IDENTIFICATION AND CHARACTERIZATION OF CrlA : A cAMP RECEPTOR-LIKE G PROTEIN-COUPLED RECEPTOR IN *DICTYOSTELIUM DISCOIDEUM*

By

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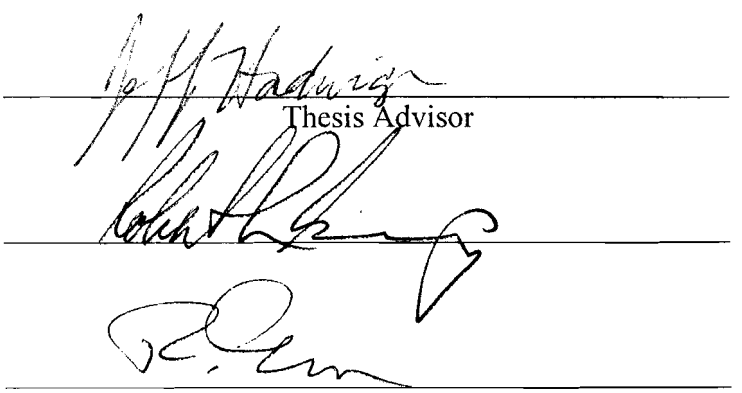

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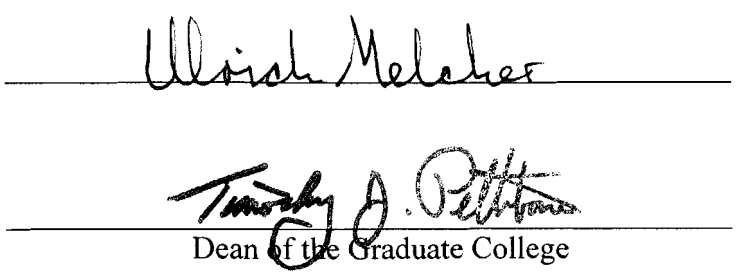

Dean of the Graduate College

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Finally, I must thank my grandparents, Alfred and Doris Martz. You are an amazing woman, Grandma, and a true inspiration. This is especially for you, Grandpa. We miss you.

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NOMENCLATURE

Bsr	Gene encoding blastocidin resistance
cAMP	Cyclic adenosine 3', 5' monophosphate
car1-4	cAMP receptor genes 1 to 4
Car1-4	cAMP receptor proteins 1 to 4
cDNA	Complementary DNA
cGMP	Cyclic guanosine 3', 5' monophosphate
crlA	cAMP receptor-like receptor A gene
CrlA	cAMP receptor-like receptor A protein
DIF	Differentiation Initiation Factor
$G418^r$	Gene encoding neomycin resistance
GFP	Green Fluorescent Protein
Gα4	Gα4 subunit gene
Gα4	Gα4 subunit protein
Gα5	Gα5 subunit gene
Gα5	Gα5 subunit protein
GPCRs	G protein-coupled receptors
KAx-3	*Dictyostelium discoideum* parental strain
LPA	Lysophosphatidic Acid
ORF	Open reading frame

PDE	Phosphodiesterase
tasA	TasA receptor gene of *Polysphondylium pallidum*
TasA	TasA receptor protein of *Polysphondylium pallidum*

CHAPTER 1

INTRODUCTION

All eukaryotic organisms must possess the ability to sense their surroundings in order to survive changes in their environment. Therefore, various external signals including light and chemicals must be properly received and transduced in order to facilitate functionally correct cellular responses. These transduction pathways often induce a cellular response by altering target proteins that control ionic composition or second messenger levels that are involved in cell growth and differentiation, chemotaxis, sensing, and endocrine function (1). Some signal transduction pathways are regulated through G protein-coupled receptors (GPCRs) that comprise the largest group of cell surface receptors in eukaryotic organisms (2). Specific binding of G protein-coupled receptors to G proteins at the intracellular surface of eukaryotic membranes permits downstream transmission of external signals (3, 4).

In humans, G protein-coupled signal transduction is necessary for some of our basic senses, protection from bacterial invasion, and proper endocrine and neural function (5). Olfactory cells, or kinocilia, in the nasal mucosa and taste buds on the tongue provide receptor surfaces responsible for transducing chemical signals that are converted to electrical impulses and permit sensing of smells and tastes (6-8). Odorant-binding proteins of the olfactory cilia extend into the nasal mucosa and form a meshwork to which chemical stimuli may bind and induce depolarization of the olfactory cells.

Stimulating substances must be partially water-soluble in order to pass through mucous to reach the olfactory cells (7). The sense of taste works similar to smell, but ligands need not cross a medium such as mucous. Receptors for taste sensation sit atop the taste buds and are readily exposed to ingested food particles (6, 9).

Vision is dependent upon specialized G protein-coupled reception (8, 10-12). Rod cells in the eye contain gated sodium channels that can bind cGMP. This maintains the rod cell in a resting, depolarized state. The rod cell also releases a neurotransmitter that is inhibitory to sensory neurons to which it synapses. Under these conditions, the neuron is maintained at a relatively low level of excitation as long as no light strikes the rod cell (13). Closing of the gated sodium channels and subsequent polarization of the rod cell occurs when a photon strikes a rod cell. Excitation lessens the amount of inhibitory neurotransmitter released by the rod cell and activates the post-synaptic sensory neurons.

G protein-coupled transduction pathways are needed for a proper immune response. Macrophages and neutrophils are able to attack and destroy invading bacteria, viruses, and other injurious agents. Neutrophils primarily function in the circulating blood and are short-lived while macrophages work in the surrounding tissues and provide protection over extended periods of time. Presentation of different substances at the site of inflammation can cause neutrophils and macrophages to chemotax (14, 15). These substances include bacterial toxins, degraded constitutive components of the inflamed tissue, complement reaction products, and plasma clotting products (14, 16, 17). Gradients created by the dispersion of these substances dictate the directional movement of the leukocytes.

Hormone and neuropeptide signals can also be mediated through G protein-coupled receptors. G protein-coupled receptors function in oxytocin signal-mediated contraction of the uterus and lactation through stimulation of myoepithelial cells (18). Some serotonin G protein-coupled receptors inhibit gastric secretion while others stimulate vasoconstriction (19, 20). The neurotransmitter bradykinin mediates smooth muscle contraction and pain reception through G protein-coupled receptors (21). Dopamine G protein-coupled receptors function in various parts of the human brain to inhibit the central nervous system (22, 23). Aberrations in this signaling pathway have been implicated in the pathology of Parkinson's disease (24).

Various components comprising signal transduction pathways in eukaryotic cells are organized in a cascade motif where downstream participants are dependent upon the function and specificity of previous cellular response facilitators (25). A high level of organization is needed for proper interpretation, especially when multiple external signals are present and collaboration among the components in different signaling pathways exists (4). While many signal transduction components are known, the functional specificity of these components and their interaction with additional constitutive moieties in different pathways remains to be determined. Further experimentation must be done to fully understand the complexity of these systems.

Unfortunately, signal transduction is difficult or impossible to study by genetic approaches in complex multicellular organisms. For this reason, simple eukaryotic systems have been employed that are more easily managed in laboratory settings and possess G protein-coupled systems that are more readily studied. Mating in the yeast *Saccharomyces cerevisiae* and egg-laying in the nematode *Caenorhabditis elegans* are

both mediated through G protein transduction systems (1, 26). The social soil amoebae *Dictyostelium discoideum* is also an excellent system to use when studying signal transduction (27). The manner by which this organism undergoes development during its asexual life cycle is dependent upon cAMP signaling through G protein-coupled receptors (28).

Dictyostelium discoideum serves as a model organism for the investigation of G protein-coupled receptors. Migration to bacterial food sources, transition from unicellular growth to multicellular development, morphogenesis of the aggregate into the mature fruiting body, and development of prespore and prestalk cells are all affected by a variety of G protein-mediated signal transduction pathways in this organism (29-34). This simple eukaryotic organism is a common inhabitant of the forest floor and typically feeds on bacteria that it locates through folic acid-stimulated chemotaxis. Cells grow vegetatively as individual amoebae until their food source is depleted. After a period of starvation, *Dictyostelium* cells aggregate and begin a social, multicellular development cycle that ultimately leads to a fruiting body comprised of a sorus or spore cap atop a supporting stalk (35). While many multicellular plants and animals grow and differentiate simultaneously, *Dictyostelium discoideum* possesses the ability to separate growth and development. This permits the analysis of developmental processes including signal transduction and chemotaxis with respect to recessive mutations in haploid cells (36, 37).

As the *Dictyostelium discoideum* genome is sequenced, more opportunities will hopefully arise that lend themselves to the investigation of G protein-coupled receptors. Compilation of genomic sequence information allows for the identification of new

receptor genes that can be targeted for investigation. The identification and characterization of new receptors that function as signaling components in *Dictyostelium* will help define the specificity of many signal transduction pathways that operate in this organism.

CHAPTER 2

BACKGROUND and SIGNIFICANCE

Introduction to G protein-coupled Receptors

G protein-coupled receptors constitute the largest family of cell surface receptors in eukaryotic cells. Working in conjunction with GTP-binding regulatory proteins, or G proteins, these surface receptors are required for converting external signals to internal cell messages. For example, the senses of taste and smell are perpetuated through G protein-coupled receptors and their ability to bind chemical ligands. Rhodopsin is a specialized G protein-coupled receptor that facilitates vision by sensing light and inducing polarization of photoreceptor cone cells within the eye. Some hormones function via these receptors to serve as neurotransmitters or regulators of cell growth, division, and differentiation. Chemotaxis, the ability of a cell to detect and move toward a chemical attractant or away from a chemical repellent, is also mediated by G protein-coupled receptors. Chemotactic ability is important for leukocyte migration during an immune response.

Basics of G protein-coupled Receptors

G protein-coupled receptors can be recognized by a variety of shared characteristics. Serpentine, seven transmembrane domains are the most easily recognized trait (2, 9) (Figure 1). Additionally, the transmembrane domains typically possess a disproportionate composition of hydrophobic amino acid residues relative to the

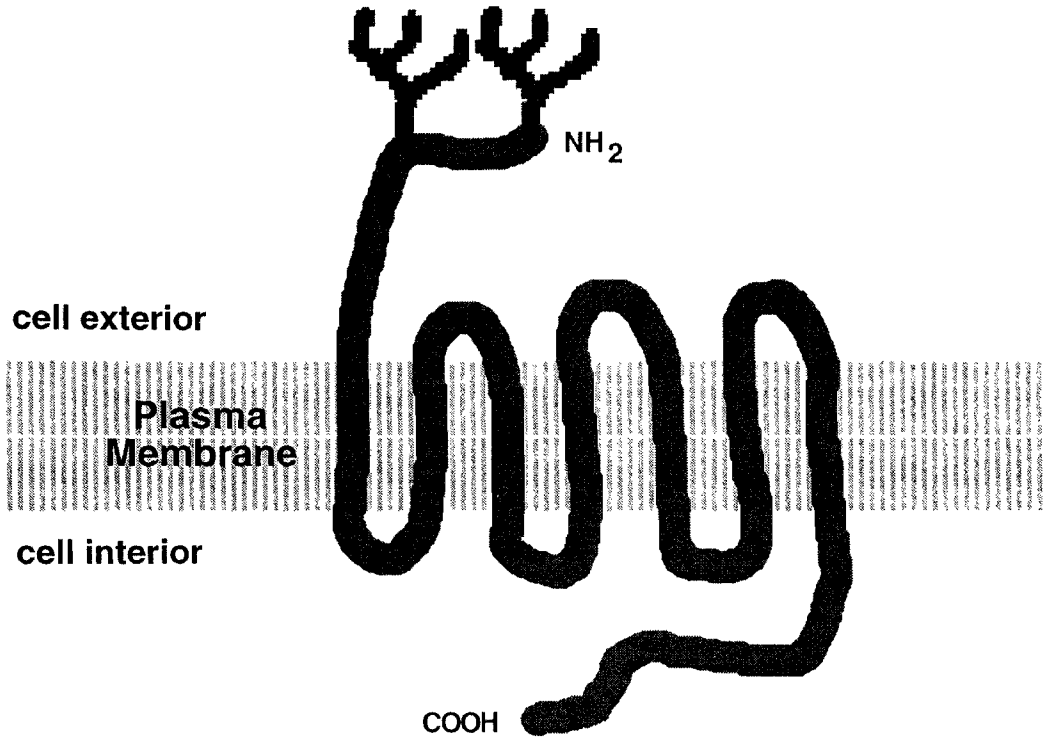


Figure 1 - G protein-coupled Receptor Structure

Seven transmembrane (TM) domains characterize these proteins. Conservation of hydrophobic amino acid residues within the transmembrane regions permits grouping of individual G protein-coupled receptors with similar function into families. The cAMP receptor family in *Dictyostelium discoideum* is an example.

remainder of the protein that does not span the cell membrane. Within the transmembrane domains, higher levels of sequence identity between different G protein-coupled receptors have been used to group the receptors into families (9, 28). All G protein-coupled receptors possess an intracellular carboxyl terminus and extracellular amino terminus. Serine residues present within the carboxyl tails are needed for ligand-induced phosphorylation (38). These general observations have led to a prediction of common topology for the G protein-coupled receptors and can be a useful tool in their identification.

G protein-coupled Receptors and Heterotrimeric G proteins

Through their intermediary action, G protein-coupled receptors are responsible for inducing intracellular responses by a vast number of extracellular signals. In addition to binding ligand outside the cell, the receptor must also affiliate with membrane-associated GTP-binding proteins located on the intracellular surface of the cell membrane. These G proteins are heterotrimeric in nature - consisting of α, β, and γ subunits (25, 39). When a transduction pathway is inactive in the absence of ligand, the G protein is bound to the requisite G protein-coupled receptor via its α subunit and GDP is found in the guanine nucleotide-binding pocket of the α subunit (40). When an appropriate ligand binds to the G protein-coupled receptor, GDP dissociates and is replaced by GTP on the α subunit. This causes the heterotrimeric G protein to dissociate into an active α subunit with bound GTP (2) and an active $\beta\gamma$ subunit dimer (41). While detached, both moieties are free to diffuse along the membrane interior and can directly interact with effectors located along the plasma membrane (Figure 2).

The magnitude and duration of a signal depend upon how long a membrane target

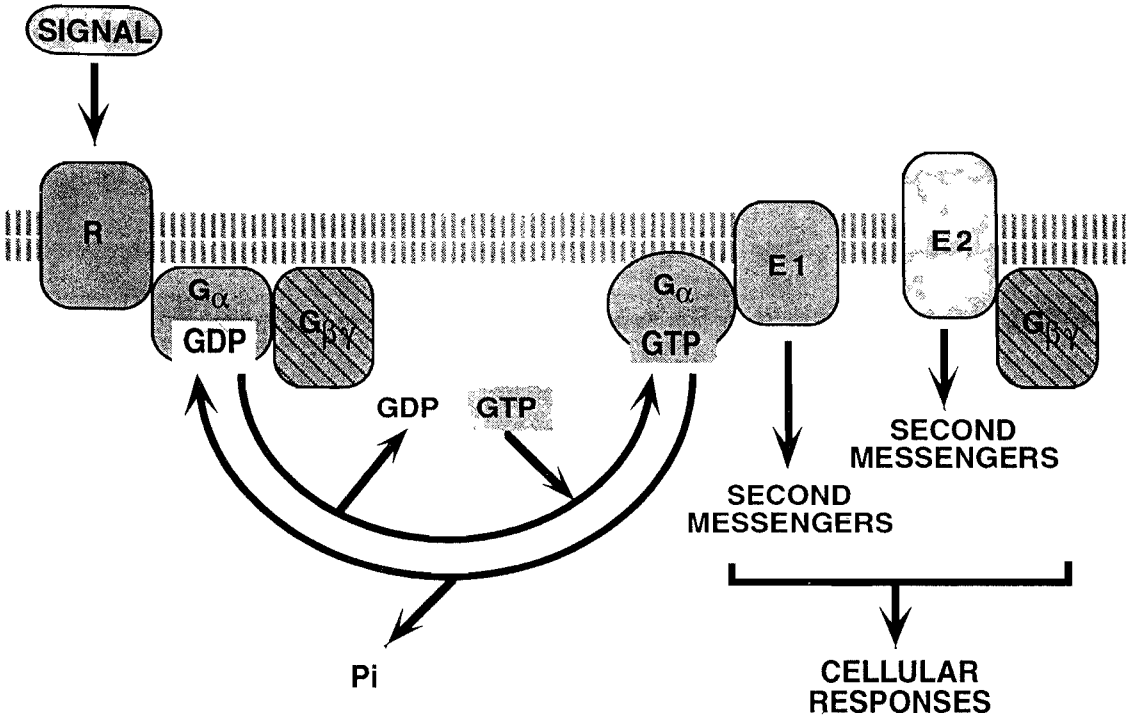


Figure 2 - G protein-mediated Signal Transduction

Activation of the a pathway occurs when an external signal binds to a receptor. Heterotrimeric G protein subunits associate with the intracellular surface of the receptor and react with downstream effectors or secondary messengers that lead to cellular responses. The inherent GTPase activity of Gα subunits determines the duration of excitation.

has an activated α subunit or βγ subunit dimer bound. This activation is dictated by the Gα subunit. The inherent GTPase activity of the α subunit hydrolyzes the bound GTP to GDP and the α subunit re-associates with a free βγ subunit dimer (40). Thus, the signal is switched off.

Dictyostelium discoideum as a Model System for Development

Wild-type development of *Dictyostelium discoideum* requires chemotaxis, morphogenesis, and regulated gene expression. Activation of these processes through downstream second messengers is instigated at the cell surface by the binding of ligand to G protein-coupled receptors (27). A comprehensive and complete understanding of the various signal transduction pathways in *Dictyostelium discoideum* could have future ramifications in the investigation of embryonic development, pharmacology, and immunology.

Developmental Life Cycle of *Dictyostelium discoideum*

The relatively rapid asexual life cycle of *Dictyostelium* furnishes a convenient system for studying developmental processes (35) (Figure 3). Cells grow as individual amoebae in the presence of a sufficient food source. During vegetative growth in the wild and laboratory settings, the amoebae phagocytize bacteria they locate through folic acid gradients (42). Additionally, axenic cultures of *Dictyostelium* acquire nutrients from liquid media (HL-5) through pinocytosis .

Depletion of nutrients induces aggregation in *Dictyostelium* cells (43). In starving cells, pulses of cAMP are released every six minute (44). Recognition of a cAMP gradient by a cell helps to orient that cell as well as induce secretion of cAMP from that cell. This intercellular signaling network causes a population of cells to stream to an

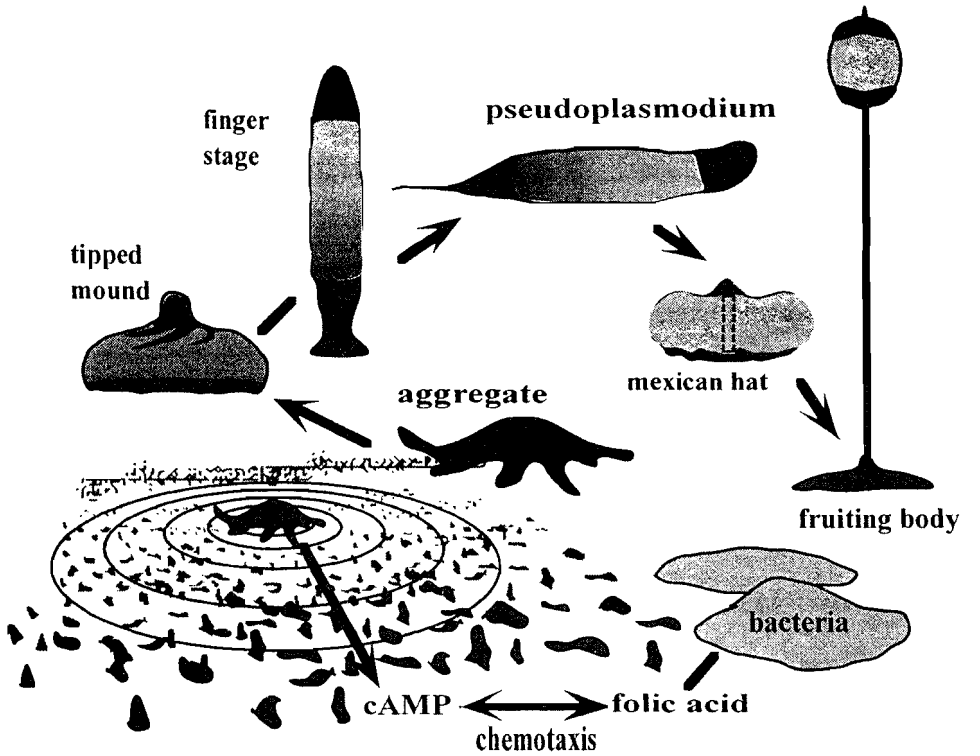


Figure 3 - Development of *Dictyostelium discoideum*

Individual amoebae grow vegetatively in the presence of a food source. Upon starvation, cells aggregate and progress through several multicellular stages that culminate in a fruiting body with spores. Wild-type development under normal conditions requires 24 hours.

aggregation center established by the initial starved cells (45). Stimulation of the aggregation pathway is not constant from an individual receptor and resensitization occurs when an external phosphodiesterase (PDE) degrades the signal (46, 47). This prepares the cell for the next wave of cAMP.

Cytodifferentiation and cell-to-cell adhesion begin when ~$1x10^5$ streaming cells reach an aggregation center. Amassed cells continue to differentiate and form a small vertical tip from the pinnacle of the aggregate. The tip elongates and stretches the aggregate into the erect finger structure. At ~16 hours post-starvation, the finger structure falls over and becomes a mobile slug or pseudoplasmodium. The slug is capable of chemotaxis to light and lays down a slime trail while migrating. By 24 hours post-starvation, cells of the pseudoplasmodium have fully differentiated and a fruiting body, or sorocarp, with a cellulose-coated stalk and spore cap forms.

Prior to cytodifferentiation, two cell types are present as the *Dictyostelium* amoebae aggregate. The anterior quarter of the slug consists of cells that will become stalk cells (48). These prestalk cells can be distinguished from the prespore cells of the posterior three quarters through gene expression analysis (49). Previous research examining how this early differentiation arises has led to two theories. The first is that the position of a cell in the slug can affect it's fate - those in the anterior become stalk cells and those in the posterior become spores. The second theory is that at aggregation there is some early differentiation of the cells that induces their sorting-out in the cell mass. For these cells, their position is determined by their early differentiation (50). Differential chemotaxis to cAMP is thought to play a significant role in the sorting-out of these cells (36).

Apoptosis, or programmed cell death, is an essential component of development in many invertebrate and vertebrate multicellular organisms (51). *Dictyostelium* cells selectively destroy themselves during development as well as in vegetative growth (52). During development, stalk cells undergo vacuolization and chromatin condensation and soon become non-viable as evidenced by their inability to replicate when placed in fresh media. This seems to be promoted by cAMP and DIF (52). During vegetative growth, some cell types experience blebbing and a reduction in size indicative of apoptosis.

Growth and Culturing of *Dictyostelium discoideum*

In order to study *Dictyostelium* development in a laboratory setting, vegetative cells are sustained in petri dishes of HL-5 media. Old media is aspirated and replaced every three to four days. Moisture and temperature can also affect the growth of *Dictyostelium* cells. Wild-type KAx-3 cells have a doubling time of eight hours when cultured in shaking nutrient-rich media where they can grow to a very high density (53). Cell lines are usually stored as frozen spores that can be germinated by simple hydration with nutrient-rich media.

Molecular Genetics of *Dictyostelium discoideum*

Dictyostelium discoideum is an ideal system for studying G protein-coupled signaling pathways. This organism has seven chromosomes of various sizes that comprise its 40 MB genome (54). Although a diploid state exists during the sexual cycle, most experimentation is performed in haploid cells of the asexual cycle. This permits relatively easy genetic manipulation and assessment of recessive phenotypes.

Manipulation of the *Dictyostelium* genome permits this organism to be used for studying signaling pathways. Electroporation is an efficient means of introducing

exogenous DNA into these cells (55). Selection of subsequent transformants is achieved through genes conferring drug resistance (56). These include the neomycin phosphotransferase and blasticidinS deaminase genes. Exogenous DNA can be incorporated into the *Dictyostelium* genome with integrating vectors.

Reverse genetics by creating gene disruptions is a useful method of studying developmental genes in *Dictyostelium*. Due to the haploid nature of *Dictyostelium*, homologous recombination can be efficiently used to create disruptions in genes of interest and complementation studies can be employed for rescuing null phenotypes (57).

Gene products from other organisms can be expressed in *Dictyostelium* with retention of function. Mammalian gene products can be efficiently produced in *Dictyostelium* due to the likeness in glycosylation systems. Membrane proteins that are difficult to express in bacteria or non-functional in yeast can also be expressed in *Dictyostelium*. Spatial and temporal expression patterns of developmentally regulated genes can be followed with reporter proteins such as Green Flourescent Protein (GFP) or β-galactosidase (57, 58). Cells can also be labeled with dyes and followed during development in chimeras to investigate dispersion and localization (59).

Cell Biology of *Dictyostelium discoideum*

In addition to its amenable genetic characteristics, *Dictyostelium discoideum* has several features that make it a superior system for studying signal transduction pathways when compared to other simple eukaryotes. Intercellular communication mediated by G protein-coupled receptors is necessary for aggregation and cell sorting during *Dictyostelium* development. Folic acid and cAMP stimulate chemotaxis of *Dictyostelium* cells through G protein-coupled receptor-dependent pathways and facilitate development

and scavenging of food sources, respectively. These mechanisms of signal transduction and the short, defined life cycle of *Dictyostelium* make it a suitable model for studying G protein-coupled receptors. Additionally, the ability to form chimeras in *Dictyostelium* allows temporal and spatial investigation of intercellular signaling.

Dictyostelium discoideum and cAMP-dependent Pathways

Starvation-dependent aggregation in *Dictyostelium* requires chemotaxis and cell-to-cell signaling. These are mediated by cAMP. Individual amoebae use extruded cAMP to orient themselves with respect to others in the vicinity. This release of cAMP to the environment creates a mechanism by which *Dictyostelium* cells may locate one another and is needed for cytodifferentiation and morphogenetic movement (60). The amoebae not only increase their production of cAMP at the time of aggregation, but also a number of other factors needed for chemotaxis. These include the necessary cAMP receptors and the phosphodiesterase that converts cAMP to the chemotactically inactive 5'-AMP. There is also an increase in cell adhesion properties at this time that is necessary for proper aggregation during development.

In *Dictyostelium discoideum*, the receptors needed for binding of cAMP and subsequent morphogenesis have been identified as G protein-coupled receptors and represent a family of similar proteins (61) (Figure 4). They have been named Car1, Car2, Car3, and Car4 (cAMP receptor) to denote their involvement in cAMP recognition (32-34, 62). As with all G protein-coupled receptors, the cAMP receptors have a serpentine morphology with seven transmembrane domains characterized by stretches of hydrophobic amino acid residues. Subtle differences in their structures lead to their independent functions in differentiation and development.

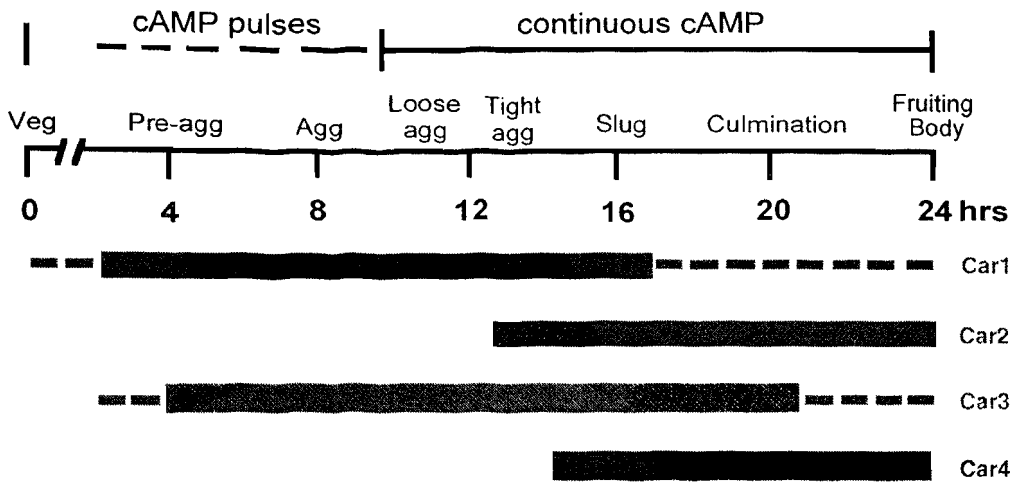


Figure 4 - Expression of cAMP Receptors During Development

The cAMP family of receptors is needed for proper cell chemotaxis, localization within the migrating slug, and formation of the fruiting body. However, the timing of maximum receptor expression is unique and coincides with their individual function throughout development. Starvation-induced pulsing of cAMP is sensed by Car1. This protein is needed for proper cell aggregation. Without Car1, development is arrested.

The Car1 receptor is maximally expressed during early aggregation, but is ubiquitous at lower levels throughout later stages (34). Disruption of *car1* results in failure to bind or sense cAMP and cells remain as a monolayer of individuals under laboratory conditions. Thus, aggregation is obstructed and development is arrested.

The Car2 receptor is predominantly and preferentially expressed in prestalk cells after pseudoplasmodium formation (33). *car2* null cells proceed through early development, but are halted in the mound stage. It is thought Car2 may be required for cAMP-directed sorting of prestalk cells during pattern formation within the aggregation mound (63, 64).

The Car3 receptor is maximally expressed during mound stage (62). However, *car3*$^-$ cells have no easily discernable phenotype as seen in the blocking of different developmental stages in *car1*$^-$ and *car2*$^-$ cells. This suggests that Car3 may contribute to a morphologically subtle phase of late aggregation whose deprivation is possibly masked by Car1 and Car2 (65).

Car4 is needed for cellular differentiation and pattern formation during late *Dictyostelium* development and is maximally expressed in pseudoplasmodia anterior regions (32). Disruption of *car4* leads to exaggerated prespore-cell characteristics such as small stalks and large spore caps.

Typically, high degrees of structural similarity exist between proteins of a receptor family (2, 9). This characteristic persists for the cAMP receptors in *Dictyostelium discoideum*. The transmembrane and loop domains of Car1 and Car2 are ~75% identical in amino acid sequence; however their carboxyl termini are quite different (33, 34, 38). The Car2, Car3, and Car4 receptors possess homopolymeric runs

of histidines and asparagines that are absent from the corresponding region in Car1 (32, 33, 62). These receptors (Car2, Car3, and Car4) share ~60% sequence identity outside of the asparagine clusters. Similar to PKA-C (catalytic subunit of cAMP-dependant protein kinase) of *Dictyostelium*, cAR4 has glutamine clusters in its carboxyl terminus (32). All members of this receptor family have clusters of serine residues in their carboxyl termini that are sites of ligand-dependant phosphorylation (32-34, 62). Various arrangements of these conserved clusters within a certain cAMP receptor type could indicate a means of their individual regulation and function.

Existence of Additional G protein-coupled Receptors

In addition to the cAMP receptors, other *Dictyostelium* GPCRs are thought to exist based upon the discovery of novel ligands, numerous Gα subunits, and sequence data (Figure 5)(Figure 6). In addition to cAMP and folic acid, lysophosphatidic acid (LPA) functions as a chemoattractant in *Dictyostelium* (66). In vertebrates, LPA induces platelet aggregation, smooth muscle contraction, and fibroblast proliferation. To date, twelve Gα subunits have been identified in *Dictyostelium discoideum* (67). Gα2 works with the cAMP receptors to transduce cAMP signals needed to begin and sustain development (28, 42). Gα4 and Gα5 function antagonistically during *Dictyostelium* development to mediate cell sorting during the slug stage (68-71). Most recently, characterization of Gα9 indicates that this G protein subunit functions to inhibit cAMP signaling and propagation during aggregate formation (67). Additional identified Gα subunits have yet to be characterized.

Sequencing of the *Dictyostelium* genome has produced data that could lead to the identification of new G protein-coupled receptors. Conceptual translations of cDNAs

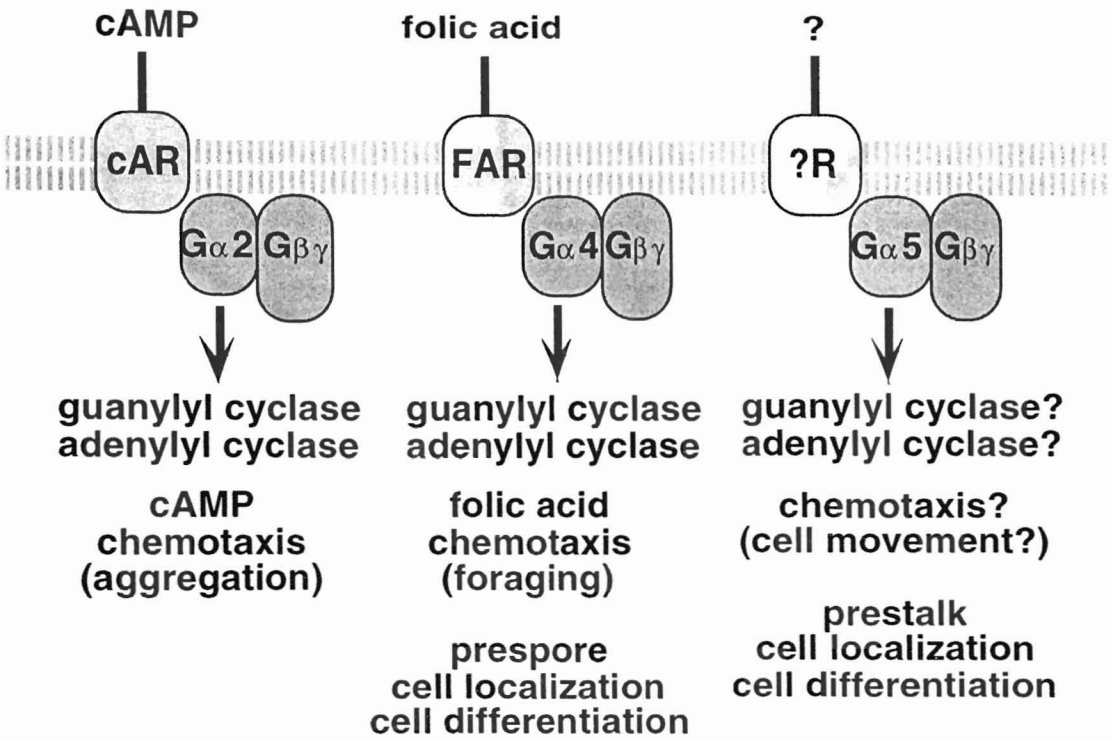


Figure 5 - Known G protein-coupled Pathways in *Dictyostelium discoideum*

The cAMP receptors work through Ga2 to promote chemotaxis. Folate is also a chemoattractant for *Dictyostelium*, but the receptor has yet to be identified. Other chemoattractants are yet to be discovered as Ga5 has no known receptors to which it associates. Due to the number of known G proteins and ligands, additional G protein-coupled receptors are thought to exist in *Dictyostelium*.

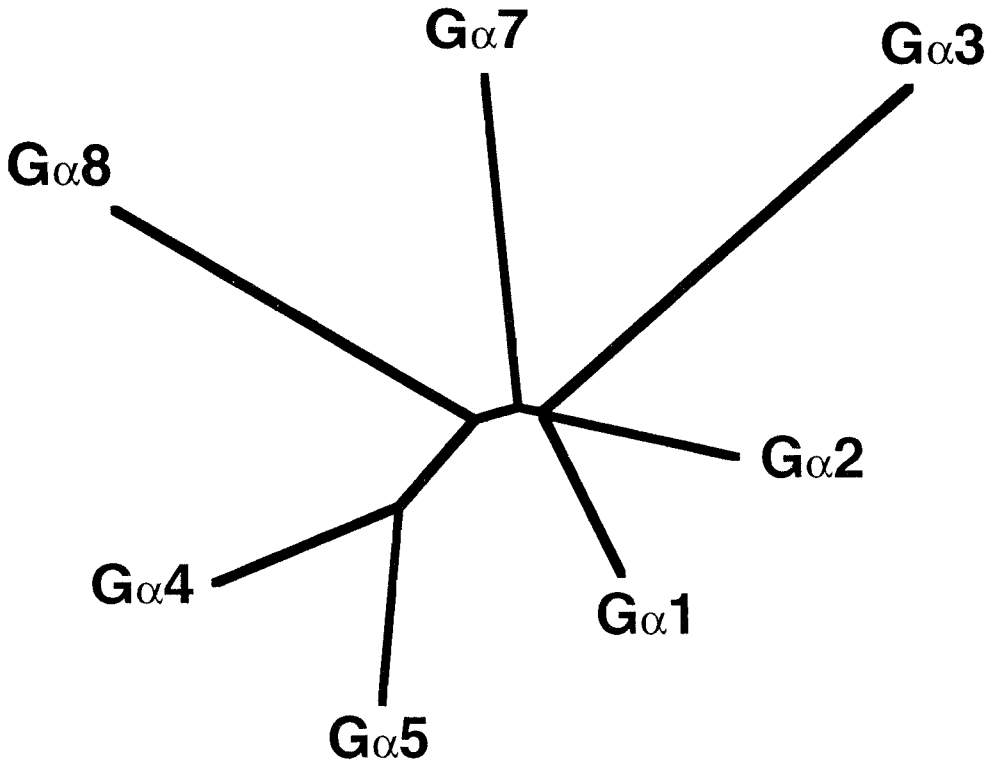


Figure 6 - Phylogenetic Tree of Identified Ga Subunits.

To date, twelve Ga subunits have been identified. For the seven Ga subunits shown in this fugure, the degree of amino acid identity was determined by the PROTDIST protein sequence program in the PHYLIP phylogeny inference package. The branch lengths are representative of the distance maps as determined by the Fitch program of the PHYLIP package.

(complementary DNAs) from available databases show transcription of potentially unknown G protein-coupled receptors. Three cAMP receptor-like receptors, CrlA-C, are being investigated due to their individual characteristics and identity with the known cAMP receptors. Here, the analysis of CrlA will be explained.

CHAPTER 3

MATERIALS and TECHNIQUES

Strains and Media

The following axenic haploid *Dictyostelium* strains were used: KAx-3 (wild-type parental strain) cells and *crlA* mutant strains created through insertion of the BlastocidinS resistance gene employing homologous recombination. Shaking cultures of vegetatively growing cells were maintained at room temperature in HL-5 media. Na^+/K^+ phosphate buffer (12 mM NaH_sPO_4 adjusted to pH 6.1 with KOH) was used to suspend cells in shaking culture under nonnutrient conditions.

For developmental assays, strains were grown on *Klebsiella aerogenes* bacterial lawns supported by SM+/3 nutrient agar. This results in plaque formation by *Dictyostelium* cells as bacteria are cleared from the plate. Cells were also plated on non-nutrient Na^+/K^+ phosphate buffer agar allowing the immediate onset of development.

Standard recombinant DNA techniques were employed to characterize CrlA function. All cloning procedures were carried out in *Escherichia coli* strain NM522. DNA constructs created for this project were introduced to *Dictyostelium* cells through electroportation following protocol described by Dynes and Firtel incorporating a BioRad Gene Pulser set at 1.3 kV and 3.0 μF (72). Clonal transformants were selected and transferred into 24 well plates for culturing and further analysis.

Genomic Sequence Information

Sequence data, cDNAs, and *Dictyostelium* genomic fragments for CrlA were obtained from a variety of sources. The cDNA sequence SSD449 was made available by the *Dictyostelium* Developmental cDNA Project (Morio *et al.*, 1998) in Tsukuba, Japan. Using this data, the *crlA* locus was examined by Southern blot. *Dictyostelium* sequences inserted into the *Sma*I site of pUC18 provided genomic fragments necessary for sub-cloning of the putative receptor gene. *crlA* sequencing vectors from Baylor Sequencing Center - *Dictyostelium* Genome Project (http://dictygenome.bcm.tmc.edu/), IIBCP1D0339 and IIBHP1D2904, primarily provided promoter and upstream sequence data. Sequencing vector JC2d106a03 from Genome Sequencing Centre Jena - *Dictyostelium* Genome Project (http://genome.imb-jena.de/dictyostelium/) provided downstream sequence information. Hereafter, these *crlA* sequencing vectors will be referred to as pBR07 (JC2d106a03), pBR08 (IIBCP1D0339), and pBR10 (IIBHP1D2904). Contiguous sequence data from contig14634 produced by Baylor Sequencing Center - *Dictyostelium* Genome Project also provided important information concerning this putative receptor.

DNA Constructs

Assembly of the CrlA ORF was the first endeavor undertaken for the characterization of this putative receptor. Inspection of pBR08 and pBR10 restriction digests and sequence data revealed relatively identical base sequences with the exception of an additional 300 bps at the 5'-end of pBR08. Hydrophilicity analysis of the conceptual protein encoded by the addition of upstream pBR08 sequence to downstream pBR07 sequence showed hydrophobic amino acid regions typical of the seven

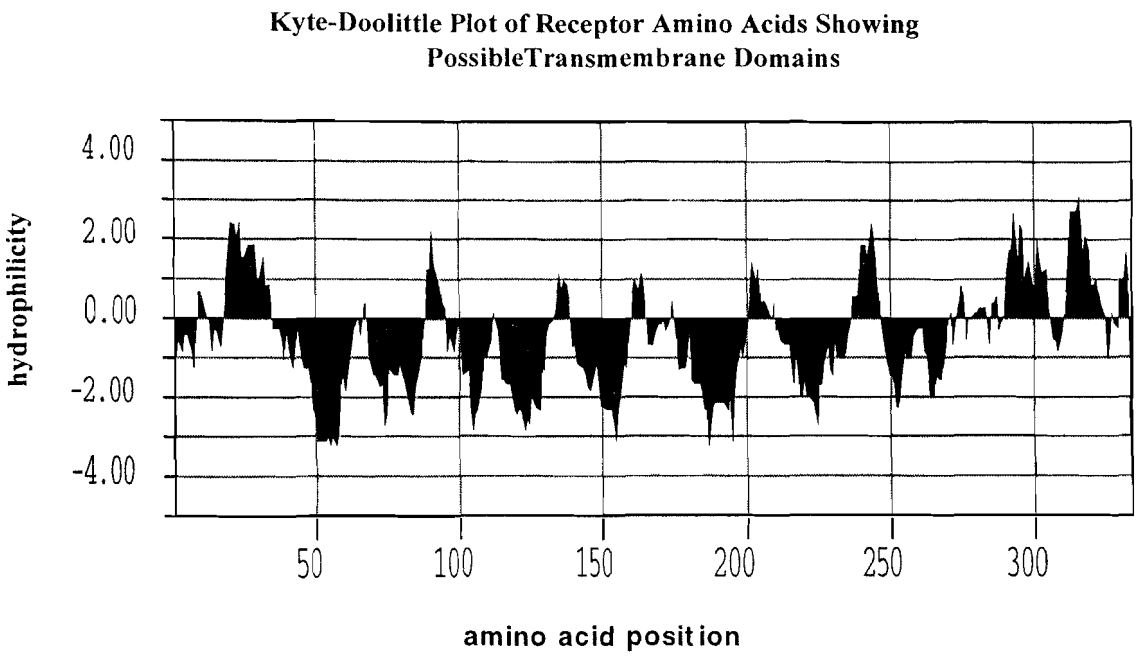


Figure 7 - Kyte-Doolittle Plot of Predicted CrlA Amino Acid Sequence

G protein-coupled receptors are characterized by seven transmembrane (TM) domains consisting of disproportionate hydrophobic residue composition relative to the remainder of the protein. Here, the conceptual open reading frame of CrlA is analyzed for its hydrophobic amino acid composition.

transmembrane domains seen in known G protein-coupled receptors (Figure 7). Analysis with pBR10 and pBR07 revealed a similar hydrophilicity plot except at the extreme upstream region. The additional 300 bps upstream to the CrlA ORF in pBR08 potentially contains promoter sequences for the *crlA* gene. To unite the upstream and downstream sequences, the *Xba*I/*Eco*RI fragment of pBR08 was inserted into the same sites of pBluescriptIISK$^+$. Following blue/white screening and restriction digestion, this construct was confirmed and named pBR05. The *Hin*dIII/*Spe*I fragment of this construct was then inserted into the same sites of pBR07. The resulting 5.4 kb construct, pBR06, contains the entire CrlA ORF and 0.3 kb of suspected promoter sequence. This corresponded to contig14634 released by the Baylor Sequencing Center - *Dictyostelium* Genome Project soon after this construct was completed. In addition to pBR06, the *Hin*dIII/*Spe*I fragment of pBR10 was subcloned into pBR07 giving the same construct as pBR06 less the suspected promoter region. This construct was termed pBR04.

To create a construct through which the *crlA* gene could be disrupted by homologous recombination, the 1.4 kb BlastocidinS resistance gene was excised as an *Xba*I fragment from pJH380 (*Hin*dIII/*Kpn*I fragment from pUCBsrDBam into the same sites of pBluescript II SK$^-$), a derivative of pBsr2 (73), and inserted into the unique *Spe*I site of pBR06. This created two disruption constructs based upon the orientation by which the selectable marker was incorporated into the predicted second exon of the *crlA* gene. By restriction digest analysis, construct pBR14 has the putative receptor and Bs^r genes oriented in the same direction. The selectable marker in construct pBR15 is inserted in the opposite direction. Transformants were selected in HL-5 medium containing blastocidinS as previously described (74)

Additionally, constructs were created to over-express CrlA in different cell types. The *Eco*RI/*Bgl*II fragment of pBR07 was replaced by the *Eco*RI/*Bam*HI fragment of pJH1012 that carries the act6 promoter fused to the G418^r gene (act6::G418^r) in order to create a 6.4 kb construct termed pBR11. The *Hin*dIII/*Spe*I fragment of pBR06 was inserted into the same sites of pBR11 to manufacture a 6.7 kb construct, pBR16, with a pUC18 backbone that possesses the putative receptor and G418^r genes reading in the opposite direction (Figure 8). Resistance to G418 is conferred in proportion to the vector copy number.

To create a construct with *crlA* and G418^r genes in the same direction, the *crlA* gene was excised as a *Bgl*II/*Eco*RI fragment of pBR06 and used to replace the *Bam*HI/*Eco*RI fragment of pT7T3 18U (Pharmacia). This was termed construct pBR24. The *crlA* gene was then excised as a 1.7 kb *Hin*dIII/*Kpn*I fragment from pBR24 and inserted into the same sites of pJH1012 that contains the act15::G418^r gene to create pBR25 (Figure 8). Similar to pBR16, resistance to G418 is conferred in proportion to the vector copy number.

An uncharacterized frameshift mutation was created in the CrlA ORF of construct pBR16 to ensure expression of the CrlA receptor was responsible for any observed phenotype and not attributed to vector sequence of the expression constructs. Here, pBR16 was digested with *Spe*I and the ends filled by the Klenow fragment of DNA polymerase I. This fragment was then ligated and termed pBR28 (Figure 8).

Expression constructs were also created using the BlastocidinS resistance gene as the selectable marker. Here, the BlastocidinS resistance gene was excised from pJH413 (*Hin*dIII/*Kpn*I fragment from pUCBsrDBam into the same sites of pBluescript IISK$^-$) as a

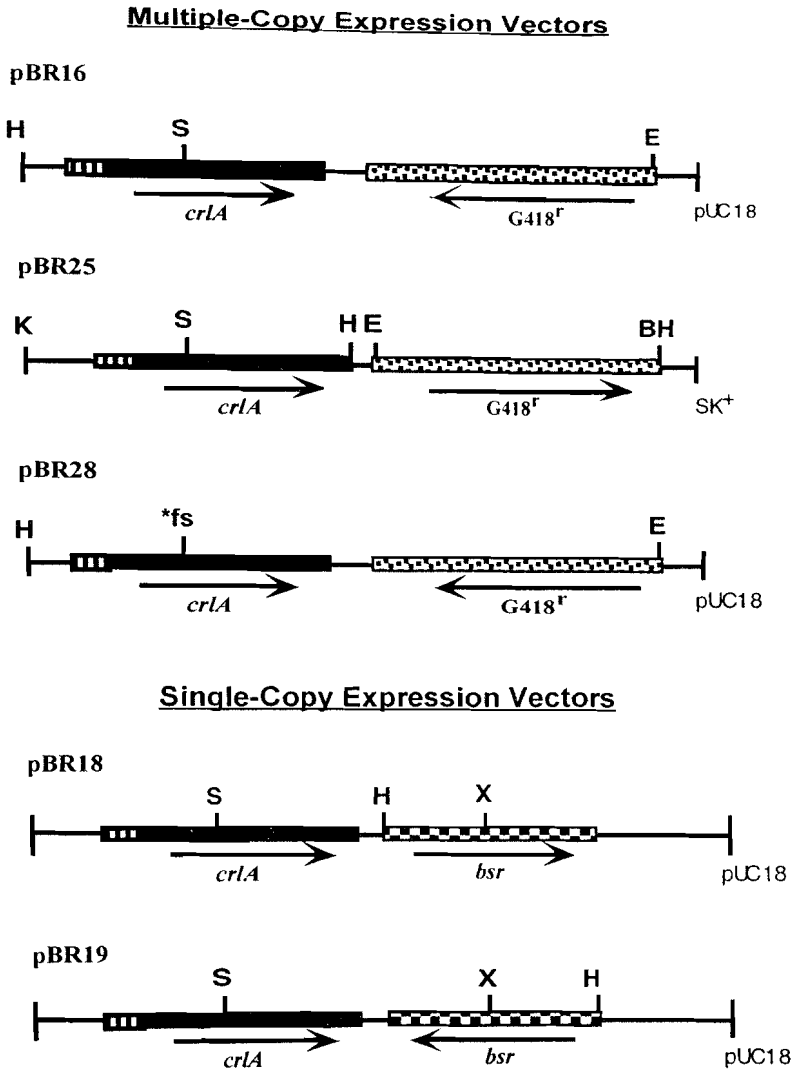


Figure 8 - Expression Constructs

The Neo^r gene(G418) was used to analyze cell response to multiple copies of the CrlA receptor. pBR16 and pBR25 have the suspected *crlA* promoter and open reading frame in identical and opposite orientations to the G418 gene, respectively. A frameshift (*fs) mutation was created at the *Spe*I site in pBR28. The BlastocidinS gene was also used as a selectable marker for overexpression. These constructs (pBR18 and pBR19) differ only in the orientation of *bsr*. Symbolism : suspected *crlA* promoter (vertical lines), CrlA open reading frame (closed), G418 resistance gene (speckled), BlastocidinS resistance gene (checkered). Restriction sites : *Hin*dIII (H), *Spe*I (S), *Eco*RI (E), *Kpn*I (K), and *Bam*HI (BH).

*Bam*HI fragment and inserted into the *Bgl*II site of pBR6. This resulted in two new constructs based upon the orientation of the *bsr* gene relative to the CrlA open reading frame. The construct with *crlA* and *bsr* oriented in the same direction was called pBR18. Consequently, the construct having the genes oriented in opposite directions was called pBR19 (Figure 8).

DNA and RNA Blots

DNA blots were performed to confirm disruption of the *crlA* locus. Northern blots of vegetative and developmental RNA were used to determine temporal expression of CrlA. Development for RNA isolation was conducted on filterpads as previously described (75). Genomic DNA blots and RNA blots were conducted as previously described (30). Radioactive probes were generated using a random primers method as described (76). DNA fragments were excised from pBR06 as *Kpn*I fragments then labeled with radioactive dCTP [^{32}P].

Developmental Assays

Clonal cells were initially assayed for their ability to undergo normal *Dictyostelium* development before subsequent experimentation was performed. Cells were grown to mid log phase (~2x10^6 cells/mL) at 200 rpm in HL-5 shaking culture and twice washed in Na^+K^+ phosphate buffer (12 mM NaH_sPO_4 adjusted to pH 6.1 with KOH). After being resuspended in phosphate buffer at ~1x10^7 cells/mL, cells were spotted on non-nutrient plates (1.5% granular agar in phosphate buffer). In the same manner, transformants were plated on bacterial lawns of *Klebsiella aerogenes* (Ka). Cells were assayed for their ability to aggregate and form fruiting bodies.

Cells were plated for development after shaking in nonnutrient media suspended cultures. Cells were initially grown to ~$2x10^6$ cells/mL in HL-5 then harvested by centrifugation. After two washes in phosphate buffer, cells were suspended in phosphate buffer at ~$2x10^6$ cells/mL and shaken at 200 rpm. Aliquots of the culture were collected and concentrated to ~$2x10^7$ cells/mL by centrifugation. Concentrated cells were plated on nonnutrient agar for development. Cells were assayed for their ability to aggregate and form fruiting bodies.

Cells from cultures treated in the previously described manner were used to make chimeras in order to access *crlA* null cell localization and sorting within migrating slugs and fruiting bodies. Wild-type KAx-3 and *crlA*$^-$ cells from axenic shaking cultures were mixed at a 9:1 ratio to a total cell concentration of ~$2x10^6$ cells/mL. In either cell mixture, the less numerous strain was labeled with pTX-GFP from the Thomas Egelhoff lab to distinguish between the cell types (77). Cells were thoroughly mixed by vortexing and concentrated to ~$2x10^7$ cells/mL in phosphate buffer through centrifugation. Cells were then plated for development on nonnutrient agar plates.

Chemotaxis Assays

Clonal cells were tested for their chemotactic ability in response to folic acid and cAMP as previously described (70). Cells were grown to ~$2x10^6$ cells/mL in HL-5 shaking culture, then washed once in phosphate buffer. After being resuspended at ~$2x10^7$ cells/mL in phosphate buffer, the cells were spotted in 1 µL droplets on nonnutrient phosphate plates. Immediately following cell deposition, a 1 µL droplet of either 1mM folate or 100 µM cAMP was placed on the phosphate plate at a distance of 2 millimeters from the edge of the cell droplet. After 3 hours, cells were checked for net

movement of cells toward the chemoattractant source.

Growth Curves

Mixed cultures were used to assay cell autonomous vegetative growth characteristics of the *crlA*$^-$ cells. Strains of interest were shaken overnight in HL-5 nutrient media and diluted back to a specified cell concentration in HL-5. KAx-3 cells were mixed with *crlA*$^-$ cells at either 9:1 or 1:9 ratios at defined total cell concentrations and shaken in HL-5. In any mixed culture, one strain was labeled with pTX-GFP to distinguish the cell types. Cells were observed and counted over a period of 4 to 5 days.

Axenic strains were grown long term in HL-5 shaking culture to access vegetative growth and ability to undergo development. Sterile HL-5 was inoculated with cells from overnight shaking cultures where cells did not exceed ~$2x10^6$ cells/mL. To attain the desired cell concentration, cells were counted on a hemacytometer, washed twice in phosphate buffer, and resuspended in a calculated volume of fresh HL-5. Cells were counted over a 4 to 5 day period. Additionally, cells were harvested and plated for development on nonnutrient agar plates. Aliquots of each culture were harvested by centrifugation and washed twice in phosphate buffer. Cells were then suspended in phosphate buffer at ~$2x10^7$ cells/mL and plated on nonnutrient agar for development. Strains were assayed for their ability to aggregate and form fruiting bodies.

CHAPTER 4

RESULTS

Identification of cAMP Receptor Homologues

Sequences for the known cAMP receptors in *Dictyostelium* were used to cull the available *Dictyostelium* cDNA and genomic databases to identify genes encoding proteins with the aforementioned characteristics of G protein-coupled receptors. In collaboration with the Dale Hereld lab at the University of Texas Health Science Center - Houston, we were able to identify three putative G protein-coupled receptors. These were designated CrlA, CrlB, and CrlC (cAMP receptor-like). According to established *Dictyostelium* nomenclature, the corresponding genes were designated *crlA*, *crlB*, and *crlC*. All of the putatuve Crl receptors possess seven transmembrane domains. However, CrlA has the highest sequence identity with the previously described Car receptors. Due to this limited sequence identity between receptors and the varying intron positions within corresponding genes, the Crl receptors may represent one or more new receptor families. Here, the characterization of CrlA will be described.

Description of the *crlA* Locus

Mapping restriction sites of the *crlA* locus was initiated by genomic DNA blots of wild-type cells. A cDNA clone, SSD449, was used as a probe and showed the *crlA* locus to be located on a 4.4 kb *Eco*RI segment. In addition, the locus appeared to be bisected by a unique *Spe*I restriction site. This was indicated by a doublet band visualized on the

Southern blot that corresponded to an *Eco*RI/*Spe*I digest of the wild-type genomic DNA. With respect to the available genomic sequences and cDNA SSD449, the *crlA* locus contains three predicted open reading frames for this receptor of 0.5, 0.3, and 0.2 kb that are separated by two introns of approximately 0.1 kb each. In addition, a segment of this locus upstream of the *crlA* gene was identified that may contain all or part of the *crlA* promoter based upon the expression of the *crlA* gene from recombinant vectors (see below).

Disruption of the *crlA* Gene

Homologous recombination employing a double cross-over strategy was used to disrupt the *crlA* gene (Figure 9A). *Eco*RI/*Hin*dIII and *Eco*RI/*Xho*I restriction digests of genomic preparations from suspected *crlA*$^-$ transformants verified insertion of the 1.4 kb *Xba*I fragment of the *bsr* gene into the *Spe*I site of the *crlA* locus. The doublet band seen with the *Eco*RI/*Xho*I restriction digest is due to the presence of a *Xho*I site within the *bsr* gene. This results in two bands of approximately 3.0 kb each (data not shown). The *Eco*RI/*Hin*dIII digestion results in bands of 3.7 kb and 2.3 kb consistent with the introduction of a *Hin*dIII site from the *bsr* gene in the gene disruption (Figure 9B).

Expression of CrlA

RNA blotting was used to assess temporal expression of CrlA in vegetatively growing and developing cells to decipher if the CrlA receptor functions during either phase of the *Dictyostelium* life cycle. Based on the RNA blot analysis, *crlA* is expressed throughout both vegetative growth and development with increased expression during the later stages of development (Figure 10). This expression pattern is similar to Gα4 and Gα5 genes which encode previously characterized *Dictyostelium* G protein subunits.

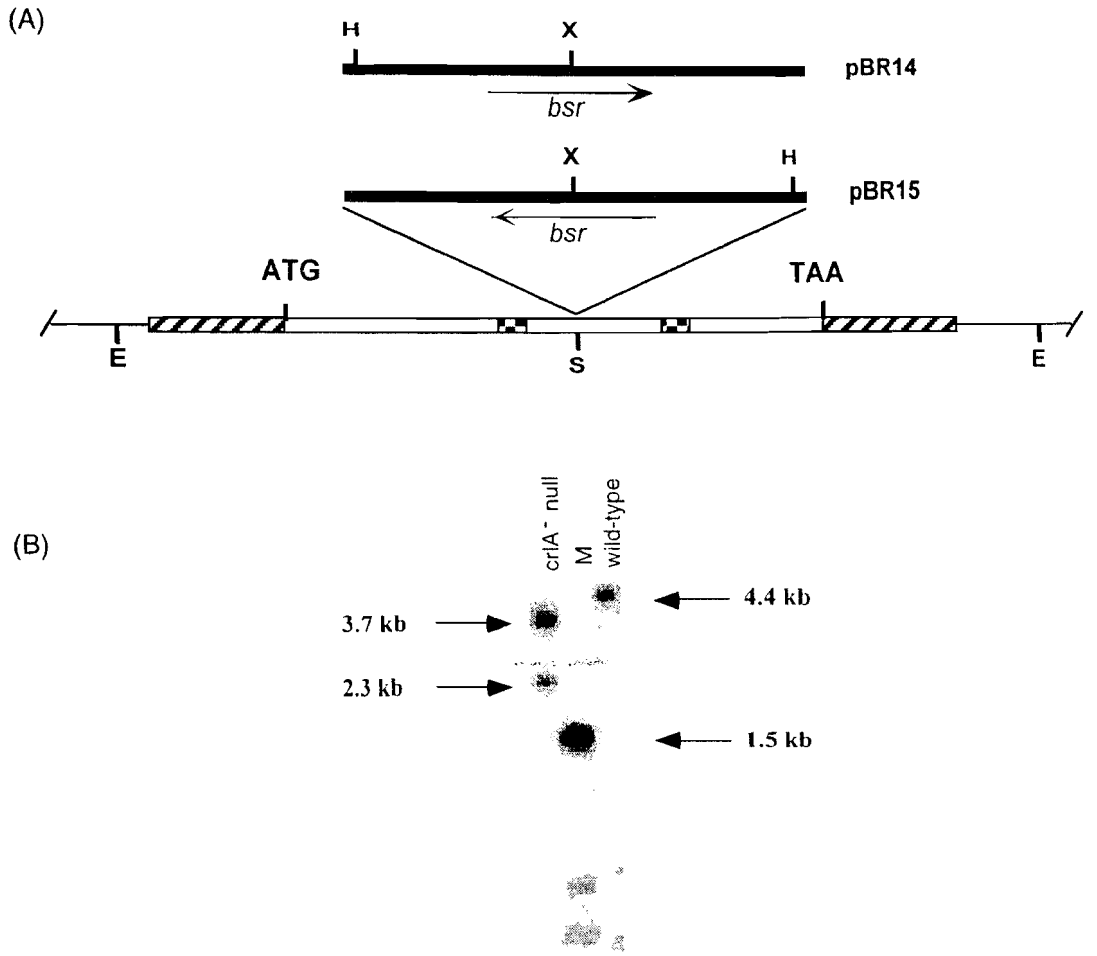


Figure 9 - Disruption of *crlA*

(A) Map of the disrupted *crlA* locus. The *crlA* gene was disrupted by inserting the 1.4 kb BlastocidinS resistance gene in either orientation. (B) Digested genomic DNA of gene disruption mutants resulted in two 3.0 kb *Eco*RI/*Xho*I fragments (not shown) and 3.7 and 2.3 kb *Eco*RI/*Hin*dIII fragments as determined by genomic DNA blots hybridized with a *crlA* specific probe. Symbolism: *crlA* open reading frame (open), introns (checkered), BlastocidinS resistance gene (closed), cloned extragenic sequence (striped) and uncloned intergenic regions (lines). DNA ladder (M)
restriction sites: *Eco*RI (E), *Spe*I (S), *Xho*I (X), and *Hin*dIII (H)

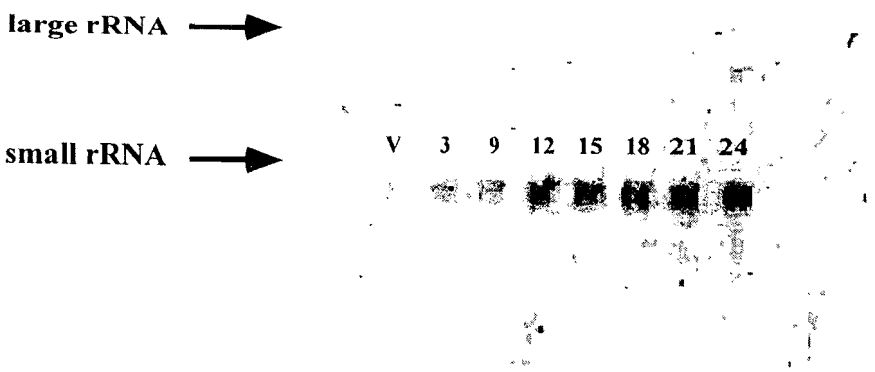


Figure 10 - Temporal Expression of CrlA

Vegetative and developmental RNA was used to assess CrlA expression. Blot analysis shows expression throughout both stages of the *Dictyostelium* life cycle with increased expression during the latter stages of development. This is similar to two previously characterized G proteins needed for cell sorting during the slug stage (pseudoplasmodium) of development. Vegetative RNA (V) is located in the first lane. Sequential numbers represent RNA isolated t_x hours after the onset of development.

The transcript was difficult to detect relative to rRNA standards under ultraviolet light suggesting a low level of *crlA* expression.

Developmental and Chemotaxis Assays

Clonal transformants were assayed for development on bacterial lawns supported by $SM^+/3$ nutrient agar. *crlA* null cells deposited on *Klebsiella aerogenes* lawns exhibited normal development compared to wild-type KAx-3 cells. Plaque growth rate was similar to that of wild-type cells and fruiting bodies appeared normal. Individual fruiting bodies were comprised of normal proportions of stalk and spore cap cells.

Clonal transformants were assayed for development on nonnutrient Na^+/K^+ phosphate agar that allows immediate onset of development. Initiation of synchronized development was observed. However, *crlA* null cells formed larger aggregates than wild-type cells and were delayed for approximately two hours in formation of the anterior tip (Figure 11). Mutant aggregates completed development without additional delays or observed structural phenotypes.

Cell responses that are dependent upon G protein-coupled receptors to known *Dictyostelium* chemoattractants were assayed using *crlA* mutants (data not shown). Consistent with normal plaque growth, *crlA* null cells are capable of chemotaxis to folic acid. Normal chemotaxis to cAMP by *crlA* null cells is consistent with proper aggregation.

Chimeric Studies of CrlA

Aberrations in signal reception necessary for proper aggregation size and tip formation could result in the developmental delay observed for *crlA*$^-$ receptor mutants. Such a defect would be expected to be cell-autonomous. To assess the nature of this

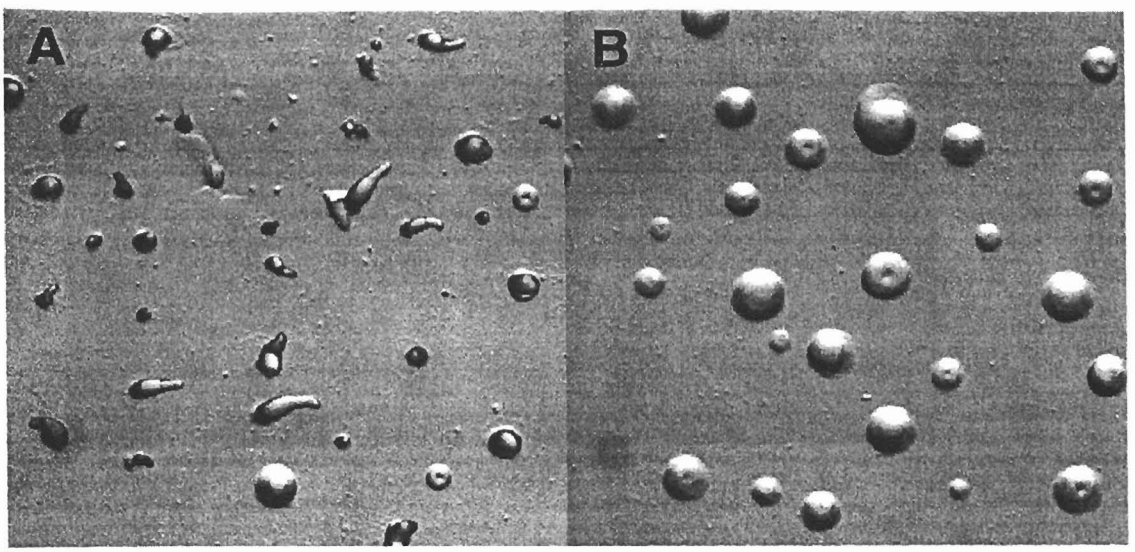


Figure 11 - Development of *crlA* Null Cells on Nonnutrient Phosphate Agar

(A) When plated under nonnutrient conditions, wild-type *Dictyostelium* cells immediately initiated development and proceeded through normal development. (B) Similar to this, *crlA* null cells began immediate development, but formed large aggregates and are delayed approximately two hours in formation of the anterior tip. Despite this defect, the mutant cells were able to complete development and form mature fruiting bodies.

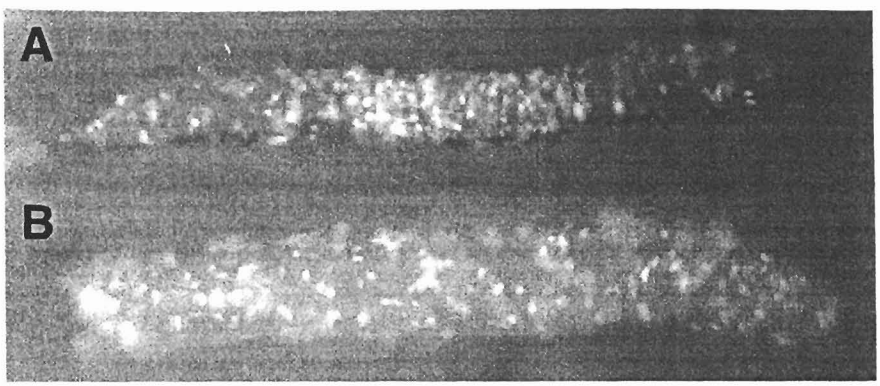


Figure 12 - Heterologous Distribution of *crlA* Mutants

(A) GFP-labeled mutants were mixed with KAx-3 cells at a 1:9 ratio and developed as chimeras. Under these conditions, *crlA*- null cells were consistently under-represented in the anterior prestalk regions of developing pseudoplasmodia. (B) As a control, GFP-labeled mutants were mixed with unlabeled mutants at a 1:9 ratio and developed as chimeras. This resulted in a homogenous distribution of labeled cells.

developmental defect, *crlA*$^-$ receptor mutants were labeled with a GFP expression plasmid and mixed at a 1:9 ratio with unlabeled KAx-3 cells prior to development to create chimeric aggregates (Figure 11). After co-aggregation and pseudoplasmodium formation, the *crlA*$^-$ receptor mutants were consistently under-represented in the anterior prestalk regions. As a control for this heterologous localization, GFP-expressing *crlA*$^-$ receptor mutants were mixed at a 1:9 ratio with unlabeled *crlA*$^-$ receptor mutants. Under these conditions, GFP-expressing cells were homogenously distributed throughout chimeras.

Overexpression and Complementation of *crlA*

Like gene disruptions, overexpression of signal transduction components can provide data for characterization of gene function. Here, a vector with a CrlA genomic fragment and G418^r gene (pBR16) was introduced to KAx-3 cells to assess CrlA function in relation to G418 resistance efficiency. Because a single copy of *G418* provides poor resistance even at low levels of selection, multiple copies of the resistance gene are expected in viable cells as the level of drug selection increases. Conversely, a single copy of *bsr* provides sufficient resistance even when selection is at relatively high levels. After electroporation and culturing in HL-5 with 5-7μg/mL of G418, small colonies of resistant cells were observed. However, most all of these colonies disappeared even when the drug was removed on the fourth day of drug selection. Cell cultures transformed with G418 resistance vector lacking the *crlA* gene were able to sustain normal growth suggesting cells cannot tolerate the *crlA* gene expression vector.

To determine whether the lethality was due to *crlA* expression or presence of vector sequence, KAx-3 cells were transformed with a *crlA* mutant expression vector

(pBR28) in which the *crlA* gene contained a reading frame mutation. Cells possessing this vector maintained viability even under the highest levels of G418 selection (>10μg/mL) suggesting that lethality was indeed due to *crlA* expression and not *crlA* sequences. Additionally, viable *crlA* expression vector transformants were observed with lower levels of G418 selection (2-4μg /mL) or by employing low-copy-number vectors conferring resistance to blasticidinS. This indicates cells can tolerate low copy numbers of *crlA* expression vectors.

Complementation and rescue of the *crlA*$^-$ phenotype was performed through transformation of receptor null cells with pBR16 and selection of transformants at low G418 concentrations (3μg/mL). Efficient transformation resulted in the isolation of several clonal transformants that displayed a normal rate of tip development compared to KAx-3 cells and accelerated tip development compared to a control of untransformed *crlA*$^-$ receptor mutants. Relative to *crlA*$^-$ receptor cells, aggregation size in the rescued transformants showed no apparent decrease suggesting pBR16 did not completely rescue all mutant phenotypes possibly due to incomplete promoter sequence in this expression vector (Figure 13).

Vegetative Growth in HL-5 Media

Axenic suspensions of *crlA*$^-$ receptor cells in shaking HL-5 media were cultured to assess vegetative characteristics since expression of CrlA occurs throughout vegetative growth. Relative to KAx-3 cells, comparable concentrations of receptor null cells were achieved up to mid-log phase (~$2x10^6$ cells/mL) from cultures that started at ~$1x10^5$ cells/mL. In late log and stationary growth phases, *crlA*$^-$ receptor cells were able to reach concentrations twice that of KAx-3 cells suggesting that cell density at growth saturation

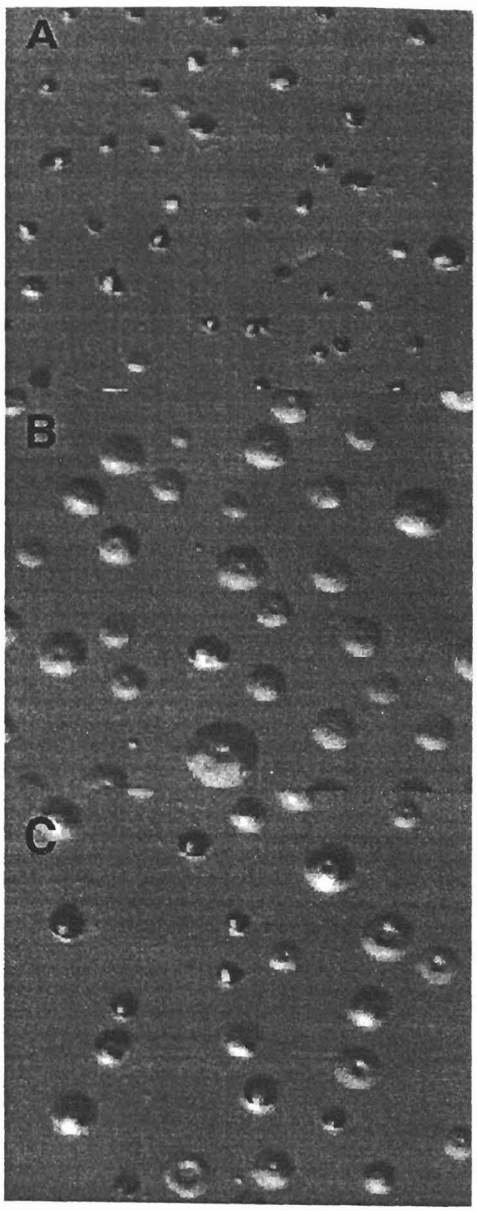
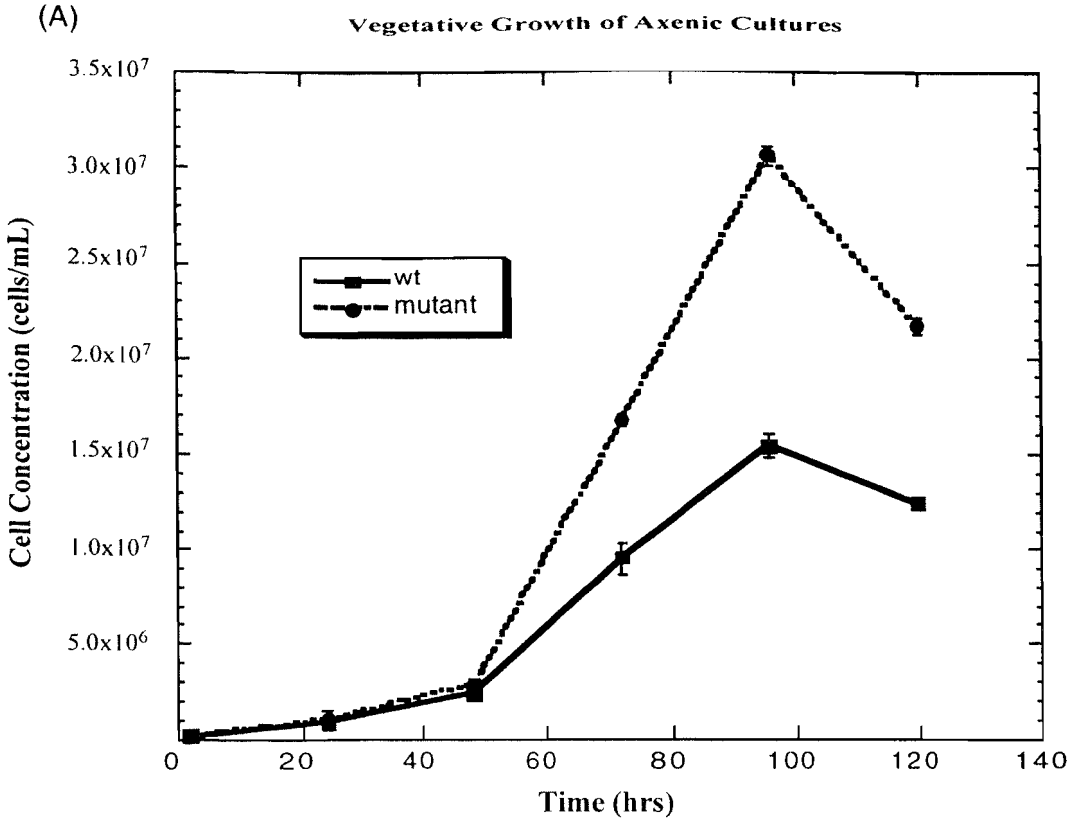


Figure 13 - Complementation and Rescue of the *crlA*- Phenotype
An attempt to rescue the mutant phenotype was done using pBR16 and selection of transformants at low G418 concentration. Cells were plated on phosphate agar plates.
(A) Wild-type KAx-3 development.
(B) *crlA*- mutant development showing large aggregates and delay in anterior tip development.
(C) Rescued *crlA*- mutant development. Anterior tip in these cells appears normal compared to KAx-3 cells and accelerated compared to null cells. However, aggregation size of the rescued mutants shows no apparent decrease suggesting pBR16 did not completely rescue all mutant phenotypes.

is influenced by CrlA function (Figure 14A).

This was also seen in mixed cultures of wild-type and *crlA*$^-$ cells. KAx-3 cells were mixed at a 9:1 ratio with GFP-labeled *crlA*$^-$ mutants and shaken in HL-5 media where initial total cell concentrations were ~$1x10^5$ cells/mL. Conversely, *crlA* null cells were mixed at a 9:1 ratio with GFP-labeled KAx-3 cells. Cells maintained their inoculated proportions up to mid-log phase. After this, the proportion of *crlA*$^-$ mutants increased with respect to wild-type counterparts (Figure 14B). This observation suggests the regulation of cell density during stationary phase is a cell autonomous effect.

(A)



(B)

9 KAx-3 : 1 GFP-labeled *crlA*⁻ Mutant Cell Mixture

	total cell #	GFP-labeled cell #
T_2	$1.7x10^5 \pm 0.2x10^5$	$1.3x10^4 \pm 0.3x10^4$ (8%)
T_{96}	$2.0x10^7 \pm 0.09x10^7$	$4.5x10^6 \pm 0.2x10^6$ (22%)

9 *crlA*⁻ Mutant : 1 GFP-labeled KAx-3 Cell Mixture

	total cell #	GFP-labeled cell #
T_2	$1.7x10^5 \pm 0.2x10^5$	$1.8x10^4 \pm 0.1x10^4$ (10%)
T_{96}	$3.1x10^7 \pm 0.2x10^7$	$7.1x10^5 \pm 0.9x10^5$ (2%)

Figure 14 - Analysis of Vegetative Growth

(A) Because CrlA is expressed during vegetative growth, null cells were suspended in HL-5 medium and counted over several days. Both KAx-3 and mutant cells grew at relatively similar rates up to mid-log phase (~2x106 cells/mL). However, *crlA*- mutants were able to reach concentrations twice that of wild-type cells during late log and stationary phase. This implies that cell density at growth saturation is influenced by CrlA function. (B) In mixed cultures with KAx-3 cells, mutants exceeded their inoculated proportion of the total cell number. This suggests that regulation of cell density during stationary phase is a cell autonomous effect.

CHAPTER 5

DISCUSSION of RESULTS

Discovery of CrlA

Scanning of *Dictyostelium* genomic and cDNA information was instigated by previous findings that suggested the existence of additional G protein-coupled receptors. The likelihood that the multitude of characterized G proteins all functioned through the known Car family of receptors was remote. This led to the discovery of the cAMP receptor-like receptors (CrlA, CrlB, and CrlC) which could represent a new family of G protein-coupled receptors. This idea was supported by the limited sequence identity between the Crl receptors and known cAMP receptors. Additionally, null strains of the new receptors were capable of normal chemotaxis and aggregation to exogenous cAMP and folic acid sources suggesting none of the Crl receptors are individually necessary for chemotaxis to these signals. Investigation of CrlB and CrlC beyond this point has yet to be completed, however results for CrlA will be described here.

Relation to cAMP Receptors and TasA

Of the Crl receptors, CrlA appears to be more closely related to the Car family of receptors based upon sequence identity. Similarities in these sequences span intracellular, transmembrane, and extracellular regions indicating CrlA perhaps shares structural and functional characteristics with Car receptors on either side of the plasma membrane. One phenotypic similarity between *crlA*$^-$ and *car2*$^-$ mutants is altered tip

development. However, the *car2*$^-$ mutants terminate development at this stage and *crlA*$^-$ mutants possess the ability to proceed past this defect and form normal stalks of mature fruiting bodies (33). Additionally, the phenotype observed for *crlA*$^-$ mutants appears somewhat similar to that of TasA receptor mutants of *Polysphondylium pallidum*, another cellular slime mold. In this organism, *tasA*$^-$ mutants are delayed approximately four hours into development compared to PN500 parental strains (78). Unlike *crlA*$^-$ mutants, the *Polysphondylium pallidum tasA*$^-$ mutants are not able to form proper fruiting bodies and development results in fruiting bodies with amorphous stalks.

Developmental Phenotypes

As observed through developmental assays, CrlA appears to function in regulating aggregate size and timing of anterior tip development. However, the signal received by the CrlA receptor remains to be identified. Potential candidates for this signal are factors that regulate aggregate size or tip development. Signals such as countin and countin2 are known to regulate aggregate size (79, 80). Countin is thought to limit the maximum number of cells in an aggregate while countin2 works antagonistically to limit the minimum number of cells in an aggregate. Together, they regulate the appropriate number of cells needed for proper aggregation and formation of a fruiting body. Consequently, the timing of tip formation could be influenced by aggregate size if intercellular signaling or cell sorting within the aggregate is impeded by increased aggregate volume. Alternatively, a delay in prestalk cell differentiation could potentially result in larger aggregate sizes and slower rates of cell movement to the site of tip development because prestalk cells sort to this portion of pseudoplasmodia (49, 81-85).

Like CrlA, the Gα4 and Gα5 subunits of *Dictyostelium discoideum* G proteins are expressed during vegetative growth and development. However, neither signaling component is needed for proper development until the aggregate stage (30, 68, 69). Gα4 is needed for scavenging bacteria through folic acid gradients, but Gα5 has not had a vegetative role assigned to it. Signaling components such as CrlA, Gα4, and Gα5 that are expressed during both vegetative and developmental stages of the *Dictyostelium* life cycle are not expressed specifically in prespore or prestalk cells. Rather, they are expressed in cells that can be recruited to potentially form either prestalk or prespore cells throughout development (48, 85, 86).

The ability of *crlA*$^-$ mutants to produce mature fruiting bodies despite their delay during tip formation suggests supplementary mechanisms exist that defeat this temporary block in development. The cell autonomous nature of this defect is evident when considering the under-representation of these mutant cells in the anterior prestalk region of chimeric aggregates. This indicates the mutant cells are not as competent as KAx-3 cells in forming anterior prestalk cells and is consistent with the role of CrlA as a receptor of external signals. Similar patterns of mutant cell distributions in chimeras have been reported for cells lacking the Gα5 G protein subunit or the ERK1 protein kinase suggesting these cell components perhaps function in the same or related pathways (74, 87). Like the *crlA*$^-$ mutant phenotype, the loss of Gα5 function results in large aggregates with delayed tip formation (30).

Lethality of CrlA

Aberrant development associated with the *crlA*$^-$ mutants can at least be partially rescued with *crlA* gene expression vectors, but the apparent lethality associated with

these vectors at high copy number indicates *Dictyostelium* cells do not tolerate either increased or altered *crlA* gene expression. The cell death phenotype could possibly result from a CrlA-mediated termination of vegetative growth if the receptor function is important for the transition from growth to development. A role for CrlA function in this transition is consistent with the increased cell number of *crlA*⁻ mutants compared to wild-type cells in mixed shaking cultures if mechanisms exist to regulate cell division when nutrients become limited. The CrlA receptor is unique in this regard since no other losses in cell viability have been associated with the vector expression of any other *Dictyostelium* G protein-coupled receptors.

Additionally, these results are similar to those of *yakA*⁻ null cells (88). YakA is a protein kinase required for transition from growth to development in *Dictyostelium* and is a homolog to Yak1p in *S. cerevisiae*. YakA is expressed throughout cell growth at very low levels and high-copy numbers of *yakA* have been shown to induce arrest of *Dictyostelium* growth in G_2 phase of the cell cycle. The *yakA*⁻ null cells also divide faster than wild-type cells. Considering the phenotypic similarities of *crlA*⁻ null cells and *yakA*⁻ null cells it is reasonable to suspect they function in the same signal transduction pathway.

Methods to Identify Additional GPCRs

The four Car and three Crl receptors cannot account for all of the G protein-coupled receptors in *Dictyostelium* based upon previous studies. Therefore, additional receptors are likely to exist. Sequence diversity among G protein-coupled receptors can be quite drastic and this characteristic may reduce the efficiency of identifying new receptors from the genomic databases using only sequence identity. Future searches

incorporating algorithms developed on quasi-periodic features, such as seven transmembrane domain structure, might prove useful as previously demonstrated in a search for *Drosophila* G protein-coupled receptors from sequence databases (89).

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APPENDIX A

Derived Amino Acid Sequence of CrlA Aligned
to the Predicted Transmembrane Domains of Car1

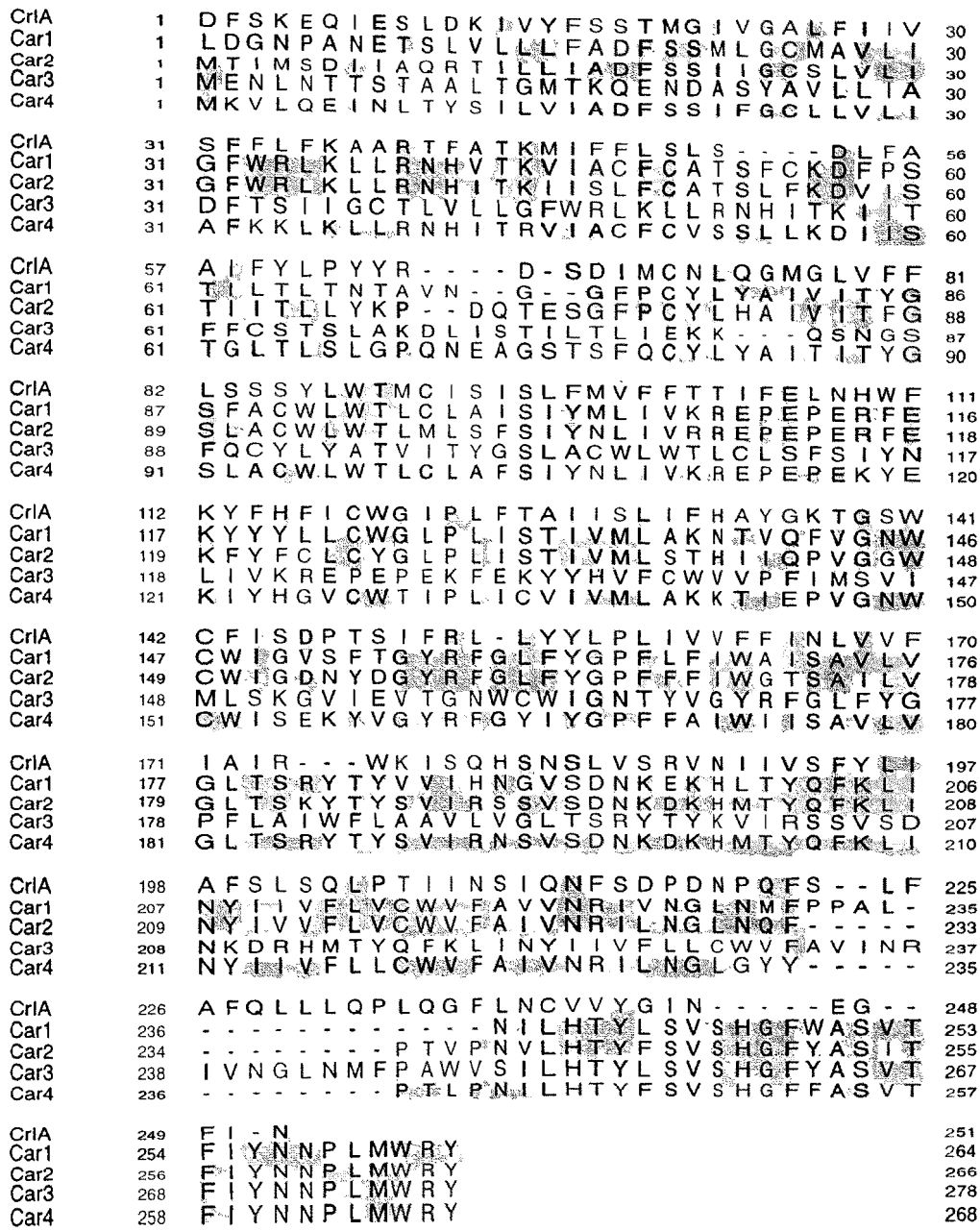
TM1
MINNFLTTITTNDTIIIKETESPNDYDFSKEQIESLDKIVYFSSTMGIVGALFIIVSFFLFKA
TM2 TM3
ARTFATKMIFFLSLSDLFAAIFYLPYYRDSDIMCNLQGMGLVFFLSSSYLWTMCISISLFMVF
TM4 * TM5
FTTIFELNHWFKYFHFICWGIPLFTAIISLIFHAYGKTGSWGFISDPTSIFRLLYYFPLIVGF
TM6
FINLVGFIAIRWKISQPSNFLGSRVKIIGSFYLIAFSLFQLPTIINSIQNFSDPDNPQFSLFA
* TM7
FQLLLQPLQGFLNCVVYGINEGFINHYVEFFEKYIFRCRCRKSRELKEIESDKTSLLVDYENS

DDEEGFDGMDKLIIDDYNRV

* intron position within CrlA coding region

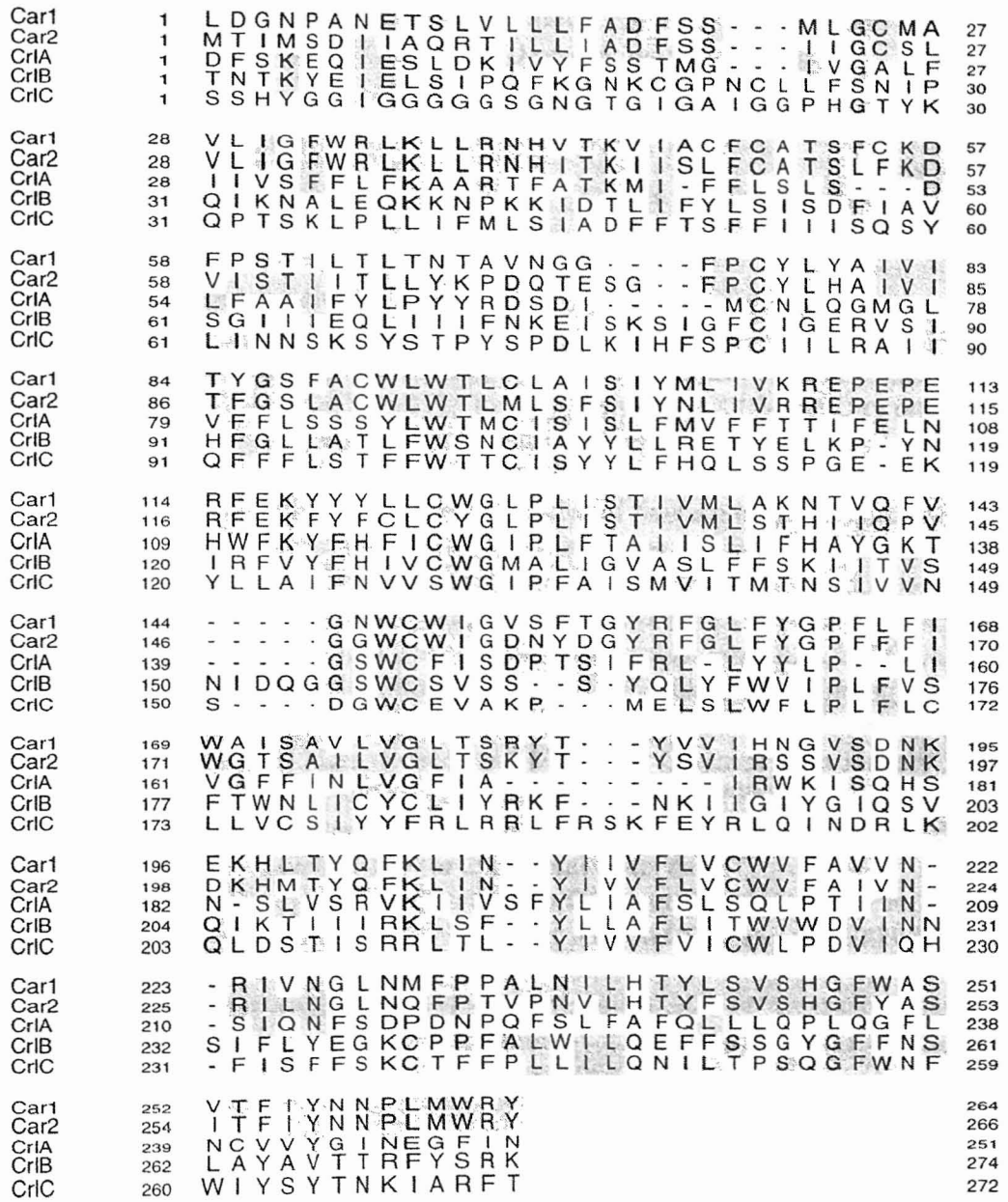
APPENDIX B

Alignment of Predicted Transmembrane Domains of CrlA to Car Family of Receptors



APPENDIX C

Alignment of Crl Receptors to Car1 and Car2
Predicted Transmembrane Domains



VITA 2

BRENT SCOTT RAISLEY

Thesis : Identification and Characterization of CrlA : a cAMP Receptor-like G protein-coupled Receptor in *Dictyostelium discoideum*

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