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MOLECULAR GENETICS OF SIGNAL TRANSDUCTION IN *DICTYOSTELIUM*

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ABSTRACT

In conditions of starvation, the free living amoebae of *Dictyostelium* enter a developmental program: The cells aggregate by chemotaxis to form a multicellular structure that undergoes morphogenesis and cell-type differentiation. These processes are mediated by a family of cell surface cAMP receptors (cARs) that act on a specific heterotrimeric G protein to stimulate actin polymerization, activation of adenylyl and guanylyl cyclases, and a host of other responses. Most of the components in these pathways have mammalian counterparts. The accessible genetics of this unicellular organism facilitate structure-function analysis and enable the discovery of novel genes involved in the regulation of these important pathways.

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PERSPECTIVES

Signal transduction is involved in nearly all physiological events, and defects in signal transduction pathways often give rise to disease. These processes are difficult to study in complex multicellular organisms. Fortunately, studies of microorganisms show that certain signaling strategies have been conserved throughout eukaryotic evolution. In *Dictyostelium*, G protein–linked signal transduction events, in particular, are essential for chemotaxis, cell aggregation, morphogenesis, gene expression, and pattern formation. Many of the proteins involved in these events have mammalian counterparts (1). Thus, the sensing of extracellular stimuli is a fundamental cellular process. Just as the yeast genetic system is useful for studies of cell division and secretion, the genetics and biochemistry of *Dictyostelium* provide powerful tools for the study of signal transduction and chemotaxis (2). Null mutants are constructed to assess the roles of receptors, G-protein subunits, and various effectors. Phenotypic rescue of these mutants provides a convenient screen for random mutagenesis, a technique that can be used to study mammalian as well as endogenous proteins. Insertional mutagenesis is used to discover new genes that feed into these pathways.

This review focuses on signaling in the early stages of development: the transmembrane signal transduction events occurring about 4 h after the initiation of starvation. An in-depth examination of events occurring in late development, as well as cell-fate decision making and morphogenesis, is provided in several recently published reviews (3–7).

INTERCELLULAR SIGNALS IN GROWTH AND DEVELOPMENT

Dictyostelids are free-living protozoa that have developed strategies to survive during starvation. Their life cycle consists of two distinct phases: (*a*) a vegetative or growth stage in which individual amoebae use phagocytosis or pinocytosis to ingest bacteria or liquid media and (*b*) a starvation-induced developmental stage in which amoebae aggregate and, within 24 h, differentiate into

a resistant form consisting of spores atop a stalk of vacuolated cells. When adequate environmental conditions recur, the spores germinate and the cycle is repeated. The most well-characterized species is *Dictyostelium discoideum.*

The transition from single cells to multicellularity is mediated by a variety of signaling molecules. One of them is the ubiquitous messenger adenosine 3′-5′ cyclic monophosphate (cAMP). Its synthesis, detection, and degradation are exquisitely regulated. Within 4 h of starvation, when the necessary components are maximally expressed, cells begin to secrete the nucleotide. It binds to surface receptors leading to chemotaxis, the synthesis and secretion of additional cAMP (signal relay), and increased early gene expression. Cyclic AMP is produced at 6-min intervals— a specific form of extracellular phosphodiesterase serves to degrade it between pulses. In a cell monolayer these oscillations in the levels of cAMP generate propagating waves. Each passing wave provides a gradient that directs the cells further toward the aggregating center (Figure 1*a*). Cells are attracted to each other and form streams as they assemble. By 10 h, up to 10^5 cells have formed a loose aggregate. At this stage, high, constant levels of cAMP activate specific transcription factors, and the cells within the aggregate differentiate. As a migrating slug appears, the prestalk cells (15%) and the prespore cells (75%) sort to the front and back of the structure, respectively. "Anterior-like" cells (10%) form isolated islands in the prespore region. The cell-type decision is governed by position in the cell cycle at the time of starvation and through the action of specific morphogens including differentiation-inducing-factor (DIF), adenosine, and ammonia. These compounds direct a series of morphological rearrangements, and culmination into a mature fruiting body finally occurs (Figure 1*b*).

Even in the vegetative and preaggregatory stages, these free living amoebae display a high degree of interaction by secreting and sensing specific signaling molecules. Pterins bind to specific receptors on the cell surface, activate guanylyl and adenylyl cyclases and actin polymerization, and induce chemotactic responses similar to those induced by cAMP in early aggregation (8–12). The cells can move toward bacteria by sensing folic acid. Growing amoebae secrete a glycoprotein called prestarvation factor (PSF), which accumulates in proportion to cell density, serving as a sensor for the availability of nutrients (13–15). At high PSF/bacteria ratios, a prestarvation response is initiated. The expression of several genes involved in early aggregation is partially increased, thereby preparing cells to enter the developmental program. How PSF mediates these effects is not known; the components used for cell-cell signaling during aggregation are not required (16).

Once the cells have starved, other diffusible molecules are secreted, presumably also for sensing cell density (17). One of these, conditioned medium factor (CMF), is involved in regulating cAMP signaling (18–20). CMF has been cloned, and cells transformed with antisense genes are unable to undergo

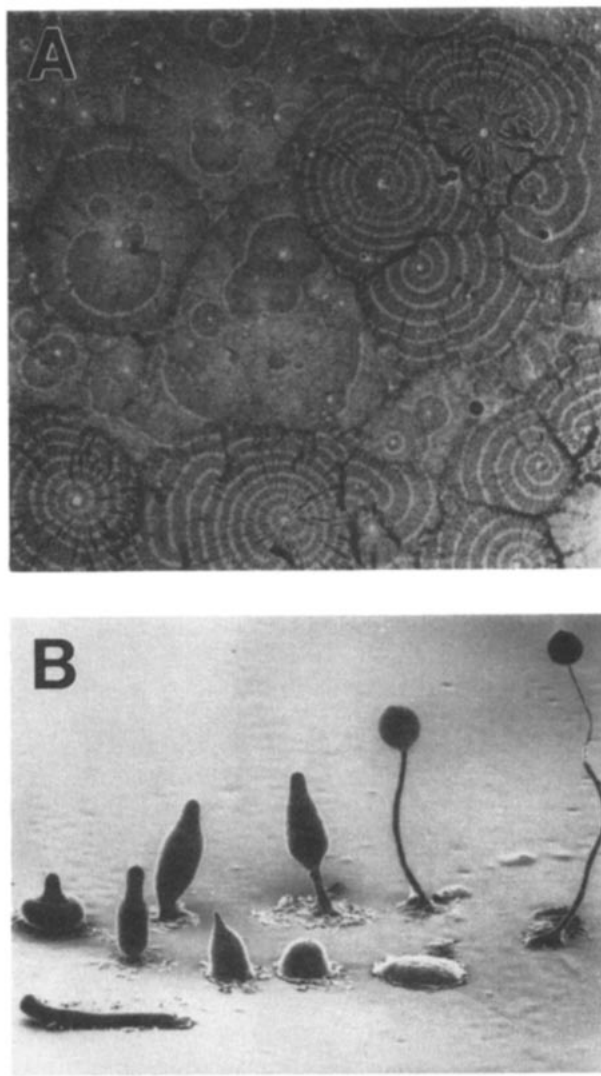

Figure 1 *Dictyostelium* chemotaxis and morphological development. (*a*) Dark field photography of aggregating cells (5 h after the onset of starvation). Each territory of 1–2 cm in diameter contains approximately 1×10^6 cells. (*b*) A composite picture depicting the various developmental stages of *Dictyostelium*. Clockwise from bottom right corner: cell aggregate, mound, tipped mound, first finger, migrating slug (off set; 2–3 mm in length), early-, mid-, late-culminant and fruiting body (2–3 mm tall). Photographs *a* and *b* courtesy of Drs. Peter C Newell and Larry Blanton, respectively.

development because they cannot respond to cAMP (21, 22). When recombinant CMF is given to the antisense cells, cAMP-mediated responses are restored within 30 s indicating that CMF is required, not for expression, but for function of one or more components of the signaling apparatus (23). Cells also respond to various other activators including platelet activating factor (PAF), lysophosphatidic acid (LPA), and yeast extract (24–26). In summary, throughout the life cycle, intercellular communication plays a central role in defining both growth and developmental outcome whether the cells are dispersed (vegetative stage) or assembled in a multicellular structure (developmental stage).

BIOCHEMICAL AND GENETIC TOOLS

GENOME *Dictyostelium's* genome is made of 40,000 kbp organized in six linkage groups (27). Through mutagenic and genetic studies, the total number of genes present in the genome is estimated to be around 7000 (28; WF Loomis, personal communication). Mutations in about 300 genes yield viable cells that display aberrations in development. A parasexual genetic system is used to assign mutants to linkage groups and to cross mutations into a single strain (29). Megabase-restriction and yeast artificial chromosome (YAC) contiguous maps spanning the entire genome have recently been completed. These can be used to rapidly localize the position of any gene and clone the surrounding locus. Completion of the maps has paved the way for sequencing of the genome (WF Loomis & A Kuspa, personal communication).

CELL CULTURE AND MUTANT HANDLING The availability of wild-type and axenic strains makes it possible to grow cells on bacterial lawns or in liquid cultures of defined media with doubling times of 4 and 12 h, respectively (30). Over 10^{11} clonal cells in 10 liters can be grown in a few days. The vegetative and developmental stages are completely independent, and switching between the two states is trivial. Since the process is readily reversible, developmental mutants can be easily selected and then propagated by returning them to liquid media (31). Mutant storage is accomplished by freezing amoebae or spores at −70° C or by desiccating spores (30).

HOMOLOGOUS RECOMBINATION The disruption of endogenous genes by homologous recombination is efficient and predictable. Close to 100 genes involved in signal transduction, cell motility, and cell differentiation have been targeted (WF Loomis, personal communication). Double and triple knockouts are readily achieved in single or tandem transformation steps. Most signal transduction genes are not essential for growth. Since the cells are free living, gene deletions that might be lethal in an organism can often be studied.

HIGH-EFFICIENCY TRANSFORMATION Establishment of permanent cell lines is achieved within two weeks of transformation. There are six different available selectable markers (neomycin, hygromycin, bleomycin, blasticidin, uracil, thymidine). Endogenous plasmids, present in *Dictyostelium*, have been used to construct shuttle vectors carrying specific promoters (32–35). These vectors segregate and have transformation efficiencies of 10^{-3}. They are used to carry out mutagenic analysis of selected genes by complementation of null mutants: Randomly mutagenized libraries are constructed in these extrachromosomal expression vectors, transfected into the mutants, and the resulting transformants are screened for phenotypic abnormalities (see below). Heterologous expression of mammalian genes with retention of function is also possible, allowing the use of *Dictyostelium* genetics to be applied to the study of mammalian proteins (36–39).

RESTRICTION ENZYME–MEDIATED INTEGRATION Restriction enzyme–mediated integration (REMI), first described in yeast, has been successfully used to isolate greater than 20 new gene products involved in aggregation and late development (40, 41; WF Loomis, personal communication). REMI is performed by cotransforming a linearized plasmid with a restriction enzyme and generating random insertions of the plasmid within the genome at the corresponding restriction sites. The cloning of the DNA flanking the insertion is achieved by genomic DNA digestion, circularization, and transformation into *E. coli*. To verify that the recovered DNA sequence is responsible for the phenotype, the rescued plasmid is used to recreate the genotype by homologous recombination.

SIGNAL TRANSDUCTION IN EARLY DEVELOPMENT

A panoply of signaling components involved in cell-cell communication have been cloned and characterized, and the genes for most of these elements have been deleted. The resulting developmental and biochemical phenotypes have provided a detailed picture of how signaling leads to multicellular development. The expression pattern and properties of these genes will be discussed. For many of the signaling elements, multiple minor transcripts are expressed throughout development— the predominant ones are illustrated in Figure 2. Figure 3 depicts a model of the signaling pathways.

Receptor Subtype Switching Programs Development

Four cAMP receptors (cAR1–cAR4) are sequentially expressed throughout development (42–45). cAR1 is expressed early in development in all cells (Figure 2). A second cAR1 transcript, encoding the same protein, is expressed

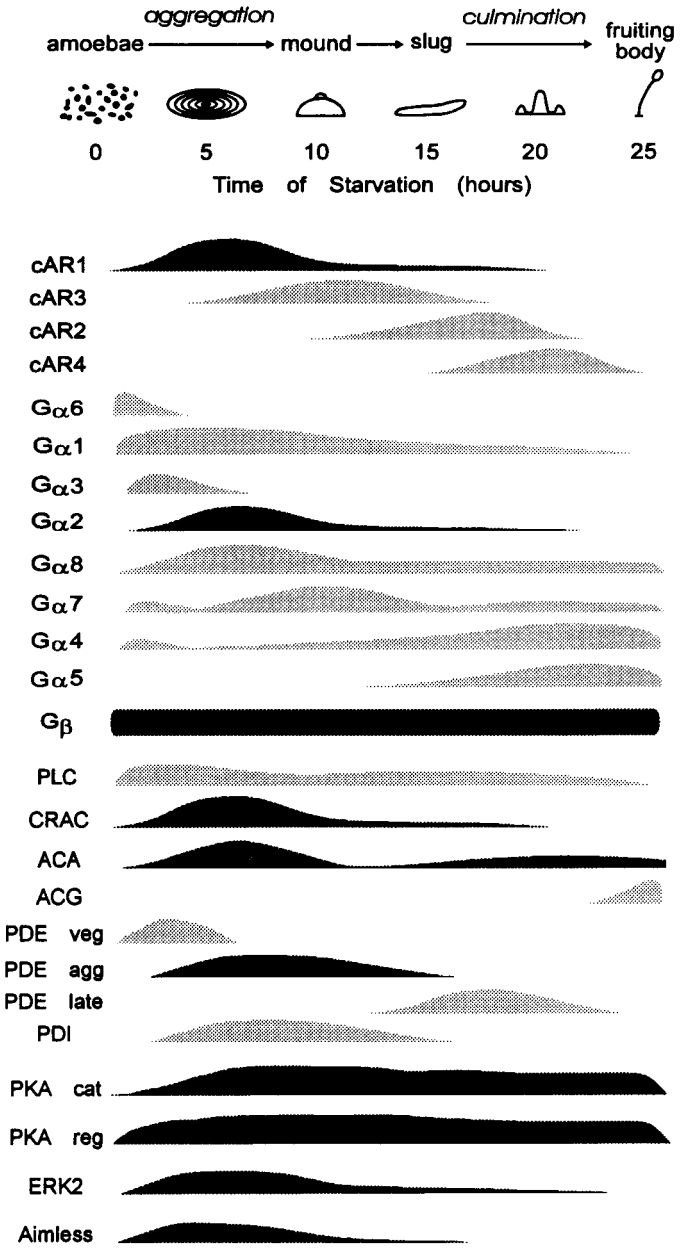

Figure 2 Time course of expression of major RNA transcripts. Top panel illustrates the developmental stages appearing after the onset of starvation. Receptors, G proteins, regulators, and several effectors are shown below. Components essential for early development are heavily shaded. See text for details.

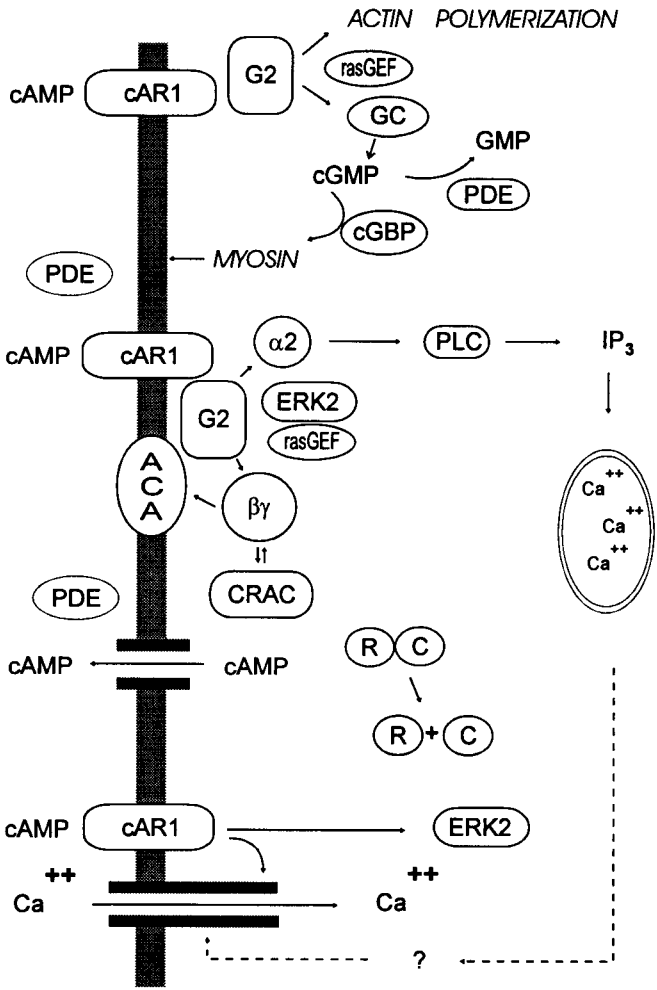

Figure 3 Signal transduction pathways. Model illustrating the proposed events following activation of surface receptors by cAMP leading to chemotaxis, signal relay, and gene expression. See text for details.

at low levels during the multicellular stages. The expression of the early transcript depends on cAMP pulses present in early development: The late transcript is induced by the presence of continuous levels of cAMP (46, 47). cAR3 appears next and is confined to prespore cells. cAR2 and cAR4 are expressed in the prestalk cells of mounds and slugs (45, 47a, 47b) (Figure 2).

The deduced amino acid sequence of each receptor conforms to the seven transmembrane topology common to members of the superfamily of G protein–coupled receptors. The proteins are 60% identical within their transmembrane domains and connecting loops (48). The C-terminal cytoplasmic domains differ extensively in sequence and twofold in length. cAR1 and cAR3 display high-affinity cAMP binding sites with Kds of about 200 and 700 nM, respectively. cAR2 binds cAMP with a Kd in the μM range (49). This range of affinities mirrors the cAMP concentrations thought to be present in the early and late developmental stages (50).

Deletions of individual and/or multiple receptors have been examined. Cells that lack cAR1 are aggregation deficient and, in response to low concentrations of cAMP, do not activate adenylyl cyclase and do not carry out chemotaxis (51, 52). However, *car1*$^-$ cells will respond to higher concentrations of cAMP and can be forced to differentiate into fruiting bodies (51). Deletion of both cAR1 and cAR3 renders the cell completely insensitive to cAMP, suggesting that cAR1 and cAR3 are functionally redundant (53). Recent studies show that cAR2 and a series of cAR1/cAR2 chimeras, when ectopically expressed in *car1*$^-$/*car3*$^-$ cells, can trigger all the signal transduction events typically mediated by cAR1 and cAR3 (see below) (54; JY Kim & PN Devreotes, unpublished results). Cells that lack cAR2 or cAR4 aggregate normally but then arrest at multicellular stages of development. The *car2*$^-$ cells form tipless mounds; the *car4*$^-$ cells form abnormal slugs and fruiting bodies (43, 45). Taken together, these observations suggest that the cARs are linked to the same signaling pathways. The differential expression of four receptors with different affinities for the same ligand may allow particular cell types to respond differently to the same external cAMP signals. These differential responses might be used to drive differentiation and the morphological movements of the cells.

Responses Mediated by Chemoattractant Stimulation

The binding of cAMP to cells leads to multiple events having distinct time courses and patterns of regulation (reviewed in 55). Within 10 to 30 s after the addition of a stimulus, inositol triphosphate (IP_3) and guanosine 3′-5′ cyclic monophosphate (cGMP) levels increase and a dramatic rise in filamentous actin occurs. The light chains of myosin I and II are phosphorylated; myosin II is translocated to the cytoskeleton where its heavy chain is phosphorylated (56, 57; M Titus, personal communication). After a 5 s lag, a transient influx of calcium occurs and the level of cytosolic calcium rises (58–60). Within 30 s, a talin homologue is recruited to the tips of filopods (61). Translocation of a cytosolic regulator of adenylyl cyclase occurs, and accumulation of cAMP peaks within one minute (62). A MAP kinase is activated and a G protein

α-subunit is phosphorylated with a similar time course (62a). As with other G protein–coupled receptors, cAMP binding to cAR1 results in persistent phosphorylation of the receptor and subsequent desensitization (see below). Some of these events presumably come together to mediate chemotaxis; others are involved in cell-cell signaling or in gene expression.

Many G Protein α-Subunits: Functional Diversity or Redundancy?

Eight G protein α-subunits have been isolated (63–67). The mRNA for each has been examined throughout development. Gα6 is present exclusively in growth stage cells; Gα1, Gα2, Gα3, Gα7, and Gα8 are expressed in early development; and Gα4 and Gα5 appear predominantly in the multicellular stages (Figure 2). All the α-subunits are proposed to couple to a unique constitutively expressed β-subunit (Figure 2). The γ-subunit has yet to be cloned. The eight α-subunits are ~35–50% identical to each other and their mammalian counterparts (68). They do not fall into distinct classes nor within any of the Gs, Gi, or Gq subfamilies. Most contain all of the highly conserved features of the guanine nucleotide binding domains found in mammalian α-subunits. Gα8 differs: It has a much longer and divergent C-terminal region, the putative site of receptor interaction.

The functions of seven of the α-subunits have been assessed by gene targeting (Gα6 remains to be analyzed) (67, 69–71; RA Firtel, personal communication). Only Gα2 and Gα4 show striking phenotypes, discussed in detail below. The deletions of the others have not displayed major growth or morphological aberrations. *gα1*$^-$ cells show slight abnormal morphogenesis and conditional defects in phospholipase C (PLC) regulation (69, 72). Cells lacking Gα5 do not display morphological abnormalities but seem defective in developmental timing (RA Firtel, personal communication). Moreover, in an experiment that might address redundancy, expression of constitutively active forms of Gα1 and Gα7 produced developmental abnormalities (67, 69). Additional experiments of this kind are needed to delineate the functions of the individual subunits.

Null mutants lacking functional Gα2 genes do not aggregate or differentiate (73). These mutants show no cAMP-induced stimulation of adenylyl cyclase, guanylyl cyclase, PLC, or actin polymerization. Moreover, membranes derived from *gα2*$^-$ cells do not exhibit guanosine triphosphate (GTP) regulation of cAMP binding affinity or guanosine-5′-0-(3-thiotriphosphate) (GTPγS) activation of PLC (74–76). Perhaps because of persistent desensitization, both dominantly active and inactive point mutations in Gα2 yield essentially loss-of-function phenotypes (77). Taken together, these data demonstrate that Gα2 is the G protein coupled to cAR1 and cAR3 and that this complex mediates the cAMP-dependent events in aggregating cells (Figure 3).

The *gα2*⁻ cells have also been used to test whether receptors for other agonists use G2. Folic acid–induced responses are normal in *gα2*⁻ cells (74, 78). PAF, on the other hand, seems to mediate some of its effects through the Gα2 subunit. The intracellular concentration of PAF increases following the addition of cAMP (24). Exogenously added PAF amplifies cAMP-dependent cellular oscillations as well as activation of adenylyl and guanylyl cyclases. In addition, PAF can independently stimulate a net calcium uptake, an effect which is abolished in *gα2*⁻ cells. Through the use of selective inhibitors, it has been proposed that PAF stimulates calcium influx via the IP_3 signaling pathway (79, 80).

As reported for several mammalian G protein α-subunits, Gα2 is phosphorylated in response to agonist (81). The phosphorylation occurs at physiological concentrations of cAMP, depends upon cAR1, and coincides with the time course of activation of adenylyl cyclase. Analysis of a series of Gα1/Gα2 chimeras and site-directed mutants of Gα2 demonstrated that serine 113 is the exclusive site of phosphorylation (82). The physiological significance of this receptor-mediated modification remains unknown— expression of the S113A mutant in *gα2*⁻ cells appears to restore the phenotype to that of wild-type cells (82).

Null mutants of Gα4 show both growth and developmental phenotypes (71). On bacteria, cells lacking Gα4 grow slower than wild-type cells. Upon starvation, they aggregate and differentiate normally to the mound stage but show abnormal late development and a low level of spore production. These phenotypes are consistent with the expression pattern of the Gα4 subunit: low in vegetative cells, absent in aggregation, and at high levels again at the mound stage (Figure 2). Biochemical analysis of wild-type and Gα4 null cells revealed that Gα4 couples to specific subtypes of folic acid receptors expressed in vegetative and differentiated cells (83). Consequently, the cells use folic acid not only as a chemoattractant, but also as a signal for multicellular differentiation in later development. Although *gα4*⁻ cells lose folic acid–stimulated responses, they retain normal cAMP-mediated adenylyl and guanylyl cyclase activation, as well as chemotaxis (71).

G Protein β-Subunit: A Single Gene Mediates All Responses

The unique G protein β-subunit in *Dictyostelium* is over 90% homologous to its *C. Elegans*, *Drosophila*, and mammalian counterparts (84). The homology extends over the seven characteristic conserved sequences designated WD repeats located in the C-terminal. Every WD repeat is more similar to those in other species than to neighboring repeats. The recent crystal structure of mammalian heterotrimeric G proteins shows that these repeats form the blades of a propeller structure (84a). Although the functional significance of these

repeats is not understood, their high degree of conservation does imply functional equivalence (85).

The Gβ null mutants have been useful for evaluating the role of heterotrimeric G proteins in a variety of processes. G proteins are not essential for cell-cycle and growth-related processes (84). The $g\beta^-$ cells are normal in size and display typical growth in liquid culture. Although the cells show apparently normal motility on agar surfaces and glass, they do grow more slowly on bacterial lawns, suggesting a defect in phagocytosis. Upon starvation, $g\beta^-$ cells fail to aggregate. cAMP-mediated adenylyl cyclase, guanylyl cyclase, PLC activation, and actin polymerization are completely absent. The cells do not carry out chemotaxis to any chemoattractant tested and do not accumulate cGMP in response to folic acid (86; PN Devreotes, unpublished observations). Moreover, membranes of $g\beta^-$ cells display only low affinity, GTP-insensitive, cAMP binding sites. Taken together, these data show that all G protein–dependent pathways are mediated through this unique β-subunit (Figure 3). Whether this subunit dimerizes with a single γ-subunit or with a family of multiple γ-subunits, as observed in mammalian cells, remains to be seen.

Adenylyl Cyclase Activation Involves a Novel Cytosolic Regulator

Two distinct forms of adenylyl cyclase are expressed during development, adenylyl cyclase for aggregation (ACA) and adenylyl cyclase for germination (ACG) (Figure 2) (87). ACA is expressed during aggregation. It shares homology and a typical 12-transmembrane topology with the *Drosophila* and mammalian G protein–coupled adenylyl cyclases. This enzyme is responsible for the synthesis of cAMP required for cell-cell signaling. Cells lacking ACA are devoid of chemoattractant-induced adenylyl cyclase activity and, of course, will not spontaneously aggregate. These cells demonstrate normal cAMP-mediated guanylyl cyclase activation and show chemotaxis towards cAMP, indicating that the upstream signaling components are present and functional. Even though ACA topologically resembles a "transporter," aca^- cells expressing ACG are still capable of secreting cAMP, suggesting that there are independent cAMP transporters (87). ACG is a novel form of adenylyl cyclase, predicted to have a single transmembrane helix separating large intracellular and extracellular domains. ACG is normally expressed exclusively during germination (Figure 2). When expressed in aggregating cells the enzyme is constitutively active and insensitive to GTPγS. Nevertheless, ACG suppresses the aggregationless phenotype of the aca^- cells and produces miniature fruiting bodies (87). Recent evidence shows that ACG can be activated by changes in osmolarity and, for this reason, ACG is proposed to be involved in osmosensing in spores (P Schaap, personal communication).

Even though ACA resembles the mammalian enzymes, its mechanism of activation is novel. As described above, both $g\alpha2^-$ and $g\beta^-$ cells lack agonist-induced adenylyl cyclase activity. However, GTPγS will stimulate ACA in membranes of $g\alpha2^-$ cells, indicating that Gα2 does not directly activate the enzyme (73). The GTPγS activation appears to be mediated through the βγ-subunits since $g\beta^-$ cells are completely insensitive to GTPγS (86). Point mutations in Gβ specifically prevent activation of the enzyme without preventing coupling of G2 to cAR1 (L Wu, T Jin & PN Devreotes, unpublished observations). It has been proposed that in $g\alpha2^-$ cells, GTPγS releases βγ-subunits from G proteins other than G2 (Gα1, Gα3, Gα7, or Gα8), which are then able to activate ACA (86). In this respect, ACA is analogous to the mammalian type II and IV adenylyl cyclases which are conditionally activated by G protein βγ-subunits (88). Unlike other effectors/regulators that interact with Gβγ-subunits, such as the β-adrenergic receptor kinase 1 and 2 (βARK1, βARK2), PLC, phosducin, and the atrial K^+ channel (GIRK1), adenylyl cyclases do not have pleckstrin homology (PH) domains (89, 90). A short sequence common to the mammalian type II and IV enzymes has been suggested as the βγ-subunit contact site (91). In *Dictyostelium*, however, a novel cytosolic protein containing a PH domain is essential for ACA regulation.

The discovery of this protein, named CRAC for cytosolic regulator of adenylyl cyclase, came from analysis of an aggregation-deficient mutant that lacked receptor- and G protein–stimulated adenylyl cyclase activity. GTPγS-induced activation of ACA in mutant lysates could be restored by the addition of cytosol derived from wild-type cells (92). This assay was used to purify the protein and obtain N-terminal sequence (93). The gene for CRAC was obtained from a REMI mutant displaying the same phenotype as the original mutant. The deduced amino acid sequence of 698 residues is hydrophilic and rich in threonine and serine residues— the PH domain is in the N-terminus. There are no other obvious sequence motifs (62). The original mutant bears an in-frame 53–amino acid deletion in the C-terminal region of the protein (P Lilly & PN Devreotes, unpublished observations). Like ACA and cAR1, CRAC is maximally expressed in early aggregation (Figure 2) (62).

The mechanism by which CRAC activates ACA is intriguing. The protein is translocated to membranes following chemoattractant stimulation of intact cells or during GTPγS activation of lysates. The GTPγS-induced relocalization does not require ACA, cAR1, or Gα2; it occurs in lysates of cells lacking each of these genes. However, this relocalization does not take place in mutants lacking the G protein β-subunit (94). These results suggest that CRAC binds directly to activated βγ-subunits or that the generation of its binding sites depends on βγ-subunits (Figure 3). Given what is known about PH domains, the N-terminal of CRAC likely mediates the βγ-subunits-CRAC association. Consequently, the mode of recruitment of CRAC to the membrane could be

analogous to that of βARK's interaction with released G protein βγ-subunits (95). Since the G protein–coupled receptor cascade is highly conserved from mammals to *Dictyostelium*, we propose that a mammalian homologue of CRAC exists and that this novel mode of regulation is important for activation of adenylyl cyclases in higher eukaryotes.

Ras and MAP Kinase Regulate Adenylyl Cyclase Activation

The pathway leading from cAR1 to the activation of ACA displays further complexity (Figure 3). This pathway involves one of two *Dictyostelium* MAP kinase homologues, extracellular signal–regulated kinase 2 (ERK2) (96). ERK2 was isolated as an aggregation-deficient REMI mutant. Northern analysis reveals that ERK2 mRNA is present in vegetative cells and in early development, peaking at ~4 h after the onset of starvation (Figure 2) (96). Surprisingly, cells lacking ERK2 are specifically defective in cAMP-stimulated adenylyl cyclase activation. Receptor-mediated activation of guanylyl cyclase is unaffected in these cells (96). Recent experiments have demonstrated that ERK2 is transiently activated by chemoattractants. This is a receptor-mediated response: It is completely lost in cells lacking both cAR1 and cAR3 (62a). It is not clear whether ERK2 acts directly, for instance, by phosphorylation of CRAC, ACA, or G protein βγ-subunits, or more indirectly, by inducing the expression of yet another essential component required for the activation of ACA.

Another component required for activation of ACA was also originally identified as an aggregation-deficient REMI mutant. The gene is called *aimless* (*ale*) because the mutant is impaired in chemotaxis to cAMP, but shows normal random motility. Molecular cloning revealed that Aimless is a homologue of the yeast cdc25 gene, a ras guanine exchange factor (rasGEF) (173). Aimless displays strong homology to other known rasGEFs in its C-terminal region. Northern analysis shows that Aimless is present in growth and in early development (Figure 2). In lysates prepared from *ale*$^-$ cells, GTPγS-mediated adenylyl cyclase activation is defective and cannot be corrected by the addition of exogenous CRAC. This suggests that the defect lies either in the generation of CRAC binding sites or in the capacity of ACA to respond to activated βγ-subunits and/or CRAC. The nature of the defect in chemotaxis is unknown.

The target of Aimless must be a ras-like protein. In *Dictyostelium*, at least six *ras* genes have been identified (*rasD*, *rasG*, *rasB*, *rap1*, *rasS*, *rasC*), each having a specific expression pattern (97–101). They share the four well-conserved GTP-binding domains as well as the C-terminal CAAX box, although rasC and rasS are considerably divergent from the mammalian consensus. Since specific null mutants are not yet available, little is actually known about

the physiological functions of the ras family of proteins in *Dictyostelium*. The overexpression of an activated form of rasD results in the appearance of multi-tipped aggregates and a decrease in the level of cAR1 expression (102, 103). In mammals, rap1A is known to revert the transformed morphology of cells overexpressing activated ras (104). A similar effect is observed in *Dictyostelium*, where the co-expression of the rap1 protein partially reverses the multi-tipped phenotype (G Weeks, personal communication). Overexpressing rap1 in wild-type cells results in amoebae displaying an enlarged actin cortex, although little effect on development is measured (105). Finally, cells expressing an activated form of rasG, which is normally expressed in growth, show similar altered cytoskeletal function but fail to aggregate (G Weeks, personal communication).

Secreted and Extracellular Membrane-Bound Forms of Phosphodiesterase Regulate Ambient Levels of cAMP

The extracellular concentration of cAMP is tightly controlled through the expression of membrane bound and secreted forms of phosphodiesterase (PDE) and a specific phosphodiesterase inhibitor (PDI) (106). The PDE gene encodes an hydrophilic 452–amino acid protein (107). Three distinct PDE transcripts are expressed during growth, aggregation, and late development (Figure 2) (108). The transcripts contain the same protein coding sequence linked to three overlapping 5′-untranslated sequences. During aggregation, PDE is found in membrane bound (mPDE) and extracellular (ePDE) forms (109, 110). The mechanism by which mPDE is associated with the membrane is not understood; the protein contains a signal sequence but no transmembrane domain. The PDI is expressed in early aggregation (Figure 2) (111). This 26 kDa cysteine-rich soluble protein tightly binds ePDE, changing its K_m for cAMP from 5 μM to 2 mM (112).

Mutants lacking phosphodiesterase activity are unable to undergo aggregation and remain as a smooth monolayer indefinitely (113). Since the cells are unable to degrade cAMP, they cannot support the cAMP oscillations essential for proper development. These mutants can aggregate following the addition of exogenous PDE, suggesting that ePDE is sufficient for degradation of cAMP (114). Previous experiments have suggested that the mPDE alone is also sufficient (110). The physiological role of PDI is unclear because its deletion by homologous recombination does not cause an obvious developmental phenotype (115).

The expression of PDE and PDI is closely regulated by extracellular cAMP. Curiously, even though ePDE and mPDE are derived from the same transcript, they are differentially regulated. The secreted form is increased most effectively by continuous applications of cAMP; the membrane-bound form is

induced by intermittent applications of cAMP and suppressed by the constant presence of cAMP (116). A post-translational modification or the presence of a membrane-associated PDE binding protein could explain these distinct expression patterns. As might be expected, the expression of the PDI gene is repressed by extracellular cAMP (111).

Is Intracellular cAMP Necessary for Gene Expression?

In *Dictyostelium* the cAMP-dependent protein kinase (PKA) is composed of a single regulatory (R) and catalytic (C) subunit (117). The 37 kDa R subunit shares extensive homology with that of mammals (118). However, the C subunit is almost twice the size of its mammalian, *Drosophila*, or yeast counterparts (73 vs 41 kDa) with similarity confined to the C-terminal domain (119, 120). The expression of the R and C subunits is developmentally regulated. Low levels are found in growing cells and expression increases throughout early aggregation (Figure 2) (119–122).

Mutants in either the C or R subunits revealed that PKA is essential for development and gene expression. Disruption of the C subunit yields viable, motile cells that are unable to aggregate and differentiate (119). A similar developmental phenotype is observed in cells constitutively overexpressing the R subunit or expressing a mutant R subunit, Rm, which cannot bind cAMP (123–125). Overexpression of Rm from either prespore or prestalk promoters essentially eliminates the corresponding cell types (126, 127). The former mutants form fruiting bodies with "glassy" heads filled with amoebae rather than spores; the latter form slugs that migrate indefinitely. Cells lacking the R subunit activity or overexpressing the C subunit progress more rapidly through the later stages of development (128, 129).

The expression of genes at all stages of development requires both cAMP and PKA. However, occupancy of PKA by cAMP is neither necessary nor sufficient to activate the genes (130, 131). Instead, most of the effects of cAMP are mediated by the cARs and do not require intracellular cAMP (132). The apparent cAMP-independent PKA activity may be due to mismatching in the levels of the R and C subunits or to stimulation by other compounds such as cGMP or calcium (130, 133).

Cyclic-GMP Is Essential for Chemotaxis

Several lines of evidence point to a fundamental role for cGMP in chemotactic orientation. Chemoattractant-stimulated guanylyl cyclase activation is very brief, peaking after ~10 s and returning to basal levels within 30 s (11, 134). The rapid turn-off may be mediated by an elevation in intracellular calcium (135). The time course of activation correlates with myosin II heavy chain phosphorylation (56). A mutant, *stm F*, defective in a specific cGMP PDE,

displays an extended cGMP response, delayed myosin II phosphorylation, and prolonged chemotactic orientation, suggesting that cGMP is important in chemotaxis (reviewed in 136; 137). The properties of a series of nonchemotactic mutants, designated KI1-KI10, more directly address the potential role of cGMP in chemotaxis. The mutants were selected as aggregation-deficient and then screened for defects in both folic acid– and cAMP-induced chemotaxis. Nine of the ten mutants are deficient in signal transduction events involving cGMP, although they all display normal cGMP phosphodiesterase activity. Genetic analysis reveals that nine mutants are recessive and belong to distinct complementation groups. One mutant, KI10, is dominant (26).

Two mutants, KI-8 and KI-10, lack folic acid– and cAMP-mediated guanylyl cyclase activation. KI-8 is defective in guanylyl cyclase activity per se, while KI-10 is deficient in the activation of the enzyme (26). Additional experiments suggest a direct link between cGMP activation and myosin II heavy chain assembly and phosphorylation: Both responses are absent in KI-10 cells (138). Interestingly, cAMP-stimulated actin polymerization takes place in mutant KI-10, suggesting that actin polymerization is not sufficient to induce a chemotactic response (138). Moreover, cells lacking or overexpressing the myosin heavy chain kinase, a homologue of mammalian protein kinase C (PKC), show altered polarization, chemotaxis, and development (139; 139a). Cyclic GMP must be involved in more than regulation of myosin II, since cells in which the myosin heavy chain has been deleted are viable and display many forms of movement, including chemotaxis (140).

Further characterization of KI4/KI5 and KI2/KI7 also points to a central role for cGMP in chemotaxis. These mutants display normal guanylyl cyclase and phosphodiesterase activities although chemoattractant-induced cGMP accumulation is low in KI4/5 and high and delayed in KI2/7. Recent experiments indicate that these mutants have defects in a cytosolic cGMP-binding protein (cGBP) (141). It has been suggested that guanylyl cyclase is inhibited by phosphorylation. This inhibition is strongly promoted by 8-bromo-cGMP. Thus, cGBP is proposed to be a kinase involved in the regulation of guanylyl cyclase (H Kuwayama & PJM Van Haastert, personal communication).

In addition to being involved in chemotaxis, guanylyl cyclase activation plays a role in mediating responses to osmotic stress (141a). In wild-type cells modest changes in osmolarity induce the synthesis of cGMP which, in turn, stimulates phosphorylation of the myosin heavy chain and leads to a series of events which together bring resistance against osmotic stress. Cells lacking myosin heavy chain or altered in cGMP synthesis (KI4, KI5, KI7, and KI8 mutants) are more sensitive to osmotic shock than wild-type cells. The signal transduction mutants *car1*$^-$/*car3*$^-$ and $g\beta^-$ display normal osmotic shock responses, suggesting that the signal to the guanylyl cyclase does not use this pathway.

A series of mutants in the phototaxis loci were isolated and found to have altered cGMP responses to light or heat stimulation while displaying normal cAMP-stimulated cGMP synthesis (142). Additional experiments need to be performed in order to fully understand the signaling pathway involved in these responses since mutant *stm F* (which is defective in cGMP PDE) exhibited wild-type light and heat responses.

PLC Activation Is Not Necessary for Chemotaxis or Gene Expression

As noted above, cAMP stimuli elevate IP_3 levels. Moreover, an antagonist of chemotaxis, 3′-NH_2-cAMP, causes a decrease in IP_3 levels (144). The only known PLC gene in *Dictyostelium* encodes a 91 kDa protein which shares homology with the mammalian PLCδ enzyme in its C-terminal domain (143). This gene is expressed throughout development and is responsible for the measurable cAMP-dependent PLC activity. Cells in which the PLC gene has been deleted by homologous recombination no longer elevate IP_3 levels in response to stimulation but otherwise show no developmental or biochemical phenotype (145). This result is surprising in view of the extensive roles that have been attributed to receptor-mediated PLC activation in mammalian cells (146).

Further studies have addressed the roles of lipids in signaling. Cells lacking PLC have tonic levels of intracellular IP_3 (145). Recent studies suggest that the IP_3 comes from a PLC-independent route of synthesis in which IP_5, synthesized through sequential phosphorylation of inositol, is degraded to IP_3 via two IP_4 isomers (147). The enzyme proposed to be involved in IP_5 metabolism displays similarity to the mammalian Multiple Inositol Polyphosphate Phosphatase (MIPP) (148; 148a). In addition, changes in the overall levels of diacylglycerol (DAG) during development have been measured (149).

DESENSITIZATION OF RECEPTOR-MEDIATED RESPONSES

Desensitization is a general term describing the waning of a response during persistent stimulation as well as diminished sensitivity to subsequent challenges. For G protein–coupled receptors, at least three processes are involved: (*a*) a loss of responsiveness without loss of cell surface binding sites, referred to as uncoupling or adaptation; (*b*) a loss of cell surface binding sites without loss of receptor molecules, referred to as sequestration or loss-of-ligand-binding; and (*c*) a loss of receptor molecules, referred to as down-regulation. Analysis of a series of mutants in *Dictyostelium* suggests that loss-of-ligand

binding and adaptation occur in the absence of changes in known second messengers, whereas down-regulation requires intracellular cAMP (150).

A great deal of evidence suggests that agonist-induced receptor phosphorylation plays a role in desensitization. According to a well-established paradigm for rhodopsin and the β-adrenergic receptor, phosphorylation by rhodopsin kinase or βARK leads to the binding of arrestin or βarrestin, a competitive inhibitor of the receptor–G protein interaction. Thus, the phosphorylated receptor is essentially removed from the reaction, terminating all responses (151). Although the role for receptor modification in uncoupling from G protein is accepted, whether phosphorylation regulates sequestration or is needed for down-regulation is not generally known. Moreover, the sites of phosphorylation are complex and vary widely for different G protein–coupled receptors (151).

Receptor Phosphorylation Causes Loss-of-Ligand Binding

For cAR1, sites of basal and agonist-induced phosphorylation have been determined by analysis of an extensive series of serine substitution mutants in the C-terminal tail (152). Eighteen serine residues, grouped in four clusters, are found in this region. The results can be summarized as follows: (*a*) clusters 2 and 3 are phosphorylated in unstimulated cells; (*b*) occupancy of the receptor in intact cells triggers a rapid addition of phosphates to clusters 1 and 2; and (*c*) within cluster 1 the phosphorylation of two specific serines residues, S303 and S304, causes an increase in the apparent molecular weight of cAR1 from 40 to 43 kDa.

Receptor phosphorylation is responsible for loss-of-ligand binding. In studies of this process, the loss of binding induced by pretreatment of the cells with cAMP was shown to be substantially due to a reduction in the affinity of the low-affinity class of cAMP binding sites (153, 154). When phosphorylation is prevented by substitution of all the serines, the loss-of-ligand binding process is completely blocked. The inhibition is due primarily to the substitutions of S303 and S304. In summary, receptors phosphorylated on these positions display about a fivefold lower affinity for cAMP than unphosphorylated receptors.

Receptor Phosphorylation Is Not Necessary for Adaptation of Adenylyl Cyclase

The affinity reduction caused by phosphorylation of cAR1 cannot account for the adaptation of adenylyl cyclase. A low concentration of stimulus leaves many nonphosphorylated receptors. The persistent presence of agonist should continue to elicit a response, yet cells adapt completely when occupancy, however low, remains constant and only regain sensitivity when the stimulus

is removed. These properties of adaptation have been modeled by assuming that all forms of the receptor contribute, to varying extents, to the overall state of activation (155–157). When the occupancy is initially increased, a preponderance of the occupied, unphosphorylated form triggers the rapid response. At steady-state, a negative contribution from the phosphorylated, unoccupied form offsets that from the more active forms and the response completely subsides. Unfortunately, in spite of the elegant models, the fully substituted or truncated forms of cAR1 in *car1*$^-$ cells support transient cAMP accumulation responses with wild-type time courses and show only subtle developmental phenotypes (D Hereld & PN Devreotes, unpublished observation).

Adaptation of adenylyl cyclase activation must therefore involve a mechanism other than receptor phosphorylation. In fact, pretreatment of cells with cAMP causes a decrease in the extent of subsequent GTPγS activation of adenylyl cyclase in cell lysates, suggesting that the receptor sends an inhibitory signal that persists in lysates (158). How is this signal transmitted and what is its target? In cells pretreated with cAMP, GTPγS can no longer generate CRAC binding sites (94). It follows that adenylyl cyclase cannot be activated. Since the generation of the CRAC binding sites requires the G protein βγ-subunits, these observations suggest that the signal from the receptor decreases the activity of the βγ-subunits. Mutant analyses show that the signal from cAR1 leading to this attenuation does not require Gα2 or CRAC (73; P Lilly & PN Devreotes, unpublished observations).

RECEPTOR-MEDIATED G PROTEIN–INDEPENDENT PROCESSES

Until recently, seven transmembrane receptors other than the sensory rhodopsin of *Halobacterium salinarium* were believed to mediate all of their physiological effects through the activation of heterotrimeric G proteins (159). However, analysis of several chemoattractant-mediated responses has revealed that these receptors are capable of activating effectors in a G protein–independent manner. The first of these responses to be identified was the receptor-operated calcium influx. Experiments performed in cells lacking Gα2 or Gβ subunits (as well as in *gα1*$^-$, *gα3*$^-$, *gα4*$^-$, *gα7*$^-$, and *gα8*$^-$ cells) demonstrate that folic acid- and cAMP-mediated calcium influx still occurs in the absence of functional G proteins (160–162). The G protein–independent calcium influx leads to a rise in cytosolic calcium levels (60). The EC_{50} for the cAR1-mediated calcium influx response is close to the Kd for the lower affinity state of cAR1. However, the maximal responses are decreased by 50% in cells lacking Gα2 or Gβ, suggesting that calcium influx is partly dependent on the standard pathway. As in mammalian cells, half of the receptor-mediated calcium influx may depend on the depletion of the IP_3-sensitive calcium store (Figure 3) (163).

Agonist-induced cAR1 phosphorylation occurs in a G protein–independent fashion: It displays the same kinetics and concentration dependence in $g\beta^-$ and in wild-type cells (162). Similar results are observed with the phosphorylation of rhodopsin in mammalian cells. Light activation is known to be sufficient to allow receptor phosphorylation: The addition of purified βγ-subunits does not change the extent of rhodopsin phosphorylation (151). In the case of the β-adrenergic receptor, phosphorylation partly depends on the presence of G protein βγ-subunits. The βγ-subunits have been shown to form a complex with βARKs, thereby targeting the kinase to the membrane where it can interact with the receptor (164).

Recent evidence suggests that G protein–independent signaling may be quite common. Indeed, several other cAR1-mediated responses including Gα2 phosphorylation (82), loss-of-ligand binding (162), ERK2 activation (62a), and various gene expression events also take place in a G protein–independent manner. Continuous levels of cAMP accelerate expression of the secreted phosphodiesterase and suppress that of its inhibitor in the $g\beta^-$ cells (164a). The transcription factor GBF can be activated by extracellular cAMP to direct expression of the immediate early genes *rasD* and *CP2* in growth stage $g\beta^-$ cells (165). No quantification is available to assess whether these responses are partially dependent on G proteins.

RAPID PHENOTYPIC SCREENS FOR RANDOM MUTAGENESIS

The *car1*$^-$, *gα2*$^-$, *gβ*$^-$, *crac*$^-$, *aca*$^-$, *pka*$^-$, *erk2*$^-$, *ale*$^-$, and *pde*$^-$ cell lines are unable to aggregate. The *car2*$^-$ and *car4*$^-$ cells arrest at later stages of development. All of these mutants regain the ability to aggregate and differentiate when transformed with plasmids constitutively expressing the respective wild-type cDNAs. Consequently, these cell lines can be used in simple biological screens for loss- or gain-of-function mutations that affect the regulation and activity of these important signal transduction components (Figure 4).

These studies rely on the development of extrachromosomal expression vectors which provide high transformation efficiencies and complete segregation [each transformant contains a unique mutated plasmid (see above)]. Randomly mutagenized genes are subcloned into these vectors, the libraries electroporated into amoebae typically yielding 5000–10,000 transformants, and screened for phenotypic abnormalities. Screening is conveniently performed by clonally seeding cells on a lawn of *Klebsiella aerogenes* (30). As each clone expands, it depletes the bacteria, forming a 1 cm plaque, and undergoes development, making visual scoring easy and unequivocal. Wild-type cells aggregate and form normal fruiting bodies; loss-of-function mutants remain as smooth monolayers or as arrested developmental structures. Sup-

pressors or gain-of-function mutants can be easily spotted as isolated positive plaques in a sea of aggregation-minus clones (Figure 4). Through biochemical analyses, the mutants can be classified and unique amino acid residues can be ascribed to specific functions. Here, the application of the technique is illustrated through the examples of cAR1 and ACA.

Identification of Distinct Activation States of cAR1

The structure-function relationships of seven transmembrane G protein–coupled receptors have been studied in various systems and several common structural features have been established (166). The existence of the four cARs as well as the description of multiple G protein–dependent and –independent responses (see above) makes *Dictyostelium* an excellent system to further study the mechanisms of agonist binding, activation/adaptation, and G protein and effector stimulation.

A region of cAR1 encompassing transmembrane span (TM) III to TMVII was randomly mutagenized. Although each sustained an average of two amino acid substitutions, 90% of the mutant receptor molecules supported wild-type development. The aggregation-deficient clones expressed mutant receptors that had defects in distinct biochemical parameters (JY Kim, MJ Caterina, KC Lin, JA Borleis & PN Devreotes, manuscript in preparation). The majority of the mutants displayed lower binding affinities but were fully functional at high agonist concentrations. These mutants clustered in distinct subclasses (classes IIIa, IIIb, and IIIc) that displayed affinities of either 5-fold, 100-fold, or >10,000-fold lower than wild type. The incremental decreases might suggest that these mutations disrupt individual ligand contact sites. The screen also gave rise to rare mutants which bound cAMP and were phosphorylated but showed neither G protein–coupling nor cAMP-mediated calcium influx (class I).

Random mutagenesis of the third intracellular loop was carried out. As noted for the larger region, the third intracellular loop could be extensively mutagenized without effect on receptor function. The few mutants that did appear displayed signaling or affinity defects (167; JLS Milne & PN Devreotes, manuscript in preparation). Most of the mutant receptors showed diminished (or absent) responses in both G protein–dependent and –independent pathways (classes IV and V).

These random mutagenesis studies provide valuable information on the structure-function relationship of cAR1. A model for receptor activation is depicted in Figure 5. In this scheme, ligand binding leads to activation of the receptor and the acquisition of an active conformation (cAR1*) which permits the interaction with the kinase and leads to receptor phosphorylation. A second

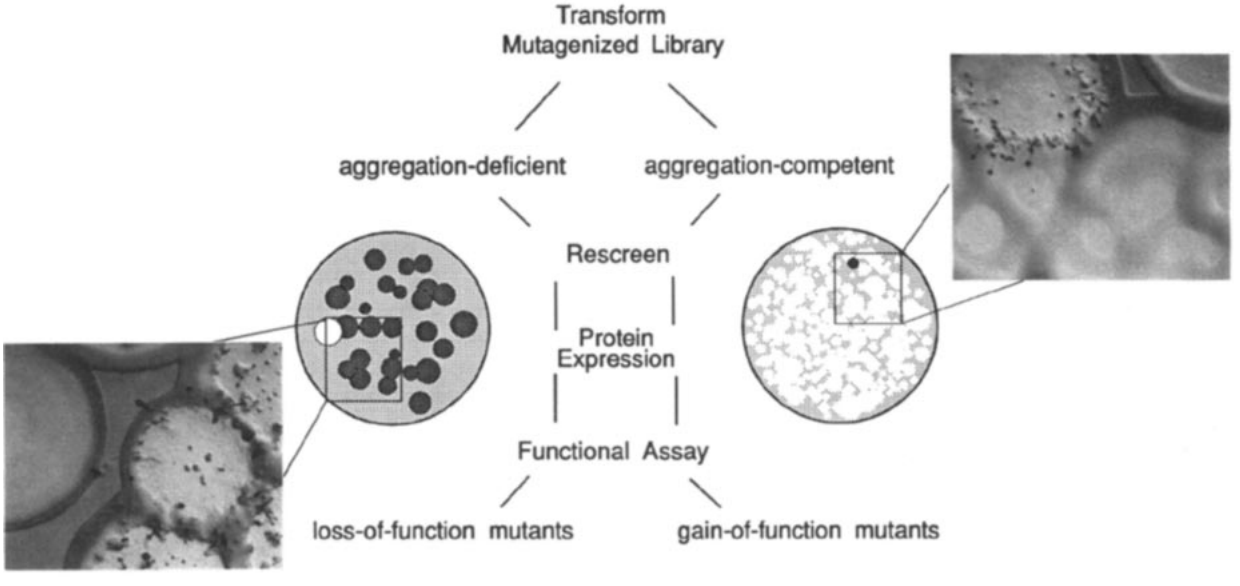

Figure 4 Isolation of loss- and gain-of-function mutants using biological screens. The mutagenized libraries are transformed into *Dictyostelium* cells and aggregation-deficient (white circles) as well as aggregation-competent (dark circles) clones are isolated from bacterial lawns. After rescreening for development and assessment of protein expression, the mutants are classified according to their biochemical characteristics.

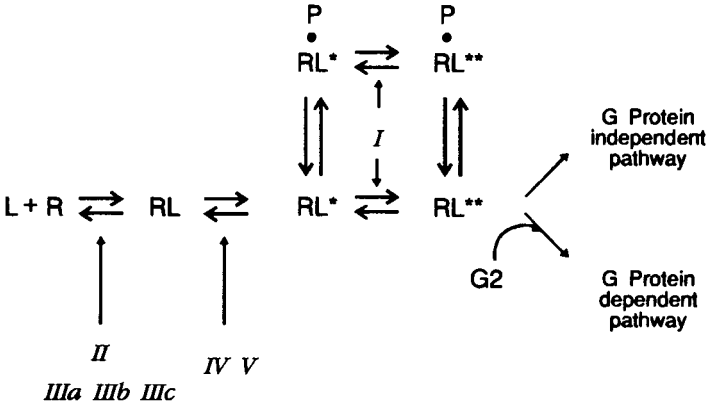

Figure 5 Model for receptor activation based on cAR1 random mutagenesis. Diagram shows interconversions between receptor states. R=cAR1, L=cAMP. The mutant classes are shown in italics. See text for details.

active state (cAR1**) enables the receptor to couple to G proteins as well as activate the G protein-independent pathway. The class III mutants lower affinity without altering activity or affecting the transitions between the activated states.

A procedure called random chimeragenesis was used to study the affinity difference between cAR1 and cAR2. As discussed earlier, under physiological conditions, cAR1 displays a much higher affinity (25 and 230 nM) than cAR2 (>2 μM). This difference disappears in the presence of ammonium sulfate; both receptors display Kds of about 5 nM (49). The residues responsible for the difference in affinity were localized to the C-terminal half of the second extracellular loop (54). In that region, there are only four nonconserved residues; two negatively charged amino acids in cAR2 appear to be the essential changes. cAR3, which has an intermediate affinity, has one negatively charged amino acid within that same region. It was proposed that the negatively charged amino acid(s) interfere with high-affinity binding by hindering the entry of cAMP into the binding pocket and that shielding by high salt eliminates this interference. Interestingly, a cAR1 mutant isolated by random mutagenesis displayed cAR2-like binding properties (class II): It carried a single amino acid substitution in the C-terminal half of the second extracellular loop. Similar results were obtained with the adenosine mammalian receptor: Residues in the extracellular loops were found to be determinants of affinity (168). These residues may comprise "gate keeper" regions modulating the accessibility of ligands to their binding pockets. The affinity differences displayed by the class

IIIa-IIIc mutants persist in ammonium sulfate and may represent differences in the binding pocket.

Isolation of Inactive, G Protein–Insensitive, and Constitutively Active Mutants of ACA

As noted above, G protein–coupled adenylyl cyclases, including ACA, form a family of transmembrane proteins predicted to span the membrane 12 times and contain large cytoplasmic domains between TMVI and TMVII and at the carboxy-terminus (C1 and C2; ~40 kDa each). Since these two cytoplasmic loops share amino acid homology with other adenylyl and guanylyl cyclases, it has been proposed that the catalytic sites are located within these intracellular domains (169). Site-directed mutagenesis and deletion analyses of the mammalian enzymes have demonstrated that catalytic activity and proper regulation require the interaction of the two cytoplasmic loops and suggested that the loops are not functionally equivalent, C1 being more important for regulation and C2 for catalysis (170, 170a).

The first five predicted transmembrane helices as well as the C1 domain of ACA were randomly mutagenized and loss-of-function mutants were isolated. These were found to be either catalytically inactive or resistant to chemoattractant and G protein activation. Of four catalytically inactive mutants analyzed, three harbored substitutions on highly conserved residues located halfway within the C1 loop. These results suggest that this region of the C1 loop is essential for substrate binding and/or catalytic activity. The G protein–insensitive mutants isolated displayed mutations that were grouped in two regions of the cytoplasmic domain: one close to the plane of the plasma membrane just after TMVI and the other halfway within the C1 loop. Mutations in the transmembrane domains led to missorting (35).

These results are interesting for several reasons. First, they suggest that, as is the case for receptors, regions of cytoplasmic loops abutting transmembrane domains are critical for G-protein interaction (171). Tang and Gilman have also demonstrated that the cytoplasmic domains of mammalian adenylyl cyclases are sufficient for G-protein activation (172). Second, these studies imply that the twelve transmembrane domains are needed for appropriate membrane association. Consistent with this possibility is the finding that three ACA mutants showed an abnormal cellular distribution following sucrose gradient fractionation. All three mutants harbored point mutations or deletions within the transmembrane domains, again suggesting that sorting is dependent on these regions.

Gain-of-function adenylyl cyclase mutants were isolated by transforming the same mutagenized libraries into *crac*$^-$ cells. Since CRAC is essential for G protein stimulation of ACA, suppression of the *crac*$^-$ phenotype screened

for mutants with high unregulated activity or for those that retain regulated activity in the absence of CRAC (CA Parent & PN Devreotes, manuscript in preparation). The characterization of these clones along with the isolation of additional mutants will bring more insight into the structure-function relationship of adenylyl cyclases.

CONCLUDING REMARKS

The recent development of powerful genetic tools for *Dictyostelium* has made it an attractive model system, uniquely suited for studies of a variety of biological processes, including chemotaxis, cell aggregation, gene expression, differentiation, and pattern formation. These events are regulated by a family of cell surface cAMP receptors that are expressed at specific stages in a starvation-induced developmental program. The pathways activated by the cARs share many features with mammalian signaling systems. Analysis of null mutants created by gene disruption has shown that none of the familiar signal transduction components are required for growth or cell motility but many are needed for various aspects of development. The phenotypes of the mutants suggest several unexpected roles and interactions among these genes. Rescue of the phenotypes of these mutants is providing simple screens for loss- and gain-of-function mutations in these important signaling proteins. Screening of populations of gene-tagged cell lines for similar phenotypes is leading to the discovery of novel genes in these pathways.

The molecular genetic analysis of signal transduction in *Dictyostelium* has answered several questions but raised many others. How do seven transmembrane receptors cause calcium influx and mediate gene expression without coupling to heterotrimeric G proteins? What regions of the cARs control the affinity of agonist and antagonist binding? How many receptor-activated states are there and which regions control the activated states? What are the mechanism(s) of adaptation that are independent of receptor phosphorylation? What additional proteins are involved in the adenylyl cyclase activation pathway? Do mammalian adenylyl cyclases have such complex activation pathways in vivo? What regions of adenylyl cyclase are involved in activation by receptors and G proteins? What are the targets of PKA that control gene expression at the various stages of development? How can PKA be active in the absence of cAMP? What is the role of cGMP in chemotactic orientation? What are the links between large and small G proteins? Future studies will undoubtedly address these and other questions.

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