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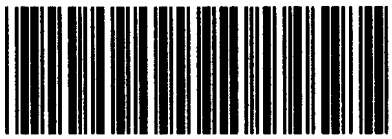

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Social senses: G-protein-coupled receptor signaling pathways in *Dictyostelium discoideum*

Ji-Yun Kim[1], Peter Van Haastert[2] and Peter N Devreotes[1]

Activation of the chemoattractant receptor of *Dictyostelium* elicits many of the same biochemical events seen when mammalian G-protein-coupled receptors are activated. Studies in this organism provide evidence for new signaling pathways that are activated by receptors of this type, and fresh insights into the mechanism of signal transduction by G proteins.

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Living cells are constantly receiving external stimuli and have evolved elegant biochemical strategies for responding to these signals. One of the most widely studied signaling pathways is mediated by a large family of G-protein-coupled receptors (GPCRs), which transverse the plasma membrane seven times. Occupancy of these receptors by agonists activates heterotrimeric G proteins, which regulate the activity of many effectors such as adenylyl cyclases, ion channels, phospholipases, phosphodiesterases and mitogen-activated protein (MAP) kinases ([1], reviewed in [2]). These signal transduction systems are conserved from yeasts to humans and transduce signals in response to pheromones, light, odorants, chemoattractants and a variety of hormones and neurotransmitters (reviewed in [2]).

The social amoeba *Dictyostelium discoideum* is an excellent model system for the study of these pathways. These free-living amoebae offer the biochemical and genetic advantages of a microorganism (reviewed in [3]), yet under appropriate conditions they spontaneously aggregate into a multicellular structure that displays cell differentiation and pattern formation reminiscent of that seen in higher eukaryotes. The resulting multicellular structures, which may contain as few as 10 or as many as 10^5 cells, undergo a series of morphogenic changes (Fig. 1). The cells first aggregate in response to cyclic AMP (cAMP) to form a 'slug', which crawls towards light and warmth before differentiating into a fruiting body. cAMP participates in the differentiation of the cells of the slug into two cell types, which sort to opposite ends of the slug: the posterior ~75 % of the cells become prespore cells, while the remainder become prestalk cells. Many of the components in the signal transduction pathways mediating these processes are cloned and characterized and can be used in genetic analyses to study their interactions and to find new genes, providing fresh insights into the more complicated mammalian systems [3].

Extracellular cAMP is important in the early as well as the late stages of development, and its function during aggregation has been extensively characterized (Fig. 1). Spontaneously secreted at six minute intervals by small groups of cells, it initiates propagated waves of cAMP that guide chemotactically sensitive cells towards central points. The wave propagation results from relay of the chemotactic signal, as cAMP induces the transient synthesis and secretion of additional cAMP. An external phosphodiesterase then degrades the signal, the system resensitizes, and is capable of responding to the next wave. This relay mechanism enables the cells to communicate over distances of several centimeters. The components that are essential for this process include cAMP receptors (cARs), phosphodiesterases, an adenylyl cyclase and the molecules that regulate their functions.

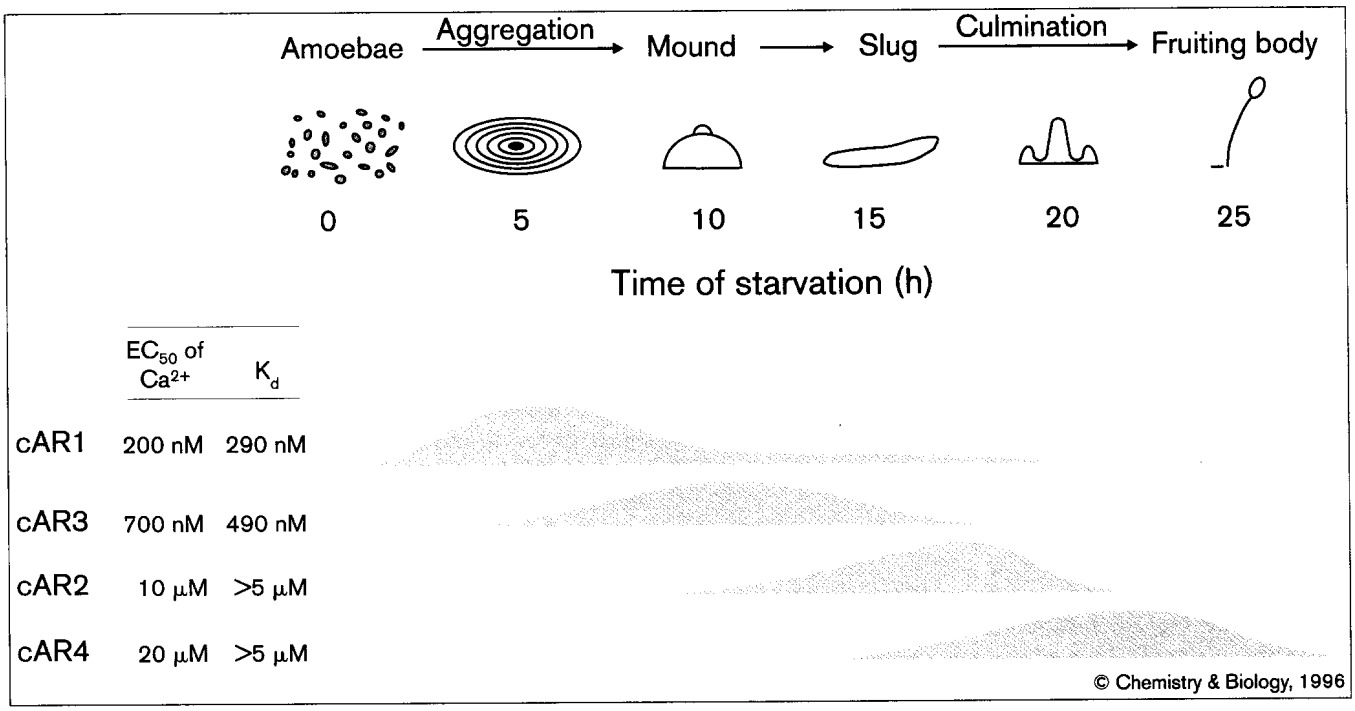
cAMP receptors are functionally redundant but differ in affinity

cAMP binds to surface receptors that are encoded by four genes, cAR1, cAR2, cAR3 and cAR4, each of which is transiently expressed at a different stage of development (Fig. 1). Deletion of cAR1 produces a cell line severely defective in the propagation of and response to cAMP signals. Due to the expression of cAR3 at a later stage, however, deletion of cAR3 is also necessary to remove all responses to exogenous cAMP [4]. Deletion of cAR3 alone has no obvious effect, suggesting that it may be redundant with cAR1. Furthermore, ectopic expression of cAR2 or cAR3 in *car1*$^-$/*car3*$^-$ cells rescues many of the functions of cAR1. Thus, each of the receptors appear to be coupled to the same signal-transduction pathways; each can mediate activation and adaptation of adenylyl cyclase, chemotaxis and actin polymerization (J-Y.K. and P.N.D., unpublished data). The EC_{50} values of these responses, however, are shifted according to the relative affinities of the receptors [5]. It seems likely that the cAMP receptors differ in affinities in order to function appropriately in the environment in which they are expressed. As the cells get closer together, they sequentially express lower affinity receptors. The appropriate response to the changes in the cellular environment is achieved by switching receptor subtype while retaining similar mechanisms of signal transduction and regulation.

Desensitization

Persistent activation of cAR1 triggers a series of events that leads not only to excitation but also to desensitization of the responses. The activated receptors undergo desensitization

Figure 1



Developmental program of *Dictyostelium*. The upper panel depicts the major stages of development starting from the initiation of starvation. The lower panel shows the time course of mRNA expression; the K_d for cAMP binding to each receptor is also indicated. Stimulation of the cAMP receptors leads to an influx of Ca^{2+}; the EC_{50} (the concentration of cAMP that elicits half the maximal release of Ca^{2+}) for this response is shown for each of the receptor subtypes.

by at least two different mechanisms: adaptation and loss of ligand binding [6]. Adaptation occurs because of a rapid and reversible uncoupling from the G protein. It is common to many GPCRs and generally considered to result from receptor phosphorylation by a G-protein-coupled receptor kinase (GRK) followed by binding of the protein arrestin. Loss of ligand binding is an agonist-induced decrease in the affinity of the receptor. This mechanism may underlie the desensitization of many other GPCR-mediated pathways; a process that has previously been attributed to sequestration of receptors away from the cell surface [7].

In response to cAMP, the cytoplasmic tail of the cAR1 receptor is phosphorylated on serine residues with a kinetics and dose-dependence very similar to those seen for both the adaptation of several cellular responses to cAMP and the loss of ligand binding by the receptor. Replacement of wild-type cAR1 with two different mutant versions that cannot be phosphorylated due to the elimination of the target serine residues completely blocks the loss of ligand binding [6]. Surprisingly, however, the kinetics of adaptation of several cAR1-mediated responses, including the activation of adenylyl cyclase, chemotaxis, Ca^{2+} influx and gene expression, are relatively unimpaired. This unexpected observation indicates that there is a 'back-up' adaptation mechanism (J-Y.K. *et. al.*, unpublished data). As the sequences of the carboxy-terminal cytoplasmic domains of the four receptors are highly divergent, it is tempting to speculate that this region has functions in addition to controlling the loss of ligand binding. It is also plausible, however, that this domain is not conserved simply because it is not essential for receptor function. If this is the case, once the four receptors appeared, presumably through gene duplication events, there would have been little selective pressure to maintain the homology.

Signal transduction by G proteins

The next step in many of the transduction pathways leading from an activated cAR is signal transduction by G proteins. *Dictyostelium* has at least eight genes encoding G protein α subunits, which are all expressed at different times during development. Deletion of some of these genes results in strong phenotypes. $G_\alpha 2$ null mutants are defective in many cAR1-mediated responses including chemotaxis and *in vivo* activation of guanylyl cyclase, adenylyl cyclase and PLCδ [8]. These mutants still respond to folic acid, however, showing chemotaxis and activation of adenylyl cyclase and guanylyl cyclase. These responses are mediated by a receptor that is also thought to be coupled to a G protein. Deletion of $G_\alpha 4$ yields a reciprocal phenotype: responses to folic acid are abolished, but cAMP-mediated responses remain normal [9]. Deletion of the G protein

β subunit, of which *Dictyostelium* has only one copy, eliminates all G-protein-mediated responses to cAMP, folic acid and a variety of other chemoattractants [10].

The absence of the β subunit prevents adenylyl cyclase activation by GTPγS, suggesting that βγ complexes activate adenylyl cyclase. In *gα2*$^-$ cells, however, adenylyl cyclase can still be stimulated by GTPγS. The following scheme of events can explain these observations. In intact cells extracellular cAMP detected by cAR1 activates G2, releasing free βγ dimers that activate adenylyl cyclase. In cell lysates, however, GTPγS can mediate the release of βγ dimers from other heterotrimeric G proteins in the absence of G2 [10].

Signal transduction downstream of G proteins

Are βγ dimers and adenylyl cyclase directly linked? A novel protein designated CRAC (cytosolic regulator of adenylyl cyclase) is essential for the activation of adenylyl cyclase both *in vivo* and *in vitro* and may function as an adaptor between βγ dimers and adenylyl cyclase. CRAC is an 88 kDa protein with a pleckstrin homology (PH) domain, and it translocates to the membrane upon activation of the pathway by GTPγS or an occupied receptor. Relocalization after treatment with GTPγS occurs in mutants lacking adenylyl cyclase and receptors, but is abolished in *gβ*$^-$ cells [11]. Proteins containing PH domains have been demonstrated to bind to βγ complexes, and the PH domain is thought to act as a molecular adaptor [12], suggesting that CRAC may work as a bridge between the βγ dimer and the adenylyl cyclase. Mammalian homologs of CRAC have not yet been found, but as mammalian adenylyl cyclases subtypes II and IV are synergistically regulated by βγ dimers in addition to regulation by the α subunit [13], a CRAC homolog might exist in these systems.

Receptor-stimulated production of inositol trisphosphate (IP_3) by phospholipase C (PLC), widely regarded as an essential response in controlling intracellular levels of Ca^{2+}, appears to be of little or no importance in *Dictyostelium*. A PLC null cell line displays no developmental abnormalities and no apparent biochemical defects other than the absence of the typical cAR1-mediated increase in IP_3 levels [14]. It is therefore possible that an as yet undiscovered route of IP_3 synthesis might regulate IP_3 levels in the absence of PLC.

Another response to chemoattractant-receptor stimulation is the rapid activation of guanylyl cyclase. This response is absent in cells lacking $G_\alpha 2$ or Gβ, suggesting that it is mediated through a subunit of G2 [8,14]. Changes in the levels of intracellular cGMP appear to be essential for chemotaxis, osmotic regulation and differentiation in early development. Of nine mutants isolated as non-chemotactic, at least six appear to be defective in either the production or detection of intracellular cGMP [15]. Particularly interesting are mutants KI-8, which lacks guanylyl cyclase activity, and KI-10, which lacks receptor-stimulated cGMP accumulation. These mutants show cAMP-induced activation of adenylyl cyclase and PLC, but no chemotaxis or aggregation. The kinases that phosphorylate the heavy chain of conventional myosin on threonine residues are not activated in these mutants, establishing the connection between the transient receptor-mediated increases in the levels of cGMP and the activation of this phosphorylation, which regulates the assembly and disassembly of myosin filaments [16]. A similar correlation between cGMP level changes and myosin heavy chain phosphorylation was shown to be responsible for osmoregulation in *Dictyostelium*. Osmotically induced activation of guanylyl cyclase does not, however, require a G protein, and displays different kinetics [15].

G-protein-independent signaling downstream of cARs

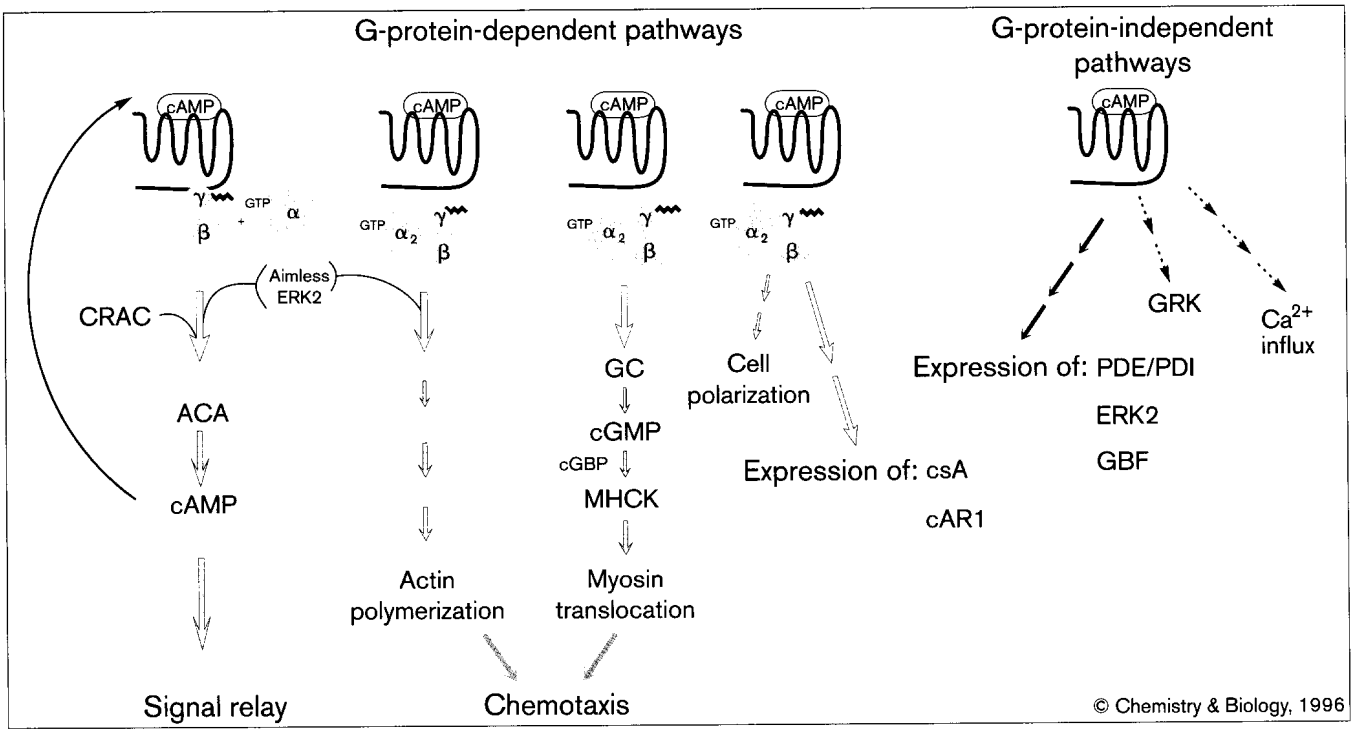
Some changes in response to binding of cAMP to cARs do not depend on the activity of G proteins, and in one case this runs counter to findings in other systems. GPCRs activate MAP kinases via the βγ subunits of various heterotrimeric G proteins in mammals and yeast, by both Ras-dependent and Ras-independent mechanisms [17]. The MAP kinase ERK2 is important in cAR-mediated signal transduction; the null mutant does not undergo development. In *erk2*$^-$ cells activation of adenylyl cyclase is markedly diminished both *in vivo* and *in vitro*, but activation of guanylyl cyclase and other responses are normal [18]. The target of ERK2 is unknown, as well as its mechanism of regulation of adenylyl cyclase activity. Surprisingly, the enzyme is activated by cARs in *gα2*$^-$ and *gβ*$^-$ cells by a G-protein-independent pathway [19].

The activation of G-protein-independent pathways can also stimulate the influx of Ca^{2+}, the kinases that phosphorylate the receptors, and the expression of an extracellular phosphodiesterase and its inhibitor. Activation of any of the four cloned receptors can lead to these responses if appropriate dose requirements are met [5], and all the responses occur in Gβ null cell lines. It is possible that G-protein-independent signal transduction through GPCRs is a general phenomenon in eukaryotic cells.

Random mutagenesis studies of signaling components

As described above, isolation of a gene that encodes a specific protein in a pathway followed by its disruption can reveal the function of the gene product and help define interactions with parallel pathways (Fig. 2). Another approach, random mutagenesis of the individual signal transduction components, has provided insights into the structure–function relationships for proteins such as cAR1, adenylyl cyclase and Gβ [20]. Extensive mutagenesis of cAR1, for example, has shown that 90 % of the mutant receptors (which have an average of two mutations per

Figure 2



Schematic view of cAR-mediated signal transduction pathways. Both conventional G-protein-linked signal pathways and novel G-protein-independent pathways are shown. The receptors are largely redundant in function and are therefore not identified by name. The Gα subunit responsible for cAMP responses is α_2. For adenylyl cyclase (ACA), βγ dimers lead to activation of the enzyme, with CRAC probably acting as an adaptor, but for actin polymerization and activation of guanylyl cyclase (GC) and gene expression it is not clear which of the G-protein subunits is responsible for activation. The activation of guanylyl cyclase leads to the production of cGMP, which, when bound to cGMP binding protein (cGBP), stimulates the activity of myosin heavy chain kinase (MHCK) and so leads to the translocation of myosin. Expression of the genes encoding adhesion contact site A protein (csA) and cAR1 is also induced by a G-protein-dependent pathway. G-protein-independent pathways are listed in arbitrary order and the causal network is not known. These pathways result in the induction of expression of the genes encoding phosphodiesterase (PDE) and phosphodiesterase inhibitor (PDI), ERK2 and G-box binding factor (GBF), a transcription factor, as well as the activation of G-protein-coupled receptor kinase (GRK) and an influx of Ca^{2+}. See text for details.

molecule) can carry out all of the functions of wild-type receptors. Many (65 %) of the remaining mutants that are impaired in function have an altered affinity for cAMP. A novel class of mutants forms high affinity cAMP binding sites but is unable to activate G proteins; nevertheless these mutants undergo agonist-induced receptor phosphorylation. Mutagenesis of adenylyl cyclase has resulted in the isolation of catalytically inactive mutants, regulation-deficient mutants in which G-protein-mediated modulation of the enzyme activity is lost, and gain of function mutants with higher V_{max} values than the wild-type enzyme (C.A. Parent and P.N.D., unpublished data).

Conclusions and future directions

Dictyostelium provides a relatively simple system where models of G-protein-linked signal transduction pathways can be tested rigorously. The capacity to isolate null mutants and carry out random mutagenesis allows the complexity of crosstalk and structure–function relationships of the components to be addressed. In addition, the recently developed technique of random restriction enzyme mediated insertion (REMI) [21] has facilitated the isolation of an increasing number of new genes in G-protein-coupled pathways. Aimless, which belongs to a family of GTP-exchanging factors for small G proteins, and ERK2 [18] were isolated using this method. These proteins are involved in the activation of adenylyl cyclase, and Aimless seems to be involved in chemotaxis pathways as well (Fig. 2). Further detailed investigations should provide exciting insights into the complex regulation of these pathways.

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