GPCR and G protein mobility in D. discoideum : a single molecule study

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Chapter 1

Chemotaxis: a mechanistic perspective

Chemotaxis is a complex interplay between numerous molecular species whose coordinated interactions culminate in highly effective directed motion in concentration gradients. Many proteins that play vital, important and minor roles have been identified and biochemically characterized. Several pathways have been recognized to act in parallel each of which contributes to, but is not essential for chemotaxis. Nevertheless a definitive answer as to how cells like *Dictyostelium disciodeum* perform chemotaxis is still unknown. Qualitative descriptions of molecular interactions have proven to be insufficient when trying to understand complex cellular cascades. New techniques such as single molecule microscopy are able to add temporal, spatial and quantitative information to the network of molecular interactions. Biophysics groups are probing the properties of cytoskeleton meshworks and tightly controlled artificial membranes *in vitro* providing information on cellular components relevant to chemotaxis which cannot be investigated in the complex environment of the living cell. Abstract simulations may give insights in the effects of noise in the biological systems and lead to new ways of interpreting old biochemical data. Here we will look at chemotaxis from a biophysicists' view, combining *in vitro*, *in silico* and *in vivo* experiments with a particular emphasis on our own single molecule work.

1.1 *Dictyostelium discoideum*

Dictyostelium discoideum is a single celled organism that can, when environmental conditions deteriorate, aggregate into a multicelled structure called a pseudoplasmodium or a slug. This pseudoplasmodium gains the ability to sense heat and light in order to guide itself towards the soil surface where it transforms into a fruiting body bearing stalk that releases spores. Taken away by the wind or passing animals, these spores are allowed to germinate in more favourable regions.

The first person that became fascinated by these organisms was the German botanist Oskar Brefeld. In 1869 he carefully described the process of cellular aggregation and culmination into a spore containing fruiting body. About 80 years later, in 1946, John Tyler Bonner showed (using axenically growing mutants) that he could manipulate the characteristic aggregation process by creating a flow in the cell medium. His experiments proved the involvement of a chemical substance in the directional movement that leads to cell aggregation. With this discovery Bonner paved the road towards extensive research in the area of chemotaxis. This process, in which cells compute the direction of a concentration gradient and initiate directional movement based on this computation, plays a role in many cellular behaviors critical to the existence of multi-cellular organisms. Examples include: embryogenesis, wound healing and the detection of infection by the immune system. The discovery of cyclic adenosine mono-phosphate (cAMP) as the chemoattractant that Bonner proposed by Konijn and others in 1967 [51] lead to a more systematic way of investigating the phenomenon. Since the advent of molecular biology, a lot has become clear as to how these cells can sense and move directionally towards cAMP sources. The publication of the genome [20] meant that many unknown factors could be easily identified and investigated using knockout techniques. Moreover, the discovery of green fluorescent protein (GFP) technology and its straightforward application in *D. Discoideum* combined with high gene sequence homology to higher eukaryotes has made it an immensely popular model organism for the study of chemotaxis.

1.2 The biochemistry of chemotaxis

The cellular response to cAMP during the aggregation stage can be divided into two facets: 1; the cells produce cAMP using adenylyl cyclase (ACA) and secrete it from their posterior [54]. 2; the cells initiate movement up cAMP concentration gradients using precise modulation of their cytoskeleton. The emergent behavior of these two distinct signaling units (which are biochemically intertwined) is highly effective aggregation through characteristic stream formation. The process is initiated by starvation which induces the expression of cAMP receptor 1 (cAR1), the first expressed and most sensitive cAMP receptor [37]. At the same time, other proteins needed for directional movement and signal relay are also expressed. Being a G protein coupled receptor (GPCR); cAR1 relays the cAMP signal via a G protein. G proteins are membrane localized heterotrimers consisting of a Gα, Gβ and a Gγ subunit. Although it was always assumed that *D. Discoideum* only has a single Gβ subunit, the genome shows that there should be two [20], knocking out only one of them is enough to interrupt chemotaxis [60]. Only a single Gγ subunit is found in the genome [102, 20], consequently, it takes part in every G protein mediated reaction. In contrast, the genome contains 12 Gα subunits [20]. The G protein Gα subunit determines the specificity for downstream effectors. Gα2 is vital to cAMP mediated responses and the principal signaling partner of cAR1 [68]. The binding of cAMP to cAR1 leads to activation of the G protein by the exchange of guanine di-phosphate (GDP) for guanine tri-phosphate (GTP) in the Gα2 subunit. Both the Gα2 and the G$\beta\gamma$ subunits then engage in signaling towards several different pathways that operate in parallel. The best studied of which is the Ras/PI3K pathway. The activation of Ras proteins by the G protein proceeds via Ras guanine exchange factors (RasGEFs). RasGEFs function as on switches for the Ras family of small GTPases, promoting the, as does cAR1 for the Gα2 subunit, exchange of GDP for GTP [7]. For chemotactic responses, RasC and RasG are the most important members of the Ras family [5].

Activated Ras molecules stimulate (among others) phosphatidylinositol-3-kinase (PI3K) which is subsequently recruited to the membrane where it phosphorylates phosphotidylinositol 4,5-bisphosphate (PI(4,5)P_2) to create phosphotidylinositol 3,4,5-trisphosphate (PI(3,4,5)P_3). PI(3,4,5)P_3 functions as a docking site for proteins that

contain a pleckstrin homology (PH) domain, these proteins include a multitude of signaling agents that play a role in cellular polarization and cytoskeleton regulation. Phosphatase and tensin homolog on chromosome 10 (PTEN) catalyses the opposite reaction of PI3K ($PI(3,4,5)P_3$ => $PI(4,5)P_2$). PTEN binds its own product, $PI(4,5)P_2$, this creates a feedback loop resulting in $PI(4,5)P_2$ rich membrane areas [41]. The same holds true for PI3K whose localization is self organising as well [74, 3]. Conspicuously PI3K and PTEN have opposing locations in a polarized cell with PI3K at the leading edge (the anterior) and PTEN lining the lateral sides and trailing edge (also called the posterior). The result of this segregation is a steep amplification of $PI(3,4,5)P_3$ signaling with respect to the external cAMP gradient. $PI(3,4,5)P_3$ enriched membrane areas such as the leading edge of a crawling cell, stimulate the generation of pseudopods [3]. For a long time, it was believed that PI3K was the key pathway that leads to cell polarization. $PI(3,4,5)P_3$ mediated signaling was thought to initiate actin polymerization at the side of the cell facing the highest concentration of cAMP but this view recently changed.

The generation of a mutant which has all five PI3Ks knocked out ended the notion that $PI(3,4,5)P_3$ signaling is vital to chemotaxis by showing that even in the total absence of PI3K mediated signaling, cells could still polarize and move directionally at near wildtype (wt) efficiencies [38]. A possible parallel pathway that *Dictyostelium* cells can address in this situation is the Phospholipase A2 (PLA2) pathway. It was shown that on inhibition of PI3K, the product of PLA2, arachidonic acid is essential for efficient chemotaxis [11, 35]. A third pathway operating downstream of the small G proteins (among which is RasC) but independent of PI3K is the Tor complex 2 (TorC2) pathway [48]. When TorC2 encounters active membrane associated activators it will phosphorylate protein kinase B R1 (PKBR1) and PKBA before returning to the cytosol. The fact that TalinB is a PKB target provides a direct link to the cytoskeleton. Talin has been shown to be important in cytoskeleton / membrane interactions [64] and cell adhesion [87]. Despite of intensive research, at the moment it is still not clear how all these parallel pathways orchestrate the cytoskeleton resulting in efficient chemotaxis. The current state of the biochemical pathways is depicted in figure 1.1. Many feedback mechanisms are in place to evoke strong, switch-like behavior and to allow cells to polarize in the absence of gradients. As is shown, feed-

back mechanisms exist that are independent of cAR1 / G protein signaling, they are in place to facilitate random movement in the absence of signaling [80]. The feedback routes that do involve either cAR1 or the G protein are probably involved in the stabilisation of pseudopods, the generation of a persistent front or the regulation of signaling. From this linear, 1 dimensional view on the signaling pathway it is not at all obvious how complex spatial patterns can arise. To explain how cells can sense, amplify, polarize and move directionally in a large variety of cAMP gradients requires knowledge of the mechanics and dynamics of each of the individual molecular players.

1.3 Signaling dynamics

A polarized *Dictyostelium* cell performing chemotaxis is a highly organised but very dynamic entity. To achieve and to maintain polarization places several interesting restrictions on the constituents responsible for the process. Let's focus only on the very first step of chemotaxis, the transduction of the cAMP gradient by the cAR1 - G protein system. At a first glance, just the linear transduction of a signal seems trivial, however in this polarized system several non-trivial constraints apply to the gradient information carriers. The "output" gradient of the receptor, cAR1, is a function of the "input" (cAMP) gradient and (more importantly) several cAR1 specific parameters. If cAR1, once activated, would be allowed to move completely around the cell, the gradient information would be washed out. It is thus of vital importance that cAR1 remains localized upon activation and does not disperse the gradient. The dispersion range, and thus the output gradient of cAR1 is consequently a function of its diffusion constant but also of its signaling off-rate. Apart from maintaining signal localization, cAR1 has to interact with the G protein, whose mobility and activation rates consequently also play a role. Moreover, in a 2D system such as the cell membrane the rate of a reaction involving multiple molecular species is directly proportional to their diffusion constant [4]. This means that high reaction rates can only be achieved at the cost of loosing gradient information by signal dispersal. A possible way around this limitation would be to confine fast moving signaling agents to domains or to a grid, indeed this seems to be a mechanism cells make use of [94]. A more detailed look

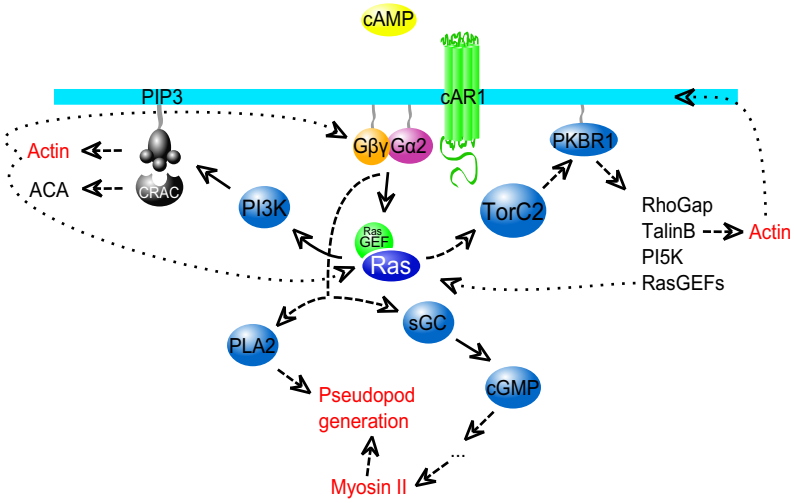

Figure 1.1: A depiction of the *D. Discoideum* chemotaxis pathway. Upon activation of cAR1 by cAMP, the G$\alpha 2\beta\gamma$ heterotrimer dissociates. Both subunits engage in signaling, G$\alpha 2$ is more important in pathways that lead to pseudopod extension whereas G$\beta\gamma$ is more important for cAMP relay involving cytosolic regulator of ACA (CRAC) and ACA [54]. The PLA2 and soluble guanilyl cyclase (sGC) pathways are activated; these pathways play important roles in the regulation of pseudopod placement [91]. RasGEFs activate Ras proteins [7]. Ras proteins and other small G proteins locally activate TorC2 which via membrane localized PKBR1 subsequently activates a multitude of factors including TalinB [48]. Talin mediates cytoskeleton - membrane interactions [64] and plays a role in cell adhesion [87]. Ras proteins also activate the PI3K pathway [79]. PI3K localizes to the leading edge where it produces PI(3,4,5)P_3 from PI(4,5)P_2. PI(3,4,5)P_3 functions as a docking site for several chemotaxis related proteins like the ACA regulator CRAC [54]. A feedback loop involving F-actin that activates Ras proteins [80] leads to the generation of pseudopods without G protein input facilitating random cell motility. We propose that there is also a feedback from actin acting on the G$\beta\gamma$ subunit specifically at the leading edge. This conceivably leads to a more persistent leading edge or the stabilisation of pseudopods. More generally, actin polymers form fences in the membrane functioning as physical diffusion barriers that influence and maintain localized signaling.

into the requirements of (polarity maintaining) signaling molecules is found elsewhere [75], the authors conlude that molecules moving at diffusion constants up to D = 5 $\mu m^2/s$ and a high off-rate are most favourable. For a fixed gradient steepness and midconcentration there are certainly optimal values for cAR1 and G protein mobility and signaling off-rates however, *D. Discoideum* cells are known to be able to chemotax in gradients that cover orders of magnitude in steepness and midconcentration. Cells are able to move directionally in gradients that cause only a minute difference in receptor occupancy over the cell body and cope with a noise that is large enough to cause the cell to experience inverted gradients for segments of time [65]. Apparently, the complex molecular mechanics in *D. discoideum* can serve as a temporal averaging filter. On the other end of the spectrum, cells can move in very steep gradients with orders of magnitudes higher mid concentrations. The properties leading to this extreme sensitivity are achieved by the tight regulation of the dynamics of all of the signaling components. signaling pathways are in principal not more than descriptive and qualitative maps of causal relations between molecules involved in the transduction of a signal. They lack the power to describe spatially organised systems such as chemotaxing *Dictyostelium* cells which requires that we take molecular properties such as mobility into account.

1.4 Biophysical techniques provide quantitative data

Fluorescent proteins, such as GFP, were traditionally used as labels in fluorescence (confocal) microscopy. At the moment however, they are also used in a variety of techniques with the ability to quantify signaling dynamics. The mobility of fluorescently tagged molecules can be determined using (among others) FRAP (Fluorescence Recovery After Photobleaching), FCS (Fluorescence Correlation Spectroscopy) and SMM (Single Molecule Microscopy). Each technique has its own set of advantages and shortcomings. FRAP is easy to implement and can report nicely on the mobility of a molecular species. Although it has difficulties dissecting multicomponent diffusion, it is able to report on complex binding/unbinding kinetics [85]. FRAP works for micrometer length scales and is thus not suited for the inspection of finer details of cell membranes. FCS is also able to report on mobility as well as

complex dynamics including multi-component systems but the error in the reported mobility depends highly on the accuracy with which one knows the used laser spot dimensions. These dimensions depend on a number of parameters making it basically impossible to estimate. The recently developed Two-focus FCS may solve some of these shortcomings though [18]. SMM, although limited to slow molecules (D = 0-10 $\mu m^2/s$, generally molecules confined to membranes or crowded spaces), is able to report very well on multi component diffusion and can even be used in 3D [39]. The positional accuracy depends only on the signal to background and the number of photons collected and thus can be arbitrarily small, in practice though, a resolution of 30-40 nm is obtained. Moreover, since the movement of molecules directly reflects the structure of their surroundings it can used to probe the underlying organization of, for example, the cell membrane at nm resolutions. Micro domains and crowding effects readily show up and in case a labeled ligand is used, SMM can report on signaling off-rates [90]. None of the before mentioned techniques can however report on molecular interactions. For such details, Förster Resonance Energy Transfer (FRET) is the appropriate technique to use. A FRET signal is extremely sensitive to the distances between a donor and an acceptor fluorophore over a range of $\sim$1-10 nm. As such it can be used to directly report on inter- and intramolecular interactions. FRET can also be used in combination with FCS to see molecular dynamics in solution [52]. When used in combination with TIR (Total Internal Reflection) illumination, FRAP and SMM are able to gain a large boost in signal to noise, TIR fluorescence microscopy is however limited to the basal membrane of a cell because the high signal to noise ratio is a direct result of its very small penetration depth (generally $\sim$100 nm).

To discern subtle changes in molecular behavior that could be key to chemotaxis, one should quantify them in a controlled environment and as a function of e.g. activation state. The tight control that is a prerequisite for precise quantification of molecular properties can be obtained using micro-fluidics. Micro-fluidic devices have been created that can make precise and stable gradient for hours, switch gradient direction very fast and allow for temporal gradient modulation [84, 78]. Nanometric, high time resolution techniques such as those described above in combination with micro-fluidics will be instrumental in solving the "problem of" chemotaxis.

1.5 The cAR1 - G protein system

Due to the existence of parallel pathways which provide considerable signaling redundancy, very few individual components apart from cAR1 and the G protein are truly essential to chemotaxis. This is one of the reasons that our group focuses on these molecules. Although in a highly polarized cell cAR1 is homogeneously distributed around the membrane, its dynamics show clear polarization. At the leading edge, cAR1 has a twofold higher cAMP off-rate [90] and its mobility is increased with respect to the posterior [17]. The first observation was G protein dependent implying that cAR1 spends less time in a G protein bound state at the leading edge, a sign of faster cycling though activation stages. The second observation is probably the result of differential cortex - membrane interactions [64]. We have established that the mobility of cAR1 is dependent on the presence of F-actin and possibly other cortex components (chapter 3). The fact that the cortex is specifically weakened at the leading edge facilitates normal (actin polymerization driven) pseudopod extension by locally reducing the cell structural integrity as well as bleb facilitated leading edge protrusion [101, 58]. The latter type of movement requires decoupling of the membrane from the stiff cortex. A faster mobility means more interactions with targets in the membrane per time unit, in this case leading to higher reaction rates specifically at the leading edge.

The G protein heterotrimer, due to its complex dynamics, lends itself for even more forms of activity modulation [97]. Like cAR1, Gα2 and G$\beta\gamma$ both exist in two mobility states and are homogeneously distributed over the cell with $\sim$70% located on the membrane and $\sim$30% in the cytosol [22]. G protein activation, as determined directly by the separation of the Gα2 and G$\beta\gamma$ subunits using FRET, is a direct reflection of cAR1 activation [44, 22]. In polarized cells, activation follows the external cAMP gradient [100]. The dogmatic view on G protein signaling dictates that upon stimulation of the GPCR, GDP is exchanged for GTP in the Gα subunit. The Gα and G$\beta\gamma$ subunits then dissociate from each other and from the GPCR and engage in signaling. In reality though, it is much less clear and many questions remain unanswered: Does the G protein decouple from the GPCR after stimulation? Are they coupled at all or do they show very transient interactions? Can a single receptor acti-

vate multiple G proteins? All these questions (and more) are important if we want to fully understand the impact and regulation of G protein signaling.

For the cAR1 / Gα2$\beta\gamma$ system several new discoveries are starting to answer the questions. Recent experiments have shown that: i; The Gα2 and G$\beta\gamma$ subunit dissociate upon activation [44, 22]. ii; Both Gα2 and G$\beta\gamma$ cycle between the membrane and the cytosol [22]. iii; Gα2 is enriched at the membrane upon stimulation whereas G$\beta\gamma$ is not [22]. iv; a majority portion of both subunits have a diffusion constant $\sim$10 fold higher then cAR1 and a small portion ($\sim$30%) matches cAR1 movement (chapter 2). v; The slow G$\beta\gamma$ immobilize upon activation in an F-actin dependent manner and this fraction increases in size, this effect is restricted to the leading edge of chemotaxing cells. The immobilization results in the loss of any fractions that match receptor diffusion. In the absence of F-actin, only the slow fraction size increase is observed and this fraction maintains a diffusion constant that matches cAR1. vi; Gα2 maintains a diffusion constant which matches cAR1 regardless of its activation state (chapter 2).

From these observations, several conclusions can be drawn. First; the default, resting state of Gα2 and G$\beta\gamma$ is the heterotrimeric form. This is confirmed by our single molecule microscopy (SMM) experiments that show that indeed the two subunits have identical movement and fraction size distributions in the cell membrane in the absence of stimulation (chapter 2). Moreover, the fact that 30% of the Gα2$\beta\gamma$ heterotrimers match the diffusion constant and type of roughly 50% of the receptors is indicative of partial receptor - G protein precoupling. The remaining 70% and the large cytosolic pool of the G protein heterotrimers cannot be coupled to cAR1 leading to the proposition that the majority fraction serves as a pool of ready-to-be-activated G proteins. Such a pool is required for initial amplification of the signal and plays an important role in polarization in shallow gradients according to the diffusion-translocation model [75]. The observation that upon activation only the Gα2 subunit increases the time spent on the membrane but not G$\beta\gamma$, implies that there must be active G$\beta\gamma$ in the cytosol. The increased membrane "on-time" of Gα2 may be the result of membrane binding or cAR1 binding. Our results suggest both take place as the Gα2 subunit's membrane fraction distribution remains unchanged after stimulation implying that both cAR1 and membrane-only associated Gα2 increase equally.

When interpreting the results of the above used techniques we must not forget their respective limitations. Elzie and others use FRAP and FRET in combination with total internal reflection fluorescence (TIRF) microscopy [22] whereas we use epifluorescence SMM. Tirf only visualizes molecules up to $\sim$100 nm (illumination intensity decreases very fast with distance) from the glass slide; this boosts the signal to noise enormously but puts heavy restrictions on the observed depth. SMM is only able to visualize molecules that are sufficiently slow compare to the illumination time, in practice this means it is limited to membrane localized molecules or molecules that have their mobility restricted otherwise. For our model system this means there are several molecular depots for which each of the two techniques cannot account. Whereas TIRF will not show cytosolic molecules more than $\sim$100 nm above the glass, Epifluorescence SMM will observe any molecule within a Z range of $\sim$1 µm but cytosolic (fast moving) molecules only contribute to the background. A very important difference between the two techniques in addition is that TIRF is limited to the basal membrane whereas our SMM measurements were done at the apical membrane. The basal part of the cell may respond differently with respect to the top membrane, especially regarding the F-actin cytoskeleton (data not shown). In the interpretation of the data it is of vital importance to incorporate the fractions that are not observed, specifically for G$\beta\gamma$ which might have an important function in the cytosol [59, 22]. Putting together the results obtained with both techniques we arrive at a model wherein cAMP binds to cAR1 causing G$\beta\gamma$ to dissociate from the cAR1-Gα2$\beta\gamma$ complex and (partly) leave the membrane to bind F-actin if present. This F-actin may very well be part of the cell cortex however since this binding is cAMP dependent and restricted to the leading edge in chemotaxing cells it most likely binds force generating F-actin fibers that are part of the Ras/PI3K/actin feedback mechanism [80]. This interaction could mean that F-actin functions as a scaffold for G$\beta\gamma$ signaling or, alternatively F-actin could attenuate the suggested inhibitory function of G$\beta\gamma$ [59] and prevent signaling to ACA. In both cases, restricting such signaling feedback to the leading edge is beneficial to cellular polarization and the stabilization of pseudopods. After activation Gα2 increases its affinity for the membrane and cAR1 leading to an increase in the net time spend at the membrane. Such dynamics are advantageous when Gα2 signaling takes place at the membrane. If the suggested

cAR1-Gα2 signaling dimer exists, the fact that cAR1 shows higher mobility at the anterior becomes more relevant to G protein signaling. A graphical representation of cAR1-G protein signaling as we envision it is shown in figure 1.2. Differences between the leading and trailing edge are also indicated.

1.6 Chemotaxis models

A chemotaxing *Dictyostelium* cell is not only complex regarding molecular interactions, dynamics and pathways but also displays intricate spatio-temporal organization of the involved molecules. The ability to organize spatially and to maintain this organization, as we discussed earlier, depends highly on the mobility parameters of those molecules. Within minutes of exposure to a cAMP gradient, cells are able to transform from being roughly symmetric into highly polarized entities.

To understand how this process can function over a very wide range of gradient parameters has been a great challenge for researchers and there have been many models that try to mimic *D. Discoideum in silico*. For the sake of modelling, the process of chemotaxis is often divided into three separate modules being; directional sensing, polarization and movement. Directional sensing is independent of the F-actin cytoskeleton and can be observed in cells which have actin polymerization completely inhibited [26]. Polarization of the cytoskeleton configuration follows the detection and amplification of the gradient and a leading and trailing edge are formed. Movement is realized subsequently by the actin dependent extension of pseudopods at the anterior and the myosin II mediated retraction of the posterior. In this dogma, once a cell has determined the gradient direction, amplified it and assumed a polarized configuration, movement is a trivial step requiring only straightforward signaling to the cytoskeleton. For this reason, models up to now focussed mainly on establishing a stable, amplified intracellular gradient or a completely polarized configuration of signaling molecules.

1.6.1 Gradient sensing

One such a model is the local excitation, global inhibition (LEGI) model [63]. This model is based on the reciprocal actions of PI3K and PTEN and can explain the

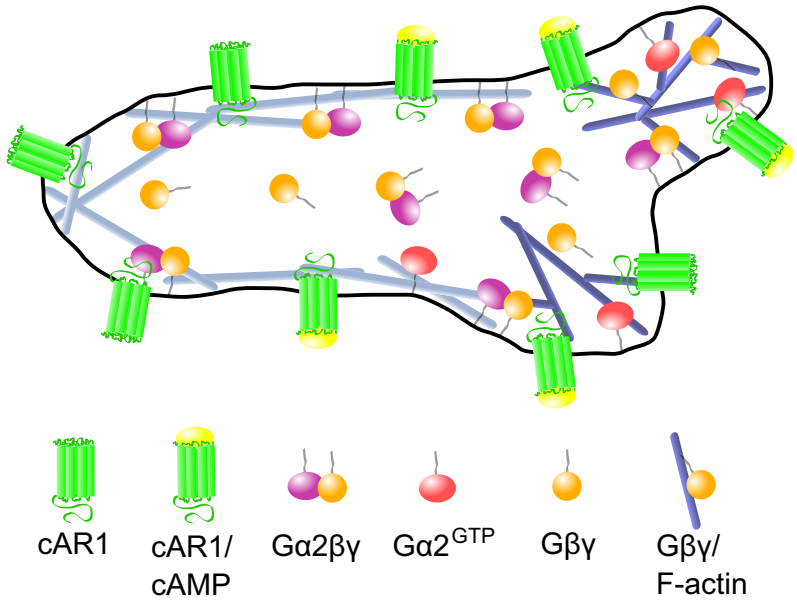

Figure 1.2: A model for cAR1 - G protein signaling during chemotaxis. The membrane is populated with cAR1, complexed cAR1-Gα2βγ and Gα2βγ; the latter is in equilibrium with a fraction in the cytosol. Upon cAMP binding by cAR1, Gβγ dissociates from cAR1-Gα2βγ. At the leading edge it binds F-actin, either at the membrane or in the cytosol, which immobilizes it. At the posterior, it simply enters the cytosol. Possibly, the immobilisation is part of an F-actin - G protein feedback loop. The function of this loop could be beneficial to the stabilisation of forming pseudopods either by F-actin functioning as a scaffold for Gβγ signaling or by inhibiting suggested "backness" signals [59]. Gα2 increases its affinity for the membrane and for cAR1 upon activation which at the same time makes is available for reactivation and allows it to better activate downstream, membrane localized signaling components. It is possible that activated Gα2 remains coupled to cAR1 in its GTP bound form. The cAR1-Gα2 complex and free cAR1 show a higher mobility at the anterior [17], this is a direct result of the fact that cortex - membrane interactions are less tight there [64]. The local attenuation of the cortex allows for faster pseudopod growth. Since the cortex is a major inhibitor of cAR1 diffusivity this leads to higher reaction rates relevant to chemotaxis at the leading edge.

observed amplification found in PI(3,4,5)P_3 signaling. In this model, upon binding of cAMP, cAR1 quickly activates downstream components (PI3K) but at the same time a slower inhibitory response is initiated (mediated by PTEN) which becomes stronger over time. This eventually results in a situation where the leading edge still overcomes inhibition while trailing edge activation diminishes. This model almost perfectly explains the polarized behavior seen in PH-domains but it lacks the ability for cells to polarize in the absence of a gradient. This is because maintenance of activation is directly dependent on cAR1 signaling however; the same molecules can be used to replicate this observation if positive feedback loops are incorporated [32]. The addition of such feedback would lead to the existence of PI(4,5)P_2 and PI(3,4,5)P_3 enriched patches on the membrane which are indeed observed [74].

1.6.2 Polarization

A more abstract model, not based on the PI3K / PTEN system is the balanced inactivation model [59]. This model is better able to explain the switch like behavior, leading to absence of activation at the anterior that is seen in many signaling components. It does so by adding a component that is fast diffusing in the cytosol ensuring its concentration is equal throughout the cell. This cytosolic component is inhibiting signaling and created at an equal rate as the activating membrane localized component. In a gradient this generates a situation in which at the posterior signaling is completely blocked but at the anterior it is not. The result is a switch like behavior that is also capable of quickly adapting to changing gradients. Interestingly, the required molecules and their characteristics correspond nicely to the cAR1 / G protein system. An important assumption is that G$\beta\gamma$ has an inhibitory function and is able to diffuse in the cytosol, the latter at least, seems to be very well possible [22].

1.6.3 Biased pseudpods

The models listed so far are compass based models. They are based on the proposition that signaling precedes the generation of well placed pseudopods. These models are a natural extension of the prevailing "gradient sensing => polarisation => movement" dogma. Several recent observations however conflict with this proposition: i; chemo-

taxis at low gradients is best described by a biased random walk. ii; Pseudopods are extended at a constant rate irrespective of the cells orientation in a gradient. iii; Unfavourable pseudopods can be retracted. iv; New pseudopods originate mostly from previous ones [6, 1]. Observations i and ii show that *D. Discoideum* cells move by default and generate pseudpods at a constant speed. In order to move directionally, regulation should take place not on when the pseudopods are generated but on where. This observation agrees with the finding that the Ras/PI3K/F-actin system does not require G protein input [80] but instead facilitates polarization leading to random movement in the absence of cAMP. When a gradient is applied, this autonomous system receives directional input and polarizes in the correct direction. Observation iii and iv suggest that not only do cells show persistence in their trajectories because of the fact that new pseudopods are (mostly) restricted to the current leading edge, a form of temporal sampling also plays a role and the decision to keep a pseudopod is made after its generation. This mechanism is reminiscent of bacterial chemotaxis which functions by a higher persistence in "correct" directions [92]. Taken together this leads to a model where instead of the gradient determining the correct direction for a pseudopod, pseudopods are positioned with a certain probability around the cell. The input parameters that govern pseudopod positioning are gradient steepness, direction and the position of the previous pseudopod. Such a model implies; i; a steeper gradient will lead to a higher directional accuracy due to more pseudopods being placed in the correct direction, ii; deviation of the cells polarity axis with respect to the gradient will lead to a corresponding bias in the probability distribution of pseudopod generation and iii; dependence of pseudopod position on the position of the previous pseudopod will lead to autocorrelation in the cells movement characterized by a certain persistence time. Indeed, the observation of thousands of cells reveals the probabilistic nature of pseudopod generation and persistence of movement nicely [6]. Additionally, it was found that cells retain the ability to generate "*de novo*" pseudopods; these are pseudopods uncorrelated from the previous ones. Because even highly developed cells can still create a *de novo* pseudopod every now and then, the ratio between correlated and *de novo* pseudopods is an important factor in the persistence of directional movement. This new view on chemotaxis, in which molecules important to chemotaxis are seen as factors influencing pseudopod

generation frequency, persistency or the probability distribution of their placement is especially good at explaining directional movement at very low gradients. Although the nature of pseudopod placement is probabilistic, it is still governed by cAR1 - G protein signaling combined with various downstream pathways and thus hard limits exist when it comes to noise and detection thresholds. We expect these realisations to generate numerous new models and discoveries which will bring us closer to a full understanding of the phenomenon known as chemotaxis.

1.7 Conclusion

The biochemistry governing chemotaxis is becoming more and more clear. Alternative pathways are being identified and the field is at a stage where it has identified a lot of key components. The recent realisations regarding pseudopod generation at low gradient strength will probably inspire a multitude of new models likely to encapsulate older models as well. Despite of the probabilistic nature, the detection is governed by the properties of signaling molecules. High time and spatial resolution techniques such as FRET, FRAP, FCS and SMM in combination with tightly controlled micro-fluidics will be instrumental in the quantification of the molecular interactions and mobilities. As quantitative information in the form of diffusion constants and reaction rates are added to the pathways, models will be become more realistic and spawn more testable hypothesis which in term will give rise to new insights. This positive feedback between biology and (bio)physics will definitely lead to a more complete and more detailed picture of eukaryotic chemotaxis.

1.8 Thesis outline

In this thesis I will focus on the mobility of the GPCR cAR1 and its associated G protein subunits, Gα2 and G$\beta\gamma$ in *D. discoideum*. Each of these three proteins has been labeled with Yellow Fluorescent Protein (YFP) which allows for the localization of individual molecules with a positional accuracy of $\sim$40 nm at a temporal resolution of 50 ms. Their respective mean squared displacements (MSDs) are measured over timelags of 50 - 400 ms. The slope of the MSD *vs* timelag plots is a proportional

to the diffusion constant while the shape reveals details on the underlying structure of the membrane and/or the cytoskeleton. In chapter 2 we focus on the G protein in its resting state, upon global stimulation and in polarized cells. We show that the behavior of G$\beta\gamma$ at the leading edge is radically different from the posterior: where at the posterior G$\beta\gamma$ behaves as in resting cells, in the leading pseudopod a fraction immobilizes in an F-actin dependent fashion and F-actin related domains form. These observations are indicative of feedback mechanisms acting directly on G protein signaling. In resting cells, $\sim$50% of the cAR1 molecules appear to be precoupled to $\sim$30% of the membrane localized G protein heterotrimers. This leaves the majority of G protein heterotrimers free to diffuse and allows cAR1 to amplify its signal. In chapter 3, we examine the mobility of cAR1. As found for the G protein, F-actin restricts cAR1 diffusion however; it appears that the cell cortex is mainly responsible instead of agonist induced actin polymerization. Our findings support the observation that cortex - membrane interactions are weaker at the anterior of a chemotaxing cell. Other factors than F-actin, related to directional sensing, also seem to be able to regulate cAR1 mobility. Chapter 4 focuses on the behavior the cAR1 and G$\beta\gamma$ in a *rasC*$^{-}$*/rasG*$^{-}$ background. In the absence of these proteins that are vital to chemotaxis we lose the polarized behavior of both cAR1 and G$\beta\gamma$. The RasC/RasG knockout cells have difficulties regulating their cytoskeleton resulting in loss of G$\beta\gamma$ immobilization and loss of spatial regulation of the actin cortex. Introduction of a functional cAR1 however, seems to restore the reported lack of chemotaxis. This implies that RasC and RasG mediate chemotaxis by induction of cAR1 expression in addition to directly functioning in the signaling pathway. Our data is important to any modelling of the system and leads to new insights on GPCR - G protein signaling.