Characterization of $GABA_B$ receptor-like proteins in *Dictyostelium discoideum*

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Yogikala Vasudeva Prabhu

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Referees/Berichterstatter: Prof. Dr. Angelika A. Noegel
Prof. Dr. Martin Hülskamp

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I take this opportunity to let several IMPORTANT people know much how they mean to me.
PS: This is the section I enjoyed writing the most☺

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Yogikala Prabhu
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Dedicated to my Parents

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Abbreviations

bp	base pair(s)
β-ME	beta-mercaptoethanol
BSA	bovine serum albumin
Bsr	blasticidin resistance cassette
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
DEPC	diethylpyrocarbonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ECL	enzymatic chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis (2-amino-ethylene) N,N,N,N-tetraacetic acid
EST	expressed sequence tag
G418	geneticin
GABA	γ-amino butyric acid
GFP	green fluorescent protein
GPCR	G-protein coupled receptors
Grl	$GABA_B$ or Glutamate like receptors
IgG	immunoglobulin G
kb	kilo base pairs
MOPS	Morpholinopropanesulphonic acid
Mw	molecular weight
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PKA	Protein kinase A
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute
RT-PCR	reverse transcript polymerase chain reaction
SDF	Spore Differentiation Factor
SDS	sodium dodecyl sulphate
SRF	Serum Response Factor
v/v	volume by volume
vol.	volume
X-gal	5-bromo-4-chloro-3-indoyl-D-galactopyranoside

1. INTRODUCTION

1.1 GPCRs (G-protein coupled receptors)

Organisms must readily respond to essential signals in their environment. Multicellular organisms greatly depend on the capacity of cells to communicate with each other and their environment. It has been recognised that membrane bound receptors devised to recognise sensory messages from the environment (light, phermones, gustative molecules) and intercellular messages such as neurotransmitters, hormones, growth and developmental factors are very similar and derive from common ancestral origins (Bockaert, 2001). They can be classified on the basis of their structure and function into different categories: 1) Channel receptors, 2) Tyrosine kinase receptors, 3) Guanylate cyclase receptor, 4) Serine/threonine kinase receptors, 5) Cytokine receptors, and 6) GPCRs.

The seven-transmembrane spanning G protein-coupled receptors (GPCRs) represent a major group of cell-surface detectors and constitute 3.5 % of the genome in vertebrates. Of the 1000 genes thought to encode GPCRs in humans, about 300-400 mediate effects by endogeneous ligands, with the remainder being sensory receptors. They are characterized structurally by an amino-terminal extracellular domain, a carboxy-terminal intracellular domain and seven hydrophobic transmembrane regions (TMDs). Generally, agonist binding at the GPCR pocket leads to a conformational change in the 7TMDs which in turn allows its association with G-proteins. The activated G-proteins initiate signalling cascade(s) within the cell eliciting a physiological response (Prinster et al., 2005). GPCRs are involved in recognition and transduction of a wide variety of signals as diverse as light, Ca^{2+}, odorants, nucleotides, amino acid residues, peptides as well as proteins (Figure 1).

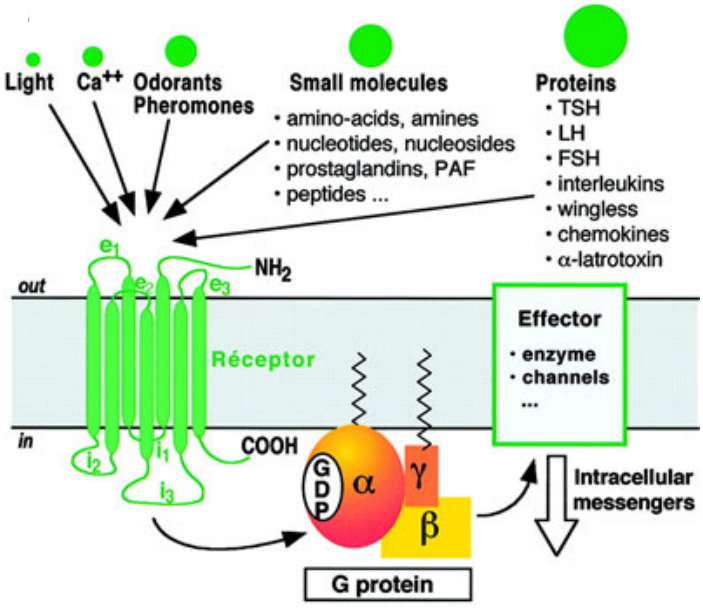


Figure 1. GPCRs as an illustration of Jacob's idea 'evolution is molecular tinkering'. (Bockaert and Pin, 1999) GPCRs have a central common core made of seven transmembrane helices (TM-I to -

VII) connected by three intracellular (i1, i2, i3) and three extracellular (e1, e2, e3) loops. The diversity of messages which activate these receptors is an illustration of their evolutionary success.

Different GPCRs differ in their length, ligands bound or sensed at their N termini, function of their intracellular and extracellular loops, C-terminal interactions and the respective G-proteins coupled. In short, a high degree of molecular tinkering renders them the capability to carry out multitude of functions (Bockaert and Pin, 1999). GPCRs are the oldest known signal transducers present in plants, animals, fungi and protozoa. They carry out signalling largely via intracellular second messenger systems such as 3', 5' cyclic AMP (cAMP); 3', 5' cyclic GMP (cGMP); 1, 2-diacyl glycerol (DAG); and inositol 1, 4, 5-triphosphate (IP_3) and also use Ca^{2+} and various inositol phospholipids embedded underneath in the membranes.

G-proteins are heterotrimeric molecules composed of α, β and γ subunits. In their active form the α subunit is GTP bound and modulates the activity of downstream effectors in most of the species, whereas in their inactive GDP bound form they are in a complex with one Gβ and one Gγ subunit. There are 20 known G-proteins in mammals each comprising an unique Gα combined with one of the 5 known Gβ and one of the 12 Gγ subunits providing an extraordinary ability to perform a wide range of signalling. These heterotrimeric proteins are also present in many other experimental models including *Drosophila melanogaster*, *D. discoideum*, *Xenopus laevis*, *C. elegans* and mouse. The role of G-proteins in development was first reported in *D. discoideum* (Pupillo et al., 1988). Presently there are 14 Gα, two Gβ and one Gγ known in this organism. However, other than coupling to G-proteins downstream, many GPCRs have also been reported to bind to many other molecules modulating the activity of the receptors, for instance arrestins or PDZ domain containing proteins to name a few (Malbon, 2005).

1.2 GABA and its receptor systems

GABA (gamma-amino-butyric acid) is the most widely distributed inhibitory amino acid derived neurotransmitter in the vertebrate central nervous system. There are two distinct types of target receptors for GABA present on peripheral nerve terminals each of which mediates synaptic inhibition: the ionotropic GABA types A and C ($GABA_{A/C}$); and the metabotropic GABA type B ($GABA_B$) receptors. Upon activation of two molecules of GABA, the pentameric $GABA_A$ receptor (ion channel) induces a rapid inhibition allowing inward influx of Cl^- and a subsequent hyperpolarization of the postsynaptic membrane, whereas activation of $GABA_B$ produces a slow, prolonged inhibition and hence is

metabotropic (Blein et al., 2000; Pagano et al., 2001). $GABA_B$ receptors, which were first pharmacologically distinguished by Hill and Bowery (1981) act presynaptically and postsynaptically based on their anatomical location and physiological functions. These receptors couple through heterotrimeric G proteins (G_I and G_o) modulating adenylyl cyclase activity and cause inwardly rectifying K^+ channels to open and voltage-dependent Ca^{2+} channels to close (Bettler et al., 1998; Blein et al., 2000) as illustrated in Figure 2. Physiologically, $GABA_B$ receptors have been implicated in synaptic inhibition, hippocampal long-term potentiation, short-wave sleep, muscle relaxation, and antinociception, which make them attractive therapeutic targets (Kaupmann et al., 1998; Bowery and Enna, 2000). The dysfunction in the GABA system leads to several neuropsychiatric disorders including anxiety, depression, schizophrenia, loss of memory, loss of sleep, epilepsy (Cyran et al., 2005). These receptors have also been implicated in several inherited neurological disorders.

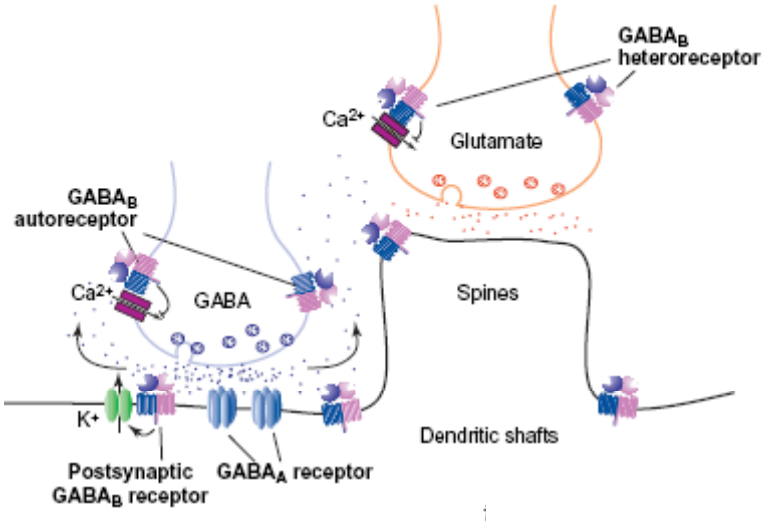


Figure 2. Schematic representation of the $GABA_B$ receptor heterodimer and its localization in the brain (Cyran et al., 2005). In the hippocampus, $GABA_B$ receptors are located presynaptically, postsynaptically and on extrasynaptic membranes. Presynaptic $GABA_B$ autoreceptors on GABA releasing terminals inhibit the release of GABA, whereas $GABA_B$ heteroreceptors inhibit the release of several other neurotransmitters (e.g. glutamate) and bioactive peptides. Postsynaptic $GABA_B$ receptors activate K^+ channels and induce slow inhibitory postsynaptic potentials, the fast component of which is mediated through $GABA_A$ receptors.

$GABA_B$ receptors belong to the family 3 of GPCRs (G-protein coupled receptors) and as such share homology with metabotropic glutamate (mGlu), Ca^{2+}-sensing (CaS), pheromone, taste and vomeronasal receptors. These proteins share 35% sequence identity. In contrast to the mGlu and CaS receptors, which form functional homodimers, the $GABA_B$ receptor is a heteromer constituted of both $GABA_BR1$ and $GABA_BR2$ proteins (Kaupmann et al., 1998).

1.2.1 $GABA_B$ dimerization

Although the concept of GPCR heterodimerization was proposed in the early 1980s, the first widely accepted evidence came from $GABA_B$ receptors. The first $GABA_B$ receptor cDNA was cloned (Kaupmann et al., 1997) and termed as $GABA_BR1$. However it was largely non-functional in most cell types when expressed alone, due to its inability to get trafficked to the cell surface (Couve et al., 1998). A year later six independent groups cloned another receptor, termed as $GABA_BR2$ (Jones et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999; Martin et al., 1999), which did not bind to GABA when expressed alone. Indeed, the coexpression of both subunits is required for a fully functional $GABA_B$ receptor (Jones et al., 1998; Kaupmann et al., 1998). Both receptors consist of an extracellular domain (ECD) and a TMD composed of seven transmembrane α-helices, loops and an intracellular C-terminus. The $GABA_BR1$ subunit binds all known $GABA_B$ ligands and is responsible for the ligand recognition by the heteromeric $GABA_B$ receptor, whereas the $GABA_BR2$ subunit is necessary for the correct trafficking of $GABA_BR1$ to the cell surface (Couve et al., 1998; White et al., 1998) by masking a signal responsible for the retention of $GABA_BR1$ in the endoplasmic reticulum (ER) (Pagano et al., 2001). While the $GABA_BR1$ ECD plays a critical role in ligand binding, however, $GABA_BR2$ also contributes to agonist binding affinity i.e. in short, both the proteins are essential for efficient ligand binding. The ECD of these proteins shares the same protein fold as bacterial periplasmic binding proteins. Ligand binding into this Venus flytrap like module causes activation of the TMD, which in turn carries out the downstream signalling. It has been shown that the $GABA_BR2$ TMD contains enough molecular determinants for coupling to G-proteins, because G-protein activation can be detected with a $GABA_B$ receptor combination containing $GABA_BR2$ TMDs only (Blein et al., 2000; Pagano et al., 2001; Grünewald et al., 2002). However, the exact role of each subunit in G-protein recognition and activation by the heterodimeric $GABA_B$ receptor is still unknown. The carboxyl-termini of the two receptor subunits were shown to physically interact with each other in yeast two-hybrid and fusion protein pull down assays (White et al., 1998; Kuner et al., 1999) but it was not essential for dimerization (Margeta-Mitrovic et al., 1999; Calver et al., 2001; Pagano et al., 2001). Infact, the N-termini and the transmembrane domain of both the receptor subunits are shown to possess enough determinants required for dimerization (Liu et al., 2004). Nevertheless, the involvement of C-termini coupling for targeting of the receptors and their function in holding the dimers strongly together through coiled coil interactions cannot be ruled out.

1.2.2 $GABA_B$ structure (schematic representation) and signalling

Upon ligand binding, the amino-terminal domain undergoes a hinge-bending motion, as in LIVBP (Leucine Isoleucine Valine Binding Pocket), which is described as a "venus fly trap" mechanism. Receptor activation produces adenylyl cyclase inhibition through the involvement of the α subunit of G_αi/o type G-proteins (Blein et al., 2000). Postsynaptic receptors cause inwardly rectifying K^+ channels to open allowing K^+ to move down its electrochemical gradient. This effect on K^+ channels is mediated by G protein β/γ subunits. It has been proposed that activation of presynaptic receptors results in the closing of voltage-dependent Ca^{2+} channels (Figure 3).

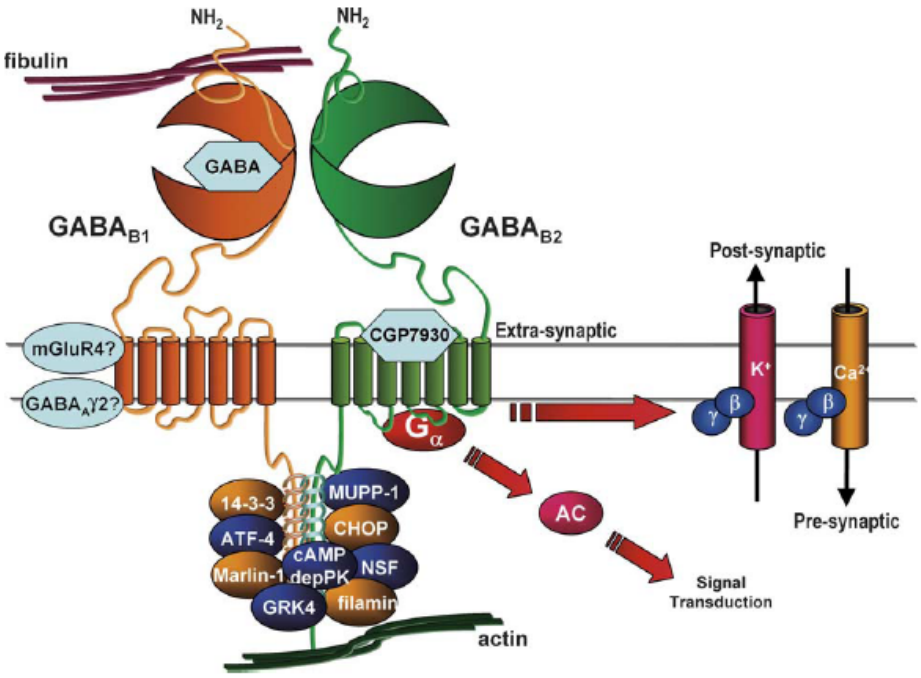


Figure 3. Schematic representation of the components of the putative $GABA_B$ receptor ''signalosome.'' (Couve et al., 2004). The figure illustrates several known interactions and signaling carried out by the functional $GABA_B$ receptors but their positions shown in the figure are only schematic.

The functional $GABA_BR1$/$GABA_BR2$ subunits were also reported to associate with a number of proteins both directly and indirectly, giving rise to a signaling complex capable of producing a diverse array of physiological and pharmacological effects. $GABA_B$ agonists such as GABA and baclofen binding to the "Venus Flytrap" structure of $GABA_BR1$ were known for a long time but interestingly, an allosteric modulator CGP7930 discovered recently appears to bind to the heptahelical domain of $GABA_BR2$. Non-canonical $GABA_B$ mediated

signaling involves some proteins that directly interact with the coiled-coil domain of either one or both the receptors, for instance, ATFx and CREB2 (cAMP responsive element binding protein-2, ATF4), which are both members of the ATF/CREB family of transcription factors. Other proteins, such as GRK4 and cAMP-dependent protein kinase, are thought to be associated indirectly to the signaling complex as illustrated in Figure 3. (White et al., 2000; Couve et al., 2004).

1.3 *Dictyostelium* as a model organism

Dictyostelium discoideum is a soil-living amoeba and feeds on bacteria in decaying vegetation. It reproduces by binary fission, as do all amoebae. They enjoy their unicellular fate as long as the food is plentiful and enter a multicellular phase on environmental pressure such as starvation. Growing amoebae chemotax toward folic acid and other nutrients, whereas starved cells undergo a developmental process that transforms them into cAMP sensing machines (Manahan et al., 2004). When synchronously starved, cells begin to spontaneously emit pulses of cyclic AMP (cAMP) - a compound used by these cells to facilitate cell-cell communication - to which other *D. discoideum* cells are attracted. As cells move towards the source of cAMP, they emit their own pulse of cAMP, thus amplifying and propagating the signal, and enabling large numbers of cells to congregate from a wide area. The cells begin to associate, forming streams of migrating cells, which merge in an aggregate consisting of up to 100,000 cells. The streams eventually come together to form a mound which develops a tip coordinating further development resulting in the formation of a slug that migrates to a favourable environment for culmination into the fruiting body.

D. discoideum, is an excellent organism for the study of the molecular mechanisms of cell motility, signal transduction, cell-type differentiation and developmental processes. *Dictyostelium* has a 34 Mb genome spread over six chromosomes encoding approximately 12,500 proteins (Eichinger et al., 2005). A significant number of genes show higher similarities to the genes of vertebrates than to those of other fully sequenced eukaryotes. This results further strengthened the view that the evolutionary position of *D. discoideum* is located prior to the branching of metazoa and fungi but after the divergence of the plant kingdom, placing it close to the base of metazoan evolution. (Glöckner et al., 2002). The most studied receptors in this amoeba are the four G-protein coupled cAMP receptors, which are induced during starvation. 48 additional GPCRs have been discovered during genome analysis.

1.3.1 *Dictyostelium* developmental checkpoints

Developmental transitions occur at two points; one is from growth to development mediated by cAMP signalling wherein many aggregation specific proteins are upregulated within a few hours and the cells gradually develop polarized cell morphology. Oscillatory pulses of cAMP with a period of 6 min propagate as waves through cell monolayers binding to the high affinity cAMP receptors (cAR1) which through their βγ subunits activate the downstream components PI3 kinase to form PIP3 and cortical myosin in turn leading to the formation of polarized cells and directional movement towards the chemoattractant (chemotaxis). cAMP also activates CRAC and adenylyl cyclase through Gα2, which in turn synthesizes more cAMP and elicits an autocrine response (Manahan et al., 2004; Strmecki et al., 2005). Aggregation results in the formation of a multicellular organism, known as a mound. A second transition occurs at the mound stage during which the precursors of the mature spore and stalk cells - prestalk and prespore cells differentiate and sort, forming a tipped aggregate or tipped mound. As development proceeds, the tip extends and an anterior-posterior axis

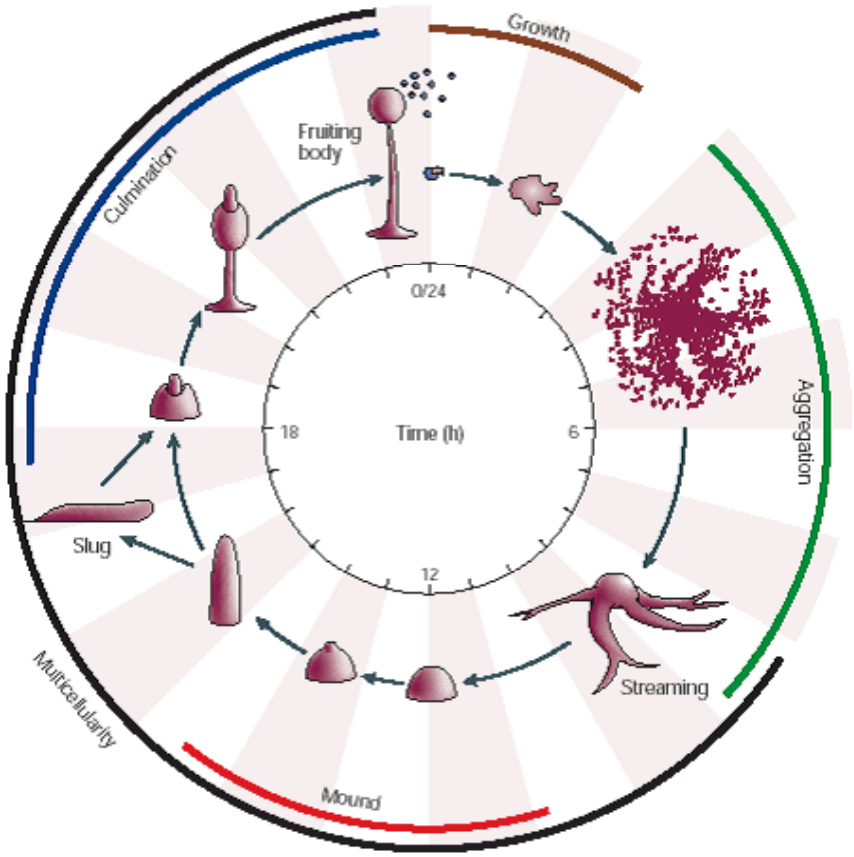


Figure 4. *Dictyostelium discoideum* morphogenesis (Chisholm and Firtel, 2004).

forms, which is maintained through the slug and early culminant stages. Culmination and the formation of the fruiting body completes morphogenesis. During this stage, the precursor

populations differentiate, producing a sorus on top of a highly vacuolated mass of stalk cells. The entire process from starvation of vegetative cells to the formation of a mature fruiting body takes 24 hours as depicted in the schematic representation of the life cycle of *D. discoideum* (Figure 4)(Chisholm and Firtel, 2004).

1.3.2 Late developmental events

A mound of developing *Dictyostelium* cells establishes asymmetry and generates the correct proportions of different cell types. Cells expressing the earliest available markers specific for prespore or prestalk cell fate appear at random locations in the mound and these cells then sort by a process which is likely to involve both differential adhesion and different rates of chemotaxis such that by the slug stage the distinct populations are found in separate locations (Williams et al., 1989; Esch and Firtel, 1991; Fosnaugh and Loomis, 1993; Ozaki et al., 1993; Yoder and Blumberg, 1994). Once the aggregates have formed, owing to high extracellular cAMP concentration there is a **developmental switch** leading to adaptation or downregulation of aggregation specific genes and activation of post aggregation genes. The high and saturating concentration of cAMP results in the induction of the transcription factor GBF which plays as a central role in controlling events pertaining to induction of post aggregation genes and cell-type specific gene expression. This immediately leads to tyrosine phosphorylation of STATa (signal transducer and activator of transcription) that in turn binds to promoter elements so as to activate the *ecmA* gene and repress *ecmB* (extracellular matrix proteins A and B respectively). The prespore specific genes are induced by cAMP and repressed by DIF-1. cAMP and DIF together are two essential morphogens required to establish a proper prestalk and prespore population to form the multicellular organism. **cAMP receptors** are involved in a complex signalling cascade in the post aggregation stages when all the four cARs are active, functional and exhibit distinct spatial expression pattern and different affinities for cAMP which helps in sorting, patterning and morphogenesis into a highly proportional organism (Johnson et al., 1993; Saxe et al., 1993, 1996; Louis et al., 1994). The extracellular cAMP has opposing effects depending on the expression levels and activation of alternate cAR receptor expression in different cell types. The cAMP mediated effect on the prespore genes and pstB (prestalk B) is dependent on a serine/threonine kinase GSK3 controlled by high affinity car3 receptor (glycogen synthase kinase), which helps to keep the *ecmB* repressed until the right time (Schilde et al., 2004). At mid culmination, the low affinity car4 inhibits and turns on the *ecmB* expression. The intracellular cAMP also regulates events to control the transition from slug to mature fruiting body and terminal differentiation via the intracellular phosphodiesterase RegA and the adenylyl cyclase ACR

which is required to control late developmental events. PKA (protein kinase A) is essentially involved in all the events governing the cell-type specification and morphogenesis (Loomis, 1998).

1.3.3 Terminal differentiation

The final morphogenesis to form the mature fruiting body requires spatial reorganization of cell populations within the slug. Slug migration is arrested by different environmental factors including low humidity, reduction of local ammonium ion concentrations followed by movement of the posterior of the slug under the tip, forming a Mexican hat like structure wherein the rearguard cells enriched with ecmB form the base and the prestalk cells present at the anterior of the slug form the tip. Fruiting body formation is initiated when apically localized prestalk cells invaginate into the prespore mass, differentiate into stalk cells and form the stalk tube (Figure 5). The first cells that enter are the pstAB cells followed by the pstA cells, which then induce *ecmB* and are committed to stalk cell differentiation. A stalk tube progressivley elongates through the prespore mass, raising it off the substratum (Jermyn et al.,1996). Simultaneously, the ALCs (anterior like cells) migrate to form part of the basal disc and upper and lower cups. As the stalk tube is formed, prespore cells enter the terminal differentiation pathway and generates spores. Terminal differentiation is greatly controlled by PKA (Aubry and Firtel, 1999).

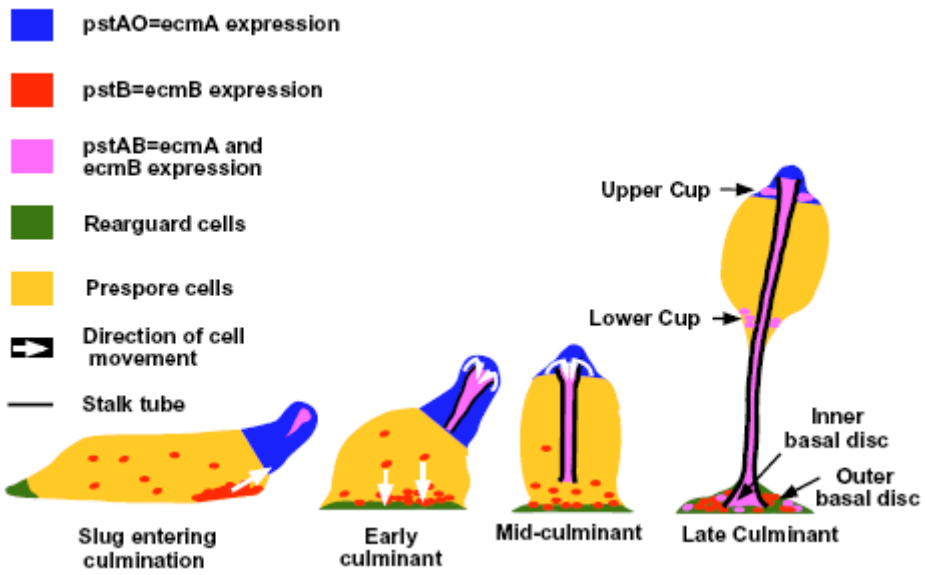


Figure 5. Diagramatic representation of the movements of ecmA and ecmB-expressing cells during culmination. (Jermyn et al., 1996)

1.3.4 Sporulation

Stalk cell differentiation and sporulation are highly interconnected. During culmination, PKA activity is highly dependent on intracellular cAMP levels (that controls the dissociation of the catalytic subunit PKA-C from the regulatory PKA-R subunit), which is produced by the late adenlyl cyclase ACR and degraded by the phosphodiesterase RegA. The two component system histidine kinase receptor DhkC and the phosphodonor RdeA activates RegA but is inhibited by another hybrid histidine kinase receptor, DhkA, which inhibits this phosphorelay via RdeA and thereby activates PKA and induces sporulation (and later by DhkB to avoid premature germination) (Loomis et al., 1998). This inhibition is mediated by a peptide, SDF-2 (spore differentiation factor) which is released by the proteolytic cleavage/processing of AcbA (Acyl binding protein) which synthesized in the prespore cells.This proteolytic cleavage is mediated by the dual function transporter/protease TagC present on the surface of the prestalk cells to release SDF-2 which then acts on prespore cells to trigger encapsulation and acts as a feed back loop in the prestalk cells to control its production (Anjard et al., 1998, Anjard and Loomis, 2005) represented in Figure 6.

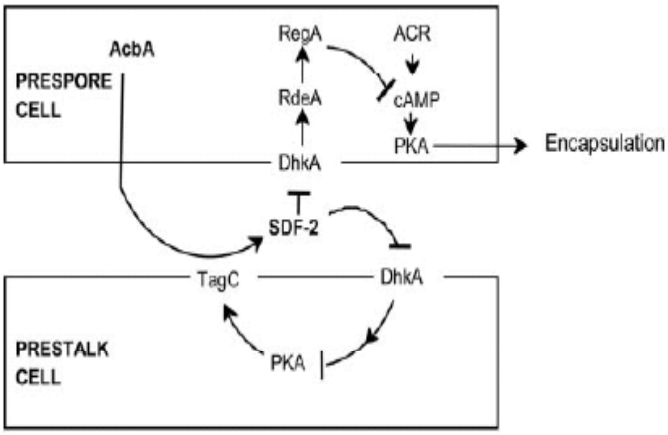


Figure 6. Proposed signaling pathway leading to sporulation (Anjard and Loomis, 2005)

The MADS-Box transcription factor SrfA (**s**erum **r**esponse **f**actor) has been largely implicated in the events regulating the spore maturation. It is known to act downstream of PKA and induces the transcription of a distinct set of genes all of which are known to function in the process of spore coat formation and its maturation (Escalante et al., 2001, 2003; Escalante and Sastre, 2002).

1.4 $GABA_B$ receptor-like proteins in *D. discoideum*

The genome sequence of chromosome 2 (Glöckner et al., 2002) gave a clue for the presence of four genes related to the metabotropic glutamate/$GABA_B$ subfamily. The complete sequence (Eichinger et al., 2005) revealed the existence of 17 genes encoding receptors belonging to Family 3 of GPCRs and all of them resembled $GABA_B$ or metabotropic glutamate like- receptors. They were recently named by Dale Hereld (*Dictyostelium* Genomics, 2005) and officially accepted to be represented as Grl (A-R) (**G**ABA_B or metabotropic **g**lutamate - **r**eceptors **l**ike) proteins. The presence of these receptors was indeed a very interesting observation as only the cAMP receptor gene family known to be present in *D. discoideum*. The presence of the animal specific (as it was thought so far) frizzled like-receptors (25 genes), the secretin family (2 genes) and 17 members representing the Family 3 of GPCRs was rather surprising (Figure 7) (Insall 2005; Williams et al., 2005).

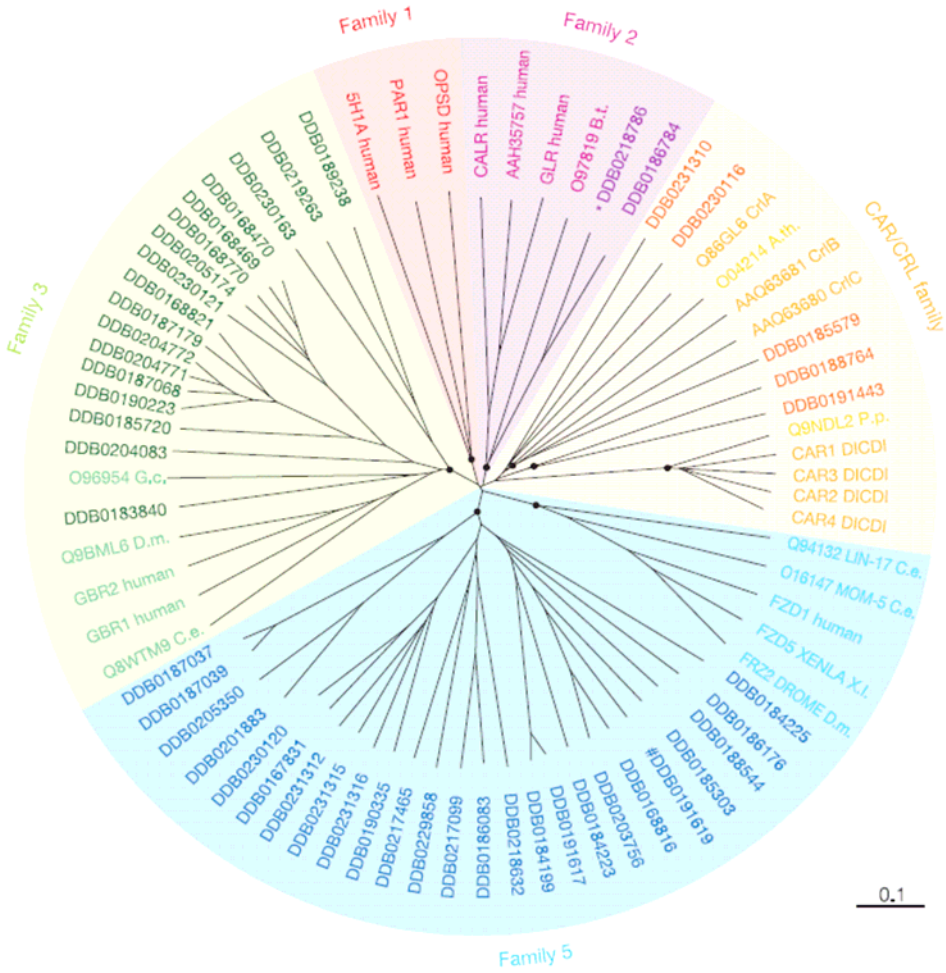


Figure 7. Phylogenetic tree of the G-protein-coupled receptor Family in *Dictyostelium discoideum*. (Eichinger et al., 2005). A CLUSTALX alignment of the sequences encompassing the seven transmembrane domains of all *Dictyostelium* GPCRs, and selected GPCRs from other organisms, was used to create an unrooted dendrogram with the TreeView program. A.th., *A. thaliana*;

B.t., *Bos taurus*; CAR/CRL, cAMP receptor/cAMP receptorlike; C.e., *C. elegans*; DICDI, *D. discoideum*; D.m., *D. melanogaster*; G.c., *Geodia cydonium*; P.p., *Polysphondylium pallidum*; X.l., *Xenopus laevis*.

We carried out a series of blast searches with Family 3 proteins and most of them displayed a close resemblance to $GABA_B$. The identity was around 25 percent to the mammalian counterparts but the Grls always clustered well amongst each other. The transmembrane domains of most of the *Dictyostelium* $GABA_B$ receptor-like proteins possess a identity and there is a high degree of conservation observed among $GABA_B$ homologs from different species with regard to their TMDs. Several amino acids in the intracellular loops 2 were conserved whereas the intracellular loop 3 of *Dictyostelium* Grls were found very similar to mammalian $GABA_B$ receptors. A further search revealed the resemblance of GrlJ, GrlF and GrlE to the receptor 1 subtypes whereas all others were slightly more closer to the sub-type 2. This observation is in contrast to what is found in mammalian $GABA_B$ receptor sub-types as they have many of $GABA_BR1$ and fewer of $GABA_BR2$ subtypes which are a result of alternative splicing. However the differences between these subtypes are only very slight and their grouping into subtypes is rather weak. Also, a few of these receptors possessed a coiled-coil domains at their C-termini but the N-terminal ligand binding domain was comparatively shorter in most of the receptors belonging to this family. Few of them also possessed a Bmp (Bacterial/ Basic membrane protein) domain at their N - termini which resembled the periplasmic binding proteins found in bacteria.

1.5 Aim of the work

$GABA_B$ receptors are widely studied amongst higher eukaryotes and mammals including rat, mouse, *Drosophila* and humans. The analysis of the *Dictyostelium* genome revealed the presence of 17 different genes encoding members of the metabotropic glutamate and/or $GABA_B$ receptor family which was surprising as this family of receptors was so far considered animal-specific. This opens up a further complexity of signaling in these amoeabe in addition to the role of the well investigated cAMP receptors. It was therefore interesting to investigate the role played by these receptors *D.discoideum*. Hence, we attempt to address the following questions:

- the function carried out by these receptors in *Dictyostelium*,
- their expression pattern and localization,
- their probable role in development, signaling pathways used by these receptors, and
- finally the dimerizing pair(s) amongst the receptor pool if any.

We have selected GrlA and GrlJ, two of the first four $GABA_B$ homologs identified in *Dictyostelium*, for the present study.

2. MATERIALS AND METHODS

Details regarding following things are not included in this section: In short,

- Standard molecular biology techniques were carried out as per (Sambrook et al., 1989). A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY, Vols. 1-3 (1989).
- Fine chemicals were obtained from Sigma as otherwise indicated.
- Standard laboratory reagents and materials were obtained from local suppliers.
- Instruments used were from the Departmental facility.

2.1 Kits

Nucleobond AX 100 and 500	Macherey Nagel
NucleoSpin Extract 2 in 1	Macherey-Nagel
NucleoSpin Plus	Macherey-Nagel
Original TA Cloning	Invitrogen
Qiagen Midi- and Maxi-prep	Qiagen
Stratagene Prime It II	Stratagene
PGEMTeasy Cloning kit	Promega
Qiagen RNAeasy Mini kit	Qiagen
High pure PCR Template preparation kit	Roche
RT-PCR	Roche
RT-PCR for Microarray	Stratagene

2.2 Enzymes, antibodies, substrates, radioactive probes and antibiotics

Enzymes for molecular biology:

Calf Intestinal Alkaline Phosphatase (CIP)	Boehringer
Klenow fragment	Boehringer
Lysozyme	Sigma
Proteinase K	Sigma
Restriction endonucleases	Amersham,Life technologies, New England Biolabs
M-MLV reverse transcriptase	Promega
Ribonuclease H (RNase H)	Boehringer
Ribonuclease A (RNase A)	Sigma
T_4 DNA ligase	Boehringer
Taq-polymerase	Life technologies/Boehringer

Antibodies:

Primary antibodies:

Polyclonal Goat anti-GST antibody	Pharmacia
Mouse monoclonal anti-GFP mAb K3-184-2	Noegel et al., 2004
Mouse monoclonal anti-actin mAb Act-1	Simpson et al., 1984
Mouse monoclonal anti-CAP mAb 231-18-8	Gottwald et al., 1996
Mouse monoclonal anti-csA mAb 33-294	Berthold et al., 1985
Mouse monoclonal anti-vacuolin mAb 221-1-1	Rauchenberger et al., 1997

Mouse monoclonal anti-comitin mAb 190-340-2	Weiner et al., 1993
Mouse monoclonal anti-interaptin mAb 260-60-10	Rivero et al., 1998
Mouse monoclonal anti-PDI mAb 221-135-1	Monnat et al., 1997
Mouse monoclonal anti-lysosmal enzyme mAb 221-342-5	Neuhaus et al, 1998
Mouse monoclonal anti-annexin mAb 185-447-3	Döring et al, 1995
LagC polyclonal antibodies	Wang et al., 2000
Dd-CAD1 polyclonal antibodies	Wong et al., 1996

Secondary antibodies:

Goat anti-mouse IgG, peroxidase conjugated	Sigma
Goat anti-rabbit IgG, peroxidase conjugated	Sigma
Sheep anti-mouse IgG, Cy3 conjugated	Sigma
Goat anti-rabbit Alexa 568 conjugated	Molecular probes

Antibiotics:

Ampicillin	Gruenenthal
Blasticidin S	ICN Biomedicals
Dihydrostreptomycinsulphate	Sigma
Kanamycin	Sigma, Biochrom
Tetracyclin	Sigma

Radiolabelled nucleotide:

α-^{32}P-deoxyadenosine triphosphate, (10 mCi/ml)	Amersham

2.3 Media and Buffers

All media and buffers were prepared with deionised water, filtered through an ion-exchange unit (Membra Pure). The media and buffers were sterilized by autoclaving at 120ºC and antibiotics were added to the media after cooling to approx. 50ºC. For making agar plates, a semi-automatic plate-pouring machine (Technomat) was used.

2.3.1 Media and buffers for *Dictyostelium* culture

Ax2-medium, pH 6.7 (Claviez et al., 1982):
7.15 g yeast extract, 14.3 g peptone (proteose), 18.0 g maltose, 0.486 g KH_2PO_4, 0.616 g $Na_2HPO_4.2H_2O$ add H_2O to make 1 liter
Phosphate agar plates, pH 6.0:
9 g agar ,add Soerensen phosphate buffer, pH 6.0 to make 1 liter
Salt solution (Bonner et al., 1947):
10 mM NaCl,10 mM KCl, 2.7 mM $CaCl_2$
Starvation buffer, pH 6.5 (Shaulsky et al., 1998):
10 mM MES, pH 6.5, 10 mM NaCl,10 mM KCl,1 mM $CaCl_2$,1 mM $MgSO_4$
SM agar plates, pH 6.5 (Sussman, 1951):
9 g agar, 10 g peptone, 10 g glucose, 1 g yeast extract, 1 g $MgSO_4.7H_2O$, 2.2 g KH_2PO_4
1 g K_2HPO_4, add H_2O to make 1 liter

Soerensen phosphate buffer, pH 6.0 (Malchow et al., 1972):
2 mM Na_2HPO_4, 14.6 mM KH_2PO_4

2.3.2 Media for *E. coli* culture

LB medium, pH 7.4 (Sambrook et al., 1989)
SOC medium, pH 7.0 (Sambrook et al., 1989)

2.3.3 Media and buffers for Yeast two-Hybrid studies

YEPD-Medium:
20 g/l Difco Peptone, 10 g/l Yeast extract
YEPD-Agar plates:
20 g/l Difco Peptone ,10 g/l Yeast extract ,18 g/l Agar agar
100 x Adenine solution:
200 mg (1,1 mmol) Adenine in 100 ml water dissolve with addition of little amounts of HCl and filter sterilized.
100 x Histidine solution:
200 mg (1 mmol) Histidine in 100 ml Water and filter sterilized.
100 x Tryptophan solution:
200 mg (1 mmol) Tryptophan in 100 ml Water filter sterilized.
100 x Tyrosine solution:
300 mg (1,7 mmol) Tyrosine in 100 ml dissolve with addition of NaOH solution and filter sterilized.
100 x Leucine solution:
1000 mg (7, 6 mmol) Leucin in 100 ml Water and filter sterilized.
100 x Uracil solution:
200 mg (1, 8 mmol) Uracil in 100 ml Water dissolve by warming in water bath and filter sterilized.
1M 3-Amino-1,2,4-triazol (3AT) solution:
8.4g 3AT in 100 ml water andn filter sterilize

10 x Amino acid solutions:
300 mg (2, 3 mmol) Isoleucine
1500 mg (1, 1 mmol) Valine
200 mg (0, 9 mmol) Arginine
300 mg (1, 6 mmol) Lysine
200 mg (1, 34 mmol) Methionine
500 mg (3 mmol) Phenylalanine
2000 mg (16, 8 mmol) Threonine,
adjust final volume to 1l and filter sterilize.

The composition of the selection media and agar plates is indicated in the following table. Agar agar and Yeast extract without nitrogen base was dissolved in water and autoclaved. The glucose solution was prepared in water and filter sterilised. The remaining stock solutions were added after cooling to 55 °C.

Reagents	SD/-Trp	SD/-Leu	SD/-Leu/-Trp	SD/-Leu/-His/-Trp/+3-AT
Yeast Nitrogen Base [g]	6,7	6,7	6,7	6,7
Agar agar [g]	20	20	20	20
Water [ml]	750	750	770	745
20% Glucose [ml]	100	100	100	100
10X Amino acid solution [ml]	100	100	100	100
100X Tyrosine [ml]	10	10	10	10
100X Uracile [ml]	10	10	10	10
100X Histidine [ml]	10	10	10	-
100X Leucine [ml]	10	-	-	-
100X Tryptophane [ml]	-	10	-	-
100X Adenine [ml]	10	10	10	10
3-AT-solution [ml]	-	-	-	25

2.3.4 Buffers and other solutions

Buffers and solutions not listed below are described in the methods section.

10x MOPS (pH 7.0/ pH 8.0):
41.9 g MOPS, 7 ml 3 M sodium acetate , 20 ml 0.5 M EDTA, add H_2O to make 1 liter
10x NCP-Puffer (pH 8.0):
12.1 g Tris/HCl, pH 8.0, 87.0 g NaCl, 5.0 ml Tween 20, 2.0 g sodium azide add H_2O to make 1 liter
1x PBS (pH 7.4):
8.0 g NaCl, 0.2 g KH_2PO_4,1.15 g Na_2HPO_4, 0.2 g KCl dissolve in 900 ml deionised H_2 adjust to pH 7.4 add H_2O to make 1 liter, autoclave
1.2 M Phosphate buffer (pH 6.8):
1.2 M Na_2HPO_4, pH 9.1 was mixed with 1.2 M NaH_2PO_4, pH 4.02 in the ratio of 2:1.
20x SSC (pH 7.0):
3 M NaCl, 0.3 M sodium citrate
TE buffer (pH 8.0):
10 mM Tris/HCl, pH 8.0, 1 mM EDTA
Tris-phenol:
1 kg phenol was melted at 60ºC in a water-bath and equilibrated with 1 vol. of 1 M Tris/HCl, pH 8.0. The equilibrated phenol was aliquoted in 50 ml Falcon tubes and stored at –20ºC.
10x TAE buffer (pH 8.3):
27.22 g Tris, 13.6 g sodium acetate , 3.72 g EDTA add H_2O to make 1 liter

2.3.5 Biological materials

Bacterial strains:

E. coli BL21 (DE)	Studier and Moffat,1986
E. coli DH5α	Hanahan, 1983
E. coli XL1 blue	Bullock et al., 1987
Klebsiella aeorgenes	Williams and Newell, 1976

Dictyostelium discoideum strain:
Ax2-214, an axenically growing derivative of wild strain, NC-4 (Raper et al., 1935) commonly referred to as Ax2.
Yeast strain:
Y190 Flick et al., 1990, Harper et al., 1993

2.3.6 Plasmids

pGEM-Teasy	Promega
pGEX-4T1	Pharmacia Biotech
pGADT7	Clontech
pGBKT7	Clontech
p1ABsr8	Gräf et. al., 2000
pDNeo II	Witke et al., 1987
pBSBsr	unpublished
EcmB-Gal	Jermyn and Williams, 1991
pspA(D19)-Gal	Dingermann et al., 1989

2.3.7 Oligonucleotides

GrlA-CTFp 5'GGAATTCAATCAAACATTTGCAACAAATACAA 3'
GrlA-CTRp 5'CGTCGACTTATACATCATCTGGATCGATTTCAGATTG 3'
GrlJ-CTFp 5' CGAATTCGAAACTTTTGCAACTAGTAC 3'
GrlJ-CTRp 5'CGGATCCTTAAACATTATTGGAATCAATTTCTGT 3'

Primers for screening knock out

GrlA-5' probeFp 5' CCTAAGAATAAATGCATTGGAGGTTC 3'
GrlA-5' probe Rp 5' ACTATTGAACAATCCATTATTG3'
GrlA-3'ProbeFp 5' GAAGCAAGAGTAAAGGCAGAATCAATA 3'
GrlA-3'ProbeRp 5' GTTAAACCAGGAACCAGTGAATGTTGTC 3'
5'intron Fp 5' CTGTAATTGTAGAATTTATACCTG 3'
Bsr actin15promoter Rp 5'GATGGGATTAATTAATTTGTAATC 3'

Primers for RT-PCR

Car2 Fp 5' GTTGGTGTTGGATTGGTGATA 3'
Car2Rp 5' CGATTGCAAATACCCAACAAA 3'
Act8 Fp 5' TTAAATCCAAAGGCCAACAGAG 3'
Act8Rp 5' TACCTGGGAACATAGTTGTACC 3'
TagC Fp 5' GTTCACTATATACCACATG 3'
TagCRp 5' CCGAAAACTACCTTTGGTGAT 3'
DhkAFp 5' ATTGAAAATGGAGACATTACCC 3'
DhkARp 5' TAAACTTTGAACCTTGACCGAC 3'

Yeast two- hybrid primers:

GrlA-CT-Fp 5'GGAATTCAATCAAACATTTGCAACAAATACAA 3'
GrlA-CT-Rp 5' CGTCGACTTATACATCATCTGGATCGATTTCAGATTG 3'
GrlB-CT-Fp 5' GGAATTCTTAAATCAAACATTCGCTTCAAGTTC 3'
GrlB-CT-Rp 5' CGTCGACTTAAAGGTTATTAGAATCAATTTC 3'
GrlL-CT-Fp 5' CGAATTCTTCTGGAAAATTTATAAACCAGTT G 3'
GrlL-CT-Rp 5' CGTCGACTTAATTATCATTTTGTGCAGCAGC 3'

GrlH-CT-Fp 5' CGAATTCCCAAAATTCTGGAGAGTATTTAGA 3'
GrlH-CT-Rp 5' GGTCGACTTAATTATTATTTTCTGAATCATTGAC 3'
GrlJ-CT-Fp 5' CGAATTCGAAACTTTTGCAACTAGTAC 3'
GrlJ-CT-Rp 5' CGGATCCTTAAACATTATTGGAATCAATTTCTGT 3'
GrlF-CT-Fp 5' CGAATTCTTCTTTAAAATGATAACAGTTGGTTTAGAAC 3'
GrlF-CT-Rp 5'CGGATCCTTAAACATCATTCGAATCAATTTCAG 3'

2.4 Methods

2.4.1 Total RNA and cDNA preparation

2.4.1.1 Isolation of total RNA from *Dictyostelium* cells

Total RNA was extracted from either Ax2 or *grlA*$^-$ or *grlJ* from different developmental stages of the *Dictyostelium* life cycle or different assay conditions using the Qiagen RNeasy Mini kit. The manufacturer's protocol for the isolation of RNA from the cytoplasm of animal cells was used for preparation. The RNA samples were used directly for northern blot analysis or after reverse transcription for RT-PCR, Real-Time PCR and Microarray analysis.

2.4.1.2 Generation of cDNA

cDNA was generated using the M-MLV reverse transcriptase, RNAse H minus (Roche) according to the manufacturers protocol. Usually 1-5µg of the respective total RNA was used for each RT reaction.

2.4.2 DNA sequencing

Sequencing of the PCR-amplified product or plasmid DNA was performed at the sequencing facility of the Centre for Molecular Medicine, University of Cologne, Cologne by modified dideoxy nucleotide termination method using a 'Perkin Elmer ABI prism 377' DNA sequencer.

2.4.3 Quantitative PCR

Total RNA was extracted at different time points of *Dictyostelium* development and cDNA was prepared as described as under (section 2.4.1.2). Primers were selected such that the expected product size was between 250-500bp. Prior to use in real time experiments the quality of the cDNA and the primers were tested by PCR. Real Time PCR was carried out with the Quantited ^{TM}SYBR$^{®}$ green PCR kit (Qiagen) according to the manufacturer's protocol. For each sample gene specific primers (10 pmole) and 1 µl of cDNA was used. As a quantification standard defined concentrations (10ng, 1ng, 100pg, 10pg and 1pg) of GrlA-C-terminal and GrlJ-C-terminal gene sequences in pGEMTeasy were used. Actin specific primers were used as positive control and to ensure comparable concentrations of cDNA in samples of wild type and mutant cells.

2.4.5 Semi-quantitative PCR (RT-PCR)

cDNA generated as described in section 2.4.1.2 and specific primers were used to carry out PCR and is mentioned in the text as and when.

2.5 Construction of vectors

2.5.1 Generation of knockout mutants and screening of transformants

2.5.1.1 Gene replacement vector for GrlA

The Blasticidin resistance gene with the act15 promoter and act15 terminator sequences were retrieved using EcoRV and XbaI from the pBs-Bsr vector and inserted into the GrlA gene such that 1.3 kb of the GrlA was replaced by the 1.4 kb Bsr gene. GrlA sequences carrying the Blasticidin resistance cassette were purified and transformed into the wild type Ax2 cells by electroporation.

2.5.1.2 Gene disruption vector for GrlJ

The neomycin resistance cassette (2.2 kb) was obtained from pDNeoII vector by EcoRV digestion and cloned into the GrlJ gene that was present in pGEMTeasy vector. The targeting vector was transformed into the wild type Ax2 cells by electroporation.

2.5.1.3 Screening of transformants

A PCR based approach was used for screening the *grlA*$^-$ cells followed by Southern blotting whereas the *grlJ*$^-$ was screened using Southern blotting. The respective transformants obtained were selected with 3 μg/ml of Blasticidin or 4 μg/ml G418 for Neomycin resistance. Single colonies were obtained by spreader dilution of the whole pool of transformants onto SM agar plates overlaid with *Klebsiella areogenes.* The single transformants were then grown with the respective selection medium in a 96-well plate and eventually transferred to a 24-well plate and a 6-well plate. The cells were used to isolate genomic DNA using the kit (High pure PCR Template preparation kit) for PCR based screening whereas amoebae spread on *K. aerogenes* were used to isolate genomic DNA for Southern blotting. However the final confirmation of the recombination event for individual single colonies for both the mutants was done using Southern blot analysis.

2.5.2 Vectors for Yeast two-hybrid interactions

Heterodimerization:

C-termini immediately after the transmembrane domains (as obtained from SMART) of the following genes, **grlA, grlB**, **grlH**, **grlL** each were cloned in frame with the binding domain (BD) of Gal4 in pGBKT7 plasmid using EcoRI and SalI respectively. The C-termini of **grlJ** and **grlF** each were cloned in frame with the activating domain (AD) of Gal4 in pGADT7 with BamHI and EcoRI respectively. Each of the (AD) contructs were cotransformed in the yeast strain Y190 with each BD fusion constructs separately and grown on SD-Trp-Leu medium plates. Negative controls were carried out with cotransformation of BD fusion constructs with the AD alone and AD fusion constructs with the BD alone. Similarly, a positive control was also performed along with by cotransforming pCL1 and pGBKT7, which are known interactors The growth of transformed yeast cells streaked on SD-Trp-Leu-His (+3AT, 60mM) were monitored over a period of 4 to 5 days.

Homodimerization:

C-termini of the respective genes, **grlA, grlB**, **grlH**, **grlL** each were further cloned in fusion with the AD of Gal4 in pGADT7 with EcoRI and BamHI, respectively. **grlA, grlB**, **grlH**, **grlL** fused with BD and AD each were cotransformed in yeast strain Y190 and selected in the similar way (see above).

2.5.3 GrlA-GFP fusion vector

The full-length GrlA was amplified as a HindIII - BamHI fragment and cloned in p1ABsr8 vector and transformed into Ax2 cells. The transformants were selected using blasticidin. The resultant cells were fixed and observed for the localization of the protein and alternatively stained with the respective antibodies for colocalization studies.

2.6. Biochemical methods

2.6.1 Western blotting (Kyhse-Andersen, 1984)

Protein samples prepared at the respective time points of *Dictyostelium* development and separated by SDS-PAGE gels (8%, 10% or 15% respectively) were blotted on to nitrocellulose membranes. They were blocked with 5 % milk in 1x NCP and probed with different dilutions of the respective primary antibodies and POD-conjugated secondary antibodies. They were then detected by ECL (Enzyme chemiluminescence) reactions.

2.6.2.Yeast two-hybrid methods

2.6.2.1 Yeast transformation

Stock solutions

50% PEG 3350 prepared in water
100% DMSO : 0.1 M Tris-HCl (pH 7.5), 10 mM EDTA
10X LiAc: 1M LiAc, pH 7.5

50 ml of YPD or SD medium are inoculated overnight with several colonies of Y190 yeast strain and incubated overnight at 30°C for 16 to 18 hours with shaking at 250 rpm to stationary phase. An overnight culture (30 ml) is transferred 300 ml of YPD or SD and incubated at 30°C for 3 hours until the OD_{600} becomes 0.4 to 0.6. The cells are centrifuged and resuspended in TE buffer or water and centrifuged again. The pellet is resusupended again in 1X TE/1X LiAc (the resuspended cells are called competent cells). 0.1 µg of plasmid DNA and 0.1 mg of herring testes carrier DNA are added to a 1.5 ml tube and mixed. 0.1 ml of competent cells is added. 0.6 ml of sterile PEG/LiAc solution is added and vortexed and incubation is at 30°C overnight. 70 µl of DMSO is added and mixed gently and the cells heat shocked at 42°C for 30 minutes. The cells are centifuged for 10 seconds and the supernatant discarded. The pellet is resuspended in 100 µl of water and plated on SD-Trp-Leu plates and the plates incubated at 30°C for 3 to 4 days. The transformed cells were further streaked onto SD-Trp-Leu-His with 3-AT concentrations varying from 25 to 60 mM and the growth monitored over a period of one week.

2.6.2.2 X-gal colony-lift filter assay

Z buffer: Na_2HPO_4 7 H_2O: 16.1 g/l, NaH_2PO_4 H_2O: 5.50 g/l, KCl: 0.75 g/l and $MgSO_4$ $7H_2O$: 0.246 g/l (pH 7.0)
X-gal stock solution: 20 mg/ml in DMF, β-mercaptoethanol
Z buffer / X-gal solution: Add 0.27 ml X-gal stock solution and 1.67 ml β-mercaptoethanol to 100 ml of Z- buffer.

Yeast colonies grown on SD-Trp-Leu plates are carefully transferred onto a nitrocellulose membrane by placing it on the yeast plate. The membrane containing the yeast colonies is soaked in liquid nitrogen and then placed on a petri plate containing a filter paper, which is already soaked in Z buffer/X-gal solution. The plate is then kept at 30°C in the dark until there is a development of blue colour.

2.7 *Dictyostelium* cell culture methods

2.7.1 Measuring cell size (Rivero et al., 1996)

Ax2 and *grlA*$^-$ and *grlJ*$^-$ cells were washed twice with Soerensen phosphate buffer and resuspended at a density of 1x10^7 cells/ ml with the same buffer supplemented with 20 mM EDTA and shaken at 160 rpm at 21^{o}C until the cells were rounded. The spherical cells were allowed to settle on a cover slip for 15 min and were then photographed using an inverted microscope (1X70, Olympus) equipped with a 40X objective and a charge coupled device (CCD) camera (CVM10, Progressive Scan, Japan). The diameters of the cells were measured from the prints. The values were processed using Microsoft Excel program.

2.7.2 Growth rate measurement

Growth in liquid nutrient medium

Log phase wild type and mutant cells were inoculated in equal volume of medium at a density of 2x10^5 cells/ml and grown at 21ºC with shaking at 160 rpm. Cells were counted at different time points using the Neubauer chamber. The experiments were carried out twice in triplicates.

Growth on SM agar plates

Ax2 and the respective mutant cells were plated onto SM agar plates overlaid with *Klebsiella aerogenes* and incubated at 21ºC for 3 to 4 days until *Dictyostelium* plaques appeared on the bacterial lawns. Cells were taken from the clearing zones of single plaques with sterile toothpicks and spotted onto fresh plates, and the diameter of growth was measured. The experiments were done in triplicates.

2.7.3 Aggregation analysis and development on phosphate-buffered agar plates or water agar plates

2.7.3.1 Aggregation in shaking suspension:

Vegetative cells grown axenically to a density of 3x10^6 cells/ml were harvested and washed twice in ice-cold Soerensen buffer. The cells were again reconstituted at a density of 5x10^7 cells/ml in Soerensen buffer and plated as monolayers on a 6 well plates (2x10^7 cells/ml/well). At different time points during aggregation images were captured using an Olympus IX70 inverse microscope.

2.7.3.2 Development on phosphate-agar or water agar plates:

Cells at a density of 2-3x10^6 cells/ml were washed twice with equal volumes of Soerensen phosphate buffer. 5x10^7 cells were then resuspended in 1 ml Soerensen phosphate buffer and evenly distributed onto a phosphate-buffered agar plate (90 mm) or water agar plate (90 mm). The plates were allowed to air dry and any excess liquid was carefully aspirated without disturbing the cell layer. The plates were then incubated at 21°C. Different stages of development were observed and the images were captured using a Stereomicroscope at the indicated time points.

2.7.4 Cytokinesis

Axenically growing cells were prepared as described in Materials and methods, 2.7.3.1 and harvested immediately after washing, fixed with methanol (Materials and methods, 2.7.14.1) and stained with DAPI, a dye that binds to DNA. Bright field images and fluorescent images were captured in parallel from different fields and the number of nuclei per cell was scored.

2.7.5 Phototaxis experiments (Wallraff and Wallraff, 1997)

Cells were harvested by centrifugation at 2000 rpm for 2 minutes and washed twice in water before placing 10^6 cells each on 1% water agar plates. The plates were incubated in a petridish storage containers (thermocol box with walls painted completely black from within) containing a vertical 3 mm wide perforation along the length of the container and were incubated in constant subdued light for 48 hours at 22°C. The slime trails left behind the migrating slugs were blotted onto nitrocellulose membranes and stained with 0.1% amido black in 25% isopropanol and 10% acetic acid (Staining solution) for 10 minutes, destained in 25% isopropanol and 10% acetic acid and washed with water and air dried.

2.7.6 Viability of osmotically shocked cells (Rivero et al., 1996)

Axenically grown cells (Ax2 and *grlA*$^-$ and *grlJ*$^-$) were harvested and washed twice each with Soerensen phosphate buffer and kept shaking (160 rpm) at 21^{o}C at a density of 3x10^7 cells/ml. After one hour 0.4 M sorbitol was added and shaking continued for 2 hours. The cells were then diluted such that 100 µl corresponding to 200 cells were plated onto a SM agar plate along with *K. aerogenes* and incubated at 21^{o}C. The colonies appearing after 3-4 days were counted and calculated for percentage of surviving cells.

2.7.7 Cell adhesion assay (Fey et al., 2002)

For adhesion assay, vegetatively growing cells (Ax2, *grlA*$^-$ and *grlJ*$^-$) were plated at 2x10^5/ml of axenic medium and incubated overnight at 21^{o}C. The next day, the cells that could not attach were counted using Neubauer counting chamber and considered as 0 min and the plates were then shaken at 60 rpm for1 h. The cells that were detached were counted at 5, 15, 30 and 60 minutes, respectively and the percentage of loose cells with respect to 0 min was calculated. The results were plotted as percentage of cells detached as a function of time. The total number of cells was determined at the end of the experiment after resuspension of all cells.

2.7.8 Neutral red staining (Raper 1940)

Ax2 and *grlA*$^-$ and *grlJ*$^-$ cells were harvested at a density of 2-3 x 10^6 cells/ml, washed twice with Soerensen phosphate buffer and were treated with equal volume of 0.1% Neutral red solution for one minute. The cells were then washed once with a large amount of buffer to remove the left over stain and 5 x 10^7 cells each were plated onto phosphate agar plate and incubated at 21^{o}C. Slugs, culminants and the mature fruiting bodies were monitored with an Olympus stereomicroscope. Slug size and the staining at the tip and base (stalk cell staining) were marked with Diskus software that allows marking of uneven surface and the slug size and dye distribution determined thereby.

2.7.9 LacZ reporter gene expression

Ax2 cells and *grlA*$^-$ cells were transformed using electroporation with the ecmB-Gal and pspA(D19)-Gal plasmids separately. The transformants were selected with 4µg/ml G418 and 3µg/ml of blasticidin was retained for *grlA*$^-$ cells. Single colonies were obtained by replica plating and they were developed on a nitrocellulose filter until slug and culminant stages and then processed for LacZ expression as described (Dingermann et al., 1989).

2.7.10 DIF induced gene expression by shaking suspension assay (Berks and Kay, 1990)

Logarithmically growing Ax2 and *grlA*$^-$ and *grlJ*$^-$ cells were harvested, washed with Soerensen phosphate buffer and plated at a density of 5x10^6 cells/cm^2 and developed until mound stage. They were then disaggregated with a syringe and a needle and divided into 4 sets to be treated differently, each with 5 mM cAMP; 100 nM DIF-1; 5 mM cAMP + 100 nM DIF-1; and none of these and incubated for two hours at 21^{o}C on a shaker (160 rpm). RNA from the respective samples was prepared using the RNAeasy kit and 20 µg each were separated over a formaldehyde agarose gel and Northern blot transfer was carried out. They

were then probed for *ecmB* and *pspA* transcripts respectively. cAMP was obtained from Fluka and DIF-1 from Biomol.

2.7.11 Spore germination assay (Ennis and Sussmann, 1975 modified)

Equal number ($5x10^7$) of cells (Ax2, *grlA*$^-$ and *grlJ*$^-$) were plated onto phosphate agar plates and spores formed at 48 h were harvested and counted, respectively. They were then treated with 0.5% Triton-X-100 for 15 min and diluted with Soerensen buffer and 100 spores each were plated onto SM agar plates in association with *K. areogenes*. Detergent resistant spores were counted as the number of plaques formed once they appeared and calculated for the viability. The experiments were carried out in triplicates.

2.7.12 Sporogenous assay (Anjard et al., 1998)

To determine concentrations of **SDF-1 and SDF-2**, cells (Ax2, *grlA*$^-$ and *grlJ*$^-$) developed until culminant and mid-culminant stages, respectively. Structures were disaggregated by vigorous shaking for 10 seconds, filters were removed and cells pelleted by 1 minute centrifugation at 4000 rpm. Supernatants were harvested for quantification of SDFs. Cells were washed in 1 ml buffer (without cAMP) and plated at $2x10^4/cm^2$ in 1.5 cm diameter wells with 0.5 ml cAMP buffer (5 mM cAMP). After 10 minutes of incubation, 10 units of SDF-1 and/or SDF-2 were added for priming and aliquots of the supernatants were harvested 5 minutes later to quantify the released factors. As a control, supernatant from noninduced cells was analysed for spontaneous release of SDFs. The level of spores in the disaggregated structures was scored after incubation for 2 additional hours.

In the **sporogenous** submerged culture **assay,** washed cells were resuspended and plated at the above indicated densities in 1.5 cm diameter plastic wells with 0.5 ml of cAMP buffer (5 mM cAMP). Spores were scored microscopically after 20 to 24 hours of incubation at 22^{o}C (Anjard et al., 1997). Purified factors (GABA, Glutamate, SDF-1 and SDF-2) were then added to cells and spores counted again 2 hours later. Every sporogenous assay included a positive control for SDF-1 and SDF-2 activity. One unit of SDF-2 activity was defined as the amount necessary to induce K-P cells (a transgenic line that contains multiple copies of PKA catalytic sub-unit gene and is sporogenous) to form 50% spores. Similarly, one unit of SDF-1 activity was defined as the amount necessary to obtain 35% spores.

2.7.13 Video imaging and chemotaxis assay

Cells were prepared as in (Materials and Methods 2.7.3.1) and starved for 5-6 h. 25-30 µl of cell suspension were diluted in 3 ml of Soerensen buffer and mixed well by pipetting (25-30 times, with occasional vortexing). This is important to dissociate cells from aggregates. 1.5 ml of the diluted cells were then transferred onto a 5 cm glass cover-slip with a plastic ring placed on an Leica inverse microscope equipped with a 10x UplanFl 0.3 objective. Cells were stimulated with a glass capillary micropipette (Eppendorf Femtotip) filled with 0.1 mM cAMP (Gerisch and Keller, 1981), which was attached to a microcontroller. Time-lapse image series were captured and stored on a computer hard drive at 30 seconds intervals with a JAI CV-M10 CCD camera and an Imagenation PX610 frame grabber (Imagenation Corp., Beaverton, OR) controlled through Optimas software (Optimas Corp., Bothell, Washington). The DIAS software (Solltech, Oakdale, IA) was used to trace individual cells along image series and calculate the cell motility parameters (Soll et al., 2001). For processing images, Corel Draw version 11 and Adobe Photoshop were used.

2.7.14 Indirect immunofluorescence of *Dictyostelium* cells

2.7.14.1 Preparation of *Dictyostelium* cells

Dictyostelium cells were grown in shaking culture to a density of 2-4 x10^6 cells/ml. Desired amounts of cells were collected in a centrifuge tube, washed twice with Soerensen phosphate buffer and finally resuspended in the same buffer at 1x10^6 cells/ml. 400 µl of the cell suspension were then pipetted onto an 12/15/18 mm acid-washed glass cover-slip lying on a parafilm covered glass plate resting in a humid-box. Cells were allowed to attach to the glass cover-slip for 15-20 min. Thereafter cells attached onto the cover-slip were fixed immediately by the fixation techniques described below.

2.7.14.2 Methanol fixation

After the cells have attached to the coverslip, the supernatant was aspirated and the coverslip was fixed by incubating the cells dipped instantaneously into the pre-chilled (-20ºC) methanol in a petri dish and incubated at -20ºC for 10 min. The coverslip was then washed with 500 µl PBS/glycine for 5 min to block free reactive groups followed by two washings with 500 µl of PBG for 15 min each. After washings, the cells were immunolabelled as described below.

PBG, pH 7.4: 0.5 % bovine serum albumin, 0.1 % gelatin (cold-water fish skin) in 1x PBS, pH 7.4

PBS Glycine: 500 ml 1x PBS, 3.75 g glycine, filter sterilize and store at -20ºC

2.7.14.2 Immunolabeling

The cover slip containing the fixed cells was incubated with 400 µl of the desired dilution (in PBG) of primary antibody for 1-2 h in the humid-box at room temperature. After incubation, the excess antibody was removed by washing the cover slip five times with PBG for 5 min each. The coverslips were then incubated for 1 h with 400 µl of a proper dilution (in PBG) of the appropriate secondary antibody followed by two washings with PBG for 5 min and then three washings with PBS for 5 min. Most of the times DAPI, a dye to stain the DNA, was incorporated along with the secondary antibody. After washings, the cover-slip was mounted onto a glass slide. Sometimes cells expressing GFP fusion proteins were directly mounted onto a glass slide after fixation and observed under a fluorescence microscope or confocal laser scan microscope.

2.7.14.3 Mounting

After immunolabeling of the fixed cells, the coverslip was swirled once in deionised water and the extra water was blotted off on a soft tissue paper and embedded carefully with gelvatol taking enough care not having air bubbles trapped in. Mounted slides were then stored in the dark at 4ºC for overnight. Thereafter, the mounted slides were observed under a fluorescence microscope or LEICA confocal laser scan microscope.

2.8 Microarray Analysis

The experimental protocol was carrired out as available at http://www.uni-koeln.de/med-fak/biochemie/transcriptomics/ from the Analysis section and the Data mining section was used to compile the data. The *Dictyostelium* DNA microarray used consists of an array of DNA probes that are derived from ESTs, published genes and controls. The array contains the sequences of 5423 non-redundant cDNA clones that were obtained from the *Dictyostelium* cDNA project (http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html), 450 partial sequences of published genes and 33 controls. Positive controls are selected partial gene sequences from *D. discoideum*, genomic DNA (100 ng/µl) and the SpotReport-10 PCR products (Stratagene). Negative controls are Fish sperm DNA (100 ng/µl), phage λ Bst E III (100 ng/µl), human Cot-1 (10 ng/µl) and SpotReport Poly(dA) (1 ng/µl). Altogether, the array contains 5906 targets, each of which was printed at least in duplicate per slide.

Set1	Slide 1	Ax2 RNA (20µg) Cy3	*grlA*⁻ RNA (20µg) Cy5
	Slide 2	Ax2 RNA (20µg) Cy5	*grlA*⁻ RNA (20µg) Cy3
Set2	Slide 3	Ax2 RNA (20µg) Cy3	*grlA*⁻ RNA (20µg) Cy5
	Slide 4	Ax2 RNA (20µg) Cy5	*grlA*⁻ RNA (20µg) Cy3
Set3	Slide 5	Ax2 RNA (20µg) Cy3	*grlA*⁻ RNA (20µg) Cy5
	Slide 6	Ax2 RNA (20µg) Cy5	*grlA*⁻ RNA (20µg) Cy3

Table 1. Experimental setup. RNA used for the experiment and the dyes used. The second line in each set represents the slide for dye swap experiment. Each row thereby depicts 6 slides each containing the respective wild type RNA with the mutant labeled with different dyes. Set 1 comprises of Slides 1 and 2; Set2: slides 3 and 4; set 3 contains slides 5 and 6, respectively.

16 h cells (Ax2 and *grlA*⁻) were harvested and RNA was isolated with RNeasy kit from three independent experiments. The different cDNAs obtained were then labeled with the respective dyes as mentioned in Table 1. The wild type and the mutant cDNA was mixed as shown in the Table 1 and hybridized with the *Dictyostelium* microarray chip. The slides were then washed and scanned at high and low intensities (ScanArray 4000XL) and quantitated (ScanArray Express version 3.3). The raw data obtained were converted to *.txt files, imported into the ArrayTools, exported to Program R for normalization and imported back to ArrayTools. The normalized data was finally imported to **SAM** (Significant Analysis for Microarray) to identify differentially expressed genes. The Ddbase (*Dictyostelium* database) ID was obtained from the DictyAccess after ruling out redundancy and the genes then obtained were separated as per 1.5 fold cutoff and above differentially expressed genes which was then clustered into genes for Biological process, Cellular component, and Molecular function using **GO Annotation Tools** (http://www.godatabase.org/dev/database)(Xu et al., 2004)**.**

3. RESULTS

3.1 $GABA_B$ receptor-like proteins in *Dictyostelium*

3.1.1 Family of $GABA_B$ receptor-like proteins in *Dictyostelium*

Analysis of the *Dictyostelium* genome revealed a family of genes, which are closely related to *$GABA_BR1$ and $GABA_BR2$* (Eichinger et al., 2005). The available information from the *Dictyostelium* database and NCBI led us to identify 17 genes, which were named recently (Hereld, 2005) as **Grl** (**G**ABA$_B$ or metabotropic **g**lutamate **r**eceptor-**l**ike) A to R. We constructed a phylogenetic tree applying the Phylodendron program from the values obtained from the ClustalW alignment using the full-length sequences of these receptors (Figure 8).

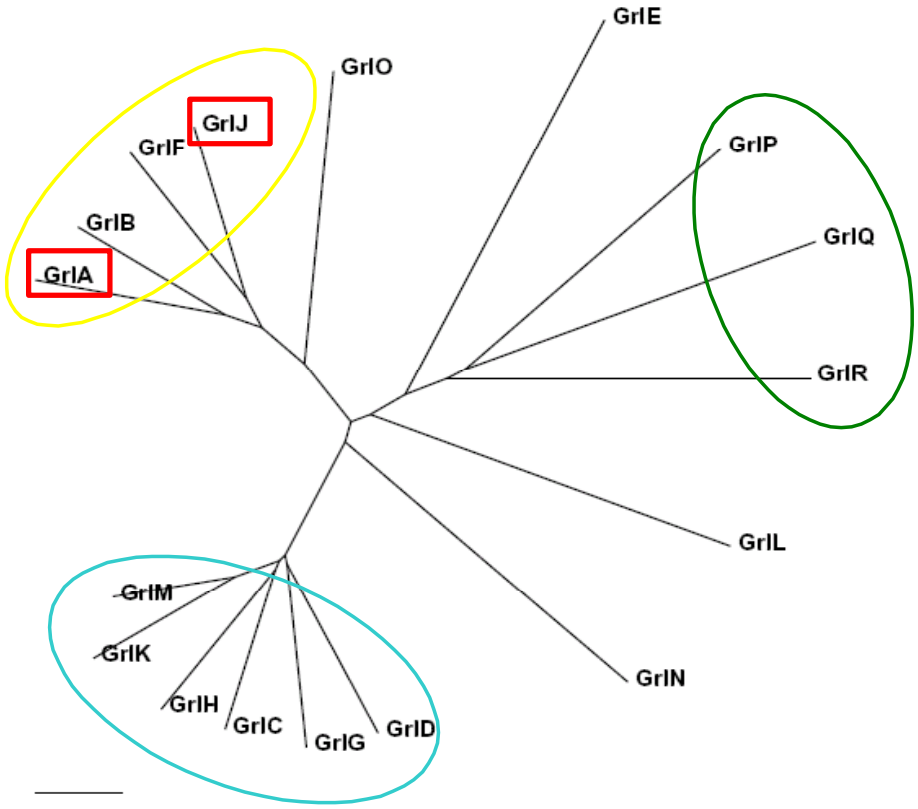


Figure 8. The family of $GABA_B$ receptor-like proteins in *Dictyostelium*. The tree was constructed using the sequence information of all Grls in the *Dictyostelium* database and by applying the ClustalW and Phylodendron (phylogenetic tree printer) programs. GrlA and GrlJ marked by red square are the topic of the current studies. Coloured circles highlight three groups of highly related receptors. Bar, 0.1 (phylogenetic distance which is expressed as the percentage of divergence divided by 100).

There were three major clusters formed and four of them (GrlO, GrlE, GrlN and GrlL) diverted out separately. We undertook the study of GrlA and GrlJ, which were two out of the first 4 $GABA_B$ receptor-like sequences identified in the amoeba (Glöckner et al., 2002). Also,

GrlA aligned slightly more with $GABA_BR2$ sub-type and GrlJ was slightly related to the sub-type 1.

3.1.2 Domain architecture of GrlA and GrlJ

GrlA is a 798 amino acids containing protein predicted to have the signature motif of GPCRs, the 7 transmembrane domain using SMART, conserved domain database (NCBI) and TMHMM prediction programs. The domain architecture of GrlJ (783 amino acid residues) was also obtained in a similar fashion. Both receptors, GrlA and GrlJ harbour a Bmp (Basic membrane protein) domain and also a domain homologous to bacterial periplasmic protein at the N-terminus apart from the TMD as depicted in the Figure 9.

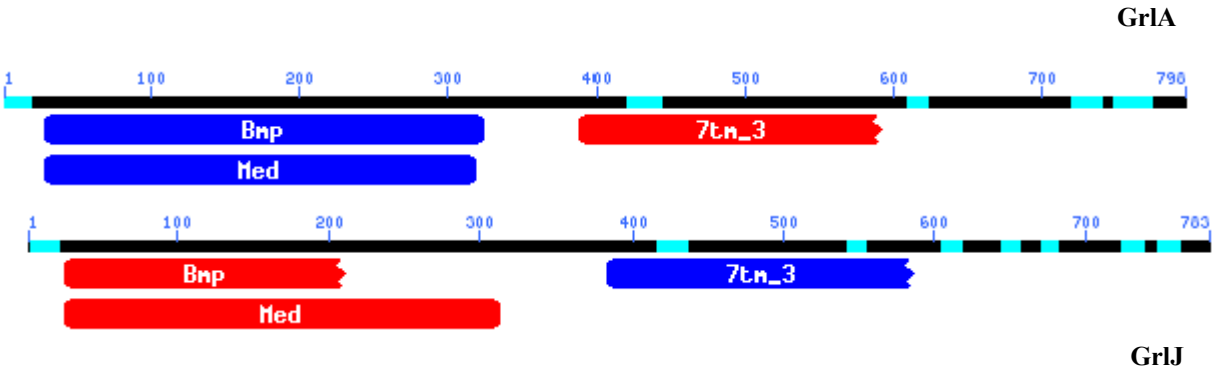


Figure 9. Schematic representation of GrlA and GrlJ. Applying the conserved domain database prediction program from NCBI, the domain architecture of GrlA and GrlJ was created. The figure depicts the schematic representation of GrlA and GrlJ illustrating the 7 transmembrane domain (7tm_3), the basic membrane protein (Bmp) domain, and an uncharacterized ABC-type transport system, represented as (Med) which is similar to the bacterial periplasmic proteins, whereas the light blue rectangles illustrates the low complexity regions.

3.1.3 Comparison of GrlA and GrlJ with $GABA_B$ receptors from higher eukaryotes

GrlA and GrlJ were further compared with several known $GABA_B$ receptor sequences from different species using the full-length sequences. The phylogenetic tree was constructed

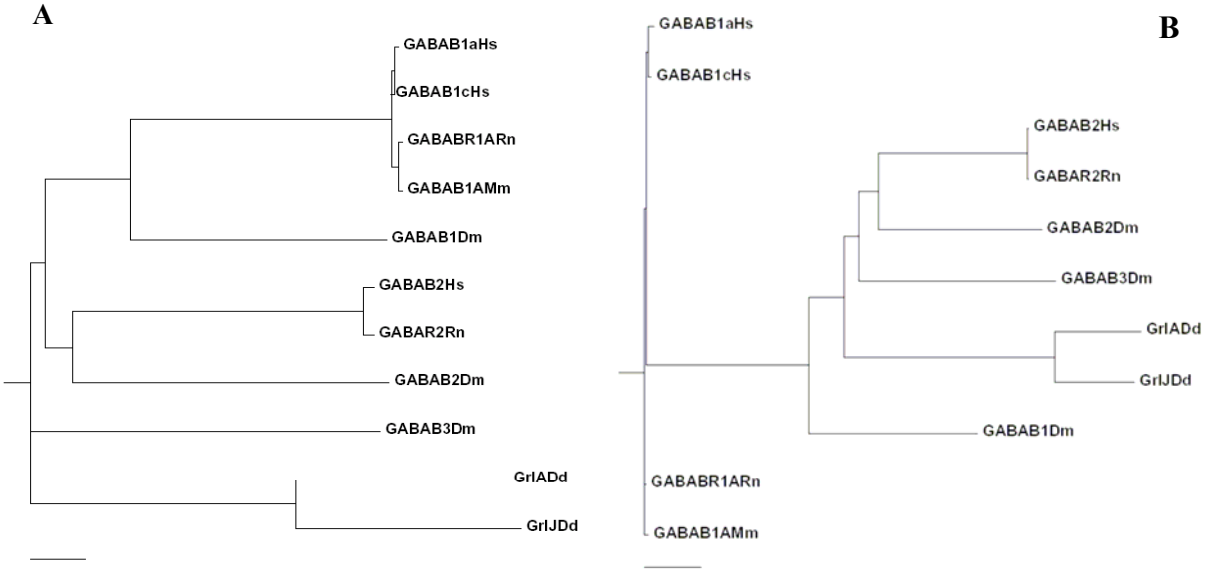


Figure 10A. Phylogenetic tree of *Dictyostelium* GABA$_B$ receptor-like proteins. The full-length sequences of GrlA and GrlJ was aligned with several GABA$_B$ sequences from higher eukaryotes with the ClustalW program. The phlyogenetic tree was constructed with the values obtained from the Clustal sequences. Accession numbers are as follows; GABAB1a Hs (Q9UBS5), GABAB2 Hs (O75899), GABAB1c Hs (NP_068704), GABAR2Rn (O88871), GABABR1a Rn (CAA71398), GABAB1aMm (AAD22194), GABAB3Dm (AAK13422), GABAB2Dm (AAK13421), GABAB1Dm (AAK13420). **10B**. The TMD regions of the aforementioned receptors were used to generate the phylogenetic tree. Abbreviations, Dm: *Drosophila melanogaster*; Rn: *Rattus norvegicus*; Hs: *Homo sapiens*; Ms: *Mus musculus*. Bar, 0.1.

applying the ClustalW program and Phylodendron (phylogenetic tree printer) programs. The *Dictyostelium* GrlA and GrlJ clustered closely with each other but separated out from the GABA$_B$ receptors from other higher eukaryotes (Figure 10A). However, when only the transmembrane regions were used for construction of the tree they were found not only again closely clustered with each other but also with the GABA$_B$R2 sequences from *Drosophila*, human and rat (Figure 10B).

3.1.4 Multiple alignment of the TMDs of GrlA and GrlJ and GABA$_B$ receptors from higher eukaryotes

We therefore analysed the transmembrane region of GrlA and GrlJ with GABA$_B$ from

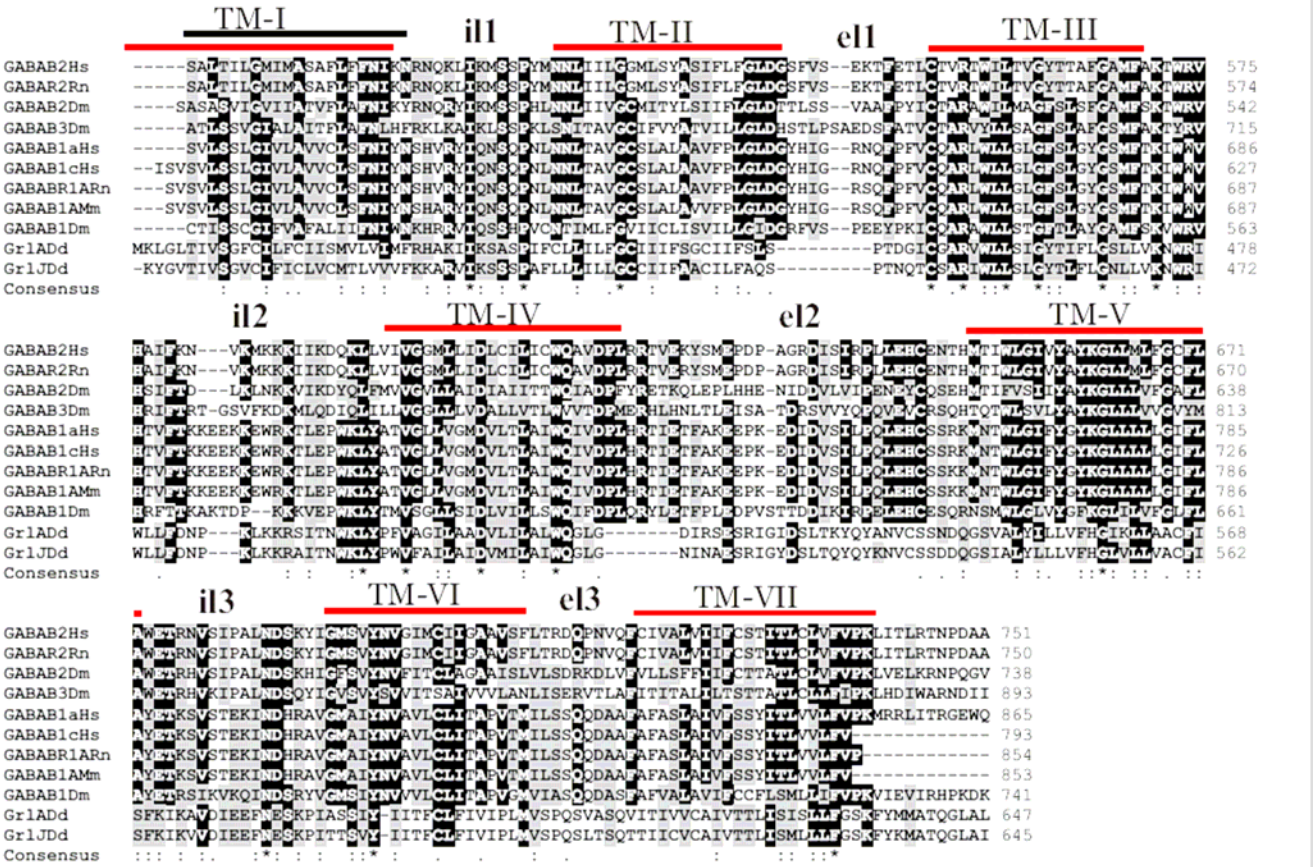


Figure 11. Multiple alignment of TMDs. The transmembrane regions obtained by SMART output were used for alignment which was done with the ClustalX program. The accession numbers of the protein sequence can be obtained from the previous figure (Figure 10). The amino acids marked in black signify the highly conserved regions in all the species whereas grey ones depict partially conserved residues. The red horizontal red bars depict the approximate transmembrane regions named as TMD I-VII (as it differed slightly in different species) whereas both the black and the red bars over the TMD-I covers the TMD-I. The intracellular and the extracellular loops are named as il1-3 and el1-3 respectively.

human, mouse and rat. The TMD regions that were used for the alignment were obtained using the SMART program and the Clustal X program. The alignment shows highly or partially conserved regions amongst the receptors under study. GrlA and GrlJ displayed regions of identity to both the $GABA_BR1$ or $GABA_BR2$ subtypes. These were located at different regions in the transmembrane domain and also in the intra- or extracellular loops. But there was no strikingly closer similarity observed to a particular subtype.

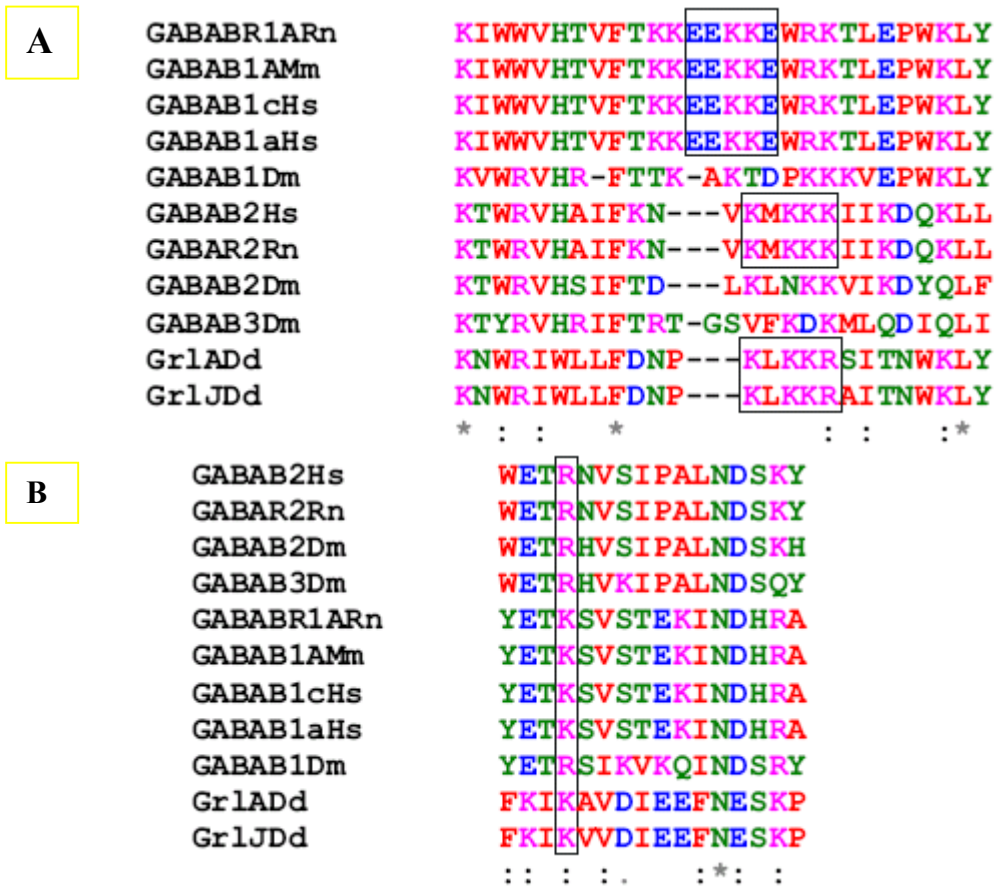


Figure 12. Multiple alignment of the intracellular loops of $GABA_B$ receptors. The intracellular loops 2 and 3 from the sequences used in Figure 10 were used for alignment. Figure 12A illustrates the alignments of intracellular loops 2 and Figure 12B represents that of intracellular loops 3. Consensus residues are shown in the last line in both the figures, wherein stars depict completely conserved residue in all the species shown here, double dot signifies high similarity whereas a single dot represents partial conservation. The black boxes represent certain important residues for G-protein coupling. Similar types of amino acids have the same colour code.

In GPCRs the intracellular loop 3 has specific residues that help in determining the G-protein specificity whereas the intracellular loop 2 has enough molecular determinants for G-protein activation. We therefore compared the loop 2 and 3 regions from GrlA and GrlJ with other known $GABA_B$ receptors. The intracellular loop 3 in between the TMD-V and TMD-VI possesses a basic Arginine residue, which is important for coupling to G proteins and is conserved in the mGlu (metabotropic Glutamate receptor) family (Franscesconi and Duvoisin, 1998) and also in $GABA_BR2$, whereas this residue is replaced by a Lysine in $GABA_BR1$. GrlA and GrlJ possess a lysine residue at this key position (Figure 12B). Of all the *Dictyostelium* Grls, 6 had a conserved Lysine and 11 of them had Arginine at the intracellular loop 3 (data not shown). As depicted in figure 12A, the intracellular loop 2 of the $GABA_BR1$ has highly conserved glutamate residues, represented as **EE KK E** motif, which are replaced by Lysine in $GABA_BR2$ represented as **KM KK K** (Robbins et al., 2001; Havlickova et al., 2002). In GrlA and GrlJ, we found basic residues, Lysine and Arginine **KL KK R** (Figure 12A) probably allowing them to couple to G-proteins as observed for $GABA_BR2$.

3.2. Expression and localization of GrlA and GrlJ

3.2.1 Expression profile of the *grlA* and *grlJ*

The temporal expression pattern of genes encoding $GABA_B$ receptor-like proteins was examined in growing and developing cells by RT-PCR and real time PCR to determine when the receptors might function. As indicated clearly in the RT-PCR analysis, both receptors were expressed during the vegetative growth and throughout development with a relatively higher expression level in the later developmental stages (Figure 13A). Overall *grlJ* message was more abundant than the *grlA* message.

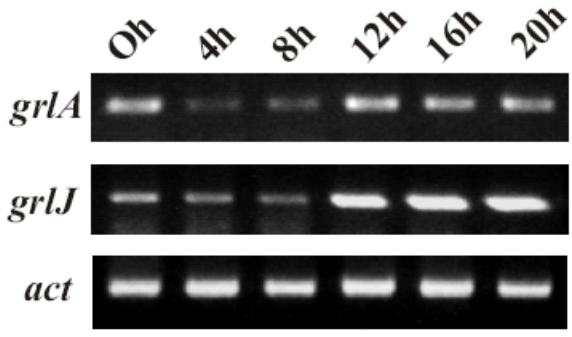


Figure 13A. RT-PCR analysis *of grlA and grlJ*. Total RNA was used as a template for the synthesis of single-stranded cDNA by reverse transcription as mentioned in Materials and methods 2.4.1.2. Gene specific PCR products were then amplified using 1 µl of each cDNA as template. Amplification of actin was used as a contol for the experiment.

To quantitate these data we carried out Real-time PCR experiments. The abundance in expression of these receptors was compared to the standards and control (actin was used as a control as it is expressed throughout vegetative and developmental phase). These data confirm that both receptors were expressed during vegetative growth and throughout development. Furthermore, *grlJ* was expressed at higher levels as compared to *grlA*, however the levels of both transcripts increased by 2-3 fold during development (Figure 13B). Both transcripts were hardly detected by northern blot analysis.

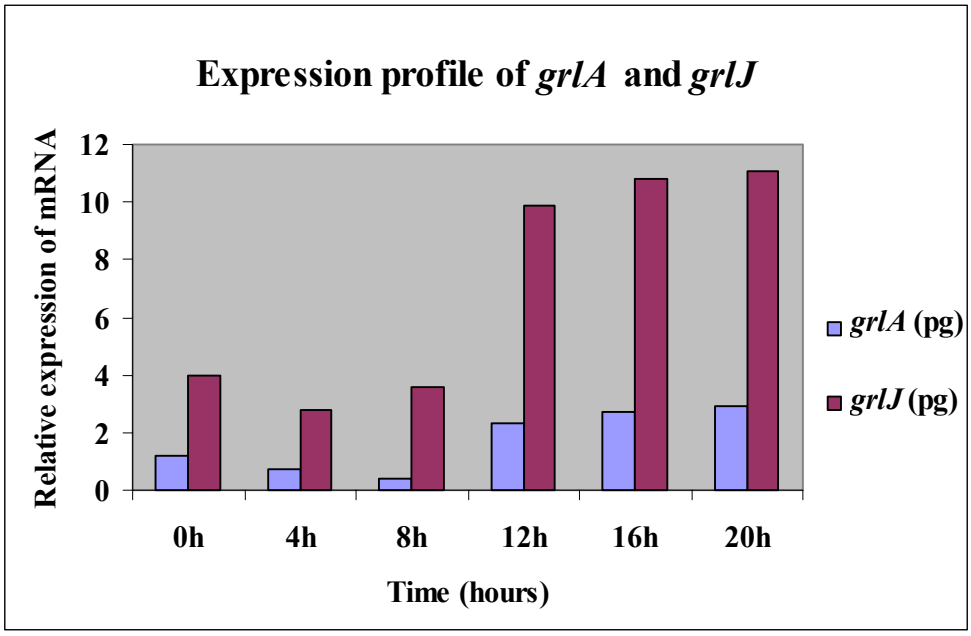


Figure 13B. *grlA* and *grlJ* expression pattern as revealed by Real time PCR. Total RNA was used as a template for the synthesis of ssDNA (Materials and methods 2.4.1.2). The Opticon Real-time PCR machine was used to quantitate the receptor expression as compared to the standard (data not shown) and the control. The experiments were carried out twice independently and in duplicates.

3.2.2 Subcellular localization of GrlA

To study the subcellular localization we expressed a full-length GrlA-GFP fusion protein in Ax2 wild type cells. Cells expressing the fusion protein were monitored for the localization of the fusion protein using a confocal microscope. Additionally we also stained the cells for membrane proteins and various intracellular compartments. GrlA-GFP was partially targeted to the plasma membrane shown by its colocalization with Annexin C1, primarily a plasma membrane protein (Döring et al., 1995). A major part of the protein remained in the cytoplasm predominantly at the perinuclear region (Figure 14A) where it colocalized with the ER marker PDI (protein disulfide isomerase) (Monnat et al., 1997). We further monitored whether GrlA-GFP colocalizes with Comitin (Weiner et al., 1993), a protein present on vesicles and Golgi membranes, Interaptin, a nuclear envelope (Rivero et

al., 1998), VatA (A subunit of vacuolar ATPase) (Jenne et al., 1998), lysosomes (mannose-6-sulfate containing carbohydrate epitope present in lysosomal enzymes) (Neuhaus et al., 1998), and Vacuolin (Rauchenberger et al., 1997) that stains vacuoles to test the possibility of its localization to any of these compartments. At the nucleus the GFP fused protein colocalized well with the nuclear envelope protein interaptin and also with PDI around the nucleus. At the plasma membrane we found an overlap with Annexin C1.

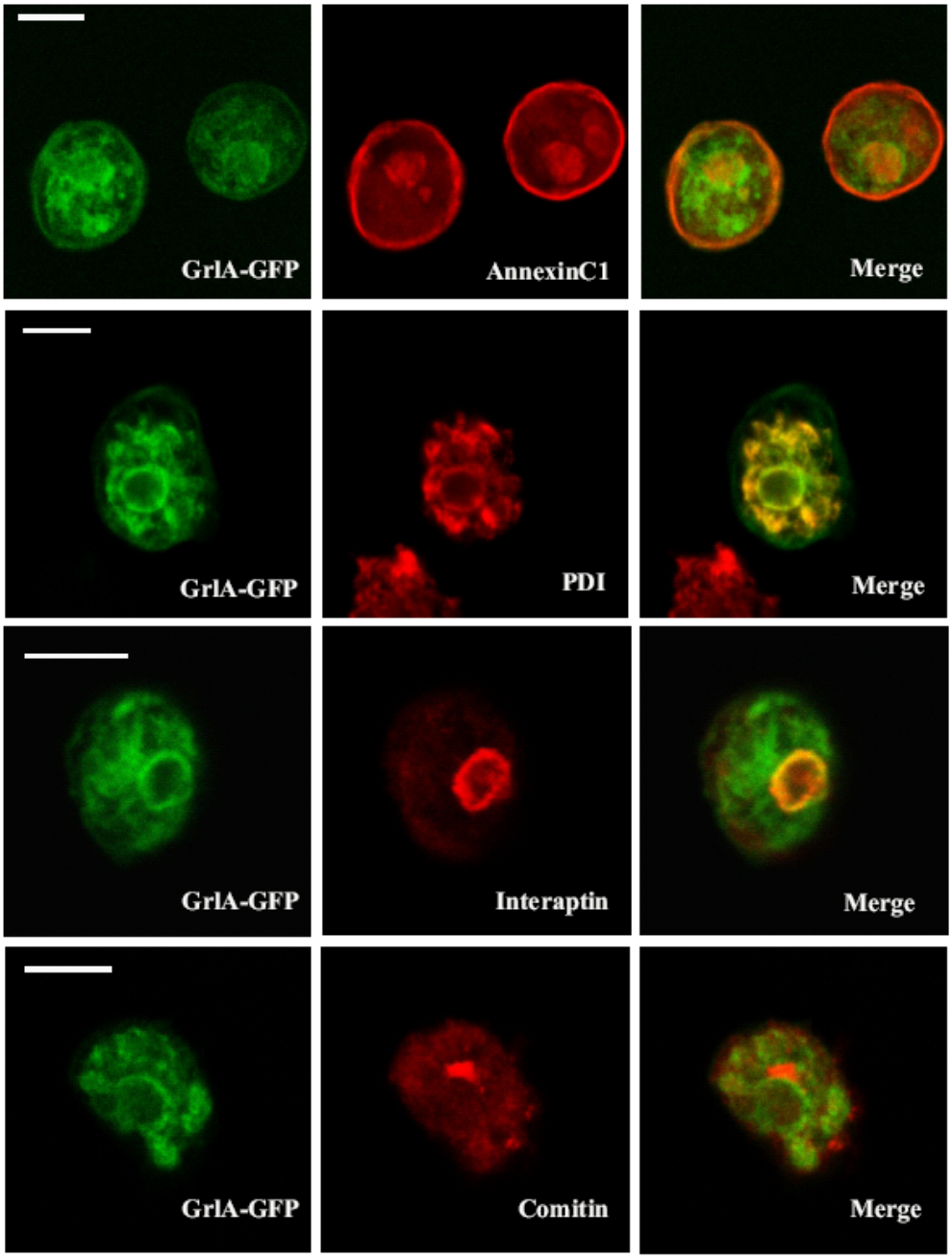


Figure 14A. Subcellular localization of GrlA. GrlA was tagged with C terminal GFP and expressed in the Ax2 cells which were fixed and stained for the membrane marker Annexin C1 (mAb 185-447-3) and markers for ER, PDI (mAb 221-135-1); Comitin (mAb 190-340) for staining the Golgi, and Interaptin (mAb 260-60-10) that predomimantly stains the nuclear envelope. Secondary antibody

conjugated with Alexa 568 was used to study the localizations. Images were captured using a confocal microscope. **Bar, 5 μm.**

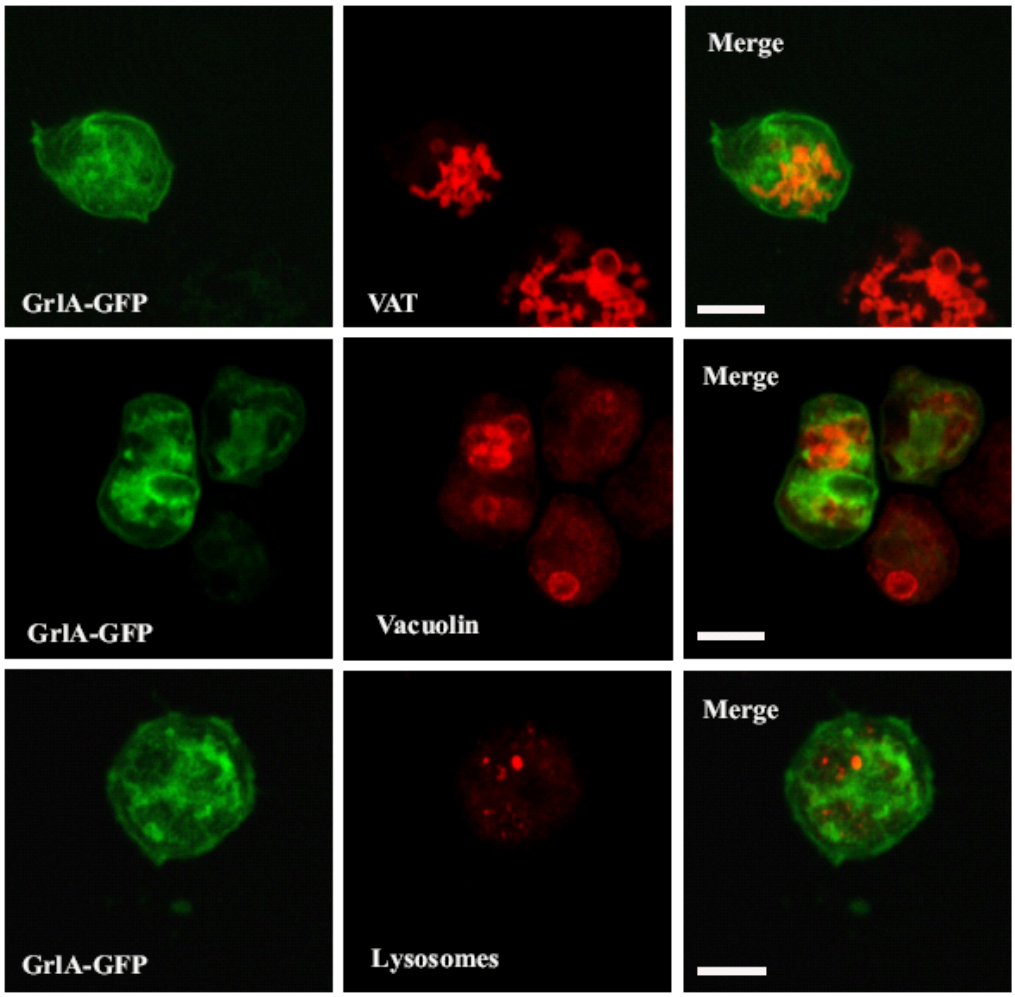


Figure 14B. GrlA-GFP cells were also stained with antibodies that detect the VatA subunit of the vacuolar ATPase (mAb 221-35-2), Vacuolin (mAb 221-1-1) that stains the vacuoles, and with an antibody against a lysosomal anitigen (mAb 221-342-5). The secondary antibody conjugated with Alexa 568 was used to detect respective staining. Confocal images were captured. **Bar, 5 μm.**

3.3. Dimerization profile of family 3 of *Dictyostelium* GPCRs

GPCRs are reported to be fully functional as homo- or heterodimers in higher eukaryotes and mammals and $GABA_B$ was the first among the GPCRs to be identified as an obligate heterodimer (Kaupmann et al., 1998). We therefore searched for the dimer pair(s) of $GABA_B$ in the known pool of receptors, which are more similar to either $GABA_BR1$ or $GABA_BR2$ type of receptors in *Dictyostelium* and subsequently assayed for a putative interaction using the yeast two-hybrid system.

Interactions	-Trp-Leu	-Trp-Leu –His (+3AT)	β-gal
GrlA-CT GrlJ-CT	++	–	–
GrlB-CT GrlJ-CT	++	–	–
GrlH-CT GrlJ-CT	++	–	–
GrlL-CT GrlJ-CT	++	–	–
GrlB-CT GrlF-CT	++	–	–
GrlA-CT GrlF-CT	++	–	–
GrlH-CT GrlF-CT	++	–	–
GrlL-CT GrlF-CT	++	–	–
pCL1+pGBKT7 (+ve control)	++	++	++

Table 2. Dimerization profile of several GPCRs by yeast two-hybrid experiments. The first column shows the different combination of C-termini used for yeast transformation. The second confirms the presence of cotransformants and the third one shows the results of growth of yeast cells on SD-Trp-Leu-His+3AT(60 mM) plates. The fourth column shows the results from the respective β -gal assays. "++" was assigned for good growth on plates and high colour intensity in the β -gal assay whereas "- " when there was no growth and no blue colour development.

We used the C-termini of the two receptors that appeared more similar to type 1 receptors in fusion to the activating domain (AD) of Gal4 in pGADT7 and several that appeared more similar to the type 2 receptors in fusion with the binding domain (BD) of Gal4 in pGBKT7, respectively. Yeast transformation and selections were carried out as described in Materials and methods (2.6.2.1). All the transformants including the positive and negative control cells grew well on the co-transformation control plate, SD-Trp-Leu plates respectively. However, colonies appeared on the stringent selection plate SD-Trp-Leu-His (+3AT) only in the positive control cotransformants depicting the receptors under investigation did not interact with their C terminus. We then studied for any possibility of homodimerization with the C-termini of 4 of the receptors which was already cloned in BD domain vector in fusion with the AD in the yeast two-hybrid system. Table 3 reveals that there was no homodimerization amongst GrlA, GrlB, GrlH or GrlL with their C- termini.

Interactions	-Trp-Leu	-Trp-Leu –His (+3AT)	β-gal
GrlA-CT	+++	–	–
GrlB-CT	+++	–	–
GrlH-CT	+++	–	–
GrlL-CT	+++	–	–
pCL1+pGBKT7 (+ve control)	+++	+++	+++

Table 3. Analysis of homodimerization amongst several GPCRs by yeast two-hybrid experiments. The first coloumn shows C-termini in fusion with the AD and BD of the respective vectors and used for yeast transformation. The second confirms the presence of cotransformants and the third one shows the results of growth of yeast cells on SD-Trp-Leu-His+3AT (60 mM) plates. The fourth coloumn shows the results from the respective β -gal assays. "+++" symbol was assigned for very good growth on plates and high colour intensity in the β -gal assay whereas "- " when there was no growth and no blue colour development.

Taken together, these results led us speculate that the C-termini of the receptors under investigation do not interact. Alternatively, a further line of work would be to clone the full-length receptors of this family and study their behaviour ideally by expressing these receptors in a heterologous system.

3.4. Generation of *grlA*$^-$ and *grlJ*$^-$ mutants and their characterization

3.4.1.1 *grlA and grlJ* gene disruption by homologous recombination

To gain insight into the *in vivo* functions of these proteins, we generated mutants carrying the disruption of *grlA and grlJ* gene individually. A targeting vector was constructed (Figure 15A) that allowed replacement of a 1.3 kb fragment of the *grlA* gene consisting of a part of exon 1, the following intron (76 bp) and a part of the second exon (consisting of half the N terminal region and first 4 TMDs) with the blasticidin resistance cassette (1.4 kb). This vector was transfected into the wild type Ax2 cells by electroporation. Transformants were tested by PCR, Southern blot analysis at the DNA level and by RT-PCR at the RNA level.

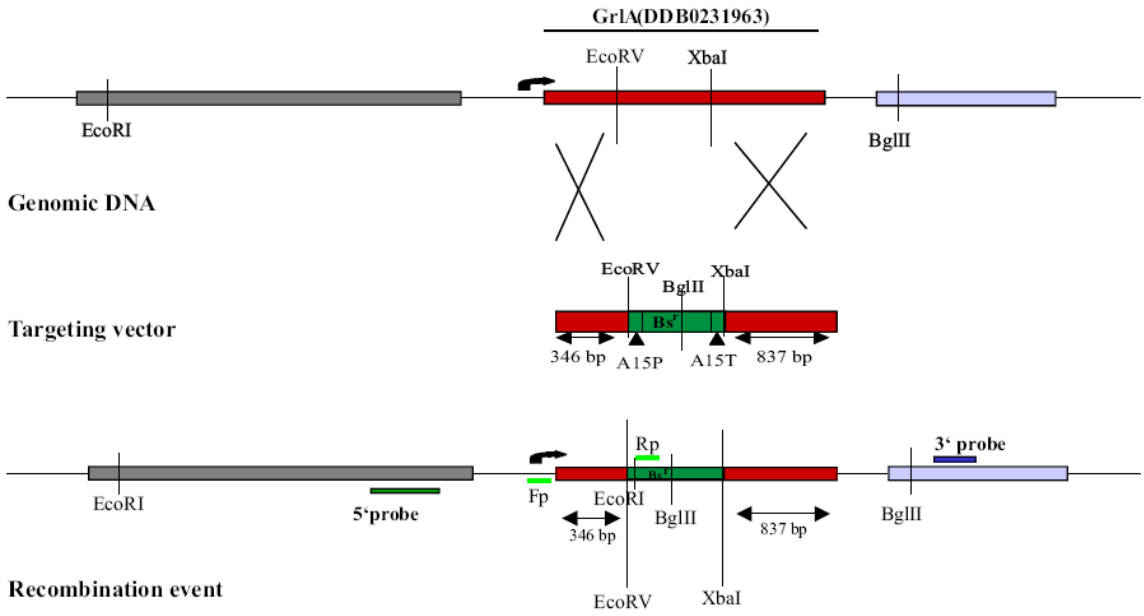


Figure 15A. Schematic representation of the homologous recombination event in the *grlA* locus. Genomic DNA organization of the locus and the targeting vector are shown. The Bsr cassette (1.4 kb) replaces a 1.3 kb EcoRV/XbaI fragment in the gene. Primers outside the sequences contained in the targeting vector were chosen for PCR analysis that would confirm the recombination event. The location of 5' and 3' probes is indicated.

Similarly, a 2.2 kb neomycin resistance cassette was inserted into the EcoRV site at the *grlJ* locus in the genome (Figure 15B) thereby disrupting the gene after the first transmembrane

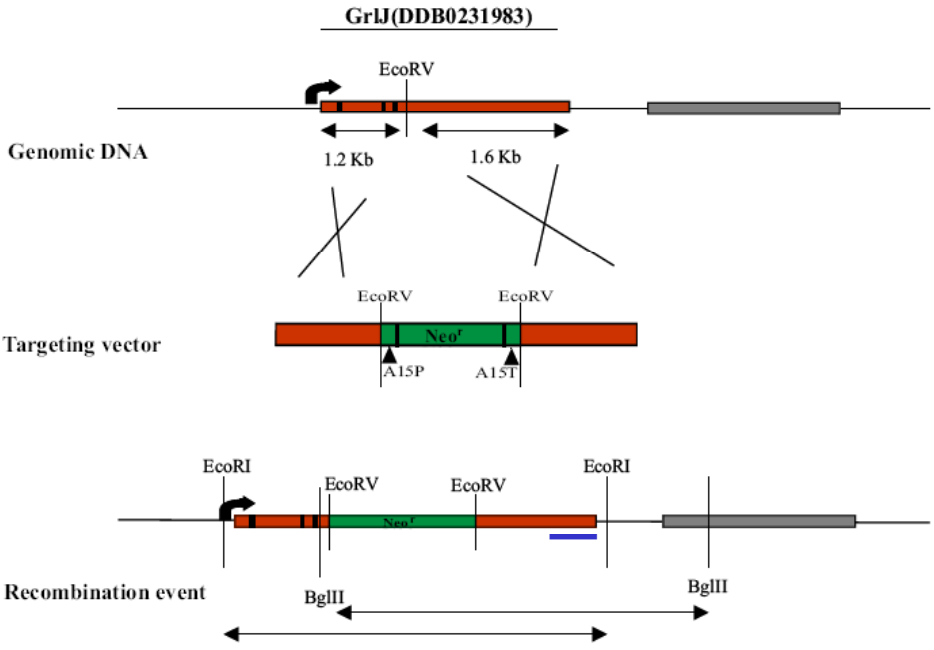


Figure 15B. Schematic representation of gene organization and homologous recombination events at the *grlJ* locus. The targeting vector was constructed so as to insert the neomycin resistance cassette at the EcoRV site to disrupt the gene after the first transmembrane domain. The strategy to confirm the recombination event was using different enzymes each restricting at least once in the

genome loci and by using a C-terminal probe for Southern blot analysis. The blue bar depicts the location of the probe (440 bp C terminal region of GrlJ) used for Southern blot analysis.

domain region such that the read-through message would also be non functional. A C-terminal probe was used for screening the transformants. The positive clones were confirmed by Southern blot analysis.

3.4.1.2 Recombination event confirmed with PCR and Southern blot analysis and RT-PCR

To investigate the occurrence of recombination event in the *grlA* locus in the genome, PCR analysis was carried out. Genomic DNA isolated from the wild type Ax2 cells and transformants was used as template for PCR. The forward primer (5'intronFp) located in the non-coding region just before the ORF of the *grlA* gene and reverse primer located in the promoter of the blasticidin resistance cassette were used for screening which should lead to the production of a 550 bp amplification product in the transformants in case of a correct recombination event. No PCR product was obtained in case of Ax2 wild type genomic DNA.

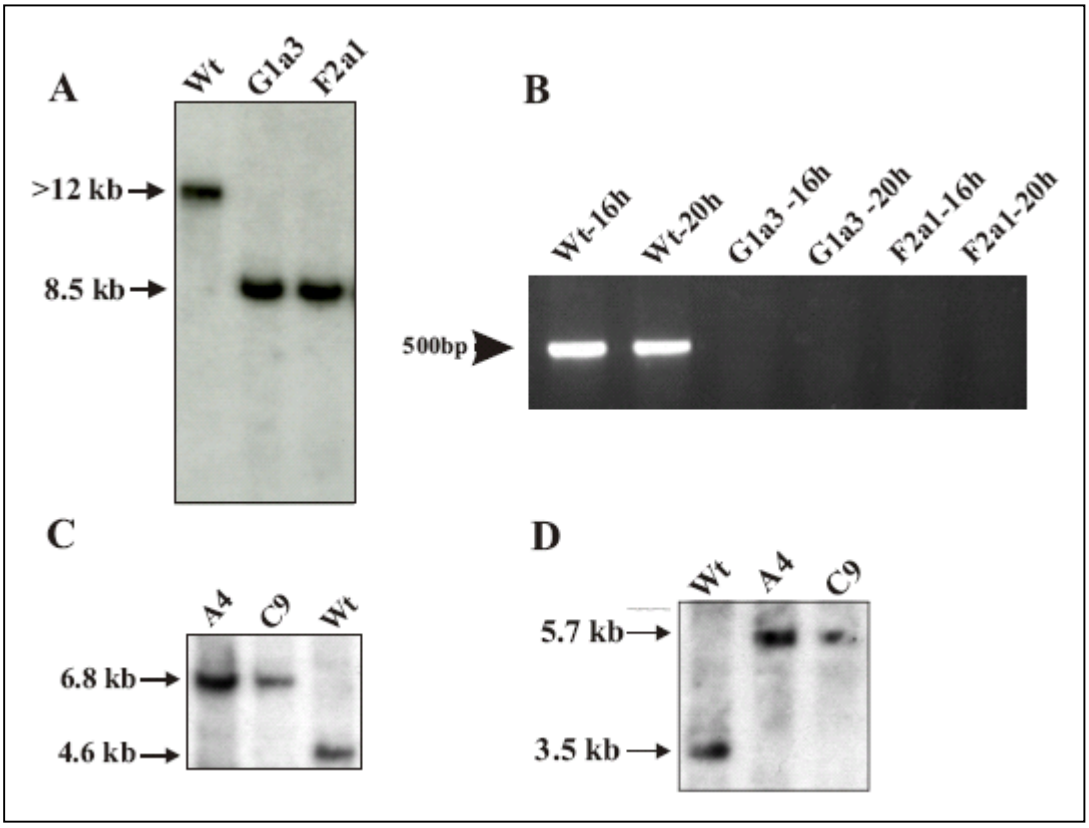


Figure 16. PCR and Southern blot analyses of null mutants. A) Disruption of the *grlA* gene in Ax2 wild type cells by homologous recombination confirmed by Southern blot analysis. EcoRI digested genomic DNA of the wild type and mutant cells confirmed that the recombination event had occurred, as the replacement of the gene sequence by the knock-out vector causes the shift from >12 kb signal in wt cells to a 8.5 kb signal in null mutants using the 5' probe. B) RT-PCR analysis using forward

primer in the ORF and reverse primer at a region which was replaced by the blasticidin resistance cassette in the mutants yielded the expected amplification of a 500 bp fragment in case of Ax2 cells and no amplification in the mutants. Both the *grlA*$^-$ clones (G1a3 and F2a1) were tested for transcripts at 16 h and 20 h of development. C) Southern blot depicting the band shift of 6.8 kb in case of the two independent *grlJ*$^-$ knockout clones (A4 and C9) with wild type displaying a signal at 4.6 kb after BglII digest. D) represents the confirmation of the same clones digested with EcoRI confirming the recombination event at the *grlJ* locus in the genome displaying a band shift of 2.2 kb with regard to the wild type due to insertion of the G418 resistance cassette. The probes are specified in Figure 15A and B respectively.

Southern blot analysis was also performed on the transformants to verify the recombination event at the right site. Genomic DNA isolated from Ax2 wild type cells and transformants was digested with EcoRI and probed with the 5' probe. EcoRI restricts at the begining of the *Bsr* gene giving a fragment of 8.5 kb in case of a recombination event whereas a fragment larger than 12 kb is obtained in case of the intact gene in wild type cells. Several positive clones were obtained which were further confirmed by digesting with BglII which cleaves the *Bsr* gene at the center. When probed with the *Bsr* gene as a probe a fragment of 3.0 kb and another at higher size (unknown) were obtained (data not shown). This served as the control at the C terminal end. Two independent clones G1a3 and F2a1 were selected for further studies. Similarly, the transformants for *grlJ*$^-$ were selected for several days with 4 µg/ml of G418 and cells were screened as described for the *grlA*$^-$. Digestion with EcoRI that cuts at either side of the gene in the genome locus gives a band that is 2.2 kb larger than the wild type cells due to the insertion of the G418 resistance cassette and BglII that cuts once in the gene and once outside the gene at the 3' end. Southern blots using the C-terminal probe confirmed the recombination event, which occurred in 95% of the transformants (Figure 16). Two independent clones A4 and C9 were used for further analysis.

3.4.2 Characterization of *grlA*$^-$ and *grlJ*$^-$ cells (Mutant Analysis)

3.4.2.1 Growth

Cell growth is the result of an interplay between various cellular processes and alteration in any of these processes affects the growth rate. We studied the growth behaviour of the mutants under different conditions. First we tested the growth of the mutants on bacterial plates with *Klebsiella aerogenes* as food source. The increase in the diameter of the colony was taken as a relative measure for growth. Both *grlA*$^-$ and *grlJ*$^-$ mutants exhibited growth on *Klebsiella aerogenes* comparable to Ax2 cells with a doubling of colony size of 24 hours. The results are from a representative experiment conducted twice in triplicates (Figure 17A).

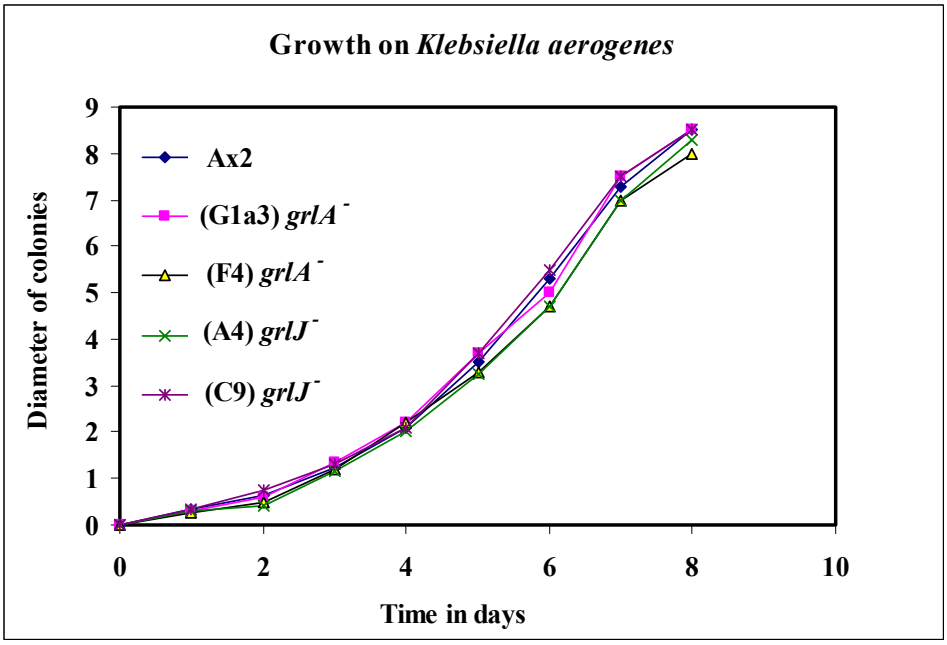


Figure 17A. Growth of *grlA*⁻ and *grlJ*⁻ on a bacterial lawn. SM agar plates were spread evenly with *Klebsiella aerogenes* and either Ax2, *grlA*⁻ or *grlJ*⁻ cells taken from the clearing zone of the respective strains were spotted at the center of the each plate. The diameter of the colony was taken as a measure of growth (Materials and Methods, 2.7.2).

Additionally both mutants displayed similar growth characteristics in suspension culture in axenic medium under similar conditions (Figure 17B) except that *grlJ*⁻ grew slightly faster and at a higher rate than the wild type cells.

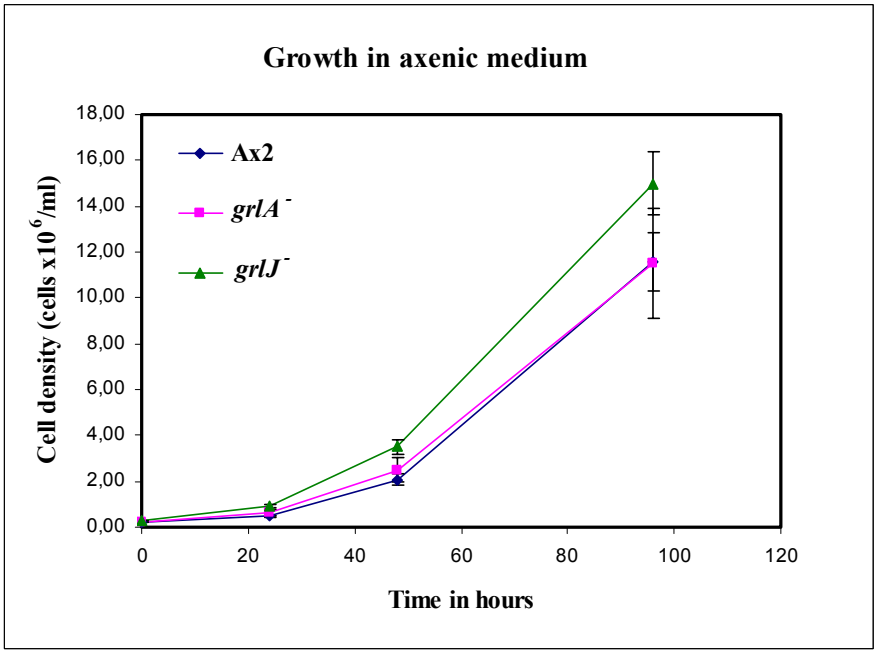


Figure 17B. Growth of *grlA*⁻ and *grlJ*⁻ in axenic medium. Cells at a starting density of $2x10^5$ cells / ml were allowed to grow at 21° C at 160 rpm and the cells were counted every 24 hours. The results

are obtained from three different sets of experiments carried out in triplicates (Materials and Methods 2.7.2).

3.4.2.2 Cytokinesis in *grlA*⁻ and *grlJ*⁻

Cytokinesis is one of the important processes required for cell survival. It was studied by counting the nuclei/cell after fixing the cells and staining with DAPI, a dye to stain the

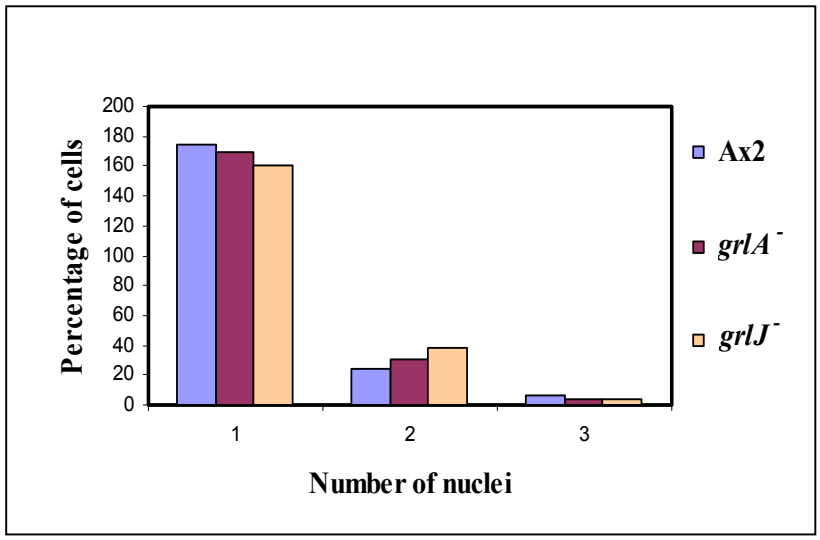


Figure 18. Quantitation of nuclei in *grlA*⁻ and *grlJ*⁻. A histogram was obtained by scoring the cells (200 each from wild type and the respective mutants) for the number of nuclei they possess (Materials and methods, 2.7.4).

nuclei. The number of nuclei per cell was quantitated and compared to wild type Ax2 cells using a fluorescent microscope. Most of the cells were mononucleated and a relatively decent number of cells was binucleated or multinucleated comparable to the wild type cells. These observations suggest that loss of GrlA or GrlJ does not affect the process of cell division in the amoeba.

3.4.2.3 Measurement of cell size in the mutants (Rivero et al., 1996)

Dictyostelium strain Ax2 and the respective mutants were grown to a density of 2-3 x 10^6 cells / ml and treated as described (Materials and Methods 2.7.1). As observed in Figure 19, *grlA*⁻ cells were slightly smaller in size as compared to Ax2 cells but the size difference was not very significant. Lack of GrlJ had no significant effect on the cells size either. Also, the cell morphology remained unaltered in both the mutants (data not shown).

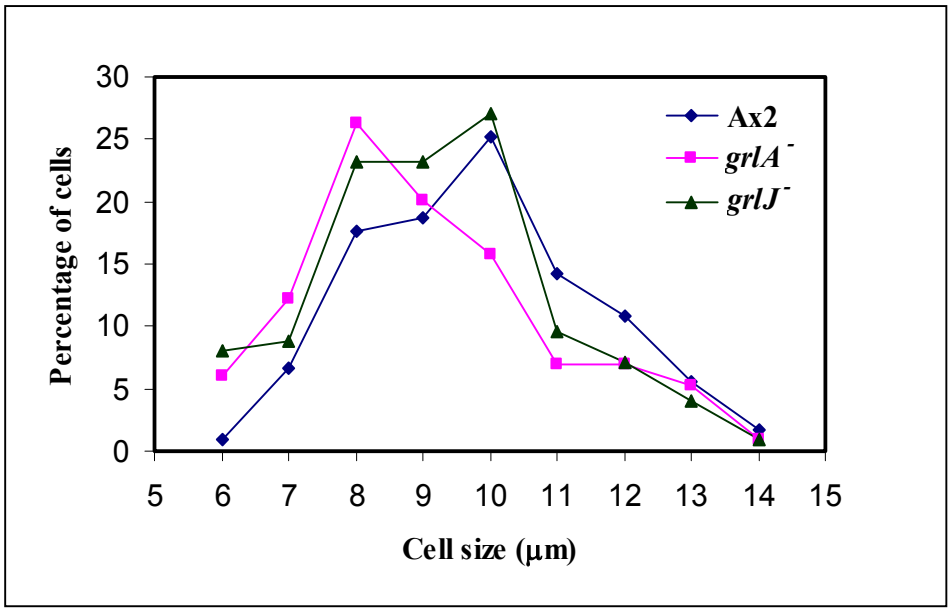


Figure 19. Cell size determination. Log phase cells (wild type and the respective mutants) were harvested and treated as described in Materials and Methods, 2.7.1. The results depicted are from a representative experiment.

3.4.2.4 Resistance to osmotic shock in the *grlA*⁻ and *grlJ*⁻ cells

Dictyostelium discoideum lives as a natural phagocyte in soil and feeds on yeast and bacteria. Therefore fluctuations in the environmental temperature, humidity and osmolarity pose physiological challenges to growth and survival of these freely living organisms. With

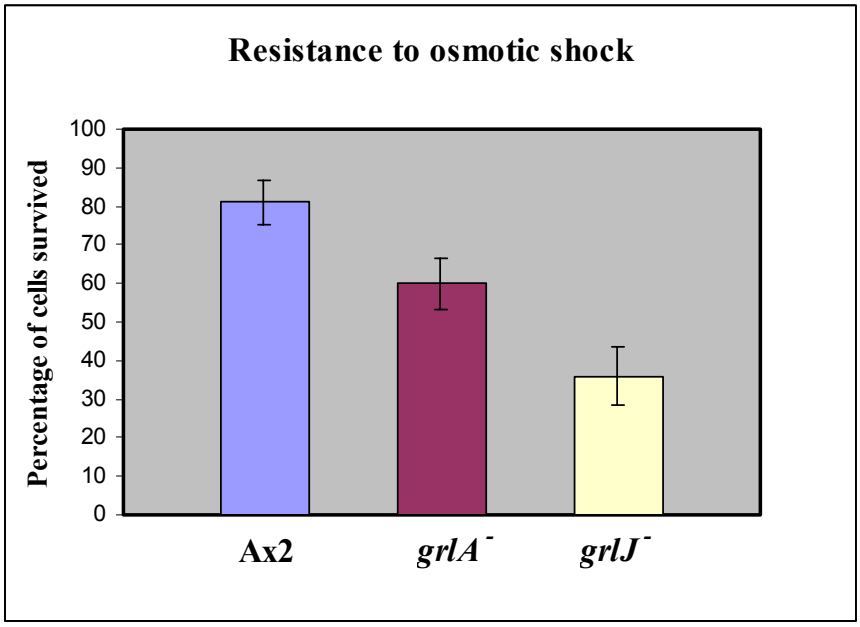


Figure 20. Response of Ax2 and *grlA*⁻ and *grlJ*⁻ to osmotic shock. The viability of the cells was observed after treatment with 0.4 M Sorbitol for 2 h followed by dilution in low osmolarity buffer and

plating 200 cells per plate along with Klebsiella aerogenes (Materials and Methods 2.7.6). The values are obtained as a result of three independent experiments carried out in triplicate. Bars indicate standard deviation.

the help of complex set(s) of proteins during growth, early development and morphogenesis, *Dictyostelium* facilitates repair and recovery of cells in response to high osmolarities (Kwon and Handler, 1995). We therefore studied the response of the mutants towards osmotic shock in comparison to Ax2 wild type cells to learn whether the receptors under investigation are involved in the osmotic shock respone in these amoebae (Materials and methods, 2.7.6). *grlJ*$^-$ were highly sensitive to osmotic shock (Figure 20) as only 36% of the cells survived treatment with 400 mM sorbitol compared to Ax2 cells of which 81% recovered after a similar shock. *grlA*$^-$ were also more sensitive towards osmotic shock response (60% survival rate) suggesting a possible role of these receptors in shock response.

3.4.2.5 *grlA*$^-$ and *grlJ*$^-$ sense folic acid as a chemoattractant

Folate and its analogues are long known chemoattractants for *Dictyostelium.* The folate-mediated pathway acts both as foraging response to identify food (bacteria) and to control certain aspects of multicellular differentiation (van Ophem and van Driel., 1985). There are two classes of developmentally regulated FARs (Folic Acid Receptors) with different binding specificities that are coupled to the $G_\alpha4$ (Hadwiger et al., 1994). Growth stage cells have a class of FARs that binds folate but not monapterin, whereas 14 h starved cells (tipped aggregate stage) have two classes of receptors: one that is compatible with

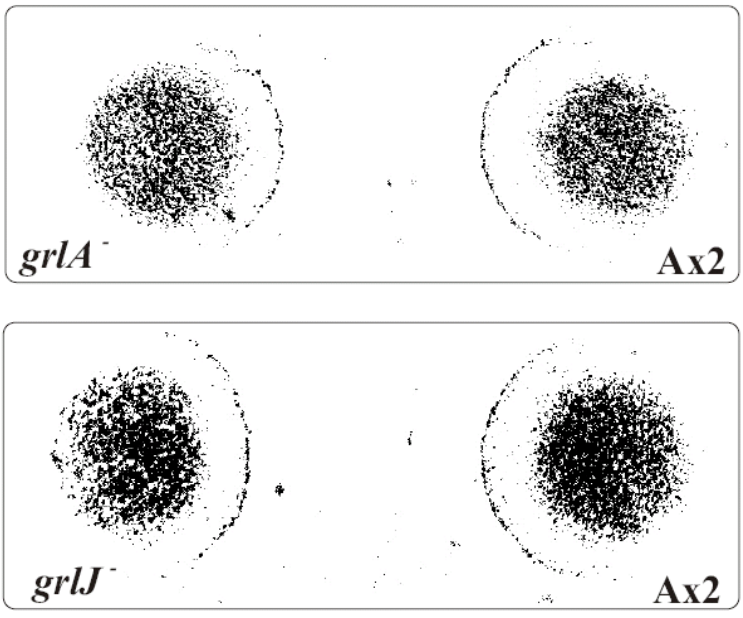


Figure 21. Folate chemotaxis displayed by two-drop assay. Ax2 cells and the respective mutants were harvested at the log phase of growth and resuspended at a density of $1x10^7$ cells/ml. 1 µl cells ($1x10^6$) was spotted on either side of the drop containing 1 mM folic acid on phosphate agar plates and allowed to dry slightly. Chemotaxis towards folate was monitored for a period of two hours using an Olympus stereomicroscope.

monapterin and the other only with folate. The early class is represented as αFAR and the receptors that bind monapterin are being classified as βFAR. Although the existence of folate receptors is known for a long time, they have not been identified. We therefore studied whether the cells lacking any of these GPCRs under investigation were capable to sense folate as a chemattractant. We carried out a simple two-drop assay wherein a drop of folate (1 mM) was placed at the center of drops on either side one from the wild type Ax2 cells and the other from the respective mutants. Equal densities of cells ($1x10^6$) were placed at equidistance from the spot of folate. Movement towards the chemoattractant source was monitored for a period of two hours and images were captured using an Olympus stereomicroscope. Our results from the qualitative two-drop assay revealed that both GrlA and GrlJ were not involved in the folate-mediated response during growth. The importance of these receptors in the signal transduction during *Dictyostelium* morphogenesis has not been addressed.

3.4.2.5 GrlA and GrlJ are not involved in phototaxis

Chemotactic aggregation of starving *Dictyostelium* cells leads to formation of a motile, multicellular organism, the slug, whose anterior tip controls its phototactic and thermotactic behaviour (Fisher et al., 1997). We therefore studied the behaviour of these

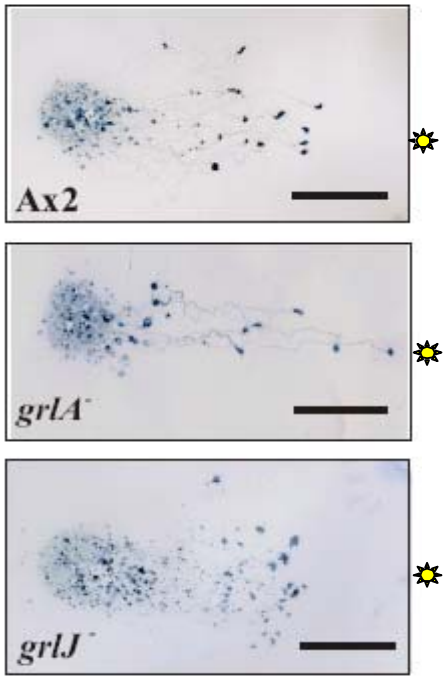


Figure 22. Phototaxis in *grlA*$^-$ and *grlJ*$^-$ cells. Wild type Ax2 and the respective mutant cells were harvested at log phase of growth and resuspended at $1x10^8$ cells/ml and 10 µl containing $1x10^6$ cells were placed at the center of water agar plates and were allowed to dry slightly. The plates were then placed in a dark box containing a slit of 3 mm and Incubated at 21^o C for 48 h. The slugs were transferred to nitrocellulose membranes and slugs and their slime trails were stained with amido black dye and studied for their movement towards the light source. The sun at the right hand side depicts the direction of the light source. **Bar, 1 cm.**

amoebae in the absence of either GrlA or GrlJ. All the strains were capable of phototaxing directionally towards the light source. The directional movement of the mutants was visually comparable to the one of Ax2 cells. The results implied that neither GrlA nor GrlJ are involved in phototaxis (Figure 22).

3.4.3 Development of *grlA*$^-$ and *grlJ*$^-$

Dictyostelium have a tendency to live as a single celled organism until they encounter unfavourable conditions like starvation upon which they undergo development wherein they aggregate into multicellular mounds that differentiate to form a migratory slug and then eventually a fruiting body (a mass of spores on top of a stalk) (Coates and Harwood, 2001).

3.4.3.1 Aggregation on solid surface

Wild type Ax2 cells and the mutants (*grlA*$^-$ and *grlJ*$^-$) were developed under submerged conditions at equal densities (Materials and Methods, 2.7.3.1) and monitored every hour under the microscope and images were captured subsequently. Both the mutants were able to form aggregates and stream towards an aggregation centre comparable to the wild type cells (Figure 23). This indicates that the cAMP synthesis and relay are not disturbed in the absence of either GrlA or GrlJ during early aggregation.

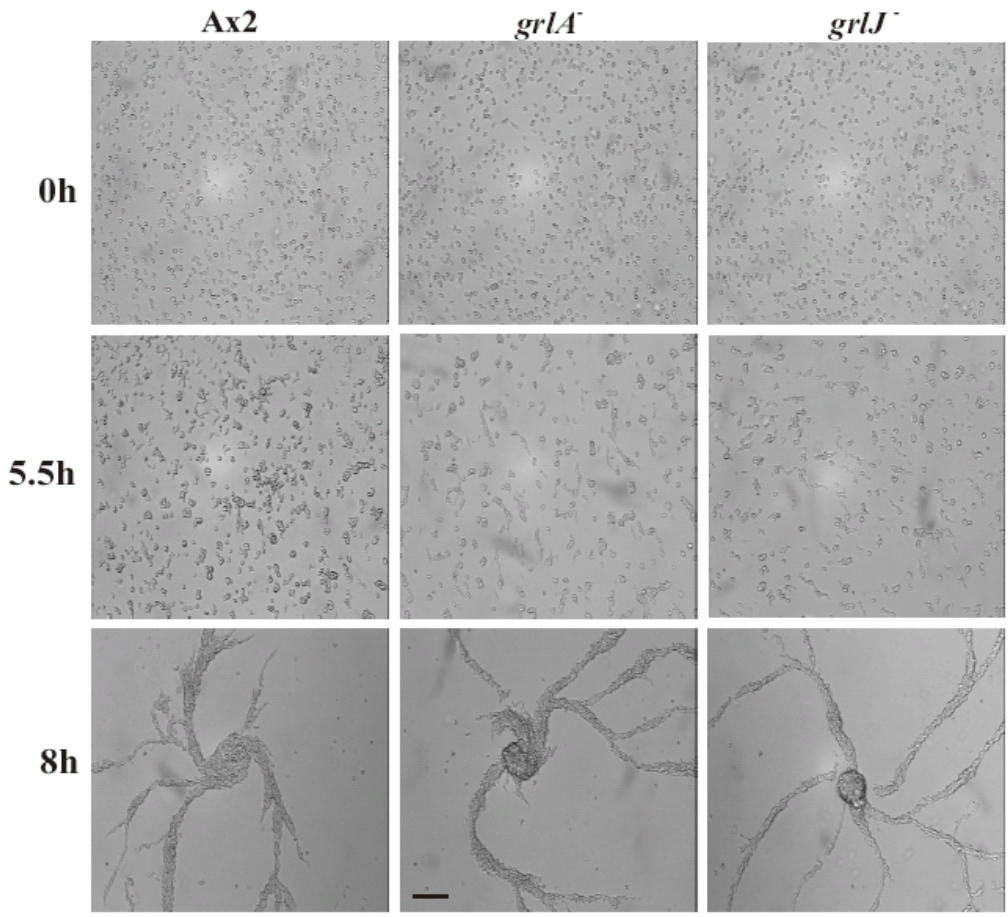


Figure 23. Aggregation on plastic surface. Cells (Ax2, *grlA*$^-$ and *grlJ*$^-$) at equal densities (2 x 10^5 cells/cm^2) were starved submerged on plastic plates and monitored for aggregation. Images were taken every hour (10X magnification) until 8 hours. **Bar, 100 μm.**

3.4.3.2 Cell motility in *grlA*$^-$

We further went on to analyse cell motility and behaviour in response to exogenously provided cAMP. These studies were carried out for Ax2 and *grlA*$^-$ cells. Aggregation competent cells were allowed to migrate towards a micropipette filled with 0.1 mM cAMP (Figure 24) and time-lapse image series were captured to generate migration paths and calculate various parameters like speed, persistence, directionality and direction change (Table 4). Within seconds of exposure to cAMP, the cells were able to orient themselves towards the chemoattractant source and then they moved towards the point source and eventually organized into streams in a head to tail fashion. Both strains were polarised, formed streams and migrated at a speed of 10.72 +/- 3.6 μm/min and 10.35 +/- 3.45 μm/min, respectively. Directionality towards the source was around 0.58 for the mutant and 0.61 for the wild type. Likewise the persistence values were similar (3.12 +/- 1.6 for *grlA*$^-$ as

compared to 3.14 +/- 1.6 for Ax2). Taken together, chemotactic motility is not altered in *grlA*$^{-}$ cells.

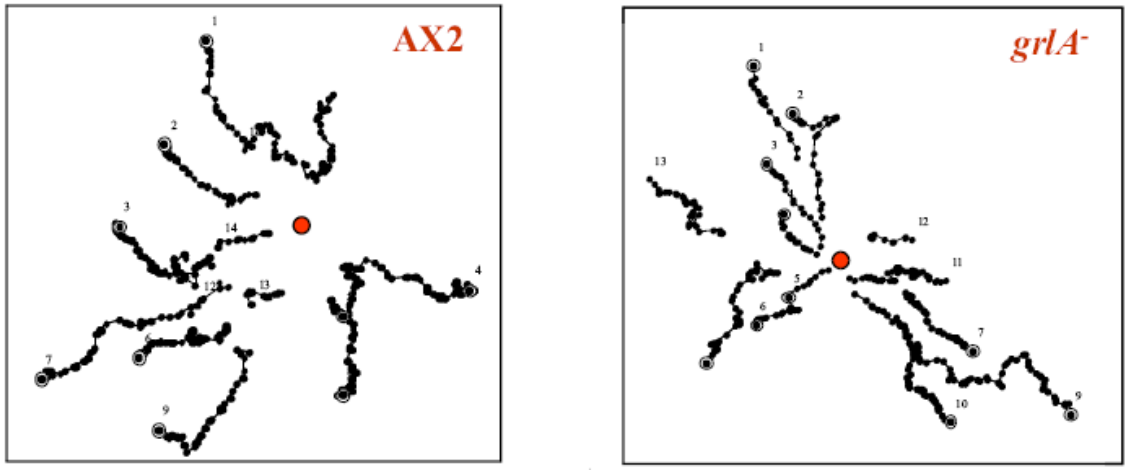


Figure 24. Chemotaxis of Ax2 and *grlA*$^{-}$ cells. Respective log-phase cells were harvested, washed and developed for 5-6 h in shaking suspension to render them aggregation competent before they were challenged with cAMP in a chemotaxis assay. The time-lapse images captured were processed using DIAS software. The cell paths illustrated are representative of three independent experiments and the red dot signifies the position of the micropipette filled with cAMP.

cAMP	**Ax2**	***grlA*$^{-}$**
Speed (µm/min)	10.729 +/- 3,6	10.346 +/- 3.45
Directionality	0.6135 +/- 0.21	0.5872 +/- 0.2
Direction Change (deg)	32.688 +/- 13.4	36.073 +/- 15.22
Persistence (µm/min-deg)	3.1414 +/- 1.64	3.1280 +/- 1.6
Buffer	**Ax2**	***grlA*$^{-}$**
Speed (µm/min)	5.233 +/- 1.9	6.11 +/- 2.26
Directionality	0.34 +/- 0.2	0.34 +/- 0.16
Direction Change (deg)	53.79 +/- 14.42	51.78 +/- 13.70
Persistence (µm/min-deg)	1.29 +/- 1.07	1.45 +/- 0.75

Table 4. Analysis of cell motility in *grlA*$^{-}$. Cells were prepared as mentioned before (Figure 20B). Time-lapse image series were captured and stored on a computer hard drive at 30 sec intervals. The DIAS software was used to trace individual cells along image series and calculate motility parameters. **Persistence** is an estimation of movement in the direction of the path. **Directionality** is calculated as the net path length divided by the total path length and gives 1.0 for a straight path. **Directional change** represents the average change of angle between frames in the direction of movement. Values are mean +/- standard deviation of around 50 cells from at least 3 independent experiments.

3.4.3.3 Unaltered expression of aggregation-specific genes in *grlA*⁻ and *grlJ*⁻

Additionally, we carried out Northern blot analysis to study the expression of genes required for aggregation such as the cyclic AMP receptor gene *cAR1* and adenylyl cyclase gene *ACA* which are one of the first genes upregulated after initiation of development (Figure 25A). The mRNA of the *cAR1* gene was present at very low levels at the vegetative phase and increased significantly within 4 h of the onset of starvation in both mutants as compared to Ax2. Also, the *ACA* transcript was detectable in the mutants at the first 4 h of starvation as in Ax2. GrlA and GrlJ may therefore have no specific role in aggregation. However, the *cAR* receptor mRNA was drastically reduced in *grlA*⁻ at later stages pointing out a possible role of GrlA in late development. In fact, *Dictyostelium* harbours 4 genes coding for cAR receptors which are expressed during development in a spatial and temporal pattern (Saxe et al., 1993,1996; Louis et al., 1994). Lower transcript levels as observed in northern analysis (Figure 25A) for the *cAR1* in *grlA*⁻ indicates a probability of lower expression of one or more of the late receptors for cAMP.

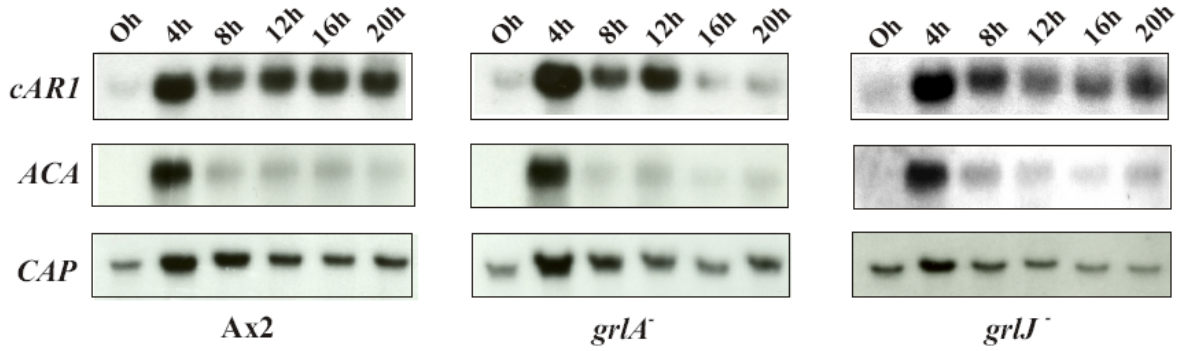


Figure 25A. Northern blot analysis of aggregation- specific genes. Total RNA was isolated from Ax2 cells and the respective mutants at indicated time points. 20 µg RNA were separated on 1.2% gels under denaturing conditions (6% formaldehyde) and transferred to membranes as described in Sambrook et al. (1989). cDNA probes coding for the indicated transcripts were used for hybridization. A CAP cDNA probe was used as a control. The CAP gene is transcribed throughout development and shows an increase in expression at the onset of development.

Furthermore the phosphodiesterase *pdeE* and the early aggregation marker contact sites A (*csA*) behaved similarly in *grlA*⁻ (Figure 25B). Taken together, *grlA*⁻ and *grlJ*⁻ cells do not display aggregation defects, as they were able to sense and relay cAMP and aggregate into streams towards the signaling center.

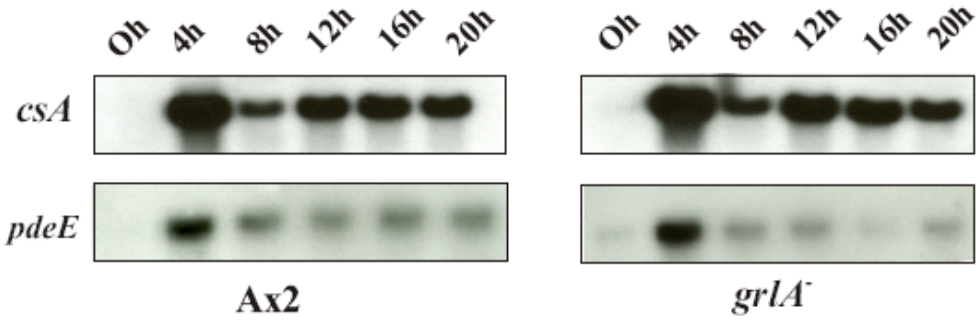


Figure 25B. Expression of contact sites A (*csA*) and *pdeE* genes in *grlA*¯. Total RNA obtained as mentioned in Figure 25A, was separated over a denaturing formaldehyde agarose gel. The same blots were used for probing with a cAMP diesterase, *pdeE,* and contact sites A (*csA*) specific probe.

3.4.3.4 Role of GrlA and GrlJ in post aggregation differentiation

3.4.3.4.1 Development on phosphate agar plates

GrlA and GrlJ are expressed throughout development with a marked induction of the expression at later developmental stages (Figure 13). In order to determine whether these receptors have a role in post aggregation stages in *Dictyostelium* development, we studied the development of mutants. Phosphate buffered agar is the commonly used substratum to study development in *Dictyostelium*. An equal number of cells ($5x10^6$ cells/ cm^2) each of *grlA*¯, *grlJ*¯ and Ax2 were plated onto phosphate agar plates and monitored for development under a stereomicroscope for a period of 24 hours and images were captured every 4 hours. As indicated in Figure 26, the wild type cells formed aggregates after 6 hours of starvation followed by formation of finger like structures (after 12 h) that migrated as slugs (16 h), then culminated (20 h) and eventually formed into mature fruiting bodies containing a stalk supporting a mass of spores after 24 h of starvation. Both mutants aggregated well and formed tight mounds similar to wild type cells but later on differences were observed. *grlA*¯ remained as mounds even after 14 h of starvation and finger like structures appeared only at 16 h and formed very few slugs at 18-19 h which culminated only at 22-24 h of starvation. Together there was a delay in development of around 4 h in *grlA*¯ from the tipped aggregate stage onwards. However, the *grlA*¯ strain formed normally shaped fruting bodies. In contrast, *grlJ*¯ showed an accelerated development after the aggregation stage and slugs were formed at 12 h after initiation of development which culminated at 16 h and formed small fruiting bodies at 18 to 20 h (Figure 26).

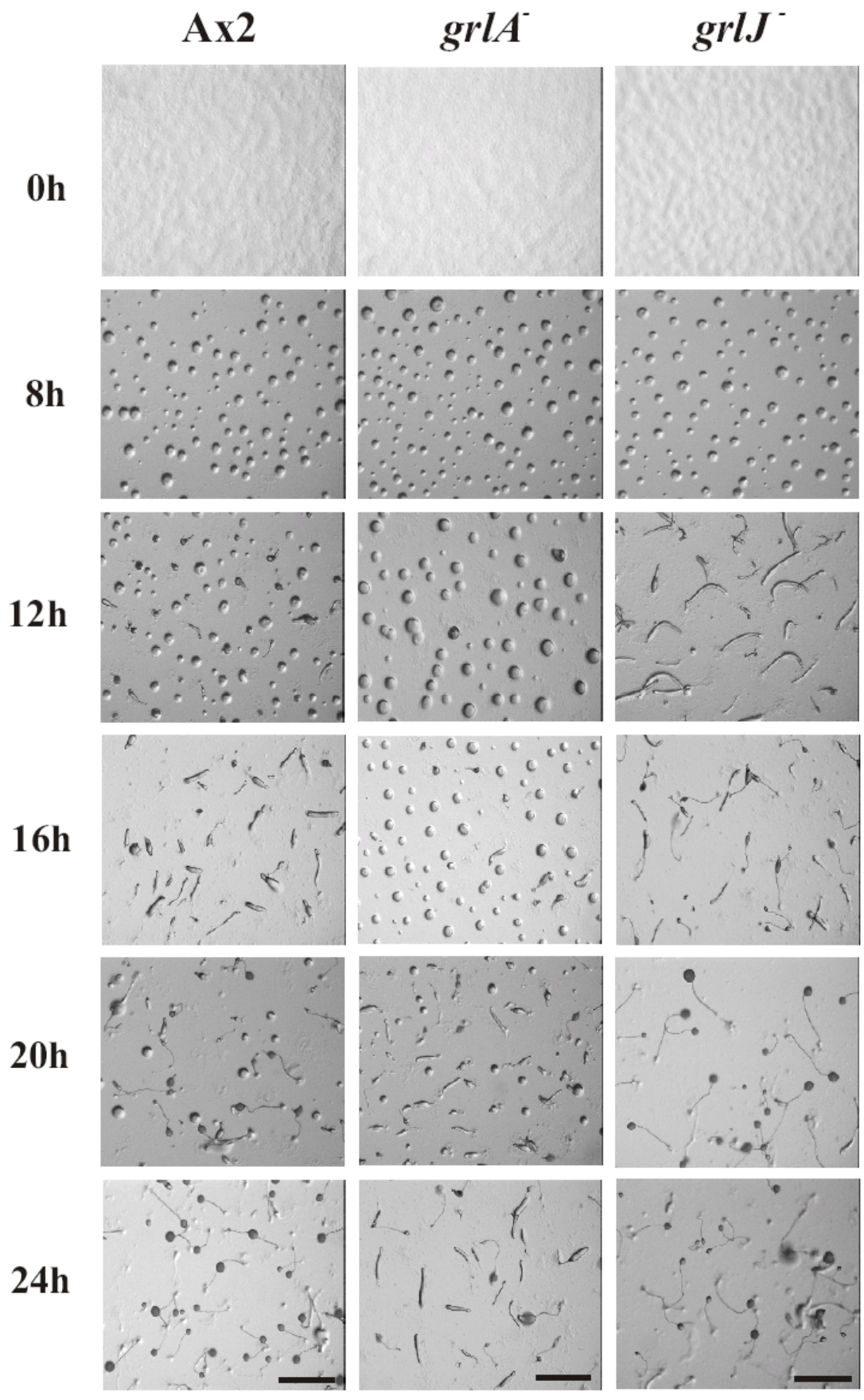

Ax2
grlA-
grlJ-
0h
8h
12h
16h
20h
24h

Figure 26. Development on phosphate agar plates. Axenically growing cells (Ax2, *grlA*⁻ and *grlJ*⁻) were prepared as described (Materials and Methods, 2.7.3.2) and plated at a density of $5x10^6$ cells/cm2 and were monitored throughout development. Images were captured every 4 hours using the Olympus Stereomicroscope. **Bar, 1 mm.**

Taken together, these observations place both the receptors under study in a developmental pathway probably acting as a positive and negative modulator of morphogenesis.

3.4.3.4.2 Gene expression profile during post aggregation differentiation

To confirm these observations further, we performed northern blot analysis for late developmental markers. cDNAs for a prestalk specific gene, *ecmB*, and a prespore-specific gene, *pspA*, were used as probes for hybridization. We detected a delay in the expression of the prestalk specific *ecmB* gene and observed also a marked reduction in the expression level even after 20 h of development in case of *grlA*⁻. *grlJ*⁻ displayed a stronger peak of expression of *ecmB* transcript at 12 h compared to the wild type cells where the transcript started appearing slightly at 12 h and shooted up at the slug stage (Figure 27A). The expression of *pspA* was timely but higher as compared to the wild type which is in agreement with the *grlJ*⁻ phenotype. Additionally, we studied the expression pattern of the late low affinity cAMP receptor, *carB*, which is first expressed in mounds and subsequently preferentially expressed in the front of the slugs where pstA cells are found (Saxe et al., 1996).

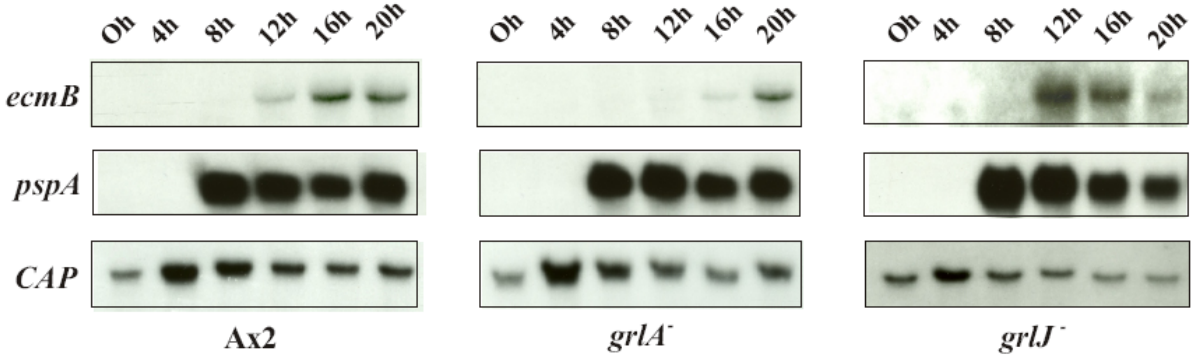


Figure 27A. Northern blot analysis of prespore and prestalk specific genes. Total RNA was isolated from the wild type Ax2 and the respective mutants at indicated time points. 20 µg RNA were separated on 1.2 % gels under denaturing conditions (6% formaldehyde) and transferred to membranes as described (Sambrook et al., 1989). cDNA probes of genes specific for the indicated transcripts were used for hybridizing the blots. A CAP cDNA probe was used as a control.

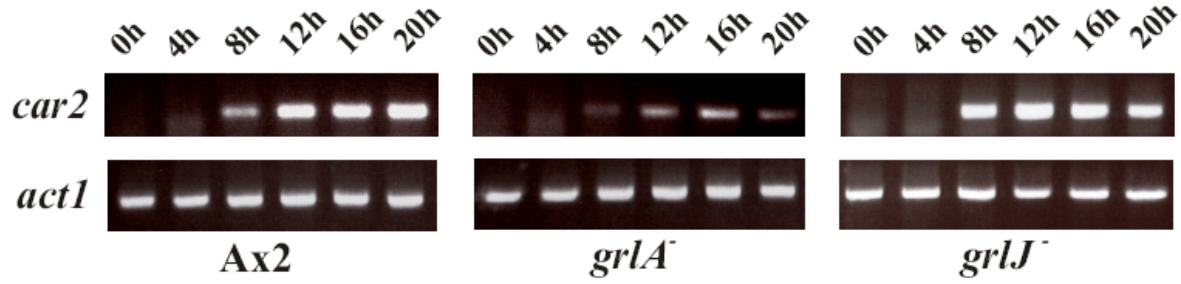


Figure 27B. RT-PCR depicting the expression of stalk specific *carB* (cyclic AMP receptor 2). RNA from the above time course experiment was used to generate single stranded cDNA (Materials and Methods, 2.4.5), which was then used as a template to amplify the *car2* transcript.

As depicted in Figure 27B, *car2* is expressed at the mound stage and the expression increases in the late developmental stages when the amoebae undergo differentiation. *grlA*$^{-}$ shows a strong reduction in the transcript level whereas *grlJ*$^{-}$ expresses *car2* much earlier at the mound stage and at a much higher level as compared to the wild type cells. Taken together, these results suggest a role of grlA and grlJ in the morphogenesis of *D. discoideum* but their action being independent of each other probably pointing towards the receptors being functional in different pathways that govern post aggregation differentiation and cell sorting.

3.4.4 GrlA and GrlJ in adhesion

Adhesion can be between cells or cell-substratum that involves cycles of making and breaking cell contacts with the extracellular matrix and while moving in tissues to make and break contacts with other cells (Bukahrova et al., 2005).

3.4.4.1 Cell-substrate adhesion in *grlA*$^{-}$ and *grlJ*$^{-}$

There are many well-studied adhesion molecules in *Dictyostelium* that play a role either during growth or development and accordingly they have a distinct pattern of expression. We analysed grlA and grlJ mutants have any significant alteration in the adhesion. As observed in Figure 28, lack of either of the receptors did not affect the cell-substratum adhesion considerably depicting no possible role of the receptors in this mode of adhesion. However, as *grlA* and *grlJ* are expressed at later developmental stages it will be further interesting to study the cell-substratum adhesion at a late developmental time point probably at the migratory slug stage.

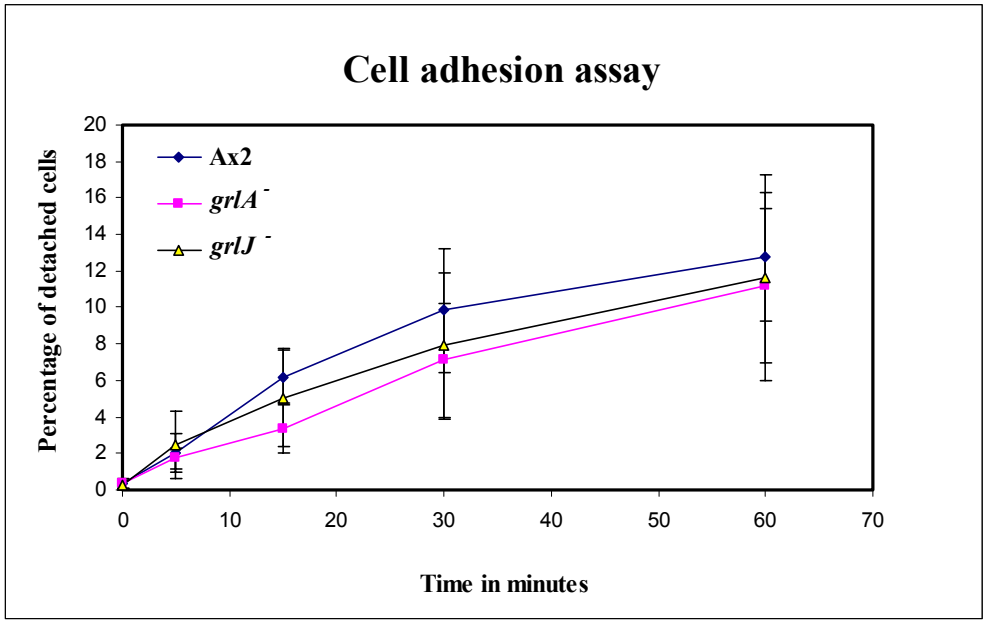


Figure 28. Cell adhesion assay (Fey et al., 2002). For the adhesion assay, vegetatively growing cells (wild type Ax2, *grlA*$^-$ and *grlJ*$^-$) were placed at 2x10^5/ml in axenic medium and incubated overnight at 21^{o}C. The assay was carried out as described in Materials and Methods, 2.7.7. Error bars represent standard deviation.

3.4.4.2 Cell-cell adhesion

Dictyostelium multicellularity is maintained by expression of several cell adhesion systems. There are two major types of cell-cell adhesion, the EDTA-sensitive and EDTA-resistant cell-cell contacts (Gerisch, 1961). During the initiation of development, DdCad-1 (gp24), a small, secreted glycoprotein with similarities to vertebrate cadherins, mediates EDTA-sensitive cell-cell adhesion (Brar and Siu, 1993; Wong et al., 1996). At the onset of aggregation, expression of gp80/contact sites A (csA) leads to EDTA-resistant cell-cell adhesion (Noegel et al., 1986; Siu et al., 1985). In the post-aggregation stage, gp150 mediates EDTA-resistant cell-cell adhesion (Gao et al., 1992; Wang et al., 2000). Upon starvation, another developmentally regulated adhesion molecule, AmpA, is secreted. This protein is thought to function as an anti-adhesive to limit cell-cell and cell-substrate adhesion during development (Varney et al., 2002).

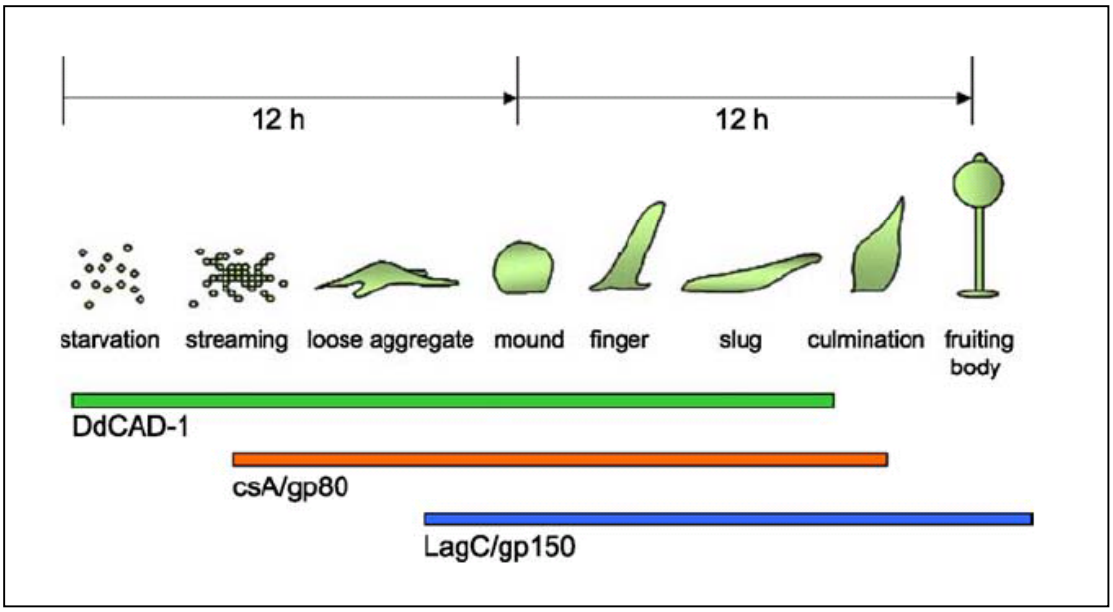


Figure 29A Ilustration of the developmental cycle of *Dictyostelium*. Expression of CAMs (cell adhesion molecules) during development is shown below: DdCAD-1 (green), gp80 (red), and gp150 (blue) **(Siu et al., 2004)**.

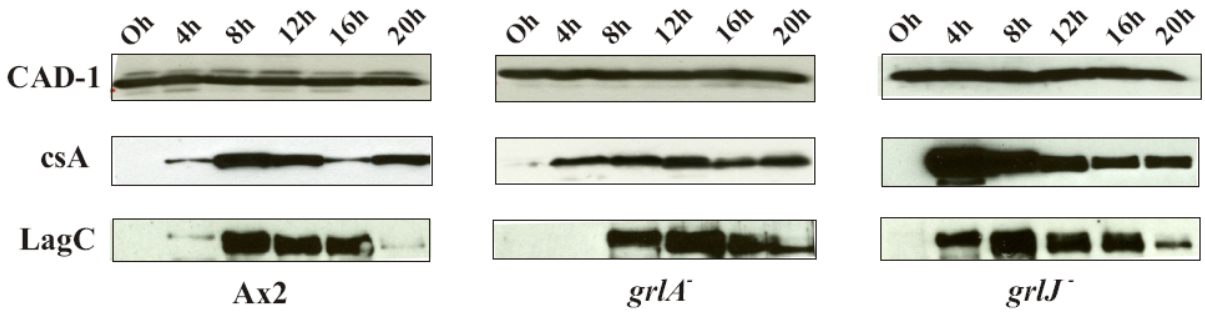


Figure 29B. Western blot analysis of cell adhesion molecules. Protein samples were obtained at different time points during vegetative growth and development from Ax2 cells and the mutants. They were separated on a SDS-PAGE gel (Materials and Methods, 2.6.1) and transferred using tank blot. The blots were then probed with respective polyclonal antibodies specific for ddCAD-1, and gp-150 (LagC), csA was recognized by mAb 33-294.

We analysed the protein lysates obtained from a time course of development and probed them with ddCAD-1 (Cadherin), csA (contact sites A) and gp150 (LagC) specific antibodies, respectively. *grlA*$^-$ and *grlJ*$^-$ displayed comparative expression of cadherin as seen from the western blot analysis, the contact sites A expression remained unchanged in *grlA*$^-$ whereas *grlJ*$^-$ displayed peak expression of the protein at 4 h itself and also LagC expression levels were comparatively high during early aggregation reflecting the premature aggregation behaviour (Figure 29B).

3.4.4.3 Localization of contact sites A in the mutants

During chemotactic migration, cells become elongated and covered with a large number of membrane protrusions. Initial contacts are made through filopodia and lammelipodia that emanate from the leading edge of migrating cells when the cells enter the aggregation stage. Subsequently, stable contacts are established through interdigitation and retraction of filopodia mediated by contact sites A/CsA/gp80. CsA is present both on end-to-end contacts and side-to-side contacts (Siu et al., 2004). When we studied the localization of CsA in the mutants during aggregation (6 h) with mAb 33-294, we found CsA properly localized to the contacts in both mutants suggesting that they would have no significant defect in cell contacts. Figure 30 is an illustration of end-to-end contact in the amoebae. Taken together, it can be concluded that grlA would have no significant role in cell adhesion while *grlJ*$^{-}$ cells show precocious expression of LagC and higher levels of expression of CsA. However, the localization of csA in *grlJ*$^{-}$ cells remains largely unchanged.

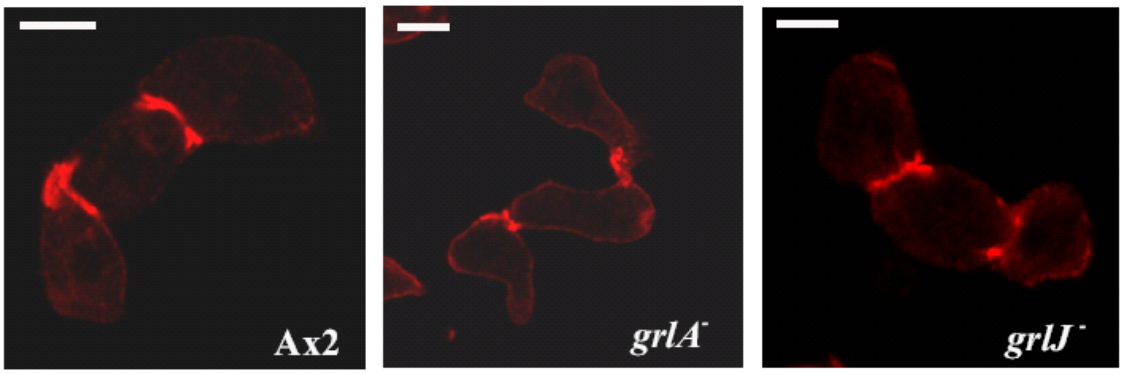


Figure 30. Localization of contact sites A in *grlA*$^{-}$ and *grlJ*$^{-}$ cells. Axenically growing cells were harvested, washed twice and resuspended at a density of 1x10^{7} cells/ml in Soerensen phosphate buffer and shaken for 6 h at 160 rpm at 21^{o}C. They were then disggregated, fixed in cold methanol and stained with mAb 33-294 for csA followed by Cy3 conjugated secondary antibody. Confocal images were captured. CsA is present at the plasma membrane and is strongly enriched at cell-cell contacts. Bar, 5 μm.

3.4.5 *Dictyostelium* morphogenesis

3.4.5.1 Slug analysis (neutral red staining) (Raper 1940)

Neutral red specifically stains the acidic vacuoles in the prestalk cells. This fact was exploited to study the distribution of highly vacuolated stalk cells in the amoebae during morphogenesis. Ax2 cells and *grlA*$^{-}$ and *grlJ*$^{-}$ were developed on phosphate agar plates and the slugs were photographed to visualise the distribution of the vital dye. The stalk cells were patterned at the anterior of the slug and neutral red positive cells were also found in the rear of the slug. We measured also the slug size using DISKUS software program that allows

determination of asymmetrical surfaces. 20-40 slugs per strains were marked and measured for their length and dye distribution at the anterior and rear end of the slugs. As depicted in Figure 31, the *grlA*⁻ slugs were comparable to the wild type and also the percentage of stalk cells at the anterior and posterior part of the slugs were similar. By contrast, *grlJ*⁻ produced long slugs. The pattern of neutral red stained cells was nearly normal except for a slightly reduced distribution at the base of the slugs.

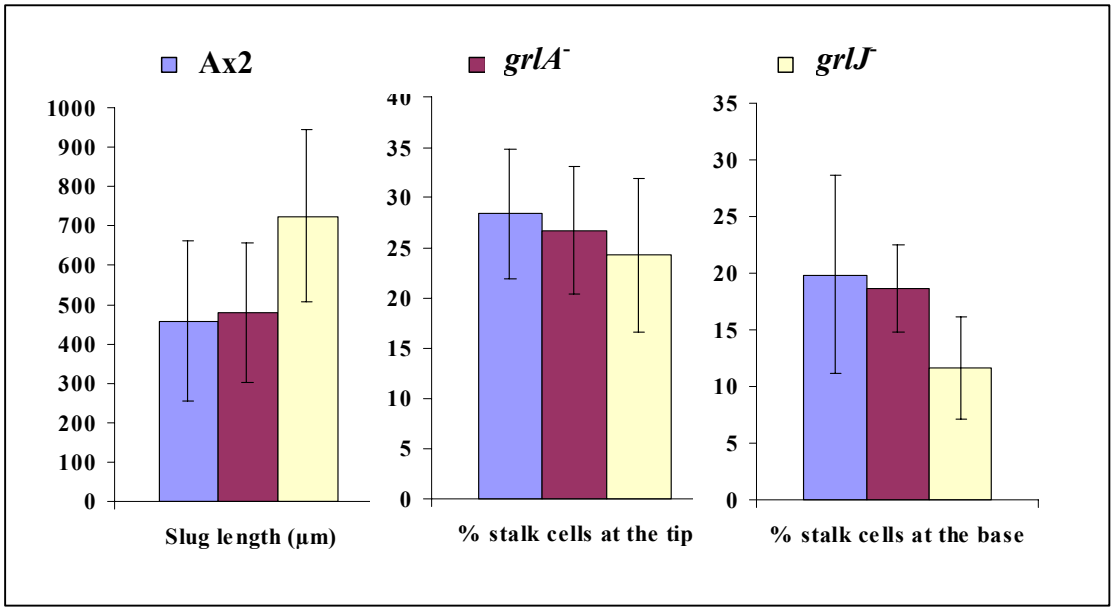


Figure 31. **Slug analysis**. Log-phase axenically growing cells were prepared as described in Materials and methods, 2.7.8. They were then photographed with an inverted stereomicroscope and around 50 slugs per strain were marked with DISKUS software program that allowed measuring uneven objects. The y-axis in the figure illustrates the length of the slugs and percentage distribution of the dye at the tip and the rear end of the respective slugs**.**

3.4.5.2 Slug migration

3.4.5.2.1 *grlJ*⁻ slugs leave behind traces during migration

Dictyostelim slugs migrate until they find a favourable condition to culminate. The stalk cells are comparatively more motile than the prespore cells and they form the tip of the slug acting as a signalling centre and organizer (Dormann and Weijer, 2001). A very high level of interaction and coordination occurs between the two cell types in the migrating slugs and they have to make a decision about the right time to stop migration and start culmination (Chisholm and Firtel, 2004). We traced the path of slug migration by performing standard phototaxis assay (Figure 22). Both strains migrated towards light as seen in Figure 32A and 32C. However, we observed an interesting pattern of breaking up of slugs in case of the *grlJ*⁻ strain which was clearly viewed in the enlarged version indicated by the arrows pointing

towards the breaks just in the path of one migrating slug. This pattern was observed for most of the slugs traced. Large aggregates usually break and so do slugs to avoid formation of bulky fruiting bodies**.** *grlJ*¯ forms long slugs as well (Figure 31) which may break up on their way to culmination (Roisin-Bouffay et al., 2000).

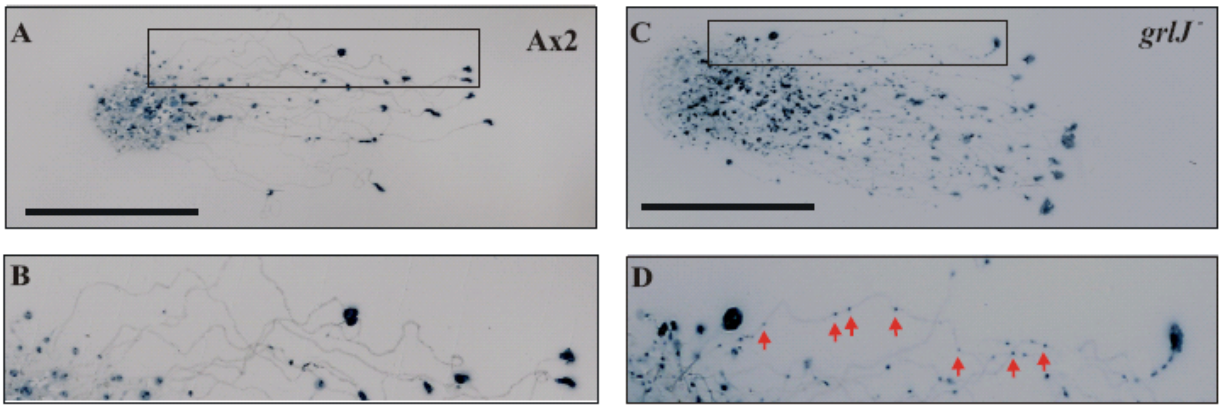


Figure 32. Slug migration by phototaxis. Ax2 and *grlJ*¯ cells were harvested and used for a phototaxis experiment as described in Figure 22. Both strains formed slugs phototaxing towards the light source. Figure **32B** depicts the enlarged version of the box in Figure **32A** which shows the path of the migrating Ax2 slugs. Whereas Figure **32D** shows the enlarged portion of the box in Figure **32C** which displays the pattern of slug movements in *grlJ*¯. As clearly indicated by the arrows, the slug broke several times on its way.

3.4.5.2.2 Breaking slugs monitored in *grlJ*¯

We confirmed the breaking pattern of *grlJ*¯ slugs during migration by applying live cell microscopy wherein we captured time-lapse images of the *grlJ*¯ development and compared it with the wild-type development. Images were captured with a CCD camera every 6 min for a period of 24 hours and were then converted into a movie using the ImageJ software from NIH. There is one slug each represented in the clips that is representative of the general pattern seen. Figure 33A, depicts the slug migration state during development of the wild type Ax2 cells. The red arrows in the figure display the rear part of a representative wild type slug that was tracked for migration. The yellow arrows show the part after the slug had migrated.

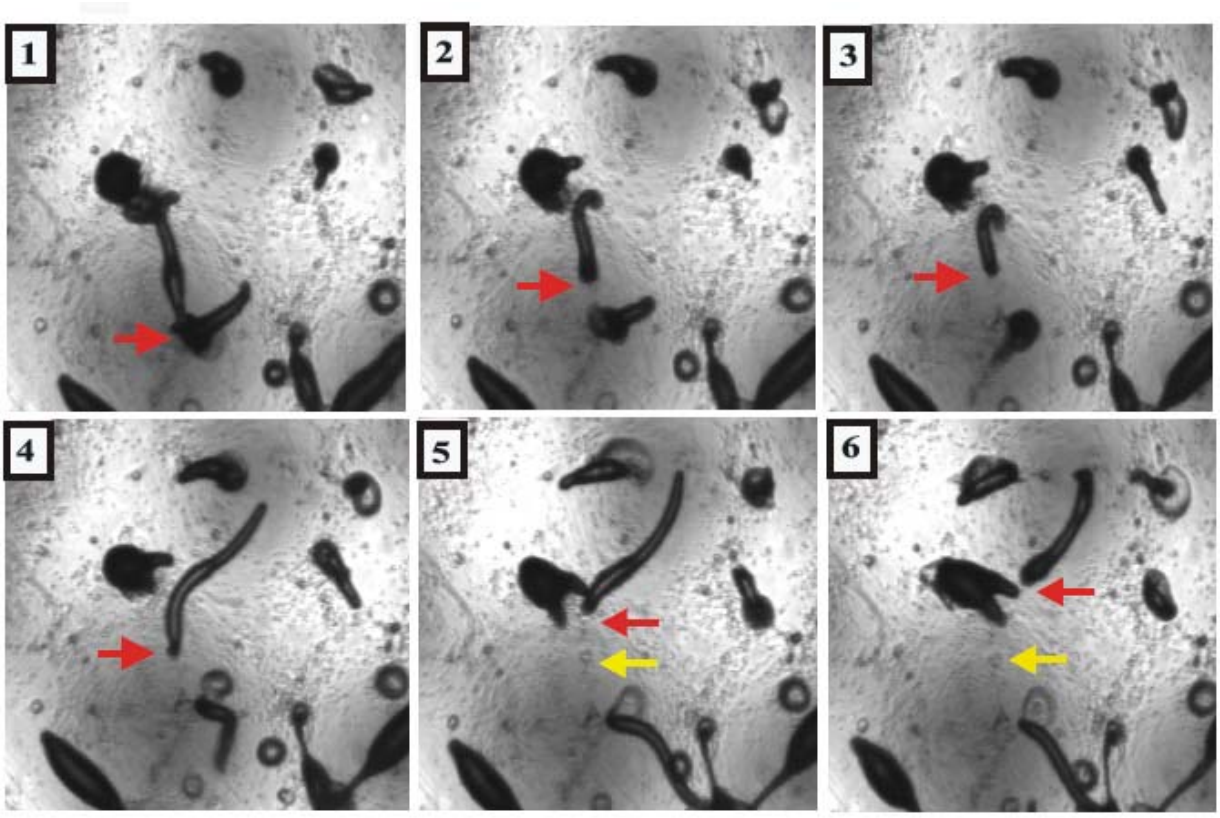


Figure 33A. Movie clips from developing wild type Ax2 cells. The images depicted in the figure are still images taken from a series of frames during the slug migration stage. 1, 2, 3, 4, 5 and 6 represent the frames selected that display slug migration. Red arrows indicate the rear portion of the slug under investigation whereas yellow arrow points to the region after the slug has migrated. The arrows depict the migration of a single representative slug.

Figure 33B is a similar illustration for slug migration in *grlJ*¯. The red arrows in all the frames depict the slug piece left behind after the slug has migrated, green arrows are the slug breaking phenomena. In all, it can be clearly ascertained that the *grlJ*¯ slugs have a tendency to break apart during migration that does not occur in the wild type cells. These studies depict a possible role of GrlJ in maintaining the integrity of slugs. *grlJ*¯ slugs break apart during migration and end up forming smaller but proportionate fruiting bodies in the absence of the protein.

It was reported earlier that surgical removal of either the anterior or the posterior part of the migrating slug can lead to the reestablishment of the original pattern within 8 h in the absence of cell division (Raper, 1940, Sakai, 1973) indicating a transdifferentiation occurring in the *grlJ*¯ to produce a smaller but properly proportional organism.

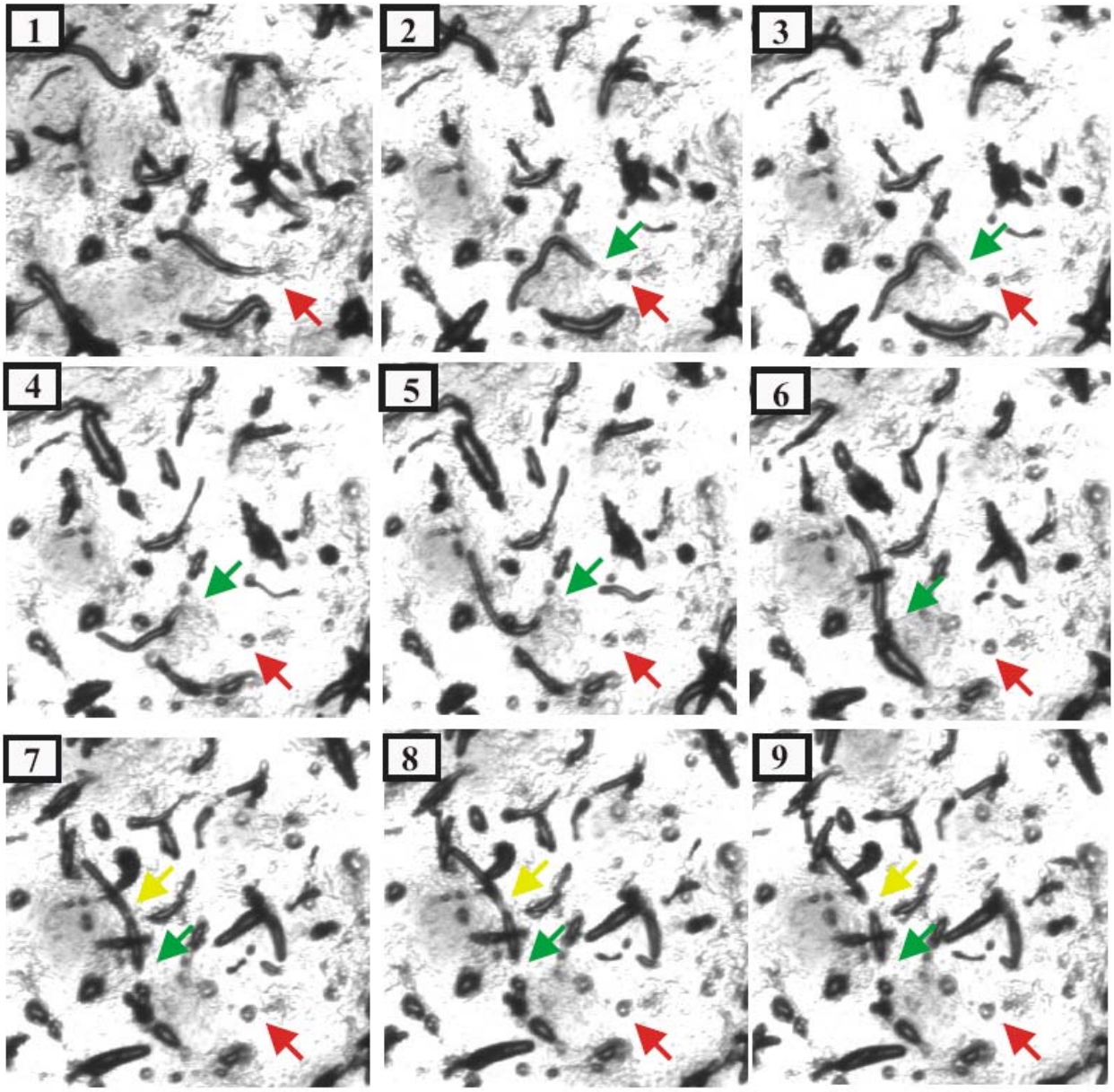


Figure 33B. Movie clips from developing grlJ⁻ slugs. The images shown are still images taken from a series of frames during the slug migration stage. 1, 2, 3, 4, 5 and 6, 7, 8 and 9 represent the frames selected that display slug migration. Red arrows in all the frames indicate the portion of slug that was left behind whereas green arrows signify the rear end of the slug after it had left behind the first portion and yellow arrows depicts the new breaking point. All the arrows show the slug breaking phenomena of one representative slug.

3.4.5.3 DIF and cAMP induction of stalk and spore specific genes

DIF (Differentiation-Inducing-Factor) and cAMP are two essential morphogens that regulate cell-type differentiation. DIF-1 is a chlorinated hexaphenone whose synthesis is induced at the mound stage by high cAMP concentrations when the prespore and prestalk

cells are largely intermixed. DIF-1 is produced by the prespore cells and acts on specific prestalk cells and is degraded in the prestalk cells by a dechlorinase so as to maintain the DIF gradient in the slug (Kay and Jermyn, 1983; Thompson and Kay, 2000). Northern blot analysis revealed a delay in the expression of the prestalk specific *ecmB* gene but comparable levels of prespore pspA expression in *grlA*$^-$ cells. We further analysed whether this delay was a mark of delayed development displayed by the mutant. We therefore studied the induction of *ecmB* and repression of *pspA* in response to DIF-1 (Thompson and Kay, 2000) with various combination of cAMP in a shaking culture assay (Berks and Kay, 1990). 12 h starved cells were disaggregated and resuspended at 1x10^7 cells/ml and divided into four sets. One set each was treated with 5 mM cAMP, 100 nM DIF, and 5 mM cAMP and 100 nM DIF respectively and the last set without any additions.

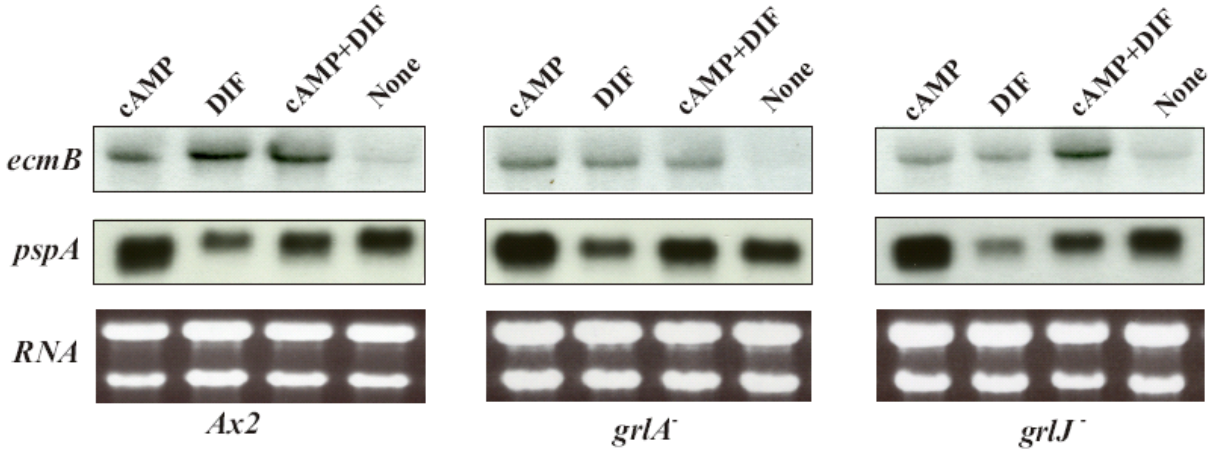


Figure 34. DIF and cAMP induced induction/repression of developmental markers. Log-phase cells (Ax2, *grlA*$^-$ and *grlJ*$^-$ cells) (Materials and Methods, 2.7.10) were synchronously starved at a density of 5x10^7 cells on 90 mm phosphate agar plates and allowed to develop at 21^{o}C. After 12 h the respective mounds or tipped mounds or slugs were harvested and treated with 5 mM cAMP, 100 nM DIF, 5 mM cAMP+100 nM DIF and none of them and allowed then to shake for 2 hours at 160 rpm at 21^{o}C. The cells were harvested for RNA isolation (Qiagen RNAeasy mini-kit). 20 µg of RNA each were loaded from differently treated cells (wild type and the respective mutants), separated and blotted. The blots were then hybridized with specific probes for the prestalk gene *ecmB* and the prespore gene *pspA*.

ecmB was induced in response to DIF as well as cAMP+DIF in Ax2 cells in congruence with published results (Thompson et al., 2004), whereas without addition of any morphogen to the disaggregated cells there was no *ecmB* expression, but with cAMP there were basal levels of *ecmB* transcript observed (Figure 34). This may be due to the fact that DIF is acting only in cells that have experienced (primed) enough and optimal levels of cAMP before. But when the mound/tipped mound integrity was lost because of suboptimal conditions (2 h shaking period), few genes may not be induced normally. Also DIF is known to repress the prespore specific gene, whereas cAMP induces it. As expected, in the wild type

cells cAMP induced the expression of the *pspA* transcript and DIF repressed the transcript levels. Interestingly, in *grlA*$^{-}$, the DIF mediated induction of *ecmB* was absent and also when both cAMP and DIF were together strongly suggesting a role of GrlA in the DIF response system. Additionally, *grlJ*$^{-}$ were also defective in the DIF response system as there was no induction of the prestalk specific, DIF inducible *ecmB* but surprisingly the induction of *ecmB* was increased with the presence of cAMP along with DIF probably directing towards requirement of cAMP priming by the cells lacking GrlJ. Both the mutants under study displayed cAMP mediated induction and DIF mediated repression in the expression of prespore specific *pspA* comparable to the wild type cells.

3.4.5.4 Localization of prestalk and prespore cells in the mutants

The developing *Dictyostelium* slugs have a clear anterior posterior pattern, with the prestalk and prespore cells arranged along this axis. Prestalk cells occupy the anterior quarter and are of two major cell types: the prestalk A cells, pstA, which are at the very front and the prestalk-O (pstO) cells just behind them (Early et al., 1993; Jermyn et al., 1989), whereas the posterior three-quarters comprise of the prespore cells with some ALCs (anterior-like cells). This spatial pattern is established at the mound stage when prestalk cells sort to the apex of the mound and organize along an anterior posterior axis as the tip elongates to form a slug.

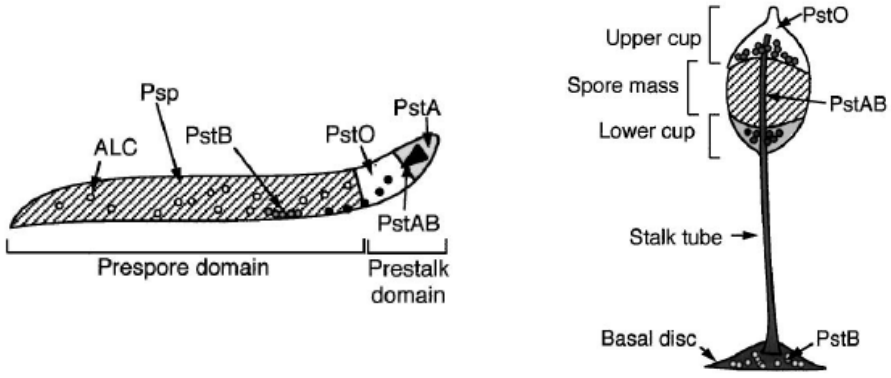


Figure 35. Schematic representation of the distribution of specific cell types in the slugs and culminants (Aubry and Firtel, 1999).

3.4.5.4.1 Patterning of pspA in *grlA*$^{-}$

Detection of the *ecmB* and *pspA* transcripts by northern blot analysis and studying their induction pattern with different morphogens gives no definitive clue about the spatial pattern of gene expression. We therefore generated *grlA*$^{-}$ strains carrying plasmids containing

promoter of *pspA* driving the expression of the β-galactosidase gene (*pspA-Gal*) into the *grlA*⁻ and monitored their patterning during development.

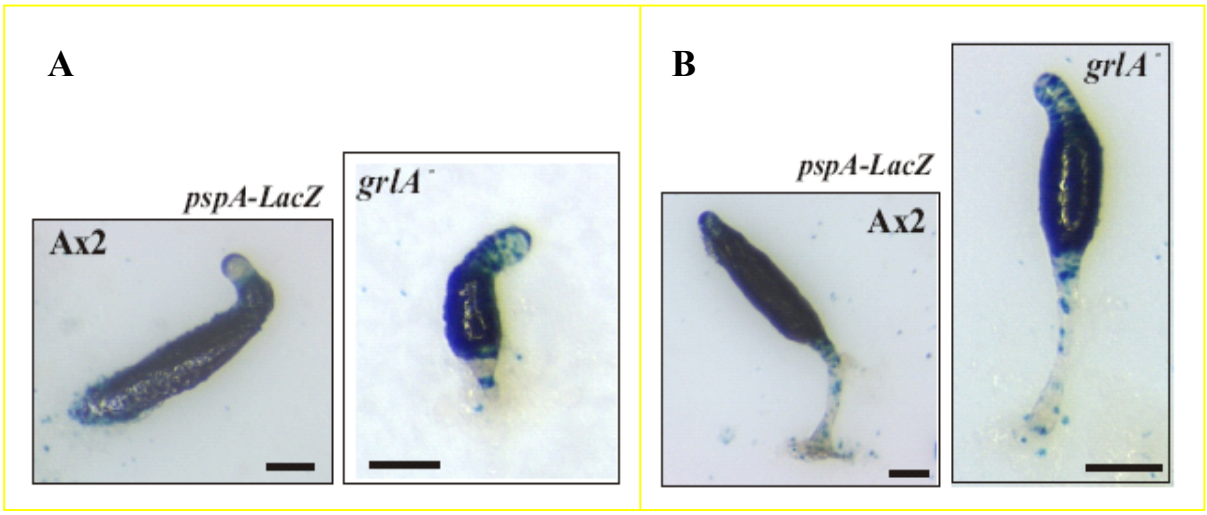


Figure 36. Localization of the prespore specific pspA in *grlA*⁻ cells. Ax2 and *grlA*⁻ cells were transformed with a *pspA-Gal* plasmid and single transformed colonies obtained were used. Log phase cells were harvested and developed on nitrocellulose filters and harvested at either slug and/or culminant stages, respectively, fixed and stained (Materials and Methods, 2.7.9) and photographed with a stereomicroscope. Bar, 100 μm.

The LacZ expression under the control of *pspA* promoter in *grlA*⁻ cells was correctly observed at the posterior of the slug whereas the prestalk cells in case of grlA- cells occupied a slightly larger region. A similar pattern was also noticed in case of culminants (Figure 36), but these differences were very slight and additional studies with the patterning of the different stalk specific markers in *grlA*⁻ under similar conditions have to be performed.

3.4.5.4.2 Localization of prestalk cells in *grlA*⁻ and *grlJ*⁻ cells

The distribution of the prestalk cells was studied with neutral red staining. The distribution of the dye was unaltered in *grlA*⁻ and *grlJ*⁻ slugs as compared to Ax2 as neutral red stained cells occupied the anterior tip and were also present at the base of the slugs (Figure 37). This was also quantitated in the slug analysis section of results (3.4.5.1). We further checked the status in culminants, which also displayed an unaltered distribution.

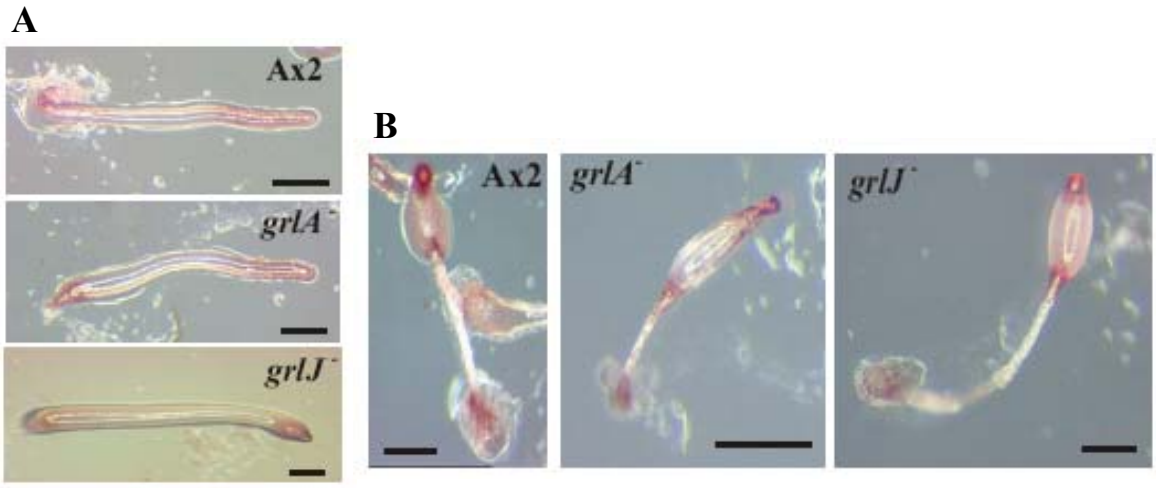


Figure 37. Distribution of neutral red stained cells in *grlA*⁻ and *grlJ*⁻ slugs and fruiting bodies. The wild type Ax2 and *grlA*⁻ and *grlJ*⁻ cells were prepared as described (Materials and Methods, 2.7.8). The slugs and the culminants were monitored for the distribution of the neutral red dye.

These results suggest that the overall localization of the stalk cells in *grlA*⁻ and *grlJ*⁻ cells is unaffected and patterning does not seem to be disturbed.

3.4.5.5 Sporulation in *grlA*⁻ and *grlJ*⁻ cells

Although prespore and prestalk cells diverge and sort out soon after aggregation, they do not form spores and stalk cells until fruiting body formation is initiated. Culmination is initiated when the slugs stop migrating and reorganize morphologically while their component cells undergo terminal differentiation (Dormann et al., 1996). Prestalk cells differentiate by entering into the stalk tube, they finally vacuolize and expand forming a growing stalk. Prespore cells differentiate into spores as they are lifted up by the stalk. Both the post aggregation and terminal differentiation require proper coordination between the two cell types working in harmony balancing events as most of the signalling components required for one cell type is provided by the other.

3.4.5.5.1 Defective spore morphology of the *grlJ*⁻ strain

Lack of GrlJ results in precocious development. Most of the strains that complete early development end up having abnormal spore morphology and are sporogenous (Chang et al., 1998). Hence we studied the spore morphology in the *grlJ*⁻ strain. The spores were obtained from the completely developed wild type Ax2 and the respective mutants, squashed under the coverslip with a little amount of buffer and bright field images were captured.

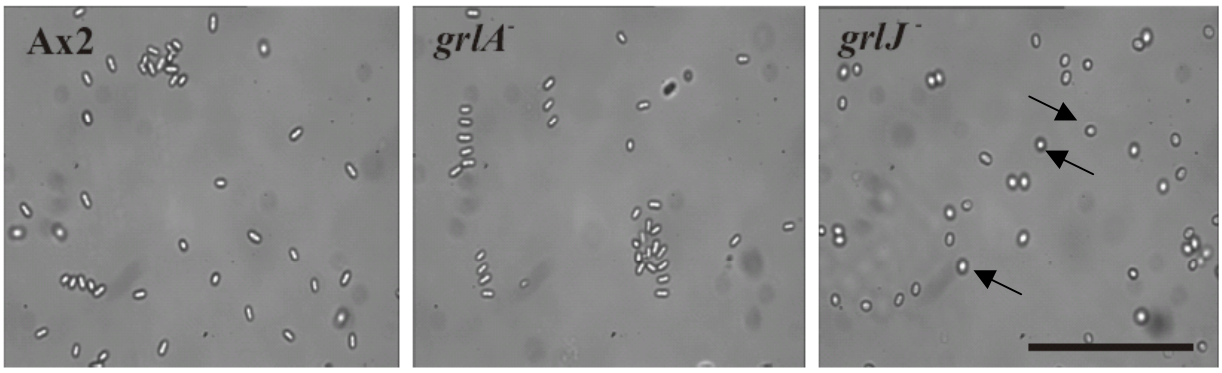


Figure 38. Defective spore morphology in *grlJ*¯. Spores were obtained from completely developed mature fruiting bodies on phosphate agar plates (Materials and methods, 2.7.3.2). They were slightly squashed with a coverslip with a little amount of Soerensen phosphate buffer and brightfield images were captured at 40x magnification. Arrows indicate representatives of malformed spores. **Bar, 100 μm.**

Ax2 cells and *grlA*⁻ produced elongated spores. In contrast *grlJ*¯ formed many rounded or mis-shaped spores (Figure 38).

3.4.5.5.2 Spore germination in *grlA*¯ and *grlJ*¯ strains

Sporulation is a tightly controlled process that involves cell-type differentiation with morphogenesis wherein the prespore cells appear early in development and progressively accumulate components of spore coat in the vesicles which are secreted to assemble the spore coat during encapsulation (Escalante and Sastre, 1998). In general, rapidly developing and sporogenous mutants form abnormal spores that are less viable. We analysed the spore germination in the mutants by plating equal amounts ($5x10^7$ cells) of axenically growing cells on phosphate agar plates and allowing them to develop for 48 hours until spore formation was complete and compared them with wild type cells. Spores were then harvested and counted to determine the number of spores produced compared to the initial number of cells plated. 100 spores from each set with/without detergent treatment were plated onto SM agar plates in association with *Klebsiella aerogenes* to check for viability. (Materials and Methods, 2.7.11). During development, inspite of losing 20% of the cells as stalk cells there is still 100 percent or slightly more recovery of the spore cells assuming that the cells undergo another round of cell division after plating. Treatment with detergents functions in several ways: one is by killing any amoebae still left on the plate, the other is by inducing germination from mature spores at a later stage, and yet another by affecting the viability of the spores if they are not formed normally or matured completely (Escalante and Sastre, 1998). The wild type cells showed recovery of 120% spores from the cells initially plated and they were all viable and

resistant to detergent treatment. *grlJ*$^-$ turned out to produce more number of spores (144%) per cells originally plated in comparison to Ax2 spores taken as 100% depicting they are sporogenous. However, lower number of spores germinated from *grlJ*$^-$ (~ 15% reduction) and they were comparatively more sensitive to detergent treatment (Figure 39) probably owing to impaired or immature spore coat that would allow diffusion of the detergent into the spore cells.

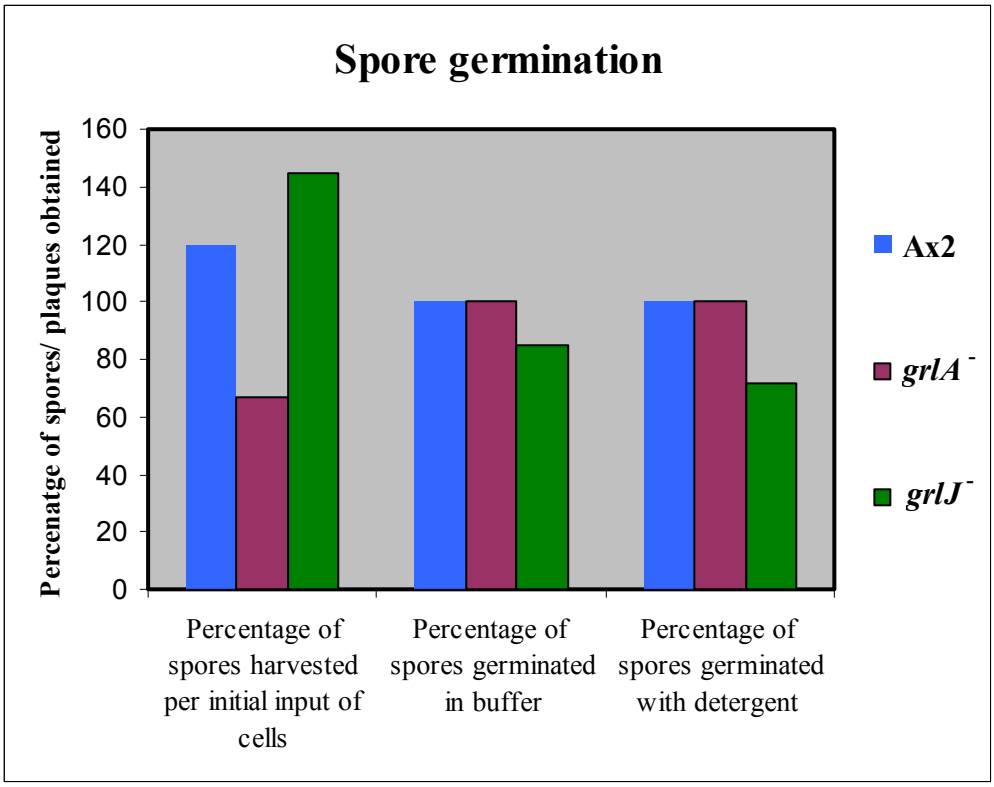


Figure 39. Spore germination test. Equal numbers ($5x10^7$) of cells were plated onto Soerensen phosphate agar plates and allowed to develop completely until 48 hours. The spores were then harvested and counted. 100 spores each were plated in association with *K. aerogenes* with and without treatment with 0.5% Triton-X-100 for 15 mins. The percentage of plaques obtained with/without detergent and the percentage of spore harvested in the mutants was calculated setting the spores harvested from wild type cells as 100 percent.

However, *grlA*$^-$ produced a lower number of spores (67%) from the initial input of cells as compared to wild type. The number of spores germinated with and without detergent treatment was comparable to the wild type strain.

3.4.5.5.3 *grlA*$^-$ does not produce SDF-2

Spore maturation is controlled by two peptides, **s**pore **d**ifferentiation **f**actor 1 and 2 (SDF-1 and SDF-2). SDF-1 accumulates during the slug stage and is released in a single burst

at the onset of culmination, whereas SDF-2 is released at the mid-culmination stage also in a single burst (Anjard et al., 1998). SDF-2 triggers spore encapsulation in responsive cells as soon as they are exposed to the signal. This response is mediated by DhkA receptors present on both the prestalk and prespore cells. SDF-1 also promote spore induction but the signal transduction pathways involved is yet uncharacterized but it involves *de novo* protein synthesis which is not the case in SDF-2 (Anjard et al., 1998, Anjard and Loomis, 2005). A spore induction assay was carried out under submerged conditions in the presence of 5 mM cAMP such that wild type cells will form neither spore nor stalk cells. Under these conditions, *grlJ*$^{-}$ were able to form spores that were not very sensitive to detergent. The supernatants obtained from this assay were used to determine the SDF-1 and SDF-2 levels (Materials and Methods, 2.7.12). *grlA*$^{-}$ cells were unable to produce SDF-2, but decent levels of SDF-1 whereas *grlJ*$^{-}$ produced both SDF-1 and SDF-2 and responded to SDF-1 and SDF-2 in a sporogenous assay. SDF-2 is homologous to the human diazepam binding inhibitor (DBI) neuropeptides which are known to bind $GABA_A$ receptors. Recently these neuropeptides and its agonist diazepam were found to induce sporulation in *Dictyostelium* (Anjard and Loomis, 2005). We therefore tested GABA and glutamate in a sporogenous assay (Materials and Methods, 2.7.12) and *grlJ*$^{-}$ responded to both GABA and glutamate like the wild type cells.

3.4.5.5.4 *grlA*$^{-}$ shows a defect in the SDF-2 production involving TagC and DhkA

SDF-2 acts through a two-component system that relays phosphate to response regulators including the cAMP phosphodiesterase, RegA. Acyl carrier binding protein (AcbA) is synthesized in the prespore cells and is proteolytically cleaved by TagC (Transporter /Serine protease) present on the prestalk cells to produce SDF-2 peptide, which then binds to the histidine kinase receptors present on the surface of both prestalk and prespore cells and in turn inhibits the phosphorelay via RdeA (phosphor-transfer protein) to RegA. RegA in its unphosphorylated form is inactive and leads to an increase in cAMP concentration by the late adenylyl cyclase ACR, and thereby induces encapsulation of the spores by activating PKA levels (Anjard and Loomis, 2005).

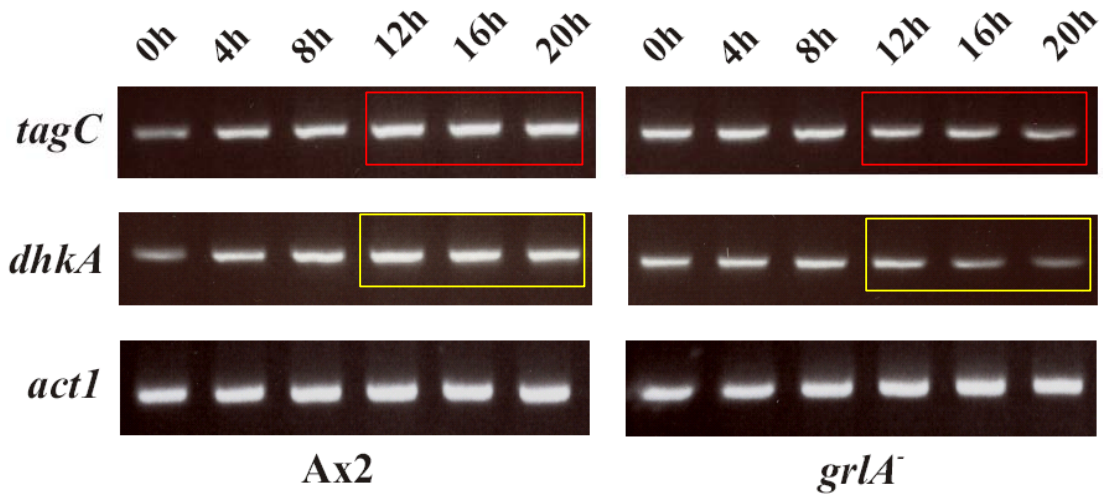


Figure 40. Expression level of *tagC* and the histidine kinase receptor, *dhkA* in *grlA*⁻ cells. RNA was isolated at different time points of development and equal amounts of RNA were used to generate single stranded cDNA synthesized (Materials and methods, 2.4.1.2). Specific primers were used to amplify *tagC* and *dhkA* sequences. Actin was used as a control, which is present uniformly throughout development.

As *grlA*⁻ cells were unable to produce SDF-2, we tested the expression profile of other components in this system of SDF-2 response i.e. *tagC*, the protease that generates SDF-2 and the SDF-2 receptor *dhkA* by RT-PCR analysis. We found down regulation of both genes during late development in the mutant (red box, yellow box), whereas in Ax2 the amount rather increased.

These results together suggest that GrlA is a key intermediate in controlling sporulation events probably by controlling the synthesis of SDF-2 indirectly by down regulating the protease that cleaves the AcbA to form SDF-2 as well as by down regulating the DhkA receptor itself.

3.4.7 Transcription profile of *grlA*⁻

3.4.7.1 Experimental design

DNA microarrays were used to investigate the transcriptional profile of the *grlA*⁻ strain as compared to the wild type Ax2. The microarray used (Materials and methods 2.8) carried sequences that represent approximately 50% of the genome. Based on the developmental characteristics we selected a time point in the later developmental stage for analysis (16 h starved cells) and carried out a comparison to Ax2 (16 h).

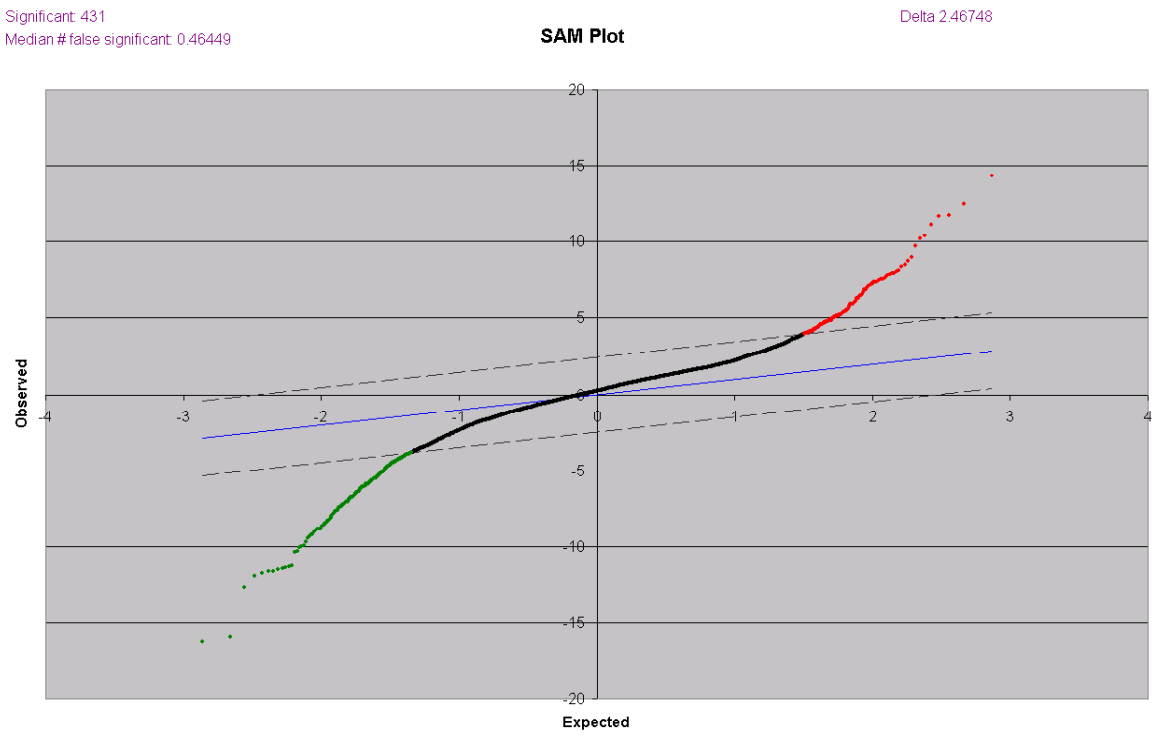


Figure 41. Transcriptional profile of differentially regulated genes in *grlA*⁻. The graph indicates the SAM (Significant Analysis for Microarray) output of significant genes. The red and green portion in the graph indicates the up and down regulation respectively in the mutant with wild type Ax2 cells used as control.

3.4.7.2 Comparison of the genes differentially regulated in *grlA*⁻ cells

From our analysis we obtained 179 genes that were at least 1.5 fold and above differentially regulated. This value constituted approximately 3.3 % of the genes studied or ~ 1.4 % of the total genes present in *D. discoideum*. Out of these 179 genes, only 57 were upregulated whereas a large repertoire of genes was downregulated in the mutant. 67 genes (~ 1.2 % of the genes under study) were found to be 2 fold and above differentially regulated in *grlA*⁻.

3.4.7.2.1 Transcriptional profile of differentially regulated genes in *grlA⁻*

Gene ID	Fold	Gene name and Putative Function	Score
SSF430	6,13	ATP binding (IEA)	11,725
SLE322	2,47	*Dictyostelium discoideum* protein-tyrosine phosphatase 3	7,909
SSA625	2,23	**pefA** , DdPEF-1	7,371
U49650	2,18	**cadA**, calcium-dependent cell adhesion molecule-1, glycoprotein gp24	9,727
SLC396	2,11	**ANN7, synexin,** DdANN7	7,012
SLI627	1,95	*D.discoideum* , transposon DIRS-1, complete, clone SB41	11,107
SSE894	1,94	**cbpI,** CBP9, calcium-binding protein, expressed in prespore cells	12,486
SLJ323	1,93	*Dictyostelium discoideum* filament-interacting protein	7,342
X15980	1,88	**pspA,** D19,cell surface glycoprotein PsA precursor, expressed in prespore cells	4,696
SSL201	1,77	**csbA,** involved in contact sites B	5,878
SSJ693	1,75	DDB0231686, AAA ATPase domain-containing protein, involved in O-glycosylation	5,151
SSM252	1,71	Similar to Opsanus tau (Oyster toadfish). cytochrome P450 1A1	7,611
SSL648	1,69	**proB**, profilin II	5,195
SLA374	1,68	**vacA1**, vacA, vacuolin A, prohibitin domain-containing protein	6,661
U61990	1,67	**thyB,** DdTK1, TDK, thymidine kinase, calmodulin-binding protein	5,586
SLD213	1,67	**tubA**, alpha tubulin,	5,035
SLI528	1,65	**cprA**, CP1, cysteine proteinase 1	7,914
SSC113	1,65	Q8BFY9 Transportin 1 (Importin beta-2) (Karyopherin beta-2)	4,948
SLG278	1,65	hspC, hsp32 ,heat shock protein, heat inducible protein	6,419
SLD169	1,64	**limE** crp, DdLim, LIM domain-containing protein, lim = LIM domain-containing protein	4,764
SLC106	1,63	H1, **hstA**, histone 1, calmodulin-binding protein,transcriptional repressor	7,383
SSB550	1,61	Similar to Arabidopsis thaliana Putative NADH dehydrogenase 10.5K chain.	7,545
SSB856	1,58	DDB0231622, H2Bv3 ,histone H2B domain-containing protein	4,700
SSD758	1,57	argS1,argS, ArgRS, arginyl-tRNA synthetase, arginine-tRNA ligase	4,602
SLF882	1,56	CG5367 PROTEIN. 6/101 (Automated)	8,345
SSG357	1,56	P13773 Cyclic AMP receptor 1. (Automated)	4,330
SLG446	1,55	**catA** ,cat , catalase	4,343
SSH136	1,53	P02889 Probable 26S proteasome non-ATPase regulatory subunit 8	4,144
SLD772	1,51	**vilA**, villin, villidin	6,368
SSD739	1,50	CD9	4,215

Table 5A. Significantly up-regulated genes of 16 h starved *grlA⁻* as compared to Ax2 cells.
Differentially regulated genes were identified using SAM program. The genes that were listed were only the ones that were 1.5 fold or more differentially regulated in comparison to the wild type cells. **GeneID** is the sequence identification number, **Score** represents the SAM value for reproducibility of the result between independent experiments, **Fold** represents statistically significant fold change in the gene induction pattern as compared to the control Ax2 cells. The **Gene name and putative function** was obtained from the ***Dictyostelium* database** and the genes that were not found or could not be identified have been deleted from the original table.

Gene ID	Fold	Gene name and Putative Function	Score
X54452	-6,08	**spiA , Dd31, spore coat protein, regulated by the MADS-box transcription factor SrfA**	-8,95
SSF323	-5,97	**pspE**,1l, 1l prespore protein,expressed in prespore cells	-16,252
SSJ167	-4,11	DDB0232102 , coiled-coil family protein, underexpressed in gskA-null strain	-8,077
L21014	-3,85	gluA, beta glucosidase	-8,819
SLE318	-3,76	rsc12	-5,994
SSL853	-3,65	DDB0231563,unknown	-8,873
SLA466	-3,64	DDB0231577, unknown, expressed in prespore cells	-11,627
SSF584	-3,55	**poxA, peroxinectin (Automated), regulated by the MADS-box transcription factor SrfA**	-11,912
SLC332	-3,04	ecmB, SC253, 253, pDd56, extracellular matrix protein	-9,938
SLB772	-2,80	Putative quinone oxidoreductase	-7,669
AF072432	-2,76	**sigB, GP63,leishmanolysin family protein, developmentally regulated expression is dependent on the MADS Box transcription factor srfA**	-11,381
X16827	-2,62	2C, coiled-coil family protein, cyclic AMP-regulated gene; underexpressed in gskA-null strain	-12,636
SSG693	-2,46	**resB, unknown, regulated by the MADS-box transcription factor SrfA during development**	-9,686
VSF366	-2,45	P90893 Putative serine protease F56F10.1 precursor	-9,484
SLF664	-2,27	**resC, unknown, regulated by the MADS-box transcription factor SrfA during development**	-7,337
SSM478	-2,25	rps5, 40S ribosomal protein S5, protein component of the small (40S) ribosomal subunit	-4,154
AB032841	-2,23	**aqpA**, aquaporin, similar to aquaporin family of water-channel proteins; expressed in prespore cells	-11,266
SSK111	-2,23	DDB0231605, esterase/lipase/thioesterase domain-containing protein, expressed in prespore cells	-11,716
SLA429	-2,17	**tenA, possible endotoxin, regulated by the MADS-box transcription factor SrfA during development**	-6,89
SSH841	-2,17	DDB0231589, unknown,expressed in prespore cells	-7,842
SSH896	-2,10	NonF-related protein	-11,461
SSM322	-2,06	**ecmD,** ShD, cellulose-binding domain-containing protein, sheathin, extracellular matrix protein	-8,492
U68246	-2,06	**wacA, aquaporin-like, member of the major intrinsic protein (MIP) family of membrane transporters**	-11,59
L08391	-2,04	rpl3, L3 , 60S ribosomal protein L3, protein component of the large (60S) ribosomal subunit	-6,069
SLA276	-2,03	rps6, 40S ribosomal protein S6, protein component of the small (40S) ribosomal subunit	-4,489
SLF442	-2,00	**masA** , malate synthase ,expressed in pstAB and pstO cells and in upper cup during culmination	-7,43
SLE422	-1,98	sahA, SAHH, S-adenosyl-L-homocysteine hydrolase,highly similar to mammalian SAHH	-5,857
X55972	-1,97	efaAII, EF1-ll, tef2, elongation factor 1 alpha protein synthesis elongation factor 1-alpha	-9,21
SSM360	-1,93	DDB0232089, coiled-coil family protein, underexpressed in gskA-null strain	-6,61
J01284	-1,91	**dscB**, dscC, discoidin I	-4,329
SLB606	-1,91	Similar to Dictyostelium discoideum; Non-receptor tyrosine kinase spore lysis A	-6,35
AF163835	-1,86	**dcsA**,Dicty Cellulose Synthase	-6,68
SLI378	-1,80	**pgtD,** rsc19, IPT/TIG domain-containing protein, IQ calmodulin-binding domain-containing proteinglycosyltransferase, identified as random slug cDNA19; expressed in prespore cells	-8,299

Gene ID	Fold	Gene name and Putative Function	Score
SLB292	-1,78	Similar to Dictyostelium discoideum, MigA	-6,744
X16959	-1,78	**7E**, coiled-coil family protein, cyclic AMP-regulated gene	-5,795
J02628	-1,77	**pdsA**,, pdeA, PDE, cAMP phosphodiesterase	-7,8
SSJ826	-1,75	**tenC, tenascin X , regulated by the MADS-box transcription factor SrfA during development**	-4,519
SSB377	-1,74	rpl9, 60S ribosomal protein L9, protein component of the large (60S) ribosomal subunit	-3,921
X62142	-1,74	**glpV,** GP1, glycogen phosphorylase 1	-4,872
SSH766	-1,73	rps18, 40S ribosomal protein S18, component of the small (40S) ribosomal subunit	-4,386
AF039748	-1,72	rps4, rp29 ,40S ribosomal protein S4, protein component of the small (40S) ribosomal subunit	-4,129
SLE367	-1,72	OSJNBb0005G07.5 protein	6,505
SLJ671	-1,70	5'-B-MODULE	-5,855
SSA403	-1,69	**pipA, DdPLC,,regulated by the MADS-box transcription factor SrfA during development**	-7,681
X55973	-1,69	efaAl, EF1-l, elongation factor 1 alpha, expressed in prespore cells	-8,474
SLB276	-1,65	**cprG**, CP7, cysteine proteinase	-4,244
SLD783	-1,65	Potential phospholipid-transporting ATPase 11	-5,126
SLE782	-1,64	Similar to Dictyostelium discoideum, coronin binding protein	-4,033
SLC892	-1,63	**galE,** UDP-glucose 4-epimerase, expressed in prespore cells, DDB0231575	-5,760
SSE255	-1,63	DDB0232098, coiled-coil family protein, underexpressed in gskA-null strain	-5,470
SSL238	-1,62	**St15,** cellulose-binding domain-containing protein,expressed in pstO cells	-4,656
SLE765	-1,61	**sigA , malic enzyme, sig = SrfA-Induced Gene**	-5,804
SSM789	-1,60	29C, Protein 29C	-3,904
SLI478	-1,59	**forB**, formin homology domain-containing protein, actin-binding protein	-4,758
SLE848	-1,57	**colC**,colossin C	-5,238
X15383	-1,57	rpl19, V14, rpgB, ribosomal protein L19, protein component of the large (60S) ribosomal subunit	-3,814
X04702	-1,57	**ubqB**, ubiquitin	-4,520
SSE875	-1,56	**culA, cullin, involved in ubiquitin-mediated protein degradation, cul = CULlin**	-7,188
SSD285	-1,55	**abcF2**, non-transporter ABC protein, consists of two ABC domains and lacks a transmembrane domain	-5,529
SSE393	-1,55	**prkA, CBS (cystathionine-beta-synthase) domain-containing protein, regulated by the MADS-box transcription factor SrfA during development**	-6,071
SSF514	-1,54	**ecmC**, ShC, cellulose-binding domain-containing protein sheathin, extracellular matrix protein	-5,401
SLK653	-1,53	Q9Y2Q0 Potential phospholipid-transporting ATPase IA	-6,593
AF151111	-1,52	**fbxA, chtA, F-Box A protein, F-box and WD40 domains containing protein**	-7,494
U82516	-1,51	**rsc22**, random slug cDNA22 protein	-6,871
SLF822	-1,51	**manF**, alpha-mannosidase, expressed in prespore cells, DDB0231616	-8,771
SLJ823	-1,51	U27537, G beta like protein, Guanine nucleotide-binding protein beta subunit-like protein	-6,438

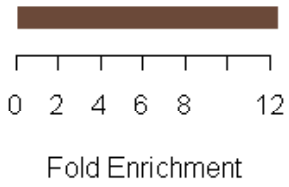
Table 5B. Significantly down regulated genes in *grlA*⁻ on 16 h of starvation as compared to Ax2 cells. Differentially expressed genes were identified using SAM program and processed the same was as mentioned in Table 5A.

3.4.7.3 GOAT (Gene Ontology Analysis Tool)

The differentially regulated genes were annotated using GO scheme (GOAT, a component of the microarray analysis tool package, extended to be used to analyze GO enrichment for gene lists from ***Dictyostelium discoideum*** expression data (http://www.godatabase.org/dev/database). Fold enrichment of genes in the *grlA*⁻ with respect to the wild type cells at the same time point of development was obtained from GOAT output

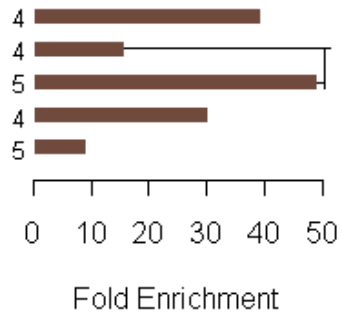
in three different organizing principles of GO: **molecular function**, **biological process** and **cellular component**. (*Dictyostelium* database). The genes that were significantly upregulated in *grlA*$^{-}$ were found to be enriched on the basis of molecular function into protein dimerization activity and calcium binding functions (Figure 42A). However, a vast array of genes were significantly downregulated in the mutant under investigation on the basis of all the three components. Out of these, one major set of genes were the ones belonging to the extracellular matrix proteins biogenesis and organization, another for phospholipase C activity, and the other for SCF ubiquitin ligase complex. Also a small portion of the sporulation specific genes were significantly downregulated in the *grlA*$^{-}$.

1.5 Fold Upregulated – Genes involved in Biological processes



list	total	p-value	Annotation
2	32	0.023	protein polymerization

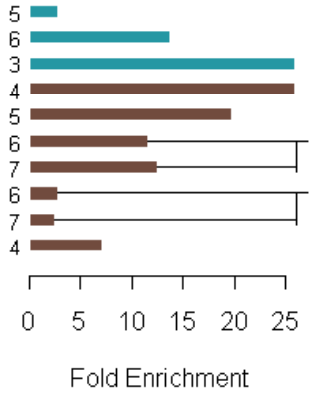
1.5 Fold Upregulated – Genes involved in Molecular function



list	total	p-value	Annotation
2	10	0.001	protein self binding
2	25	0.007	protein dimerization activity
2	8	0.001	protein homodimerization activity
2	13	0.002	calmodulin binding
3	65	0.005	calcium ion binding

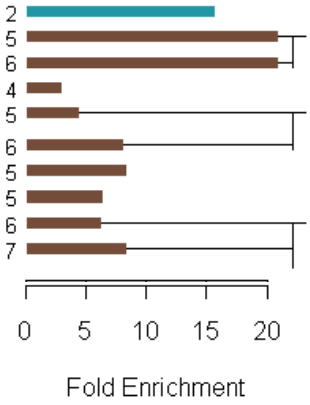
Figure 42A. GO Annotation of 1.5 Fold and above up-regulated genes organized into different GO categories. *Sequences of the genes obtained were compared to several GO-annotated genomes, and the Dictyostelium genes with significant sequence similarity were assigned the respective GO annotations (http://www.godatabase.org/dev/database). The significantly enriched gene groups (P ≤0.1) are indicated. The GO tree level of the different categories annotation is indicated to the left of each bar (2 to 8). Numbers to the right of the bars indicate the number of genes in the group (List), the number of genes with that annotation on the entire array (Total), and the P value representing the statistical significance of the enrichment. The length of the bar represents the ratio of the list frequency in the data (number of genes in the list/total number of genes in the group at that particular GO level) and the group frequency in the entire array (number of genes with that GO annotation on the array/total number of genes on the array at that particular GO level). The x axis is the scale for the ratio. In the connected bars, the lower bars are subgroups of the bars immediately above them. The bar color and pattern represent the group annotation.* The genes that were upregulated were clustered only for biological processes and molecular function of which the most enriched ones were the ones for protein polymerization/dimerization and the Calcium binding function.

1.5 Fold Downregulated - Genes involved in Biological processes



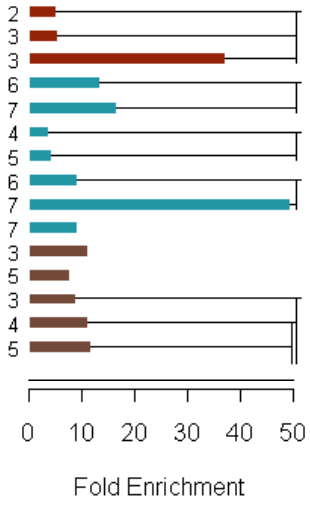
list	total	p-value	Annotation
7	174	0.014	carbohydrate metabolism
3	15	0.002	polysaccharide metabolism
3	8	0	extracellular structure organization and biogenesis
3	8	0	extracellular matrix organization and biogenesis
2	7	0.007	one-carbon compound metabolism
2	12	0.014	cellular polysaccharide metabolism
2	11	0.014	glucan metabolism
13	327	0.001	macromolecule biosynthesis
11	303	0.006	protein biosynthesis
3	29	0.012	sporulation

1.5 Fold Downregulated - Genes involved in Cellular Localization



list	total	p-value	Annotation
3	12	0.002	extracellular matrix
2	6	0.004	cullin-RING ubiquitin ligase complex
2	6	0.004	SCF ubiquitin ligase complex
8	168	0.004	ribonucleoprotein complex
8	113	0.002	ribosome
4	31	0.002	small ribosomal subunit
4	30	0.002	eukaryotic 48S initiation complex
4	39	0.004	eukaryotic 43S preinitiation complex
5	50	0.002	cytosolic ribosome (sensu Eukaryota)
4	30	0.002	cytosolic small ribosomal subunit (sensu Eukaryota)

1.5 Fold Downregulated - Genes involved in Molecular function



list	total	p-value	Annotation
12	177	0	structural molecule activity
7	95	0	structural constituent of ribosome
3	6	0	extracellular matrix structural constituent
2	11	0.014	carboxypeptidase activity
2	9	0.009	serine carboxypeptidase activity
4	82	0.026	hydrolase activity\, acting on glycosyl bonds
4	70	0.015	hydrolase activity\, hydrolyzing O-glycosyl compounds
3	24	0.006	phosphoric diester hydrolase activity
2	3	0.001	phospholipase C activity
2	16	0.021	phospholipase activity
3	20	0.004	pattern binding
2	19	0.027	translation elongation factor activity
3	25	0.007	carbohydrate binding
3	20	0.004	polysaccharide binding
3	19	0.003	cellulose binding

Figure 42B. GO Annotation of 1.5 Fold and above downregulated genes organized depending on the GO categories. (as described in 42A). A large number of genes were well represented in the three major categories of Gene Annotation. The interesting ones were the down regulation of extracellular matrix biogenesis and organizing proteins as represented by the yellow stars, the ones having phospholipase C activity (blue arrows), SCF ubiquitin ligase complex proteins and most importantly decent amount of genes related to sporulation (pink arrow).

Our microarray analysis data of significantly differentially regulated genes in *grlA*$^{-}$ and with the GO annotation further strengthened the observation for a delay in development in the absence of GrlA in *Dictyostelium.* Most of the genes involved in synthesis and organization of extracellular matrix proteins (stalk cell differentiation) and also some sporulation specific genes were found to be underexpressed. A special category of genes that were significantly downregulated in *grlA*$^{-}$ was the SrfA induced genes that are known to be play differential role in spore coat formation (encapsulation) and maturation. Another interesting category was the *fbxA*, *culA* and SCF ubiquitin ligase complex family of proteins and also a family of 2C and 7E module genes that were also found to be downregulated in *grlA*$^{-}$. The 2C and 7E module proteins were reported to be underexressed as well in the *gsk3*$^{-}$. Interesting sets of genes significantly upregulated were certain calcium binding proteins as well as certain components of the vacuoles and *pdsA*, an extracellular cAMP phosphodiesterase and contact sitesB (*cadA*).

4. DISCUSSION

4.1 $GABA_B$ receptor-like family of proteins in *Dictyostelium*

GABA, the main inhibitory neurotransmitter in the brain exerts its response by activating two major classes of receptors on the peripheral nerve terminals: the ionotropic $GABA_{A/C}$ that elicits a fast response by opening up the chloride ion channels and the metabotropic $GABA_B$ receptors that brings about slow and prolonged inhibition of neurotransmission via downstream effectors. $GABA_B$ receptors are present on the presynaptic, postsynaptic and extrasynaptic vesicles wherein they act differentially and serve to modulate fine-tuning of the CNS along with excitatory glutamate receptors. $GABA_B$ belongs to the family 3 of GPCRs together with metabotropic glutamate (mGlu), extracellular Ca^{2+} sensing, pheromone and taste receptors (Liu et al., 2004). Each of these receptors have an extracellular ligand binding domain called Venus Fly Trap (VTF), a 7 TMD which is responsible for the recognition and activation of heterotrimer G-proteins and an intracellular C terminal domain. $GABA_B$ receptors differ from the mGlu and Ca^{2+} sensing receptors which exist as homodimers, by the requirement of being obligate heterodimer.

The existence of a family of 17 genes encoding the $GABA_B$ receptor-like proteins in the slime mold, *Dictyostelium discoideum* was surprising as these kind of receptors were considered to be animal specific so far and were not found outside the metazoan branch. A recently reported distinct peak corresponding to γ-amino butyric acid (GABA) in the mass spectrum with the 6 h starved cell lysates obtained from *Dicyostelium* further supports the existence of such a family of receptors in the amoeba (Ehrenman et al., 2004). Another gene, *gadA* is expressed at 11 h of development encodes a protein with a high degree of similarity to glutamate decarboxylase, an enzyme that is known to catalyze the conversion of glutamate to γ-aminobutyric acid (Iranfar et al., 2001). Although GABA is clearly not a neurotransmitter in *Dictyostelium*, it is interesting to study if it is used as an intercellular signal and whether this signal is mediated via the $GABA_B$ receptor- like proteins. In this regard we undertook studies of two genes, which are now named GrlA and GrlJ (Hereld, *Dictyostelium* Genomics, 2005) and were amongst the first ones to be identified in the *Dictyostelium* genome (Glöckner et al., 2002). GrlA and GrlJ are highly homologous (88% identity) to each other and belong to the same cluster in the phylogenetic tree constructed from the *Dictyostelium* Family 3 proteins. The other two receptors, GrlB and GrlF, present in the same cluster were also highly related showing a similarity of around 80 percent to each other. There were three major clusters formed and few stood out from the clusters.

GrlA and GrlJ have several conserved residues in the intracellular loops 2 and 3 required for G-protein specificity and activation comparable to the mammalian counterparts

(Figure 12). They were identical to either $GABA_BR1$ or $GABA_BR2$ subtypes to a similar degree. Hence it was difficult to definitively differentiate them into different subtypes. On the basis of the TMDs, they were slightly closer to $GABA_BR2$ subtypes (Figure 10 and 11).

4.2 GrlA and GrlJ, expression, localization and dimerization

GrlA and GrlJ are expressed at comparatively higher levels at late developmental stages (12h onwards) as revealed by our RT-PCR and Real-time PCR experiments (Figure 13). A basal level of expression is observed in the vegetative growth phase and aggregation. Also *grlJ* is expressed at much a higher level than *grlA*. GrlA tagged with GFP was targeted partially to the plasma membrane as displayed by the colocalization with the plasma membrane marker protein, Annexin by coimmunofluorescence studies. It also exhibited a cytoplasmic distribution and colocalized with Interaptin, a nuclear envelope protein, and PDI, an ER marker (Figure 14 A, B). Cell fractionation studies supported the presence of the GFP-tagged GrlA as a component of the membranes (data not shown). These studies indicate that GrlA is a membrane protein but its retention in the ER may be due to its inability to reach the membrane. In the mammalian system an arginine based endoplasmic retention signal (RXRR) is present on the cytoplasmic tail of $GABA_BR1$ that is masked by $GABA_BR2$ to allow trafficking of the correctly formed dimers (Gassmann et al., 2005). However, there is no such signal found in GrlA. Another reason would be owing to incorrect folding, or the presence of a limited number of sites on the membranes to hold the overexpressed protein.

The C-termini of the $GABA_BR1$ and $GABA_B$ R2 were reported to interact in higher eukaryotes (White et al., 1998; Kuner et al., 1999). We therefore tested this hypothesis for several receptors of the Family 3 GPCRs in *Dictyostelium* using the yeast two-hybrid approach, however we found no possible interaction amongst them (Table 2). We extended this study wherein we checked for homodimerization amongst four of the receptors, which was also negative (Table 3). Our studies led us conclude that the C - termini of the receptors under investigation do not interact. These results are not unexpected because even though the $GABA_B$ receptors in higher eukaryotes interact with their C termini, they are not sufficient for dimerization. Infact, the transmembrane domain and N termini contain determinants to govern dimerization (Margeta-Mitrovic et al., 1999; Calver et al., 2001; Pagano et al., 2001; Liu et al., 2004).

4.3 GrlA and GrlJ, two GPCRs involved in late developmental stages

Phenotypic analysis unraveled some facts about the function of GrlA and GrlJ. GrlA is involved in events controlling sporulation by affecting the production of a signal, SDF-2, required to trigger prespore to spore differentiation indirectly via tagC, a protease, and by dhkA receptor itself. GrlJ may be involved in the developmental switch from aggregation to post aggregation and cells display precocious development in its absence. Lack of GrlJ also affects cell adhesion, osmotic response and affects spore morphology and germination. However the ligands are unknown as yet.

4.3.1 Charactersistics of *grlA*$^-$ and *grlJ*$^-$

To elucidate the *in vivo* function of these proteins, we generated mutants lacking *grlA* or *grlJ*. The morphology was fairly unaffected but *grlA*$^-$ amoebae were slightly smaller in size as compared to the wild type Ax2. *grlJ*$^-$ grew comparatively faster axenically as compared the wild type cells whereas the growth on a lawn of *Klebsiella aerogenes* was comparable in both *grlA*$^-$ and *grlJ*$^-$. The number of nuclei possessed by *grlA*$^-$ and *grlJ*$^-$ were also similar to the Ax2 cells (Figure 18). However, *grlJ*$^-$ was very sensitive towards 0.4 mM sorbitol treatment whereas *grlA*$^-$ was sensitive as compared to the wild type cells. These results suggested an involvement of the receptors under investigation in the osmotic shock response in *D. discoideum*. Furthermore, we found that GrlA and GrlJ were not involved in phototaxis as their slugs moved directionally towards the light souce. Folate and cAMP are known chemoattractants for *Dictyostelium*. The cAMP receptors have been known for several years and are well characterized however, little is known about folate-mediated response except that they act via Gα4 downstream. Folate acts as chemoattractant both during growth and the tipped aggregate stages (Hadwiger et al., 1994). Our two-drop assay with the axenically growing cells ruled out the possibility of the receptors under investigation to be folate receptors (Figure 21). However, the response of these receptors to folate in the tipped aggregate stage is yet to be addressed.

Strains lacking grlA and grlJ under submerged conditions formed aggregates and moved in the form of streams towards the aggregation center comparable to the wild type cells (Figure 23). Aggregation in the presence of exogenous cAMP when *grlA*$^-$ were challenged with a micropipette filled with cAMP showed the cells chemotaxed towards the source with the speed comparable to the wild type Ax2 cells. The persistence, directionality and direction change were other parameters monitored with DIAS software program, which were also found to be unaltered.Northern blot analysis revealed that the aggregation specific

genes were timely induced in *grlA*$^-$ as well as in *grlJ*$^-$. Together, our data suggests no probable involvement of the receptors in the aggregation stage as they could sense and relay cAMP signals comparable to the wild type cells.

4.3.2 *grlA*$^-$ and *grlJ*$^-$ exhibit post aggregation development defects

It was interesting to study the involvement of GrlA and GrlJ in post aggregation stages in consistence with the expression pattern of both genes as they are induced at this stage of development and expression remains high thereon. We developed mutants on phosphate agar plates. *grlA*$^-$ formed aggregates and developed normally until mound formation. Then there was a delay after 12 h i.e. tip formation was delayed and the mound stage persisted until 15-16 h when tips were formed and development was completed only after 26-27 h of starvation. A rather different phenotype was observed in case of *grlJ*$^-$. They also developed in a similar fashion until they formed aggregates and mounds. Thereafter they developed precociously by forming long, thin slugs at 12-13 h, culminated at 15-16 h and formed fruiting bodies at 18-20 h of starvation (Figure 26). Northern Blot analysis of certain late developmental markers confirmed this observation further. Interestingly, the expression of the prespore specific *pspA* was unaltered in both the mutants. The prestalk specific *ecmB* levels were rather low and the gene was expressed later in *grlA*$^-$ whereas it was expressed at a much higher level at 12 h in *grlJ*$^-$, consistent with the fact that *grlJ*$^-$ forms slugs in 12 h and *ecmB* is expressed in slugs (Figure 27A). The expression of prestalk specific *car2* transcript was also found to be altered in a manner similar to *ecmB* in both the mutants (Figure 27B). The expression of the prespore specific, *pspA* was unaltered in *grlA*$^-$ whereas it was expressed at higher levels in *grlJ*$^-$. However, the timing of expression was not affected in both the mutants. It was reported that *pspA* expression, unlike the expression of other prespore specific markers, *cotB* and *cotC*, is induced in aggregation streams and thus may be induced earlier in development via regulatory pathway that do not control post aggregation events (Schnitzler et al., 1994; Hopper et al., 1995). This can explain why *grlA*$^-$ does not show a significant difference in the expression level or pattern of *pspA* even though they display altered development pattern. These results indicate that GrlA and GrlJ have a significant role in post aggregation development.

4.3.3 Cell adhesion

Dictyostelium is an indispensable model to study motility and multicellularity. Adhesion plays a very important role in both these processes (Gerisch, 1961). During movement the cells have to gain traction towards the substrate which involves making and breaking of cell contacts with extracellular matrix and with each other (Bukahrova et al., 2005). Multicellularity in *Dictyostelium* is achieved by chemotactic migration of single amoebae to form aggregates and mounds. Cells within the mounds sort by a process which is likely to involve both differential adhesion and different rates of chemotaxis, they differentiate in the mound and undergo morphogenesis to from a fruiting body which involves two major types of adhesion, the cell-substratum and the cell-cell adhesion (Kessin, 2001; Chisholm and Firtel, 2004; Siu et al., 2004).

The cell-substratum adhesion is well studied in higher organisms and is predominantly mediated by binding of β-integrin to the extracellular matrix resulting in the assembly of focal adhesion complexes consisting of talin, vinculin, α-actinin, and paxillin that link to the actin cytoskeleton (Yoano et al, 2004; Bukahrova et al. 2005). There are no integrin like molecules in *Dictyostelium* but adhesion receptors, such as SadA that is present in the vegetative stages and may act as potential initiators of focal adhesion sites between cells and the extracellular matrix. However Talins, myosin VII, phg1 and paxillins are other putative cell-substrate adhesion molecules reported so far. Also a disintegrin domain containing protein was shown to be involved in both cell-substratum and cell-cell adhesion (Varney et al., 2002). We studied the whether GrlA or GrlJ would have any putative role in this process. Cell-substrate adhesion experiments with vegetatively growing wild type and *grlA*$^-$ and *grlJ*$^-$ mutants suggested no probable involvement of these receptors in cell adhesion, however the involvement of GrlA and GrlJ during late developmental stages cannot be ruled out.

The second type of adhesion, the cell-cell adhesion, is very well studied in *Dictyostelium* and three major cell-cell adhesion proteins that are differentially expressed at different stages in the development have been described. The first ones to be expressed are the contact sites B or cadA (cadherin), which mediate Ca^{2+} dependent, EDTA sensitive contacts and are present in the vegetative state and rapidly accumulate in development (Knecht et al., 1987; Wong et al., 1996). They are followed by Ca^{2+} independent, EDTA resistant contacts mediated by contact sites A (gp80), which are expressed at the onset of aggregation (Faix et al., 1990, 1992). The developing cells secrete a large protein complex, CF (countin factor), that acts as a negative feed back loop and regulates the expression of CadA and csA (Roisin-Bouffay et al., 2000). The third ones are the EDTA resistant and Ca^{2+} insensitive contacts

mediated by LagC (gp 150) and are expressed at low levels at mid-aggregation followed by a rapid increase after aggregates have formed (Gao et al., 1992). The levels of these molecules were unaltered in *grlA*$^-$. However there was a very interesting earlier and increased expression of Lag C in *grlJ*$^-$ and also high levels of aggregation specific contact sites A as compared to the wild type cells which remained unaltered thereon. Enhanced cell contacts in the absence of GrlJ may be one reason for precocious development in the strain as contacts mediated by LagC are in turn controlled by GBF which turns on the expression of post aggregation genes. However, it would also be tempting to speculate that high cell adhesion in *grlJ*$^-$ is due to low levels of CF which thereby results in an increase of csA, but the aggregation was fairly timely with the formation of normally sized aggregates.

Recently a paxillin homolog, paxB, was shown to be involved in a multitude of functions during development including cell sorting and migration (Bukahrova et al., 2005). It will be further interesting to explore whether a probable link exist between these receptors and paxillin.

4.3.4 Probable role of GrlA in morphogenesis

GrlA is expressed at a higher level in post aggregation stages as revealed by our Real-time PCR experiments. The strain lacking grlA displays a clear developmental delay from the mound stage onwards exactly the time when grlA transcript levels increase in the wild type cells. The tip is formed only after a delay of 3-4 h in the mutant cells and then they undergo complete development with normally shaped fruiting bodies. Northern blots indicated a delay in the expression of stalk specific genes, *ecmB*, and slightly reduced levels of *ecmA* transcript at 12 h (data not shown). We further studied whether this delay in the expression of *ecmB* was just a mark of delayed development or GrlA is involved in the induction of these genes by a shaking suspension assay wherein the induction of *ecmB* and repression of *pspA* by DIF-1 and induction of *pspA* by cAMP were assessed. Interestingly, DIF-1 mediated induction of *ecmB* was found to be absent in the mutant suggesting GrlA an intermediate component in the DIF response pathway. However, DIF was able to repress *pspA* in *grlA*$^-$. The localization of LacZ driven by *pspA* promoter was found to be unaffected in the *grlA*$^-$ except for a slight decrease in the prespore region as compared to the wild type Ax2 cells but the difference was very slight. It will also be interesting to study the expression of *ecmA* and *car2* in response to DIF and cAMP to ascertain whether GrlA is involved in the induction of prestalk specific genes in general or whether the defect is restricted to specific prestalk subtypes. When we studied the distrubution of the neutral red dye (stains the vacuolated prestalk cells) in the wild type Ax2,

grlA$^-$ and *grlJ*$^-$ strains, they showed no observable difference in the patterning of stalk cells at the slug and culminating stages. However it would be interesting to also study the patterning of each specific prestalk marker to get an insight on the localization of specific prestalk genes.

4.3.4.1 GrlA is involved in sporulation events

As envisioned from our studies, the strain lacking grlA has a defect in the induction of the prestalk gene *ecmB* and may be a patterning defect too. However, the prespore marker was expressed normally inspite of the general delay in the development exhibited by the mutant. We further examined sporulation in *grlA*$^-$. The mature fruiting bodies formed in *grlA*$^-$ were normal in appearance and spores formed also displayed normal morphology. However, the number of spores produced was less (67% in comparison to wild type cells) in *grlA*$^-$. The spores germinated comparably to the wild type cells both with and without detergent treatment.

4.3.4.2 *grlA*$^-$ has a defect in the production of SDF-2

Sporulation occurs when the stalk tube elongates progressively downwards through the spore mass lifting it up the substratum followed by differentiation of prespore cells to form spores (sporulation/encapsulation) (Weeks, 2000). As mentioned earlier, two peptides SDF-1 and SDF-2 with potential morphogenic functions induce this process. A factor (AcbA) synthesized in the prespore cells is proteolytically cleaved by a transmembrane protease (TagC) present on the prestalk cells to release SDF-2 which activates the histidine kinase receptor DhkA on the prespore cells which then inhibits the intracellular phosphodiesterase RegA, thereby increasing the cAMP levels that further increase the PKA activity and trigger the conversion of prespore cells to generate spores (Anjard et al., 1998; Loomis, 1998; Anjard and Loomis, 2005). Until the SDF-2 signal is available, the cells remain in the prespore stage by the action of another two-component system mediated by histidine kinase receptor DhkC present on the prespore cells via RdeA and RegA. Our studies with the strain lacking grlA revealed a defect in sporulation with a reduced number of spores. We further estimated the SDF-1 and SDF-2 released by the mutant cells after developing them until culminant and mid-culminant stages, respectively. The results pointed out the defect in the production of SDF-2 whereas SDF-1 was produced normally in *grlA*$^-$ as compared to the wild type Ax2 cells. However it is puzzling as to how *grlA*$^-$ can still form reasonably decent amount of spores inspite of not synthesizing SDF-2. One possible explanation may be that the timing of

spores harvested for the assay was around 48 h. It is known that discadenine, an adenine derivative that accumulates during culmination, acts on another histidine kinase receptor DhkB, resulting in the accumulation of cAMP and thereby PKA levels which may be responsible for the residual sporulation in the absence of SDF-2 signaling (Wang et al., 1999).

4.3.4.3 Defect in SDF-2 production is mediated by reduced levels of *tagC* in *grlA*$^-$

To shed more light onto the defect in the production of SDF-2 in the strain lacking grlA, we investigated another component in this process, namely the transmembrane protease that cleaves AcbA to generate SDF-2. Our RT-PCR results ascertained that the defect in the SDF-2 production was rather an indirect one i.e. via down regulation of *tagC,* the transporter/protease that cleaves AcbA to release SDF-2. Hence, this strengthens the role of GrlA at the level of generation of the signal that controls sporulation.

4.3.4.4 *grlA*$^-$ displays low levels of the SDF-2 receptor *dhkA*

We then assessed the levels of *dhkA*, the hybrid histidine kinase receptor that mediates the function of SDF-2 intracellularly via inhibition of the phosphorelay to the response regulators RdeA and RegA, which then increases the intracellular cAMP levels, activates PKA and triggers sporulation. Interestingly, we observed a low level of expression of *dhkA* in the *grlA*$^-$ as compared to the wild type cells. This provided another role for GrlA in controlling sporulation events by controlling the levels of *dhkA*, which thereby may probably keep the DhkC mediated pathway (activated by ammonia, a known inhibitor of culmination) functional for a longer time and inhibit sporulation (Singleton et al., 1998).

4.3.4.5 Transcriptional profile of genes in *grlA*$^-$

The gene expression profile in *grlA*$^-$ agrees with the developmental delay observed in the mutant as most of the prestalk and prespore specific genes were down regulated except for *pspA*, which was significantly upregulated. This can be explained by the fact that induction of *pspA* occurs much earlier than at the mound stage (Aubry and Firtel, 1999) and that *grlA* is expressed and a functional product may be formed only in post aggregation stages. A detailed study of all the genes differentially regulated in *grlA*$^-$ in comparison to the wild type cells 16 h and the fold enrichment led us to several interesting findings. The expression of genes required for extracellular matrix biogenesis and organization were downregulated. The

expression of extracellular cAMP diesterase *pdsA* was significantly increased in the mutant, which probably signifies high extracellular cAMP levels. Also *fbxA* and *culA*, part of SCF (**S**kp, **c**ullin, **F**-box) ubiqutin ligase complex, were significantly downregulated. One of the targets of the fbxA/SCF complex is RegA, an intracellular cAMP phosphodiesterase, which is ubiquitinated and degraded (Tekinay et al., 2003). These observations depict a situation where there are high levels of extracellular cAMP and low intracellular levels. A very interesting category of downregulated genes in *grlA*$^-$ is the MADS-box transcription factor *srfA* (**S**erum **r**esponse **f**actor) induced genes, of which a 6 fold down regulation was observed for its hallmark gene, *spiA*. The family of genes induced by this transcription factor is reported to be involved in spore maturation and spore coat formation in *Dictyostelium* (Escalante et al., 2004). In higher eukaryotes SRFs are well-studied as transcription factors that are involved in a multitude of processes such as synaptic plasticity, regulation of contractile proteins, mitogenesis and myogenic terminal differentiation (Nelson et al., 2005; Ramanan et al., 2005; Vlahopoulos et al., 2005). The Rho-induced stimulation of SRF mediated transcription, which is required for cell growth, contractility and migration, is controlled by a G-protein (Dutt et al., 2004). In *Dictyostelium* the upstream regulators of SrfA are not well characterized except for its induction by PKA. These results would further help us to place GrlA upstream of SrfA in *Dictyostelium*. However, these results are not definitive and we cannot rule out the possibility that the delay in the expression of SrfA induced genes in GrlA would reflect just a developmental delay. Also, a direct relation between SDF-2 and SrfA has not been shown so far as SDF-2 is not synthesized *de novo* but is cleaved from AcbA and released immediately.

4.3.4.6 Hypothetical model depicting the role of GrlA in controlling sporulation events (Figure 43)

Upon activation, GrlA may direct TagC, a protease present on the surface of prestalk cells to cleave AcbA, which is released from the prespore cells to generate SDF-2, a signal that triggers sporulation. SDF-2 then binds to the histidine kinase receptor DhkA present on both the prespore and prestalk cells that controls the phosphorylation of RegA, the intracellular phosphodiesterase via RdeA, an intermediate phosphotransfer protein. The activity of RegA is reduced when its response regulator is not phosphorylated thereby increasing the intracellular cAMP level formed by the late stage adenylyl cyclase ACR, which then binds to the regulatory subunit of PKA and frees the catalytic subunit (Anjard and Loomis, 2005). The PKA-C then triggers encapsulation indirectly by inducing the expression of *srfA* in the prespore cells. The MADS box transcription factor *srfA* in turn is involved in

the induction of several genes that are required for spore coat formation and maturation (Escalante et al., 2003). Interestingly, we also found *dhkA* (SDF-2 receptor) and *fbxA* (a component of SCF E3 ubiqutin ligase complex - functional predominantly in the prestalk cells) downregulated in *grlA*. The interplay between *fbxA*, *regA* and and *dhkA* to increase the intracellular cAMP levels are well characterized (Tekinay et al., 2003).

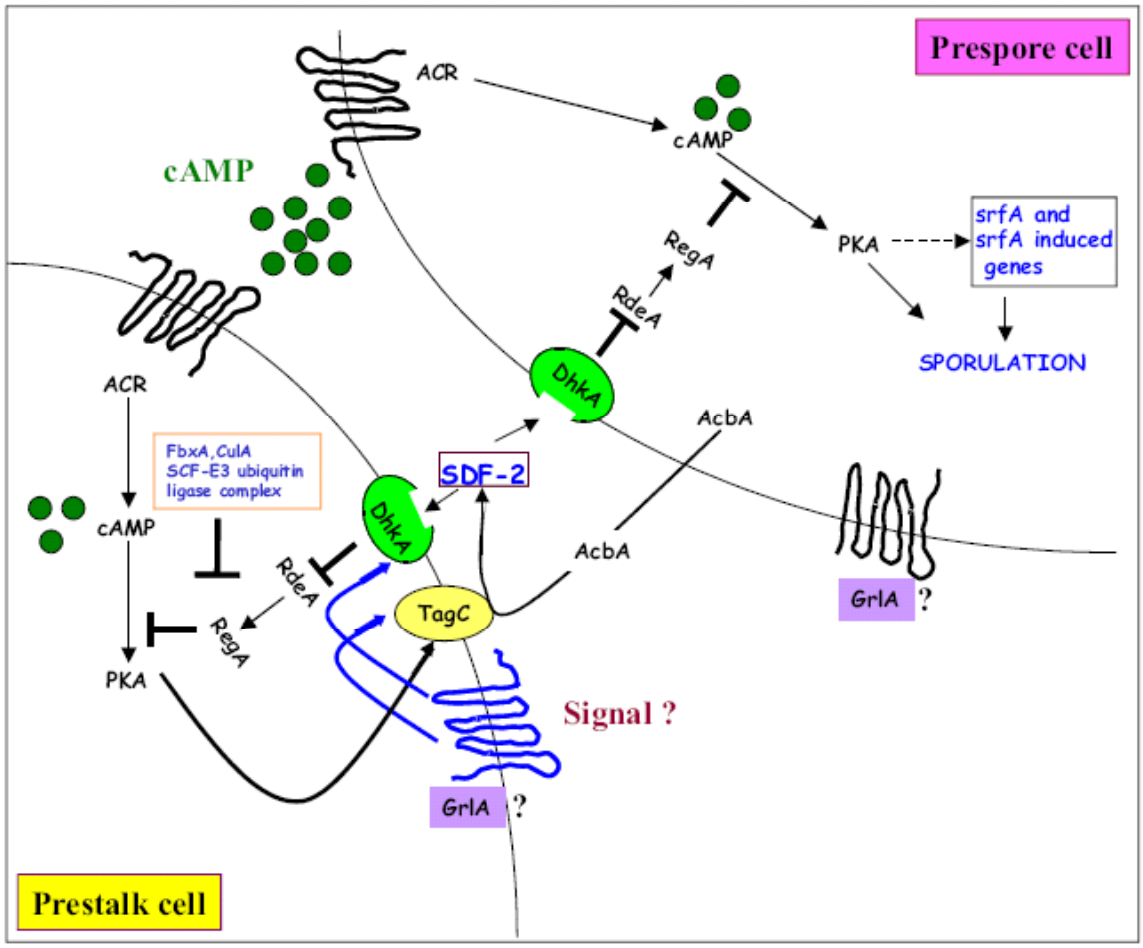


Figure 43. Schematic illustration of a probable role of GrlA in sporulation. The figure illustrates a speculative pathway where GrlA could function. Upon activation by an unidentified ligand, GrlA would help induce sporulation by activating TagC to cleave AcbA to form SDF-2, which in turn binds to the DhkA receptors present on the surface of both prestalk and prespore cells and inhibits phosphorelay via RdeA to cAMP phosphodiesterase, RegA. Thus PKA is activated which in turn triggers sporulation in prespore cells whereas it acts as a feedback loop for the extracellular activity of TagC in prestalk cells. The dotted arrow indicates indirect regulation (transcriptional regulation of *srfA* inducible genes). The blue arrows depict the pathway and the intermediates (written in blue colour) that are affected in the absence of GrlA in our present findings.

In the prestalk cells, the same pathway downstream of DhkA is functional that helps in the extracellular expression of TagC acting as a feedback loop to rapidly amplify the SDF-2 signal for encapsulation. In the absence of GrlA, TagC would not cleave to release the necessary signal SDF-2 for encapsulation by blocking the process thereby reducing the PKA levels which is also supported by the low levels of expression of dhkA and fbxA complex.

We suggest that GrlA may function in two ways in sporulation: a) it is present on the prestalk cell surface and affects the expression or activation of tagC to release SDF-2, and b) it controls the expression of dhkA, fbxA/SCF complex to increase the intracellular cAMP levels. How exactly GrlA mediates these responses have to be addressed and also whether grlA has some function before sporulation as observed from the phenotype in the mutant of grlA. However both *tagC* and *dhkA* are also expressed much earlier than sporulation. This would in one way explain the presence of *grlA* much earlier than at the onset of sporulation. Also we have no clue as to what ligand activates grlA at these developmental stages. In short, we have just touched the tip of an iceberg that is yet to be explored.

4.3.5 GrlJ in morphogenesis

Lack of GrlJ shows an altered developmental pattern at late stages in development, which is in agreement with its expression levels. *grlJ*¯ aggregates well and forms mounds at a fairly comparable time as the wild type cells. There is a boost in the development thereon. The *grlJ*¯ end up forming slugs at 12-13 h of starvation and culminate at 16 h and form mature fruiting bodies before 18-20 h of starvation. We found not only an increase in the expression levels but also earlier expression of LagC in *grlJ*¯ in comparison to the wild type cells. LagC expression is a direct measure of G-Box binding factor activity, which controls the post aggregation gene expression (Sukumaran et al., 1998). This could be one reason for the precocious development of *grlJ*¯. Also, the aggregation specific contact sites A is expressed at higher levels but the aggregation was found to be timely in *grlJ*¯. We therefore speculate GrlJ to be functional at this transition (mound to postaggregation differentiation) and cells lacking grlJ eventually end up developing precociously. The expression of certain stalk specific genes such as *ecmB* and *car2* are precocious and the prespore specific *pspA* expression was comparatively higher consistent with the *grlJ*¯ phenotype. Another interesting observation was that the grlJ mutant formed long fingers that broke up several times before culmination and eventually formed smaller but proportionate fruiting bodies. Few left over slug pieces developed further but several rounded up and remained on the substratum even until 30 h of development. One of the ways to maintain normal group size in *Dictyostelium* is by controlling cell-cell adhesion and motility. Large groups break apart from the aggregation

streams or from larger groups to form small groups, which is controlled by certain factors one of which is counting factor (Roisin-Bouffay et al., 2000). *grlJ*⁻ expresses higher levels of cell-cell adhesion molecules which may lead to the formation of larger slugs. However, they may break apart so as to maintain right proportions but in this process they break several times which may be due to a control that is lost in the absence of GrlJ (Raper, 1940, Sakai, 1973).

Additionally DIF-induced expression of *ecmB* displayed an indefinable behaviour as it was not induced by the addition of DIF-1 but only when cAMP and DIF were added together whereas the repression of *pspA* was comparable to wild type cells. This may be due to the fact that DIF acts only in cells that have experienced (primed) enough and optimal levels of cAMP before (Thompson et al., 2003) and the mound/tipped mound integrity is lost due to suboptimal assay conditions (2 h shaking period).

Several precociously developing strains (RegA, RdeA) are sporogenous and end up forming spores with abnormal morphology (Thomason et al., 1999). The precocious sporulation is mediated by increase in the intracellular cAMP levels and this is also found in the strain where the catalytic subunit of PKA is constitutively active (Simon et al., 1992; Chang et al., 1998). *grlJ*⁻ was also found to be sporogenous and yielded a higher number of spores than the wild type cells and the spore morphology was severely impaired. However fewer *grlJ*⁻ spores germinated both with and without treatment. The grlJ mutants formed SDF-1 and SDF-2 from the supernatants tested from the culminants at different culminating stages. They also were able to induce sporulation in response to SDF-1 and SDF-2. Likewise we assessed sporulation in response to GABA and glutamate, which induced sporulation in both the wild type cells and in *grlJ*⁻ in a similar manner. The impaired germination of spores may be due to a switch that is triggered before time and leads to precocious development in the absence of GrlJ.

Another important finding in the strain defective in grlJ was its sensitivity towards osmotic shock. One of the components that affect the intracellular cAMP levels is the DokA (homolog of hybrid histidine kinase) essential for osmotic stress resistance in *Dictyostelium*. DokA causes a rapid increase in the intracellular cAMP levels in response to osmotic stress via negatively regulating the RdeA-RegA pathway by acting as a phosphatase that dephosphorylates RdeA and a role for GrlJ would be its involvement in this response by negatively regulating DokA.

4.4 Concluding remarks

GrlA and GrlJ, two highly related receptors (on the basis of sequences) were found to function in post aggregation events in a very different manner. Our studies with the strains lacking these respective GPCRs, led us place them in different pathways, the GrlA as one of the mediators in sporulation and GrlJ probably involved in the events that control the transition for differentiation. However, the ligands for the receptors are not known and also the downstream mediators. There are two developmentally regulated G-protein subunits, Gα4 and Gα5 that are putative candidates. Gα5 is involved right during tip morphogenesis, the time when we see a defect in the grlA mutants, and Gα4 is known in late folate and pterin mediated response to regulate morphogenesis and spore production (Hadwiger et al., 1994; 1996).

We have explored two of the 17 known Grls in *Dictyostelium*. It would be tempting to suspect any or many of them would be GABA receptors as GABA is synthesized in *Dictyostelium* as shown by its presence in the cell lysates of 6 h starved cells (Ehrenman et al, 2004). The presence of *gadA*, the product of the gene that synthesizes GABA, and AcbA which is similar to the neuropeptide DBI (diazepam binding inhibitor), which is known to bind to $GABA_A$ receptors in higher eukaryotes (Iranfar et al., 2001), makes the possibility of the presence of GABA-like signaling in *Dictyostelium* even stronger. GABA would not carry out neuronal signaling in *Dictyostelium* but possibly some kind of intercellular signaling.

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Summary

GPCRs are a superfamily of diverse integral membrane proteins that allow detection and transduction of a large variety of extracellular signals. In *Dictyostelium* only seven members of one family, the crl (cAMP receptor like) family had been identified and studied in detail. The analysis of the genome sequence uncovered 48 additional putative GPCRs grouped into the secretin (family 2), metabotropic glutamate/$GABA_B$ (family 3) and the frizzled / smoothened (family 5) families of receptors. The presence of family 2, 3 and 5 receptors in *Dictyostelium* was indeed surprising because they had been thought to be animal-specific. GABA (gamma amino butyric acid), the principal inhibitory neurotransmitter in mammalian brain, signals through ionotropic ($GABA_{(A)}$/$GABA_{(C)}$) and metabotropic $GABA_{(B)}$ receptor systems. The functional $GABA_B$ receptor is a heterodimer of receptor 1 and receptor 2 subtypes. The *Dictyostelium* genome harbours 17 different genes encoding $GABA_B$ receptor like proteins each having slightly closer resemblance to one of each subtype. Yeast two-hybrid studies led us speculate that the C termini of these receptors alone may not be sufficient for their interactions to form homo or heterodimers. The detailed analysis of one of each type of receptors namely - GrlA and GrlJ was undertaken. Both these receptors are expressed throughout the development of *Dictyostelium* with a marked elevation in the later developmental stages. Mutant analysis revealed both GrlA and GrlJ to be involved in post aggregation morphogenesis in *Dictyostelium.* Strains lacking GrlA displayed a delay in development consistent with its expression pattern and completed development 3-4 hours later than the wild type cells. Furthermore, GrlA was found to be involved in controlling sporulation events as *grlA*$^-$ cells were defective in the generation of the signal required for spore differentiation, SDF-2. This may be due to a decrease in the levels of TagC, a protease present on the surface of prestalk cells that cleaves AcbA (formed in prespore cells and released) to form SDF-2 and the SDF-2 receptor, DhkA. Transcriptional profiling of the *grlA*$^-$ (16 h) displayed downregulation in the serum response factor (*srfA*) induced genes, which further strengthened the role of GrlA as an important component in sporulation and spore maturation. Our data suggest that GrlJ controls events during the transition from aggregation to post aggregation. *grlJ* $^-$ develops precociously after aggregation stages forming thin fingers that break several times on their way until culmination and form smaller fruiting bodies with malformed spores which are less viable. However, *grlJ* $^-$ does not exhibit any defect in the production or response, neither to the peptides controlling sporulation such as SDF-1 or SDF-2 nor towards GABA or Glutamate like the wild type cells.

Zusammenfassung

G-Protein gekoppelte Rezeptoren (GPCR) repräsentieren eine der grössten Proteinfamilien und nehmen eine Schlüsselrolle in der menschlichen Physiologie ein, da sie verschiedenste extrazelluläre Signale an die Zellen übermitteln. Ihre grosse Verbreitung weist darauf hin, dass sie in vielfältige physiologische Prozesse involviert sind und durch eine Vielzahl von endogenen Signalmolekülen wie Ionen, Geruchsstoffe, Aminosäuren, Peptide, Neurotransmitter, Hormone, Nukleotide und Fettsäuren aktiviert werden.

GPCRs sind in der Evolution früh entstanden, wobei "primitive" Organismen wie Hefe nur eine beschränkte Anzahl an und nur bestimmte Typen von GPCRs exprimieren. Umso erstaunlicher war der Befund, dass die einzellige Amöbe *Dictyostelium discoideum* Vertreter aller GPCR Familien mit Ausnahme der Familie 1 besitzt, die in Säugern die gamma-adrenergen Rezeptoren, Geruchsrezeptoren und Lichtrezeptoren umfasst. Bis zu diesem Zeitpunkt sind die Mitglieder Sekretin-Familie, die metabotropen Glutamat-Rezeptoren und die Frizzled/Smoothened Rezeptoren als Metazoen-spezifisch angesehen worden.

In dieser Arbeit haben wir zwei Rezeptoren, GrlA und GrlJ, analysiert, die zur Gruppe der metabotropen $GABA_B$ Rezeptoren gehören. Eine Mutantenanalyse zeigt für beide Rezeptoren eine Rolle in der späten multizellulären Entwicklung von *Dictyostelium*. GrlA ist in die Kontrolle der Sporulation involviert und beeinflusst die Bildung des Sporulationsfaktors SDF-2, GrlJ kontrolliert einen früheren Entwicklungsprozess, und sein Verlust führt zu einer verfrühten Entwicklung. Während die Bedeutung dieser Rezeptoren für eine korrekte multizelluläre Entwicklung von *Dictyostelium* nicht in Frage steht, ist die Identität der Signale noch nicht geklärt worden.

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit- einschließlich Tabellen und Abbildungen-, die anderen Werke im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie- abgesehen von unten angegebenen beantragten Teilpublikationen- noch nicht veröffentlicht ist, sowie, dass ich eine Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Frau Prof. Dr. Angelika A. Noegel betreut worden.

Köln,

7th December, 2005

Yogikala Prabhu

Curriculum Vitae

Name	:	Yogikala Vasudeva Prabhu
Address	:	Luxemburger Str, 124-136, Unicenter, Et-Zi: 24-33, 50939, Cologne, Germany.
Present address	:	9, Kaveri, Mukand Society, Near Anand Park, Thane (West) 400601, Thane, India E-mail: yogsjlt@yahoo.com
Date of birth	:	18.04.1976
Nationality	:	Indian

School Studies

1981-1991	:	Karthika High School, Mumbai, India
1991-1993	:	K.J. Somaiya Junior College of Science and Commerce,

University Studies

1993-1996	:	Bachelor of Science (Microbiology and Biochemistry), Mumbai University, India
1996-1998	:	Master of Science (Biochemistry) Mumbai University, India

Doctoral Studies

09.2002- 09.2005	:	PhD fellowship sponsored by "*International Graduate School in Genetics and Functional Genomics*", University of Cologne.
April 2003- December 2005	:	Faculty of Mathematics and Natural Science, University of Cologne, **Supervisor: Prof. Dr. Angelika A. Noegel**, Institute for Biochemistry I, Medical Faculty, University of Cologne, Cologne, 50931, Germany

Lebenslauf

Name	:	Yogikala Vasudeva Prabhu
Addresse	:	Luxemburger Str, 124-136, Unicenter, Et-Zi:24-33, 50939, Köln, Deutschland.
Heimataddresse	:	9, Kaveri, Mukand Society, Near Anand Park, Thane (West) 400601, Thane, India E-mail: yogsjlt@yahoo.com
Geburtsdatum	:	18.04.1976
Staatsangehörigkeit	:	Indisch

Schulausbildung

1981-1991	:	Karthika High School, Mumbai, Indien
1991-1993	:	K.J. Somaiya Junior College of Science and Commerce, Mumbai, Indien

Universität Studium

1993-1996	:	Bachelor of Science (Microbiology and Biochemistry), Mumbai University, Indien
1996-1998	:	Master of Science (Biochemistry) Mumbai University, Indien

Promotions Studium

09.2002- 09.2005	:	Stipendiat bei der "*International Graduate School in Genetics and Functional Genomics*", Universität zu Köln.
04.2003- 09.2005	:	Mathematisch-Naturwissenschaftliche Fakultat, Universität zu Köln, **Betreurin: Prof. Dr. Angelika A. Noegel**, Institüt Für Biochemie I, Medizinische Fakultät, Universität zu Köln, Köln, Deutschland