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G Protein–Linked Signaling Pathways Control the Developmental Program of Dictyostelium

Review

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Evolutionary Origins of G Protein–Linked Signaling

The chemical signals exchanged between neurons at synapses not only transmit information, but can also regulate morphogenesis and influence gene expression during development and remodeling of the nervous system. Studies of microorganisms indicate that secreted "transmitters" serve similar functions in simple multicellular organisms, such as the cellular slime mold, Dictyostelium discoideum. In the last several years, the accessible genetics and cell biology of Dictyostelium have allowed a detailed description of the strategies it uses for cell–cell communication. As described in the following sections, these signaling pathways are analogous to those involved in responses to neurotransmitters in mammals.

The actions of a wide variety of neurotransmitters are mediated by seven transmembrane helix receptors. When bound by ligand, these receptors activate heterotrimeric G proteins, catalyzing the exchange of GTP for GDP on the α subunit and the dissociation of the α from the βγ subunits. Both the α and the βγ subunits can stimulate or inhibit effectors, including adenylyl cyclases, phosphodiesterases, phospholipases, and ion channels (Gilman, 1987; Dohlman et al., 1991; Logothetis et al., 1987; Birnbaumer, 1992). The receptors for serotonin, dopamine, acetylcholine, odorants, and light are but a few examples. The G protein–linked signal transduction strategy plays an essential role in the developmental program of Dictyostelium (Devreotes, 1989; Van Haastert and Devreotes, 1993). The remarkable conservation of these systems indicates that this microorganism can be used to analyze the structures and interactions of receptors, G proteins, and effectors and to discover new components in these pathways. Furthermore, the mechanisms by which these signal transduction pathways influence morphogenesis and gene expression can be studied by genetic analysis.

In Dictyostelium, development is initiated by nutrient depletion. Within a few hours after the onset of starvation, a cell–cell communication system appears that enables about 10^5 individual cells to aggregate from distances of a few centimeters to form a millimeter-sized organism (Figure 1). This process is mediated by extracellular cAMP, which is secreted by centrally located cells at 6 min intervals. Surrounding cells respond by advancing chemotactically toward the center and by "relaying" the signal more distally as a propagated cAMP wave. The periodic stimulus also acts as a developmental timer, accelerating the pace of gene expression. Within about 10 hr, the aggregating cells coalesce into a tight mound and an elongating apical tip arises. As the multicellular structure undergoes further morphogenesis, forming a migrating slug and ultimately a fruiting body, cells in the anterior or posterior regions of these structures, under the continued influence of cAMP, differentiate into stalk or spore cells.

Many of the molecular components of these signaling pathways are known. There is a family of four surface cAMP receptors (cAR1–cAR4), which display the typical seven transmembrane domain topology (Klein et al., 1988; Saxe et al., 1993; Johnson et al., 1993). There are eight G protein α subunits ($G_\alpha1$–$G_\alpha8$), which are each about 45% identical to the others and to mammalian α subunits in general (Hadwiger et al., 1991; Wu and Devreotes, 1991). There is a single β subunit (G_β), which is 70% identical to those in other eukaryotes and, presumably, forms heterotrimers with each of the α subunits (Lilly et al., 1993). The corresponding γ subunit(s) has not been found. The effectors include an adenylyl cyclase (designated ACA) that is topologically analogous to mammalian adenylyl cyclases (Pitt et al., 1992) and a ligand-stimulated phospholipase C (PLC) (Drayer and Van Haastert, 1992). The presence of other characteristic components of the cAMP second messenger pathway—the regulatory and catalytic subunits of a cAMP-dependent protein kinase (PKA) and secreted and membrane-bound forms of phosphodiesterase (PDE)—indicates that cAMP functions as both an extracellular and intracellular messenger (Firtel and Chapman, 1990; Mutzel et al., 1987; Faure et al., 1990). The mRNA and protein levels of these components are tightly regulated, and most are transiently expressed at specific stages in the developmental program (Figure 1).

Genetic Analysis of Signal Transduction Pathways

The genetic approaches possible in Dictyostelium allow for rigorous testing of models of signal transduction pathways. For instance, do receptor-mediated increases in certain second messenger levels necessarily indicate a role in physiological processes? Studies of null cell lines described below have led to surprising findings. In addition, the expression of a wide variety of receptor and G protein isoforms in mammalian cells, such as neurons, has led to hypotheses of "networking" and "cross-talk," in which parallel pathways contain common components and products of one pathway can affect the activities of others. In Dictyostelium, the physiological significance of these kinds of interactions can be explored by genetic analysis. So far, these studies suggest that the cAMP receptor subtypes are partially overlapping in function—

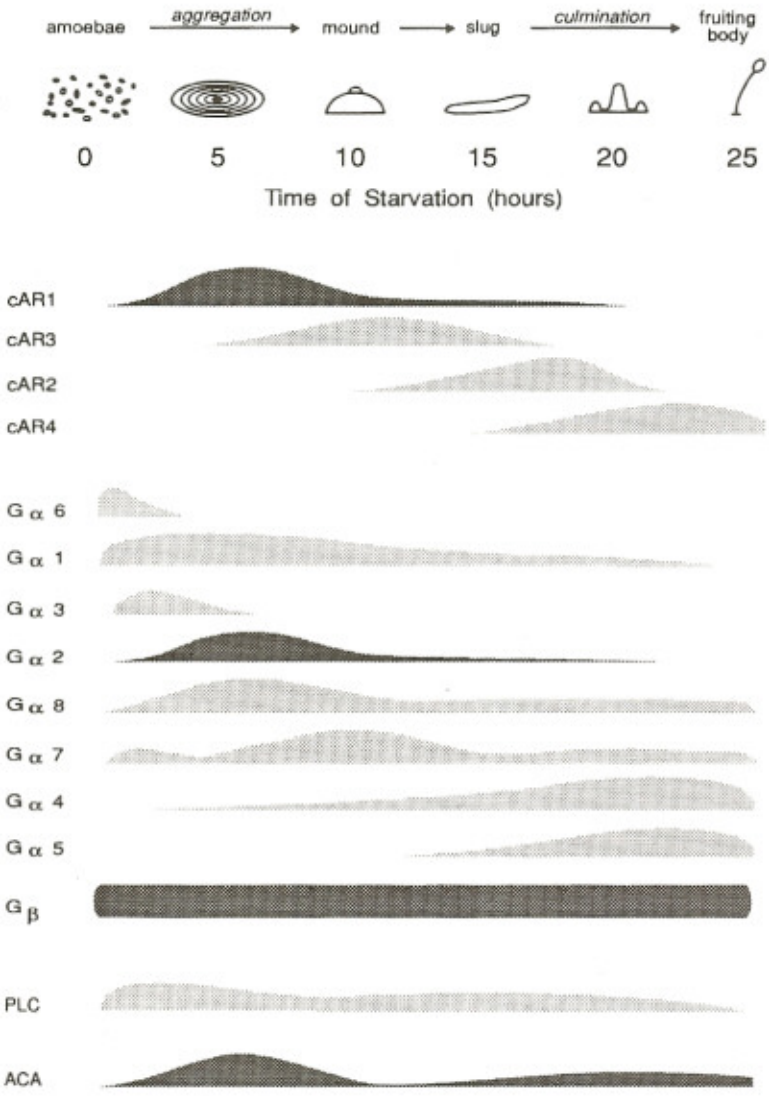

Figure 1. Developmental Program of Dictyostelium

Diagrams illustrate successive stages in development. Cells are 10 μm, aggregation territory is 1–2 cm, and multicellular structures are several 1–2 mm. Shaded profiles illustrate the time course of expression of major RNA transcripts for cAMP receptors, G protein subunits, and effectors. cAR1, $G_{\alpha}2$, G_{β}, and ACA, which play major roles in early development, are heavily shaded.

each playing a more prominent role at a specific stage in the program—but all appear to couple to only one of the eight G proteins. The remaining G proteins are likely to be linked to as yet undiscovered classes of receptors.

The phenotypes of mutants made by targeted gene disruption and conventional mutagenesis have shown that cAR1, $G_{\alpha}2$, G_{β}, ACA, PKA, and PDE play important roles in the early developmental program (Sun and Devreotes, 1991; Kumagai et al., 1989; Lilly et al., 1993; Pitt et al., 1992; Mann et al., 1992; Sucgang and Kessin, personal communication). A series of physiological and biochemical observations on these and several other mutants (Table 1), can be assembled into a schematic diagram of the signal transduction pathways involved in cell–cell signaling, chemotaxis, and gene expression (Figure 2).

Among the eight G proteins, only G2 appears to be critical in mediating responses to cAMP. Null mutants lacking $G_{\alpha}2$ ($g\alpha2^{-}$ cells) do not carry out chemotaxis or differentiate under any conditions (Coukell et al., 1983; Kumagai et al., 1989). cAMP fails to elicit the extension of pseudopods and concomitant increases in filamentous actin as well as the synthesis of cGMP, cAMP, and inositol trisphosphate (IP_3) (Hall et al., 1989; Kesbeke et al., 1988; Drayer and Van Haastert, 1992). In membranes isolated from $g\alpha2^{-}$ cells, GTP does not regulate cAMP binding affinity and GTPγS fails to activate PLC (Kesbeke et al., 1988; Bominaar et al., 1991; Snaar-Jagalska et al., 1988).

Which of these second messenger pathways activated by G2 are functionally significant? cGMP appears to play a role in chemotactic orientation: a mutant of a cGMP PDE (*streamer F*) displays an exaggerated polarization, and a series of mutants that specifically do not synthesize cGMP are unable to carry out chemotaxis (Ross and Newell, 1981; Kuyama, personal communication). In contrast, whereas external

Table 1. Physiological and Biochemical Characteristics of Dictyostelium Mutants

	car1$^-$	*aca*$^-$	*synag 7*	*gα2*$^-$	*gβ*$^-$	*plc*$^-$	*streamer F*[a]
Aggregation	−	−	−	−	−	+	+
Chemotaxis	Late	+	+	−	−	+	+
Synergy	Weak	Weak	+	−	−	ND	ND
cAMP	Weak	−	−	−	ND	ND	+
IP_3	Weak	+	+	−	ND	−	ND
cGMP	Weak	+	+	−	ND	ND	++
GTP/ACA	+	−	−	+	ND	ND	ND
GTP/PLC	+	+	+	−	ND	−	ND
GTP/BIND	−	+	+	−	ND	ND	ND
cAR1-P	−	+	+	+	+	ND	+
Ca^{2+}	−	+	+	+	+	+	+

In each case, + means the mutant behaves like wild type in that assay; − means that the function is lost. cAMP, IP_3, and cGMP refer to the cAMP-stimulated accumulation of these messengers. GTP/ACA or GTP/PLC refer to the capacity of GTPγS to stimulate ACA or PLC activity in lysates (Van Haastert and Devreotes, 1993). GTP/BIND refers to the capacity of GTP to reduce the affinity of [^{3}H]-cAMP binding to isolated membranes (Van Haastert and Devreotes, 1993). cAR1-P refers to the ligand-induced phosphorylation of cAR1 reflected in an altered electrophoretic mobility (Klein et al., 1988). Ca^{2+} refers to cAMP-stimulated $^{45}Ca^{2+}$ uptake (Milne and Coukell, 1991).

ND, not determined.

[a] Aggregation and chemotaxis occur but are abnormal.

cAMP is obviously essential for cell–cell signaling, receptor-mediated changes in intracellular cAMP levels appear to be dispensable for chemotaxis and differentiation (see below). Receptor-stimulated production of IP_3, widely regarded as a fundamentally important response in many cells, including many types of neurons, also appears to play a relatively minor role in chemotaxis and gene expression—a *plc*$^-$ cell line displays a wild-type phenotype (Drayer and Van Haastert, personal communication). Further work is needed to discover the effectors of G2 that are essential for chemotaxis and gene expression. In this regard, it is notable that $G_\alpha 2$ undergoes a rapid, cAMP receptor-mediated phosphorylation, which may transiently influence its activity toward certain effectors (Gundersen and Devreotes, 1990).

The pathway leading to activation of ACA branches at G2 (Figure 2). GTPγS will activate ACA in membranes from the *gα2*$^-$ cells, indicating that $G_\alpha 2$ does not directly confer guanine nucleotide regulation to the enzyme (Kesbeke et al., 1988; Pupillo et al., 1992). Appropriate regulation of ACA is also present in each of the *gα*$^-$ cell lines (*gα6*$^-$ has not been tested). These observations might be explained if the activation were mediated by the βγ subunit complex. In intact cells, $G_\alpha 2$ would be required to regulate the transient release of the βγ subunit complex by cAR1 excitation of G2. In lysates incubated with GTPγS, βγ subunit complexes could be released from any G protein heterotrimer. A similar mechanism is believed to regulate mammalian adenylyl cyclase subtypes II and IV. These subtypes are prevalent in brain, so this mechanism could be important in synaptic transmission (see Tang and Gilman, 1991; Federman et al., 1992).

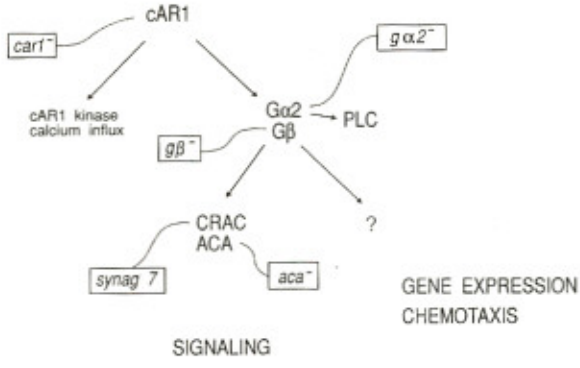

Figure 2. Signal Transduction Pathway

Relationship between major components required in cell–cell signaling, chemotaxis, and early gene expression. Null mutants described in the text are indicated.

Analysis of an aggregationless mutant, designated *synag 7*, indicates that an additional component is required to confer guanine nucleotide sensitivity to ACA (Table 1; Figure 2). In intact *synag 7* cells, cAMP stimuli do not trigger cAMP synthesis, and in lysates, GTPγS does not activate ACA. The in vitro defect can be corrected by supplementation of the assay with a protein, designated cytosolic regulator of adenylyl cyclase (CRAC), found in wild-type cytosol (Theibert and Devreotes, 1986; Lilly and Devreotes, submitted). Moreover, the aggregationless phenotype of *synag 7* can be reversed by transformation with the gene encoding CRAC (Insall, Lilly, and Devreotes, unpublished data). The early evidence suggests that the βγ subunit complex and CRAC may act synergistically to activate ACA. A mammalian homolog of CRAC might be expected to be found in neurons in which adenylyl cyclase subtypes II and IV are in abundance.

Both *synag 7* cells and *aca*$^-$ cells, which contain little or no cAMP, fail to differentiate in isolation. However,

these mutants can be induced to aggregate and differentiate by synergy with wild-type cells or by appropriate stimulation with exogenous cAMP (Pitt et al., 1992, 1993; Schaap et al., unpublished data). These observations show that receptor-mediated increases in intracellular cAMP are not required for chemotaxis and gene expression. Yet, genetic analyses demonstrate that an active catalytic subunit of the PKA is essential for later development, and it is reported that this defect cannot be bypassed by synergy or cAMP stimulation (Harwood et al., 1992; Mann et al., 1992; Anjard et al., 1992). These apparently conflicting observations might be resolved if it were found that the PKA is "cross-activated" by receptor-mediated increases in cGMP or, alternatively, that its "basal" activity is sufficient to mediate its actions.

Although G2 is required for most of the actions of cAMP on cells, certain responses, such as Ca^{2+} influx and receptor phosphorylation, persist in $g\alpha 2^-$ cells (Milne and Coukell, 1991; Pupillo et al., 1992). These receptor-elicited responses also remain in each of the $g\alpha^-$ and $g\beta^-$ cell lines, suggesting that they occur completely independently of G proteins (Milne and Devreotes, 1993; Milne and Wu, personal communication). This unexpected finding suggests that there is a novel pathway, indicated by the arrow extending from cAR1 to cAR1 kinase and Ca^{2+} influx in Figure 2, by which seven transmembrane helix receptors can transduce signals. So far, such a G protein–independent ion flux mediated by a seven helix receptor has not been reported. This might be anticipated in neurons, since ionic regulation is particularly essential.

What are the roles of the four cAMP receptor subtypes that are sequentially expressed during the developmental program (Figure 1)? Current evidence suggests that all of the cAMP receptors are linked to the same signal transduction pathways as those outlined above for cAR1 and the affinity of each subtype is matched to the ambient cAMP concentration it is required to sense. For instance, in *car1*$^-$ cells, high concentrations of cAMP can activate the adenylyl and guanylyl cyclases and can induce differentiation, indicating that another receptor can substitute for cAR1 (Pupillo et al., 1992; Soede et al., submitted; Insall et al., submitted). Furthermore, the $G_\alpha 2$-independent pathway for Ca^{2+} influx can be stimulated equally well by any cAMP receptor ectopically expressed in a *car1*$^-$ cell; the concentration of cAMP that elicits a half-maximal response depends on the affinity of the expressed receptor (Johnson et al., 1991; Milne and Devreotes, 1993). It is likely that the cAMP receptors differ in regulatory properties as well as affinities. The four sequences, although well conserved throughout the transmembrane domains and intracellular loops, are highly divergent in the C-terminal cytoplasmic domains, the regions thought to be important for desensitization (see below). Thus, the switching of receptor subtypes may permit the same pathways to be differentially regulated as required at a particular stage of development or within a certain cell type.

Desensitization of These Signaling Pathways

Persistent stimulation of cells with cAMP leads to desensitization of many of the responses mediated by cAR1. Similar phenomena are observed in many G protein–linked signaling pathways, including those activated by rhodopsin, the β-adrenergic receptor, and the yeast pheromone receptors. The process involves several distinct mechanisms, including adaptation, a rapid reversible "uncoupling" reaction, loss of ligand binding without receptor loss, and down-regulation, a ligand-induced acceleration of receptor degradation and disappearance of receptor RNA (Van Haastert et al., 1992; Dohlman et al., 1991).

The properties of adaptation have been investigated in Dictyostelium by studying transient changes in cAMP, cGMP, myosin light chain phosphorylation, and pseudopod extension in response to jumps in external cAMP (Devreotes and Steck, 1979; Van Haastert and Van der Heijden, 1983; Berlot et al., 1985; Fontana et al., 1984). Cells respond to increments in the fraction of occupied receptors and adapt when the stimulus is held constant (Figure 3). The kinetics and dose dependence of ligand-induced phosphorylation of cAR1 are strongly correlated with those of adaptation processes (Vaughan and Devreotes, 1988). For instance, a stimulus increment evokes a rapid activation of ACA and a slower increase in the extent of cAR1 phosphorylation. As the level of cAR1 phosphorylation reaches a new steady state, the activation of ACA subsides. The enzyme can be activated again by a further increment in the stimulus or by its reapplication after a recovery period (Figure 3).

The parallels between these features of adaptation and those involved in uncoupling of receptor–effector interactions in higher eukaryotic cells are remarkable. However, the mechanism of cAR1-mediated adaptation of ACA differs in its details from the standard paradigm proposed for the rhodopsin and β-adrenergic receptor systems. According to that model, ligand-induced phosphorylation of the receptors facilitates the binding of arrestins, which prevent further interaction of the excited receptors and target G proteins. In contrast, adaptation of ACA in Dictyostelium is reflected in a loss in the capacity of GTPγS, which bypasses the receptor, to activate the enzyme in cell lysates. The extent of attenuation of the GTPγS sensitivity is correlated with the amount of modified cAR1 (Figure 3). These observations suggest that adaptation involves a mechanism by which phosphorylated cAR1 sends an adaptive signal to a downstream component, such as a G protein linked to ACA or to the enzyme itself.

The phosphorylation of cAR1 occurs in a complex pattern on three clusters of serines found in its C-terminal cytoplasmic domain (Hereld et al., 1994). The serine cluster located nearest the C-terminal contains only sites of basal phosphorylation. The centrally located cluster contains basal sites as well as ligand-induced sites. The cluster located proximally, only 39

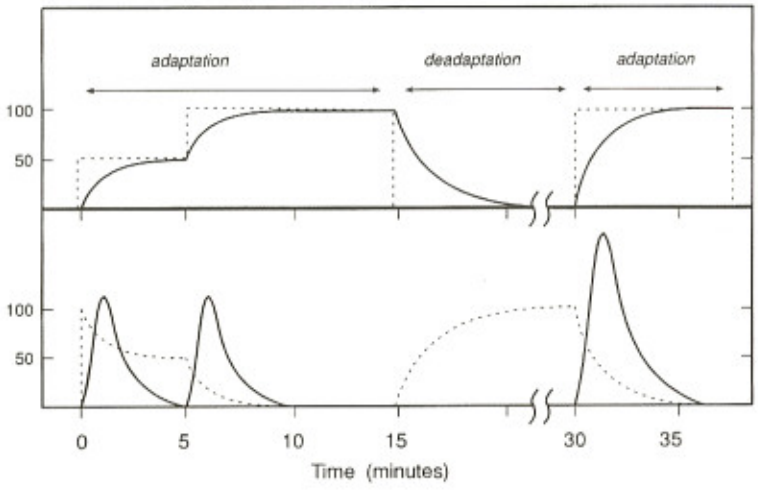

Figure 3. Properties of Adaptation

(Top) Applied stimulus, in units of fractional receptor occupancy, is indicated as a broken line. The solid line shows the corresponding changes in cAR1 phosphorylation. (Bottom) ACA activation. The solid line shows the time course of cAMP synthesis. The area below the dotted line indicates the capacity of the enzyme to be activated by GTPγS in vitro.

residues from the seventh transmembrane domain, contains no basal sites and is the major target for ligand-induced phosphorylation. The role of phosphorylation is currently being investigated by expressing mutant versions of cAR1 as well as cAR2 and cAR3, which also undergo ligand-induced phosphorylation, in *car1*$^-$ cells (Hereld et al., 1994).

Role of G Protein–Linked Signal Transduction in the Late Developmental Processes of Morphogenesis and Pattern Formation

The evidence outlined above indicates that G protein–linked signal transduction pathways are essential in early development. Extracellular cAMP also plays a key role in morphogenesis and pattern formation during the later stages of the program. For instance, the anterior tips of the multicellular structures continue to secrete cAMP, and if the structures are infused with cAMP, they immediately disassemble (Schaap and Wang, 1984). Moreover, there is an important, but poorly understood, interplay between cAMP and an endogenous steroid-like molecule, differentiation-inducing factor, which determines whether a cell differentiates as stalk or spore (Williams, 1989).

These effects of cAMP are likely to be mediated by cAR3, cAR2, or cAR4, which are expressed at the completion of aggregation or exclusively in the multicellular stages (Saxe et al., 1993; Johnson et al., 1993). As outlined above, these receptors are capable of linking to the same signal transduction pathways as cAR1, although they differ markedly in affinity and probably also in desensitization properties. Whereas cAR1 and cAR3 are expressed in all the cells, cAR2 and cAR4 are expressed only in the 20% of the cells destined to become stalk cells. cAR2 null mutants, created by targeted gene disruption, aggregate normally, but the resulting multicellular structures are markedly detained or arrested at the mound stage prior to formation of the apical tip (Saxe et al., 1993). Interestingly, the cell type–specific genes are still expressed in the arrested structures formed by the *car2*$^-$ cells, and moreover, the prespore genes are markedly overexpressed. These observations suggest that although cAR2 is important for chemotactic cell sorting, morphogenesis, and appropriate late gene expression, it is not essential, perhaps because cAR4 can substitute.

Summary

The similarity of the signal transduction systems controlling early development in Dictyostelium with those mediating the action of hormones and neurotransmitters in mammals suggests that these strategies were quickly refined as eukaryotic cells began to communicate. These simple, genetically tractable organisms thus offer a great opportunity to elucidate these pathways further. Combinations of the null mutants are being studied to address questions of redundancy, cross-talk, and networking. Since cAR1, cAR2, $G_\alpha 2$, G_β, ACA, CRAC, PKA, and PDE are essential to the program, the capacity to rescue these phenotypes also serves as a convenient screen for functional mutations in these proteins. Finally, random mutagenesis by the recently developed method of restriction enzyme–mediated insertion provides a means to isolate new genes (Kuspa et al., 1992). The clear phenotypes of the null mutants observed so far indicate that the Dictyostelium developmental program can be used as a guide to isolate novel components of G protein–linked pathways.

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