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A0005

Cyclic AMP Receptors of *Dictyostelium*

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P0005 **When confronted with starvation, the social amoeba *Dictyostelium discoideum* survives by undergoing multicellular development and sporulation. The coordination of these processes is achieved in part through intercellular communication using secreted adenosine 3′,5′-cyclic monophosphate (cAMP) and a family of cell-surface cAMP receptors (cARs). The cARs are examples of G protein-coupled receptors (GPCRs), which enable eukaryotic cells in general to sense and respond to a wide array of environmental and hormonal signals ranging from single photons to large glycoprotein hormones. Due to their involvement in diverse physiological processes, GPCR-targeted drugs are frequently employed in medicine to treat many common conditions including inflammation, hypertension, heart failure, and neurologic and psychiatric disorders. Because GPCRs and the pathways they regulate are conserved in virtually all eukaryotes examined to date, genetically tractable microbes such as *Dictyostelium* have contributed significantly to our understanding of GPCR function and regulation.**

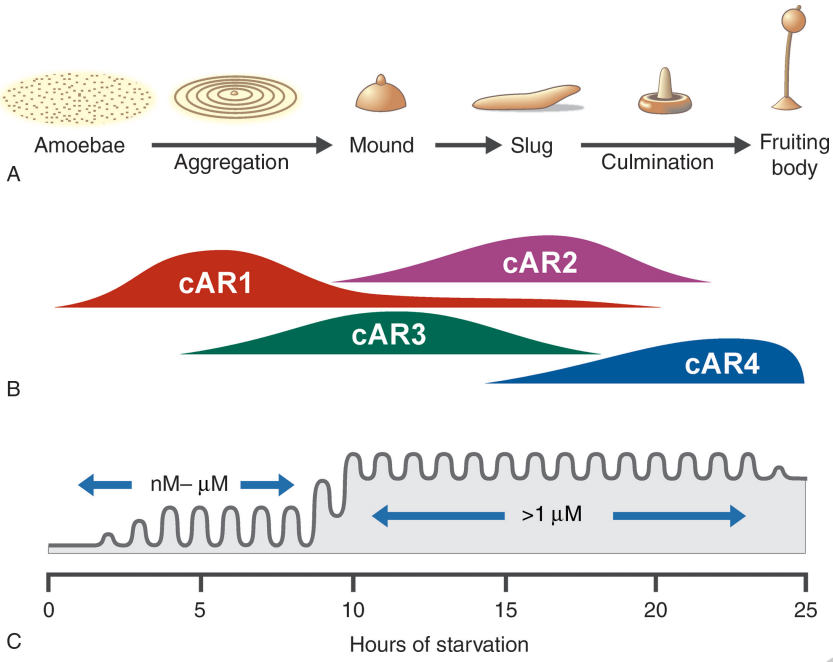
S0005 *Dictyostelium* Development and cAMP Signaling

P0010 *Dictyostelium discoideum* is an amoeba found in soil where it feeds on bacteria. In order to survive periods of starvation, 10^4–10^5 amoebae aggregate and execute a 24 h developmental program that yields a fruiting body comprised of a round mass of spores held aloft by a slender stalk (Figure 1A). When nutrients return to the environment, spores germinate to yield amoebae which resume cell division.

P0015 Not long after Sutherland and his colleagues discovered cAMP to be an important intracellular second messenger in hormonal signaling, Konijn and his associates demonstrated that cAMP is a potent chemoattractant for *Dictyostelium* and correctly speculated that it was the so-called acrasin secreted by starving amoebae, which mediates their aggregation. Shaffer had proposed that the acrasin would be emitted periodically by cells at aggregation centers and relayed outwardly as waves by surrounding cells. Indeed, exogenous cAMP was shown to elicit the transient activation of adenylyl cyclase and secretion of cAMP. Tomchik and Devreotes later demonstrated the concentric waves of extracellular cAMP waves which arise every ~6 min at aggregation centers and travel radially outward through aggregating populations. Subsequent pharmacologic characterization of these responses established the framework for the identification of the cAMP receptors.

S0010 Identification and Properties of cAMP Receptors

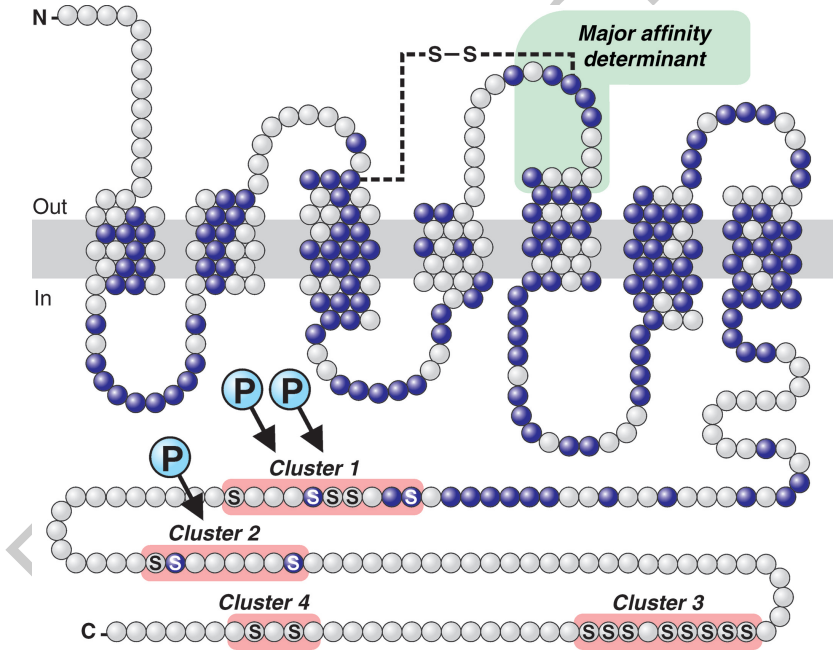
P0020 The molecular identification of the first cAMP receptor (cAR1) began with its photoaffinity labeling with the cAMP analogue, 8-azido-[^{32}P]cAMP. This approach identified a protein of either 40 or 43 kDa depending on whether or not the cells had been exposed to cAMP, suggesting the existence of a reversible, ligand-induced modification which proved to be phosphorylation. Radiolabeling of cells with [^{32}P]phosphate permitted purification of the 43 kDa phosphorylated form of the receptor. Antibodies directed against purified cAR1 lead to the isolation of a cDNA that encoded a protein of the expected size and hybridized to an mRNA, expressed transiently in early development, consistent with cAMP binding. Formal proof that the isolated cDNA did indeed encode cAR1 came from expression of the cDNA, which resulted in increased cAMP binding, and disruption of the corresponding gene, which obviated cAMP binding and cAR1-mediated responses. The deduced sequence of cAR1 possessed seven putative transmembrane domains (Figure 2) and exhibited weak homology to mammalian G protein-coupled receptors (GPCRs). cAMP stimulation of GTP binding to isolated membranes and GTP hydrolysis was further evidenced



F0005 **FIGURE 1** Correlation of: (A) developmental morphology, (B) cAR expression, and (C) extracellular cAMP levels. See text for additional details.

that cAR1 is indeed coupled to a G protein. This together with studies of yeast pheromone signaling provided the earliest indications that virtually all eukaryotes have inherited these ancient sensory mechanisms.

Three other highly homologous cAMP receptors, designated cAR2–4, were subsequently identified by hybridization with a cAR1 probe. The cARs are expressed successively during development, peaking in expression at roughly 5 h intervals in the order: cAR1, P0025



F0010 **FIGURE 2** Model of cAR1. Dark blue spheres represent amino acids conserved in all four cARs. Densely packed amino acids in the plane of the membrane (gray bar) signify the seven-hydrophobic-transmembrane helices. Nonconserved amino acids (light gray spheres) in the region labeled major affinity determinant are largely responsible for the widely differing cAMP affinities of cAR1 and cAR2 (and possibly other cARs). Clusters of serines in the C-terminal cytoplasmic domain (S), the distribution of ligand-induced phosphorylation (P), and the putative disulfide bridge (––S–S––) are also indicated. cAR2–4 differ in the lengths and sequences of their C-terminal cytoplasmic domains.

cAR3, cAR2, cAR4 (Figure 1B). Disruption of the genes encoding each cAR results in developmental defects consistent with the timing of their expression. cAR1$^-$ cells fail to aggregate. cAR2$^-$ cells arrest shortly after aggregating at the mound stage. cAR3 gene disruption has been variously reported either to have no apparent effect or to interfere with spore cell differentiation late in the mound stage and consequently yield fruiting bodies predominantly comprised of stalk cells. cAR4$^-$ cells develop normally beyond the mound stage but exhibit defects in culmination, resulting in mis-shapen fruiting bodies.

P0030 The cARs differ markedly in their affinities for cAMP. The early cARs, cAR1 and cAR3, have high affinities (i.e., low- to mid-nM K_d's), whereas those expressed later in development, cAR2 and cAR4, have low affinities (K_d's > 1 μM). These affinities are appropriate for the extracellular cAMP concentrations that exist at these stages of development (Figure 1C). During aggregation, the cAMP signal oscillates from sub-nM to near-μM concentrations. In contrast, external cAMP oscillates at elevated concentrations exceeding 1 μM in the multicellular stages. Nonconserved residues in the third extracellular loop largely determine whether a cAR has a high or low affinity (Figure 2). By analogy with rhodopsin, this extracellular loop, positioned by disulfide linkage to the extracellular end of the third transmembrane helix, is likely to lie at the entrance to the cAMP binding cleft of cARs and, thereby, influence binding.

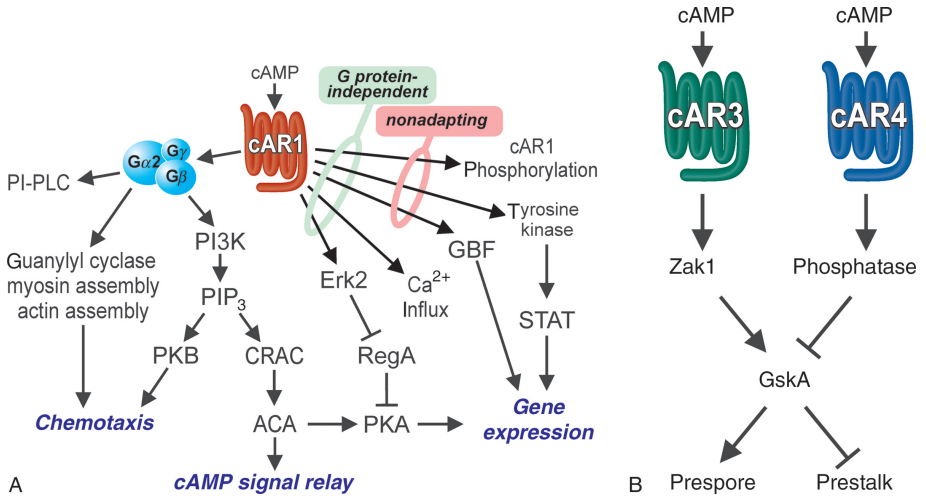
S0015 Signaling Pathways

P0035 cAR1 is perhaps the most versatile GPCR yet to be characterized as it regulates a wide range of downstream effectors and biological responses. Consequently, it serves as a valuable model for understanding diverse modes of GPCR signaling. cAR1 mediates three principal cellular responses during aggregation: (1) propagation of cAMP waves, (2) chemotaxis up the cAMP gradient of each oncoming wave, and (3) regulation of genes required for development. The pathways underlying these responses have been determined to a large extent (Figure 3A). Most striking is the dichotomy between signaling pathways that involve G proteins and those that do not. Comparatively less is known about the pathways governed by cAR2–4 in large measure due to technical challenges posed by multicellularity, although significant progress has been made towards elucidating mechanisms by which these cARs promote cell differentiation in multicellular stages.

S0020 G Protein-Dependent Pathways

P0040 *Dictyostelium* possesses at least nine heterotrimeric G proteins comprised of distinct α-subunits (designated Gα1–9) and common β- and γ-subunits. Genetic and biochemical evidence indicates that Gα2βγ is the principal G protein to which cAR1 couples. Activation of Gα2βγ by cAR1 liberates the Gβγ dimer and GTP-bound Gα2, which in turn activate various effectors.

P0045 The Gβγ dimer is believed to activate the aggregation-stage adenylyl cyclase (ACA) by a mechanism involving activation of phosphoinositide-3-kinase (PI3K), which converts the membrane phospholipid phosphatidylinositol-4,5-biphosphate (PIP_2) into phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3, in turn, binds to the PH domain of cytosolic regulator of adenylyl cyclase (CRAC), thus recruiting it to the



F0015 **FIGURE 3** cAMP receptor signaling pathways. Pointed (↓) and flat-headed (⊥) arrows indicate activation in inhibition, respectively. (A) cAR1 pathways leading to chemotaxis, cAMP signal relay, and gene regulation. G protein-independent and nonadapting pathways are indicated. In multicellular stages, cAR2–4 might activate some of the pathways shown for cAR1. (B) Role of cARs in the GskA-mediated determination of cell fate. cAR2 (not shown) might promote prestalk cell differentiation at the mound stage in the manner shown for cAR4.

plasma membrane. How CRAC then activates ACA remains to be determined. The resulting cAMP functions intracellularly via protein kinase A (PKA) to modulate gene expression and, in addition, is secreted from the cell in order to relay the cAMP signal to neighboring cells.

P0050 G$\beta\gamma$-mediated increases in PIP_3 levels also recruit other PH domain-containing proteins to the plasma membrane including protein kinase B (PKB or Akt), which has been shown to be critical for chemotaxis. In cells undergoing chemotaxis in a shallow cAMP gradient, PKB and other PH domain-containing proteins localize exclusively at the cells' leading edge where they presumably promote actin assembly and pseudopod extension. These mechanistic insights into cAR1-mediated chemotaxis, namely the involvement of PI3K activation and recruitment of PKB to the leading edge, have since been found to also pertain to the chemotaxis of a variety of mammalian cells including neutrophils.

P0055 The Gα2 subunit is implicated in the cAR1-dependent activation of guanylyl cyclase (cGMP) which provides another important input to the chemotaxis machinery. The product of cGMP regulates myosin heavy chain kinases and, thereby, promotes the assembly of conventional myosin II in posterior and lateral regions of chemotaxing cells where it can propel the rear of the cell forward upon contraction and suppress lateral pseudopod formation.

S0025 G Protein-Independent Pathways

P0060 Several cAR-mediated responses appear to be G protein-independent based on their preservation in cells lacking what is believed to be the sole G protein β-subunit gene. These include phosphorylation of cAR 1, uptake of Ca^{2+}, and activation of the mitogen-activated protein kinase Erk2, the transcriptional regulators GBF and STAT, and the GSK3 homologue GskA.

P0065 cAR1 is phosphorylated on serine residues within its C-terminal cytoplasmic domain. The 18 serines in this domain exist in four clusters (Figure 2). In unstimulated cells, cAR1 is basally phosphorylated within clusters 2 and 3. Upon stimulation with cAMP, cAR1 becomes reversibly hyperphosphorylated due to the addition of approximately two phosphates within cluster 1 and a third phosphate within cluster 2. Cluster 1 phosphorylation causes the 40–43 kDa electrophoretic shift. Other cARs also undergo cAMP-induced phosphorylation commensurate with their affinities.

P0070 Erk2 promotes the intracellular accumulation of cAMP by negatively regulating RegA, a cAMP-specific phosphodiesterase. Erk2 is presumably the third of three kinases in a typical MAP kinase cascade. The identity of the upstream kinases as well as the G protein-independent mechanism by which cAR1 activates the cascade remain to be determined.

P0075 cAMP triggers the rapid and transient influx of Ca^{2+} ions in aggregation competent cells. This is mediated largely by cAR1 due to its natural abundance at this stage in development. However, expression of cARs in vegetative cells indicates that the components required for uptake are expressed at this stage and that other cARs can also mediate Ca^{2+} uptake. The magnitude of Ca^{2+} uptake is roughly proportional to receptor level, indicating that cARs are the limiting components for this response.

P0080 G-box binding factor (GBF) binds G-rich elements in early postaggregative genes and is required for their induction by cAMP. Gene disruptions suggest that either cAR1 or cAR3 mediate the G protein-independent activation of GBF. In contrast to many aggregation-stage genes whose expression requires periodic cAMP pulses that mimic natural cAMP waves, GBF-mediated gene expression is induced by constant cAMP, indicating that it is not subject to adaptation. Thus, the GBF pathway is appropriately activated upon aggregation when extracellular cAMP rises to levels that persistently occupy cAR1.

P0085 STAT proteins have been extensively studied in the context of cytokine signaling in mammalian immune cells. In *Dictyostelium*, exogenous cAMP triggers STATa's phosphorylation on tyrosine, SH2 domain-mediated dimerization, and translocation to the nucleus where it governs prestalk gene expression. cAR1 is required for this response but it can be substituted in this capacity with cAR2, suggesting that multiple cARs might activate STATa during development. The cAR-activated tyrosine kinase involved remains to be identified.

P0090 GskA, a homologue of glycogen synthase kinase-3 (GSK3), is an important regulator of cell fate in *Dictyostelium* and cARs play key roles in regulating its activity. GskA activity promotes spore cell differentiation, whereas inactivity results in stalk cell differentiation (Figure 3B). Independent of G proteins, cAR3 activates the nonreceptor tyrosine kinase Zak1 which, in turn, phosphorylates and activates GskA. On the other hand, cAR4 (and perhaps cAR2) activates a phosphotyrosine phosphatase, resulting in the dephosphorylation and inactivation of GskA. The role of G proteins in the latter process, if any, is not known. Therefore, cAR3 signaling promotes spore differentiation and cAR2 and cAR4 favor stalk cell differentiation. Because cAR2 and cAR4 are expressed predominantly in prestalk cells, it is unclear whether these mechanisms determine cell fate or act thereafter in cell-type maintenance.

P0095 One known target of cAMP-activated GskA is STATa. GskA phosphorylates multiple serines of STATa, causing it to be exported from the nucleus. This is one mechanism by which cAR3 signaling might oppose prestalk differentiation. As has been described for other developmental systems, GskA may also determine cell fate by phosphorylating β-catenin

and thus targeting this transcriptional coactivator for destruction.

S0030 ### Mechanisms of G Protein-Independent Signaling

P0100 It remains to be determined how cAMP-occupied cAR1 communicates with and activates these effectors. By analogy with G proteins and also mammalian G protein-coupled receptor kinases (GRKs), the yet to be identified cAR kinase might interact selectively with the ligand-occupied conformation of the receptor's cytoplasmic loops. Other G protein-independent processes such as Ca^{2+} uptake could involve lateral signal transduction within the plane of the membrane by direct interaction of cAR1 with another integral membrane protein, analogous to the interaction of *Halobacterium* sensory rhodopsins (SI and SRII) with their associated histidine kinases (HtrI and HtrII).

S0035 ## Desensitization Mechanisms

P0105 In general, receptor-mediated responses are governed by various desensitization mechanisms which attenuate the cell's responsiveness. cAMP triggers the sequential phosphorylation ($t_{1/2} \sim 2$ min) and internalization ($t_{1/2} \sim 15$ min) of cAR1. cAR1 phosphorylation causes a several-fold reduction in the receptor's intrinsic affinity for cAMP, which may extend the range of cAMP concentrations to which the receptor can respond during aggregation. In addition, preliminary results indicate that phosphorylation of cAR1 is a prerequisite for its internalization as in other systems. Prolonged cAMP exposure results in down-regulation of cAR1 levels, the combined effect of diminished cAR1 gene transcription, and cAR1 degradation. Degradation presumably results from delivery of internalized receptors to lysosomes.

P0110 On a more rapid timescale, nearly all of the cAR1-mediated responses to abrupt cAMP increases are transient, returning to prestimulus levels in 30 s to several minutes despite constant stimulation. This rapid and reversible attenuation of responses is referred to as adaptation. The few responses that do not adapt include cAR1 phosphorylation and GBF activation. The mechanisms of adaptation are poorly understood and might be distinct for each pathway. cAR1 phosphorylation appears not to be involved as elimination of phosphorylated serine residues in cAR1 by site-directed mutagenesis has little impact on the kinetics of these responses.

P0115 Cells adapted to one cAMP concentration can respond to yet higher concentrations (provided the receptor is not saturated). This observation indicates that adaptation is a graded signal that is just sufficient to offset the excitatory signal, the strength of which also reflects receptor occupancy. For the adenylyl cyclase pathway, adaptation can be explained by the transient translocation of PI3K to the plasma membrane and the subsequent degradation of PIP_3 by the phosphatase PTEN. In addition, FRET experiments indicate that the G protein is persistently dissociated in adapted cells. Taken together, these findings suggest that an adaptation pathway emanates from cAR1 and acts upon the adenylyl cyclase excitatory pathway somewhere beyond the G protein, causing PI3K to be released from the membrane. Because PKB activation also depends on PI3K, the same adaptation mechanism is likely to govern chemotaxis.

Gradient Sensing in Chemotaxis S0040

P0120 Temporal challenges with fixed cAMP concentrations as described above have been invaluable for deciphering cAR-mediated pathways and revealing the existence of adaptation mechanisms. However, natural cAMP waves also contain spatial information which the cells must rapidly and accurately sense for efficient chemotaxis. Although cAR1 is uniformly distributed in the plasma membrane, shallow cAMP gradients differing by as little as 2% across the length of the cell prompt highly asymmetric localization of various proteins, indicating that the cell senses and amplifies small differences in cAR1 occupancy on this surface. Proteins with PH domains including PKB and CRAC are highly localized to the plasma membrane of cell's leading (or anterior) edge, indicative of elevated PIP_3 levels. This reflects the recruitment of PI3K to the anterior plasma membrane and its subsequent activation. The membrane translocation of PI3K is mediated by its N-terminal domain which presumably binds an entity in the plasma membrane that is generated in response to cAR1 activation. Conversely, PTEN is associated with the posterior plasma membrane via specific interactions with PIP_2. Thus, PTEN is excluded from the anterior membrane where PI3K actively converts PIP_2 to PIP_3 and instead localizes to posterior regions where PIP_2 should be more abundant. Localization of the antagonistic activities of PI3K and PTEN to opposing poles of the cell should result in a steep gradient of PIP_3 and associated PH domain-containing proteins. Precisely how the cell amplifies the directional information of a shallow chemoattractant gradient to achieve extreme gradients of activities within the cell is likely to be critically important for efficient chemotaxis and is under intensive investigation.

Glossary G0045

chemotaxis Directed movement of cells toward (or away from) the source of diffusable chemoattractant (or repellant) molecules. G0125

G0130 **G protein** Heterotrimeric proteins with inactive (GDP-bound) and active (GTP-bound) states which are activated by ligand-occupied receptors and, in turn, activate downstream targets within the cell.

G0135 **G protein-coupled receptors** Cell surface, integral membrane proteins possessing seven membrane-spanning α-helices and usually capable of activating cytosolic G proteins upon binding specific extracellular signaling molecules (also known as seven-transmembrane or serpentine receptors).

G0140 **signal transduction** Molecular events by which the perception of an extracellular signal by a cell is translated into an appropriate cellular response.

Further Reading

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