protein complex, much remains to be investigated concerning their function in Cx43 gap junctions.

Materials and Methods

Reagents
Materials were obtained from the following sources: PP2 and K252a from Calbiochem; endothelin, thrombin receptor-activating peptide (TRP; sequence SFLLRN), Cx43 polyclonal and α-tubulin monoclonal antibodies from Sigma (St. Louis, MO); Cx43 NT monoclonal antibody from Fred Hutchinson Cancer Research Center (Seattle WA); pY416 Src antibody from Cell Signalling, ZO-1 monoclonal and polyclonal antibodies from Zymed; 4G10 phospho-tyrosine monoclonal antibody from Upstate; HRP-conjugated secondary antibodies from Cell Signalling and secondary antibodies for immunofluorescence (goat-anti-mouse, Alexa488 and goat-anti-rabbit, Alexa594) from Molecular Probes. Myc monoclonal antibody was purified from hybridoma cell line 9E10.

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 diffusion of Lucifer Yellow (LY) from single microinjected cells was done as described. Typically, microinjections were started at 3 minutes after addition of endothelin, and monitored at ~8 minutes after endothelin stimulation.

RNA interference
To generate Cx43-deficient Rat-1 cells, Cx43 was knocked down by stable expression of retroviral pSuper (pRS) [55] containing the shRNA target sequence GGTGTGGCTGTCAGTGCTC. 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. Non-functional shRNA was used as a control.

Construction and expression of cDNA constructs
Mutants Δ263, Y265F and Y267F were described before . All other mutations were created by PCR based site directed mutagenesis (PCR-SDM)
(Primers:
Y247F F: GGGAAGAAGCGATCCTTTCCACGCCACTGG,
R: CCAGTGGCGTGGAAAGGATCGCTTCTTCCC;
P263A F: GCCCATCAAAAGACTGCGGATCTGCAAAATACGCCTACTTCAATGGC,
R: GCCATTGAAGTAGGCGTATTTTGCAGATCCGCAGTCTTTTGATGGGC ;
P263Q: F: GCCCATCAAAAGACTGCGGATCTCAAAAATACGCCTACTTCAATGGC,
R: GCCATTGAAGTAGGCGTATTTTTGAGATCCGCAGTCTTTTGATGGGC;
F268A F: GCGGATCTCCAAAATACGCCTACGCCAATGGCTGCTCCTCACCAACGGC,
R: GCCGTTGGTGAGGAGCAGCCATTGGCGTAGGCGTATTTTGGAGATCCGC;
Y286F F: CGCCTATGTCTCCTCCTGGGTTCAAGCTGGTTACTGGTGACAG,
R: CTGTCACCAGTAACCAGCTTGAACCCAGGAGGAGACATAGGCG;
S368A F: CCTTCCAGCAGAGCCGCCAGCCGCGCCAGCAGCAGGCC,
R: GGCCTGCTGCTGGCGCGGCTGGCGGCTCTGCTGGAAGG).
To prevent targetting of these constructs by Cx43 directed shRNA, we made two silent muta-

tions in the shRNA target site, using PCR-SDM
(primers: F: CCGCTGGAGGGAAGGTGTGGTTGTCCGTGCTCTTCATATTC ,
R: GAATATGAAGAGCACGGACAACCACACCTTCCCTCCAGCGG).
Cx43 mutant cDNA was cloned into pEntr 1A (Invitrogen) by BamHI/Xho restriction and subsequently cloned into pAd/Dest/CMV adenoviral expression vector (Gateway system, Invitrogen) by homologous recombination. Virus was produced in 293A packaging cells according to standard procedures. Supernatant containing virus particles was titrated on Rat-1 Cx43 knockdown cells to determine the amount required for Cx43 expression at levels comparable to endogenous Cx43 expression in Rat-1 cells.

SDS-PAGE, immunoblotting and immunoprecipitation
Cells were harvested in Laemmli sample buffer (LSB), boiled for 10 minutes 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 lysis buffer (10mM NaH_2PO_4, pH 7.8, 150mM NaCL, 0.5% NaDoc, 0.5% SDS, 1% NP40), supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitors (2mM $NaVO_3$, 5 mM NaF, 1mM PMSF). Lysates were spun down and the supernatants were subjected to immunoprecipitation using protein A-conjugated Cx43 antibody for 4 hours at 4ºC. Proteins were eluted by boiling for 10 minutes in LSB and analyzed by immunoblotting.

Immunostaining and fluorescence microscopy
Cells grown on coverslips were fixed in methanol for 15 minutes. Samples were blocked in PBS containing 1.5% BSA for 30 min. Subsequently, samples were incubated with primary and secondary antibodies for 30 minutes each in PBS/1.5% BSA, washed five times with PBS and mounted in Immumount (Thermo Scientific). 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).

Sequence alignment
Sequences of mouse, rat, human, cow and chicken Cx43 were retrieved from NCBI Gene, and aligned using multiple sequence alignment program Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html)

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