Characterisation of PhdB, a pleckstrin homology domain containing protein in *Dictyostelium discoideum*

INAUGURAL-DISSERTATION

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zü Köln

vorgelegt von

Dhamodharan Neelamegan

aus

Bangalore, Indien

Köln, 2004

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

Prof. Maria Leptin

Date of oral examination: 07.07.2004

Tag der mündlichen Prüfung

The present research work was carried out under the supervision of
Prof. Dr. Angelika A. Noegel, in the Institute of Biochemistry I, Medical Faculty, University of Cologne, Cologne, Germany. From June 2001 to July 2004.

Diese Arbeit wurde von Juni 2001 bis Juli 2004 am Biochemischen Institut I der Medizinischen Fakultät der Universität zu Köln unter der Leitung von
Prof. Dr. Angelika A. Noegel durchgeführt.

Acknowledgement

I wish to express my deep sense of gratitude to Prof. Dr. Angelika A. Noegel, esteemed advisor of my promotion studies, for encouraging and supporting me through out my study and for critically reviewing the thesis.

I am extremely grateful to Dr. Francisco Rivero, Dr. Akis Karakesisoglou and Dr.Ludwig Eichinger for their keen interest, advice, and encouragement through out my course of my study.

I am thankful to Rosi, Maria and Berthold for their help and cooperation during my stay here in the lab. Thanks to Bettina Lauss, for her continuous help with official works during the period of my stay in Köln. My due thanks to Dr. Budi Tunggal for his help in sorting out the computer problems encountered during my work and thesis writing.

I take this opportunity to express my humble and sincere thanks to my parents for all they have done to bring me to this level.

I am thankful to all my previous and present lab colleagues for their direct and indirect help during my work. I am also thankful to all my friends and well wishers who helped me directly or indirectly for the successful completion of my research work. I also thank Dr. Subramanya Hegde for all his help during my stay in Köln.

The financial assistance received by me from the Graduate School 'International graduate school in genetics and functional genomics' University of Cologne Germany, in the form of Stipend is highly acknowledged.
Also the financial assistance received by me from the DFG is highly acknowledged.

04 May 2004 Dhamodharan. N
Cologne, Germany.

Abbreviations*:*

AP	Alkaline phosphatase
APS	Ammonium persulphate
Bsr	Blasticidin resistance cassette
BSA	Bovine serum albumin
CH	Calponin homology
CRAC	Cytosolic regulator of adenylyl cyclase
cAMP	Adenosine- 3', 5'- cyclic monophosphate
cAR	cAMP receptor
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulfoxide
dNTP	Deoxyribonucleotide triphosphate
DTT	1,4-dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis (2-amino-ethylene) N,N,N,N-tetraacetic acid
GEF	Guanine-nucleotide exchange factor
GPCR	G-protein coupled receptor
GFP	Green Fluorescence Protein
GAP	GTPase activating protein
GST	Glutathione S-transferase
FITC	Fluorescein-5-isothiocyanate
HRP	Horse radish peroxidase
HEPES	N- (2-hydroxyethyl) piperazine-N-2-ethanesulphonic acid
IPTG	Iso-propylthio-galactopyranoside
IgG	Immunoglobulin G
Kb	Kilobase pairs
kDa	KiloDalton
MES	Morpholinoethansulphonic acid
MOPS	Morpholinopropanesulphonic acid
mRNA	messenger ribonucleic acid
mAb	Monoclonal Antibody
NP-40	Nonylphenylpolyethyleneglycol
OD	Optical density
PIPES	Piperazine-N,N.-bis(2-ethanesulphonic acid)
PMSF	Phenylmethylsulphonylfluoride
PKB/Akt	Protein Kinase B
PI3K	Phosphatidylinositol 3 kinase
PAGE	Polyacrylamide gel electrophoresis
PH	Pleckstrin homology
rpm	Rotations per minute
SDS	Sodium dodecyl sulphate
TRITC	Tetramethylrhodamine β isothiocyanate
TAE	Tris borate EDTA
WT	Wild type
X-gal	5-bromo-4-chloro-3-indolyl-D-galactopyranoside

1. INTRODUCTION

1.1 *Dictyostelium* life cycle and molecular techniques

Dictyostelium discoideum is a non-metazoen eukaryote, which lives as a unicellular amoeba in the soil and feeds on bacteria. When the food source is exhausted the amoebae aggregate into a mass of up to 10^5 cells, which differentiate and develop into a mushroom shaped fruiting body (Kessin, 2000).

In the laboratory *Dictyostelium* are grown on a lawn of bacteria or in a liquid medium. To begin development in the laboratory we remove the source of nutrition and put the cells on a moist solid substratum. The substratum can be agar or filter paper which is sufficiently moist. As no nutrients are provided the amoebae aggregate and make a fruiting body entirely using the metabolic reserves accumulated during the trophic phase. The fruiting body consists of a ball of spores supported by a stalk. The striking feature of *Dictyostelium* during starvation is that they undergo a switch in behaviour by inducing the expression of additional genes to form a fruiting body (Figure I).

Dictyostelium is an excellent organism for the study of the molecular mechanisms of cell motility, signal transduction, cell-type differentiation and developmental processes. It is haploid and therefore mutants can be easily generated. The molecular genetic techniques available include gene inactivation by homologous recombination, gene replacement, antisense strategies, restriction enzyme-mediated integration (REMI), library complementation and expression of green fluorescent protein (GFP) fusion proteins. The hereditary information is carried on 6 chromosomes with sizes ranging from 4 to 8 Mb (Cox *et al.,* 1990; Kuspa and Loomis, 1996) resulting in a total of about 34 Mb of DNA; a multicopy 90 kb extrachromosomal element that harbours the rRNA genes, and the 55 kb mitochondrial genome.

The genome sequencing is completed and the estimated number of genes in the genome is ~12,000. Many of the known genes show a high degree of sequence similarity to homologues in vertebrate species (Eichinger and Noegel, 2003). Furthermore *Dictyostelium* cells resemble human leukocytes in their motility characteristics.

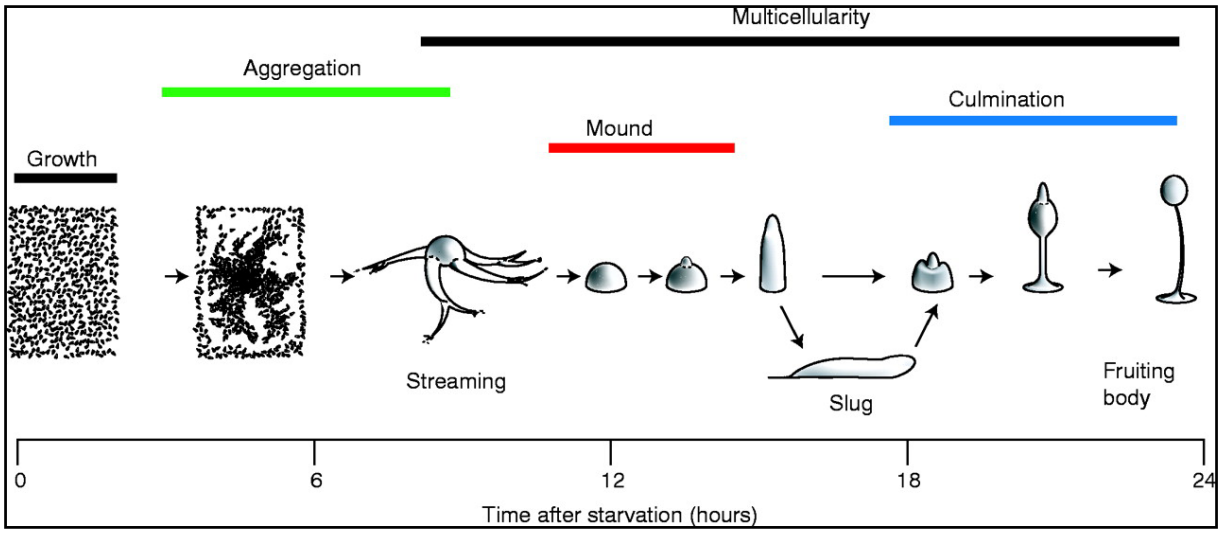


Figure I. *Dictyostelium* development. During growth phase *Dictyostelium* exists as single cell amoeba. Upon starvation *Dictyostelium* undergoes chemotaxis towards a pulsatile cAMP source secreted by the cells at the centre. During aggregation cells coalesce into adherent cells and form streams. The streams eventually come together to form the mound. The mound is the first stage in the multicellular development. It develops a tip, which coordinates further development. Next a finger like structure emerges which either immediately forms a fruiting body or a motile slug that migrates to a favourable environment for culmination into the fruiting body. The scale shows the relative timing of development (The picture was taken from Coates and Harwood, 2001).

1.1.2 Chemotaxis

Chemotaxis is a process by which cells respond to an extracellular chemical signal and guide their movement towards the chemical signal. Chemotaxis has a role in various functions of a cell, prokaryotes tracing their food, protozoa like *Dictyostelium* forming multicellular structures, axon guidance and embroygenesis is in part due to chemotaxis.

Eukaryotic cells respond to chemoattractant gradients by adapting a polarised morphology of their cytoskeleton towards the origin of the signal (Chen *et al.,* 1997). The actin cytoskeleton is an important player in this event, and allows protrusion of an actin-rich lamellipod (pseudopod). In chemotaxing cells, including *Dictyostelium* and leukocytes, the forward movement is driven by extending the leading edge through localised polymerisation of F-actin. This change is coupled to an actin-myosin mediated contraction of uropod (posterior region of a cell), which allows the uropod to be released and retracted towards the direction of movement. Small GTPases like Rac and Cdc42 have been shown to regulate the process of F-actin assembly at the leading edge of the cell (Hall *et al.,* 1989). Dominant negative Rac

prevents F-actin assembly, whereas constitutively active Rac results in excessive F-actin assembly and membrane ruffling (Rivero *et al.,* 2001). It has been shown that the Rho family members Cdc42 is required for proper orientation of the cytoskeleton towards the direction of the chemoattractant signal. Macrophages expressing dominant negative Cdc42 move in response to directional signals but the movement lacks directionality (Allen *et al.,* 1998). These observations indicate that Cdc42 may act in leading the cell movement towards a particular direction. Both *Dictyostelium* and leukocytes use G-protein coupled serpentine receptors to transduce the extracellular signal. In the mammalian system binding of chemoattractant to the receptor elicits a transient increase in phosphoinositides (PIs), cAMP, cGMP, Ca^{2+} and rearrangement of F-actin. In both leukocytes and *Dictyostelium* the receptor molecules remain uniformly distributed across the cell surface during chemotaxis (Xiao *et al.,* 1997; Postma *et al.,* 2002). Thus despite uniform distribution of chemoattractant receptors on the cell surface the cells extend their leading edge towards the source of chemoattractant. It was concluded therefore that the cell adopts an internal asymmetric signaling relative to the external gradient. In addition to the small GTPases as important players at the leading edge of the cell the phosphatidylinositol 3 kinase (PI3K) signalling pathway is another essential component. The main feature of the PI3K pathway is the recruitment of PI3K at the leading edge, which results in localised production of PI (3,4,5) P3. PIP3 serves as docking site for many pleckstrin homology (PH) domain containing proteins. Studies on this pathway have led to the identification of several PH domain containing proteins as effectors of PI3K. They include Akt/PKB, CRAC and PhdA.

Studies with Akt/PKB, CRAC (required for receptor activation of the adenylyl cylcase; Insall *et al.,* 1994) and PhdA (Funamoto *et al.,* 2001), a newly identified PH domain containing protein in *Dictyostelium,* provide evidence for localised activation of responses leading to directional cell movement. These proteins transiently localize to the plasma membrane in response to cAMP. When *Dictyostelium* cells are bathed in cAMP the GFP fusions of CRAC and Akt/PKB are uniformly distributed along the plasma membrane, indicating that the chemoattractant activates the receptors uniformly distributed on the cell surface. Further insights were obtained when the response was examined in chemotaxing cells. Under these conditions the GFP-PH domain fusion proteins of both CRAC and Akt/PKB were found to localise to the leading edge of the cells. This observation strongly indicates that receptor mediated responses are locally activated at the leading edge (Figure II; Firtel and Chung, 2000).

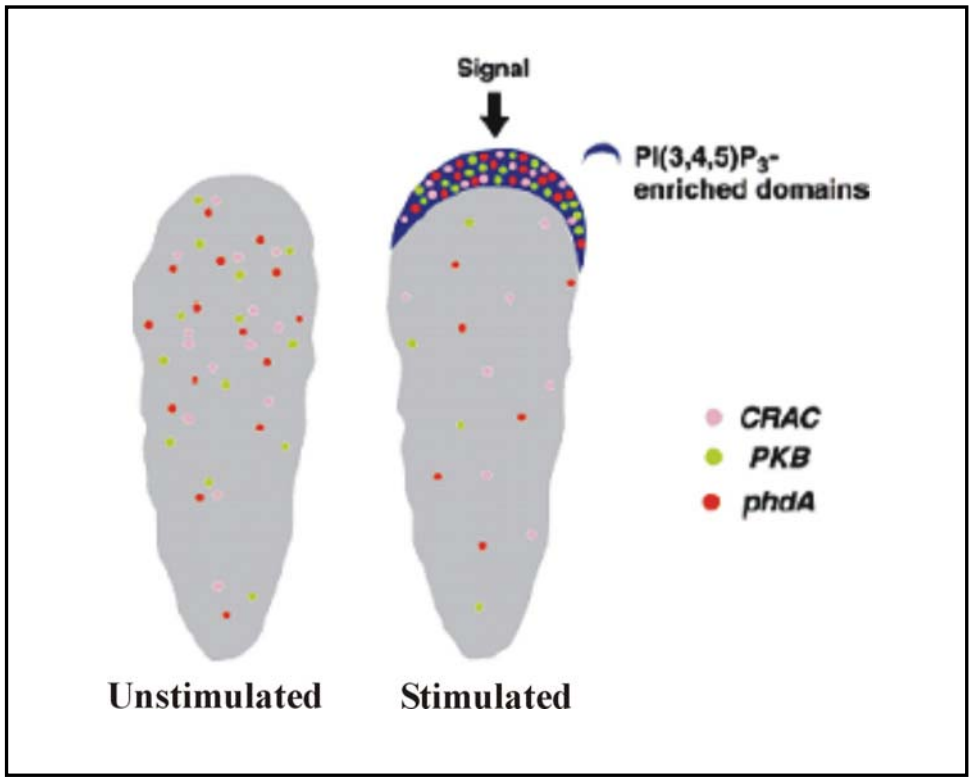


Figure II. Asymmetric accumulation of PIP(3) and PH domain containing proteins at the leading edge of the cell. In an unstimulated cell the PH domain containing proteins CRAC, Akt/PKB and PhdA are cytosolic. In chemotaxing cell, these proteins become localised to the leading edge.

In neutorophils it has been shown that PI3K, Rac and F-actin pathways are integrated and function through a positive feed back loop, by which localised signals are amplified to allow formation of the leading edge (Leevers *et al.,* 1999; Weiner *et al*, 2002).

In mammals four isoforms of PI3Ks are known, α, β, γ and δ. But only leukocyte specific PI3Kγ has been tentatively implicated in translocating signals from G protein linked receptors into specialized responses such as chemotaxis (Janetopoulos *et al.,* 2001). Studies with knock out mice lacking the catalytic subunit of PI3Kγ, the p110γ subunit, indicate the possibility of PI3K signalling pathway (Zhou *et al.,* 1995) involved in directional movement. The leukocytes lacking PI3Kγ failed to move directionally in response to chemotactic stimulation. This is also in accordance with the response of cells expressing dominant negative Cdc42. This observation also suggests a co-operative relationship between these two processes (Allen *et al.,* 1998).

In addition to the GTPases and PI3K role at the leading edge of a cell, recently in leukocytes it has been shown that PAK1 and PIXα are recruited to the leading edge which are down stream of the heterotrimeric G protein coupled receptor. The recruitment and activation of

PAK1 is mediated by direct binding of PAK1 to Gβγ. The interaction of Gβγ and PAK1 also brings PIXα into the complex. As a result Cdc42 is activated, leading to the activation of PAK1 (Daniels *et al.,* 1999). Studies with PIXα knockout mice reported that the mice were normal, however neutrophils isolated from these mice exhibit severe defect in chemotaxis and chemotactic signaling (Li *et al.,* 2003) .

Based on the recent findings chemotaxis can be broadly divided into three modules as shown in figure III. Module 1 represents the event at the leading edge when the chemoattractant receptor is activated, small GTPases along with GEF/GAP factors regulate modulation of F-actin polymerization at the leading edge. Module 2 represents the activation of PI3K signalling that contributes to the localised accumulation of phosphoinositides, which mediates actin polymerization by recruiting PH domain containing proteins. Module 3 represents the myosin based retraction of the uropod which is regulated by cGMP signalling at the posterior end of the cell.

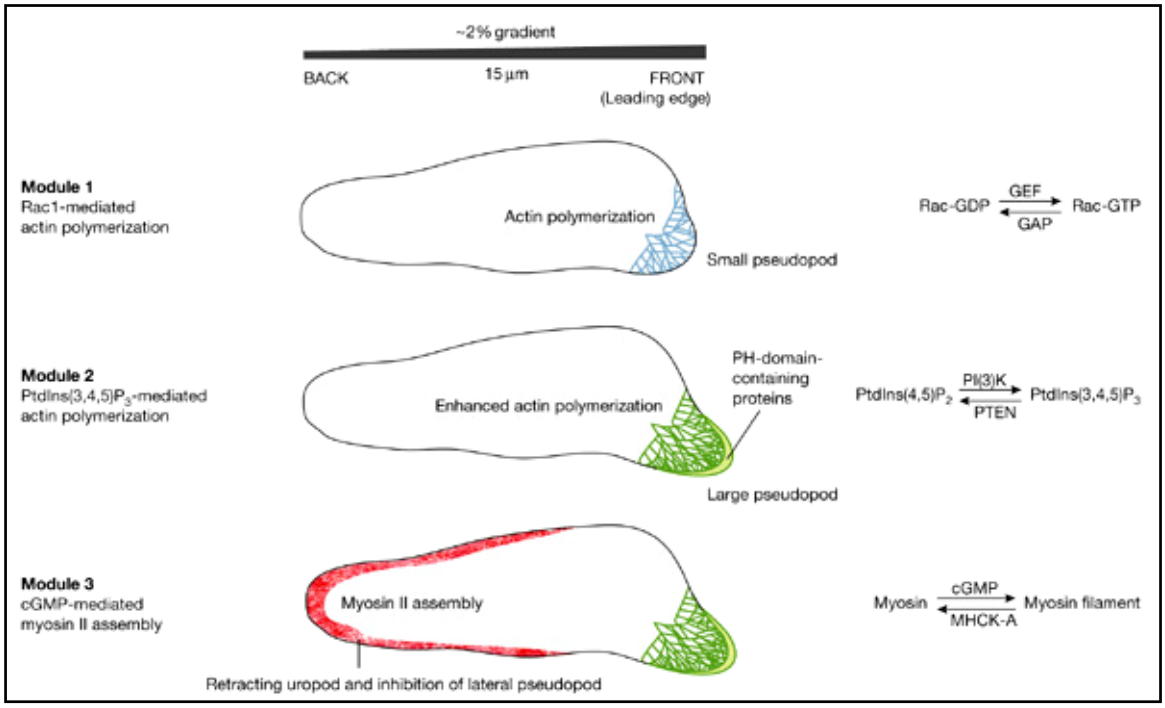


Figure III. Module 1, activation of the chemoattractat receptor leads to formation of active small G proteins of the Rho/Rac group, leading to actin polymerisation and a small pseudopod is extended at the front of the cell. It is proposed that specific activators of GTPases, guanine exchange factors (GEFs) and inhibitors GTPase-activating proteins (GAPs) are preferentially recruited at the higher chemoattractant concentrations. The polarity generated by the first module is used by the second module to trigger a patch of phosphatidylinositol-3,4,5 trisphosphate (PtdIns (3,4,5) P_3) at the leading edge, which enhances actin polymerisation and extension of the pseudopod. The third module inhibits the formation of pseudopodia at the back and sides of the cells by cementing the uropod with myosin II assembly. Increase in cGMP concentration leads to myosin filament formation at the uropod (Graphic from Postma *et al.,* 2004).

1.1.3 *Dictyostelium* chemotaxis

cAMP plays an important role in the development of *Dictyostelium.* Early in the developmental cycle, when cells are starved some cells begin to secrete pulses of cAMP. The surrounding cells respond by undergoing chemotactic migration towards these cells. When extracellular cAMP binds to the cell surface receptor, signals are transduced and this results in the formation of intracellular cAMP, which is then secreted into the medium eliciting cAMP responses in other cells further away from the aggregation centre.

The cAMP signalling is mediated through a cell surface receptor, which activates G-protein dependent signalling, which finally activates adenylyl cyclase (Van Haastert *et al.,* 1991).

The signalling pathway leading to the activation of the adenylyl cyclase ACA during aggregation are well studied. In addition to the Gβγ subunit, which appear to be the direct activator of ACA, proteins like, Pianissimo, CRAC (Cytosolic Regulator of Adenylyl Cyclase) and MAP kinase ERK2 play important roles in controlling the production of cAMP, but do not affect chemotaxis (Wu *et al.,* 1995; Chen *et al.,* 1997; Firtel, 1996). Many of the components of aggregation stage signalling pathways are induced by nanomolar pulses of cAMP via an autoregulatory loop, by ACA. By this chemotactic mediated mechanism, cells are able to form mounds of ~ 10^5 cells. Also cAMP is an important regulator of gene expression. At the early aggregation stage of development, pulses of low concentration of cAMP control the expression of a number of genes. At later stages higher concentrations of cAMP are required for gene expression.

The chemoattractant signal is perceived by G-protein coupled receptor (GPCR) cAR1. Using GFP tagged cAR1 it was demonstrated that the receptor remains evenly distributed along the plasma membrane in highly polarised chemotaxing cells (Servent *et al.,* 1999). This observation indicated different activation of signalling pathways in the front and back of the cells, which is not dependent on differential distribution of the receptor. The G-protein β subunit was shown to exhibit a shallow anterior-posterior gradient similar to the extracellular chemoattractant. This reflects a shallow receptor occupancy gradient. Studies with GFP fusions of PH domain containing proteins including CRAC and the PI3K effector Akt/PKB demonstrated that proteins carrying PH domains that bind to the PI3K products PtdIns(3,4,5)P_3 / PtdIns(3,4)P_2 rapidly translocate to the plasma membrane in response to a uniform stimulation with the chemoattractant cAMP (Parent *et al.,* 1998). Analysis of *Dictyostelium* PI3K1 and PI3K2 localisation revealed that in vivo these two proteins rapidly and transiently translocate to the plasma membrane in response to stimulation by cAMP. Both PI3Ks localise to the leading edge during chemotaxis. The localisation and activation of PI3K

at the leading edge could thus mediate generation of a steep PtdIns(3,4,5)P_3 gradient (Zhou *et al.,* 1998). In cells lacking the Gα2 or the Gβ subunit (Lilly and Devreotes, 1995) the translocation of PI3Ks to the leading edge does not occur. This observation shows that the localisation of PI3K is a downstream event of the heterotrimeric G protein. The interacting partners of PI3Ks at the membrane are presently not known. The persistence of the steep PtdIns(3,4,5)P_3 gradients indicates that a mechanism must restrict the PtdIns(3,4,5)P_3 at the leading edge. One possibility is that PtdIns(3,4,5)P_3 is degraded along the lateral side of the cell. A candidate enzyme is the tumour suppressor PTEN a phosphatase, that dephosphorylates PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 to PtdIns(4,5)P_2 / PtdIns(4)P, respectively (Lee *et al.,* 1999). *Dictyostelium* has provided more evidence for a direct role of PTEN in regulating chemotaxis (Funamoto *et al.,* 2002). These studies revealed that in resting cells PTEN is localised to the plasma membrane and uniformly distributed around the cell. In response to global stimulation the phosphatase PTEN transiently delocalises from the plasma membrane. In chemotaxing cells, PTEN is excluded from the leading edge but is present along the sides and the back of the cell. Thus PI3K and PTEN exhibit opposite patterns of spatial localisation during *Dictyostelium* chemotaxis.

The defects of PI3K1/PI3K2 null cells are more severe than those of Akt/PKB null cells. PI3 kinase presumably has downstream effectors in addition to Akt/PKB that are required for chemotaxis. Towards this, PH domain containing proteins have gained importance, as they may have an essential role in the PI3Kinase activated localised signalling.

1.1.4 Cell-cell adhesion during *Dictyostelium* development.

Two mechanisms of cell-cell adhesion, characterised as EDTA sensitive and EDTA resistant aggregation have been shown to operate at different stages of development in *Dictyostelium.* Upon starvation the cells undergo a developmental programme where cells migrate chemotactically towards regions of higher concentration of cAMP and form multicellular aggregates. Within 24 h morphogenesis takes place and the cells eventually form a fruiting body consisting of two major types of cells, spore cells and stalk cells. During the first few hours of starvation, the cells produce and accumulate a glycoprotein of 24 kDa (gp24). Expression of gp24 confers cells to acquire EDTA sensitive binding sites (Knecht *et al.,* 1987) that are disrupted by low concentration (1 mM) of EDTA. The cells acquire EDTA resistant cell binding sites a few hours later, at the early aggregation of development (Gerisch *et al.,* 1985). The adhesion sites of these cells are stable in the presence of 10 mM EDTA. The

adhesion in these cells are mediated by the homophilic interaction of an 80 kDa glycoprotein (gp80) also known as contact site A (csA; Muller and Gerisch, 1978; Siu and Kamboj, 1990). The first phase of csA expression is in the very early stage of aggregation and results in a low basal level of expression. The mechanism involved in the induction of this low level expression of csA is not known. The low level of expression of csA does not seem to be dependent on cAMP (Ma and Siu, 1990). The second phase requires cAMP and results in high level of csA expression. As the chemotaxing *Dictyostelium* cells respond by secreting cAMP into the medium, the secreted cAMP augments the expression of csA. In this cAMP signal pathway, cAMP binds to the cell surface cAMP receptor (Klein *et al.,* 1988) and this binding leads to the G- protein coupled activation of adenylyl cyclase. The activated adenylyl cyclase now produces intracellular cAMP (Snaar-Jagalska *et al.,* 1988). The cAMP is then secreted into the medium and binds to the cAMP receptor on adjacent cells. Since the cAMP receptor is desensitised soon after the ligand (cAMP) binding, cAMP is produced and secreted in a pulsatile manner. This pulsatile cAMP production and release leads to the differential activation of certain aggregation stage specific genes, including the regulation of csA expression. The expression of csA begins by transient activation of the cell surface cAMP receptor. The receptor which is coupled to the G-protein, Gα2 subunit transduces the down stream signalling (Newell *et al.,* 1988; Pupillo *et al.,* 1989). Also it was shown that adenylyl cyclase which is the down stream effector of cAMP receptor activation, is not involved in this pathway, and that the intracellular cAMP is not required for csA expression (Ma and Siu, 1990).

In the present study we have identified a novel PH domain containing protein from the cDNA and genomic DNA databases of *Dictyostelium* the protein is referred as PhdB (Pleckstrin homology domain containing Protein B). We also generated *Dictyostelium* mutants, in which the PhdB gene was inactivated by homologous recombination, and analyse its role in *Dictyostelium* aggregation.

1.2 AIM OF THE STUDY

This study was initiated since the *Dictyostelium* genome sequencing was in progress and information on potential novel genes was available in the databases.

Dictyostelium is haploid and simple, and most of the signalling pathways as well as chemotactic behaviors like those seen in white blood cells are highly conserved throughout evolution. Hence *Dictyostelium* genome offers a great value to understand and determine the functions of novel genes as well as homologous genes from other species.

The aims set for the study were to characterise novel actin binding proteins, by developing tools like monoclonal and polyclonal antibodies against the new protein, and to generate *Dictyostelium* mutants which lack the expression of the gene of interest by homologous recombination to understand the exact function of the new gene.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Laboratory materials

Cellophane sheet, Dry ease	Novex
Centrifuge tubes, 15 ml, 50 ml	Greiner
Coverslips (glass), ∅ 12 mm, ∅ 18 mm, ∅ 55 mm	Assistent
Corex tube, 15 ml, 50 ml	Corex
Cryo tube, 1 ml	Nunc
Electroporation cuvette, 2 mm electrode gap	Bio-Rad
Microcentrifuge tube, 1.5 ml, 2.2 ml	Sarstedt
Micropipette, 1-10 µl, 10-200 µl, 100-1,000 µl	Gilson
Needles (sterile), 18G–27G	Terumo, Microlance
Nitrocellulose membrane, BA85	Schleicher and Schuell
Nitrocellulose-round filter, BA85, ∅ 82 mm	Schleicher and Schuell
Nylon membrane, Biodyne B	Pall
Parafilm	American National Can
Pasteur pipette, 145 mm, 230 mm	Brand, Volac
PCR soft tubes, 0.2 ml	Biozym
Petri dish (35 mm, 60 mm, 100 mm)	Falcon
Petri dish (90 mm)	Greiner
Plastic cuvette, semi-micro	Greiner
Plastic pipettes (sterile), 1 ml, 2 ml, 5 ml, 10 ml, 25 ml	Greiner
Quartz cuvette, Infrasil	Hellma
Quartz cuvette, semi-micro	Perkin Elmer
Saran wrap	Dow
Slides, 76 x 26 mm	Menzel
Syringes (sterile), 1 ml, 5 ml, 10 ml, 20 ml	Amefa, Omnifix
Syringe filters (Acrodisc), 0.2 µm, 0.45 µm	Gelman Sciences
Tissue culture flasks, 25 cm^2, 75 cm^2, 175 cm^2	Nunc
Tissue culture dishes, 6 wells, 24 wells, 96 wells	Nunc
Whatman 3MM filter paper	Whatman
X-ray film, X-omat AR-5, 18 x 24 mm, 535 x 43 mm	Kodak

2.1.2 Instruments and equipments

Centrifuges (microcentrifuges):	
Centrifuge 5417 C	Eppendorf
Centrifuge Sigma	B. Braun Biotech Instruments
Cold centrifuge Biofuge fresco	Heraeus Instruments
Centrifuges (table-top, cooling, low speed):	
Centrifuge CS-6R	Beckman
Centrifuge RT7	Sorvall
Centrifuge Allegra 21R	Beckman
Centrifuges (cooling, high speed):	
Beckman Avanti J25	Beckman
Sorvall RC 5C plus	Sorvall
Centrifuge-rotors:	

JA-10	Beckman
JA-25.50	Beckman
SLA-1500	Sorvall
SLA-3000	Sorvall
SS-34	Sorvall
Dounce homogeniser, 10 ml	B. Braun
Electroporation unit, Gene-Pulser	Bio-Rad
ELISA reader	Lab Systems
Fluorescence spectrophotometer	Photon Technology International
Freezer (-80°C)	Nunc
Freezer (-20°C)	Siemens, Liebherr
Gel-documentation unit	MWG-Biotech
Heating block, DIGI-Block JR	neoLab
Hybridising oven	Hybaid
Incubators:	
CO_2-incubator, BBD 6220, BB 6220	Heraeus
Incubator, microbiological	Heraeus
Incubator with shaker, Lab-Therm	Kuehner
Laminar flow, Hera Safe (HS 12)	Heraeus
Model 422 Electro-Eluter	Bio-Rad
Microscopes:	
Light microscope, CH30	Olympus
Light microscope, DMIL	Leica
Light microscope, CK2	Olympus
Fluorescence microscope, DMR	Leica
Fluorescence microscope, 1X70	Olympus
Confocal laser scan microscope, DM/IRBE	Leica
Stereomicroscope, SZ4045TR	Olympus
Oven, conventional	Heraeus
PCR machine, PCR-DNA Engine PTC-2000	MJ Research
pH-Meter	Knick
Refrigerator	Liebherr
Semi-dry blot apparatus, Trans-Blot SD	Bio-Rad
Sonicator, Ultra turrax T25 basic	IKA Labortechnik
Speed-vac concentrator, DNA 110	Savant
Spectrophotometer, Ultraspec 2000, UV/visible	Pharmacia Biotech
Ultracentrifuges:	
Optima TLX	Beckman
Optima L-70K	Beckman
Ultracentrifuge-rotors:	
TLA 45	Beckman
TLA 100.3	Beckman
SW 41	Beckman
UV-crosslinker, UVC 500	Hoefer
UV- transilluminator, TFS-35 M	Faust
Vortex, REAX top	Heidolph
Waterbath	GFL

2.1.3 Kits

Advantage cDNA PCR kit	Clontech
Nucleobond AX	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

2.1.4 Enzymes, antibodies, substrates, inhibitors and antibiotics

Enzymes used in the molecularbiology experiments:

Calf Intestinal Alkaline Phosphatase (CIAP)	Boehringer
Deoxyribonuclease I (DNase I)	Boehringer
Klenow fragment	Boehringer
Lysozyme	Sigma
Proteinase K	Sigma
Restriction endonucleases	Amersham, Life technologies, New England Biolabs
Reverse transcriptase, Superscript II	Life technologies
Ribonuclease H (RNase H)	Boehringer
Ribonuclease A (RNase A)	Sigma
S1-nuclease	Amersham
T_4 DNA ligase	Boehringer
Taq-polymerase	Life technologies/Boehringer

Primary antibodies:

Goat anti-GST antibody	Pharmacia
Mouse anti-Actin monoclonal antibody, Act 1	Simpson *et al.*, 1984
Mouse anti-CAP monoclonal antibody, 231-18-8	Gottwald *et al.,* 1996
Mouse anti-csA monoclonal antibody, 33-294	Berthold *et al.,* 1985
Mouse anti-Filamin monoclonal antibody, 82-382-8	Brink *et al.,* 1985

Secondary antibodies:

Mouse anti-goat IgG, alkaline phosphatase conjugated	Sigma
Goat anti-mouse IgG, peroxidase conjugated	Sigma
Goat anti-mouse IgG, alkaline phosphatase conjugated	Sigma
Goat anti-rabbit IgG, peroxidase conjugated	Sigma
Mouse anti-goat IgG, peroxidase conjugated	Sigma
Sheep anti-mouse IgG, Cy3 conjugated	Sigma

Substrates:

BCIP/NBT	Promega
pNPP, Sigma Fast tablet sets, 5ml, 20 ml	Sigma

Inhibitors:

Diethylpyrocarbonate (DEPC)	Sigma
Leupeptin	Sigma
Pepstatin	Sigma
Phenylmethylsulphonylfluoride (PMSF)	Sigma

Antibiotics:

Ampicillin	Gruenenthal
Blasticidin S	ICN Biomedicals

Chloramphenicol	Sigma
Dihydrostreptomycinsulphate	Sigma
Geneticin (G-418)	Life technologies
Kanamycin	Sigma, Biochrom
Tetracyclin	Sigma

2.1.5 Chemicals and reagents

Acetic acid (98-100%)	Riedel-de-Haen
Acrylamide (Protogel: 30:0,8 AA/Bis-AA)	National Diagnostics
Adenosine triphosphate (ATP)	Sigma
Agar-Agar (BRC-RG)	Biomatic
Agarose (Electrophoresis Grade)	Life technologies
3-Aminophthalhydrazide	Fluka
Bacto-Agar	Difco
Bacto-Peptone	Difco
Bacto-Tryptone	Difco
Boric acid	Merck
Bovine serum albumin (BSA)	Roth
5-Brom-4-chlor-3-indolyl-β-D-galactopyranoside (X-Gal)	Roth
Bromophenol blue (Na-Salt)	Serva
Caesium chloride	Biomol
Calcium chloride-dihydrate	Merck
Chloroform	Riedel-de-Haen
Coomassie Brilliant Blue R 250	Serva
p-Coumaric acid	Fluka
cyclic Adenosine monophosphate	Sigma
Cyclohexamide	Sigma
Deoxyribonucleotide triphosphates (dNTP)	Sigma, Roche
Dimethylformamide	Riedel-de-Haen
Dimethylsulfoxide (DMSO)	Merck
Dithiothreitol (DTT)	Sigma
Ethanol	Riedel-de-Haen
Ethidium bromide	Sigma
Ethylene diamine tetraacetate (EDTA), disodium salt	Merck
Ethylene glycol-bis [2-aminoethylether]- -N,N,N',N'-tetraacetate (EGTA)	Sigma
Formamide	Merck
Gelatin (Teleostean), cold-water fish skin	Sigma
Glucose	Merck
Glycerine	Riedel-de-Haen
Glycine	Riedel-de-Haen
Hydrochloric acid, 32%	Fluka
Hydrogen peroxide (H_2O_2), 30%	Merck
Imidazole	Merck
Isopropanol	Merck
Isopropypl-β-D-thiogalactopyranoside (IPTG)	Loewe Biochemica
Magnesium acetate-tetrahydrate	Merck
Magnesium sulphate-heptahydrate	Merck
Maltose	Merck
β-Mercaptoethanol	Sigma

Methanol	Riedel-de-Haen
Morpholino ethane sulphonic acid (MES)	Merck
Morpholino propane sulphonic acid (MOPS)	Gerbu
N- [2-Hydroxyethyl] piperazine-N'-2--ethanesulfonic acid (HEPES)	Biomol
Nonylphenyl-polyethyleneglycol (NP-40)	Fluka
Peptone	Oxoid
Phenol	Roth
Phosphoric acid	Merck
Piperazine-N, N'-bis [2-ethane sulphonic acid] (PIPES)	Sigma
Polyethyleneglycol 4000 (PEG 4000), solution	Sigma
Polyoxyethylene-sorbitan monolaurate (Tween 20)	Roth
Polyvinyl alcohol, Av. MW 30,000-70,000	Sigma
Polyvinylglycol MW 10,000	Sigma
Ponceau S Concentrate	Sigma
Potassium acetate	Fluka
Potassium chloride	Fluka
Potassium dihydrogen phosphate	Fluka
di-Potassium hydrogen phosphate	Merck
Sodium acetate	Fluka, Merck
Sodium azide	Merck
Sodium chloride	Fluka
Sodium citrate	Fluka, Merck
Sodium dihydrogen phosphate-dihydrate	Merck
Sodium dodecyl sulphate (SDS)	Serva
di-Sodium hydrogen phosphate	Merck
Sodium hydroxide	Riedel-de-Haen
Sodium lauryl sarcosinate	Sigma
Sorbitol	Fluka
Sucrose	Fluka
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Merck
Trichloroacetic acid	Merck
Tris [hydroxymethyl] amino methane	Fluka, Riedel-de-Haen
Triton X-100	Merck
Trypan blue	Merck
Xylene cyanol	Fluka
Yeast extract	Oxoid

Radiolabelled nucleotide:

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

2.1.6 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.

2.1.7 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

2.1.8 Media *for E. coli* culture

LB medium, pH 7.4:
(Sambrook *et al.*, 1989)

10 g bacto-tryptone
5 g yeast extract
10 g NaCl
adjust to pH 7.4 with 1 N NaOH
add H_2O to make 1 liter

For LB agar plates, 0.9% (w/v) agar was added to the LB medium and the medium was then autoclaved. For antibiotic selection of *E. coli* transformants, 50 mg/l ampicillin, kanamycin or chloramphenicol was added to the autoclaved medium after cooling it to approx. 50ºC. For blue/white selection of *E. coli* transformants, 10 µl 0.1 M IPTG and 30 µl X-gal solution (2% in dimethylformamide) was plated per 90 mm plate and the plate was incubated at 37ºC for at least 30 min before using.

SOC medium, pH 7.0:

(Sambrook *et al.*, 1989)

20 g bacto-tryptone
5 g yeast extract
10 mM NaCl
2.5 mM KCl
dissolve in 900 ml deionised H_2O
adjust to pH 7.0 with 1 N NaOH

The medium was autoclaved, cooled to approx. 50ºC and then the following solutions, which were separately sterilized by filtration (glucose) or autoclaving, were added:

10 mM $MgCl_2.6H_2O$
10 mM $MgSO_4.7H_2O$
20 mM Glucose
add H_2O to make 1 liter

2.1.9 Media for hybridoma cells

Freezing medium:

80 ml Normal media
10 ml foetal calf serum, heat inactivated (Roche)
10 ml DMSO, hybri-max (Sigma)

3x HAT medium:

572 ml Normal media
36 ml 50x HAT supplement (Biochrom)

1x HAT medium:

572 ml Normal media
12 ml 50x HAT supplement (Biochrom)

1x HT medium:

572 ml Normal media
12 ml 50x HT supplement (Biochrom)

Normal media (NM):

500 ml RPMI 1640 (Biochrom)
55 ml Foetal calf serum, heat-inactivated (Roche)
11 ml Kanamycin, 5 mg/ml
6 ml 1mM β-mercaptoethanol, freshly made

RPMI 1640:

Readymade RPMI 1640 medium (1x) containing 25 mM HEPES, 0.532 g/l L-glutamine, 5.5 g/l NaCl, 5 mg/l phenol-red and 2.0 g/l $NaHCO_3$ was obtained from the company Biochrom.

RPMI 1640 (w/o HEPES, w/o glutamine): Same as RPMI 1640 except that it is without HEPES and L-glutamine. It was obtained readymade (1x) from the company Biochrom.

2.1.10 Buffers and other solutions

The buffers and solutions that were commonly used during the course of this study are mentioned below-

Hepes-phenol:

1 kg phenol was melted at 60ºC in a water-bath and equilibrated with 1 vol. of 1 M Hepes, pH 7.5. The equilibrated phenol was aliquoted in 50 ml Falcon tubes and stored at –20ºC.

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

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

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, pH 8.0

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

2.1.11 Biological materials

Bacterial strains:

E. coli BL21 (DE)	Studier and Moffat,1986
E. coli DH12S	Life technologies
E. coli DH5α	Hanahan, 1983
E. coli JM 83	Vieira and Messing,1982
E. coli MC1061	Wertman *et al.*, 1986
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)

2.1.12 Plasmids

pBluescript II SK(+/-)	Stratagene
pBsrGFP	Unpublished
pGEX-43T	Pharmacia Biotech
pIMS	Simon *et al.,* 1988

2.1.13 Primers

dh6	5’ATGCAACCCAAAGATTATATG 3’
dh7	5’TTGTTGTTCTTGTAAATGATC 3’
dh8	5’TTAATGAGTTGTATGAGAAGA 3’
dh16	5’TGTGCAGAATGTGGAGCATCA 3’
dh29	5’TCTTGGAGATCAAAGAAATTC 3’
dh30	5’TCCAATTTCTTTTTAATTGAT 3’
Gefrp	5’ACTATTGTAACGATGGATGAT 3’
kobsrrp	5’TCTACTAATTCTAGATCTTG T 3’
kofp	5’AACAACAATAACAACACAGAT 3’

2.2 METHODS

2.2.1 General methods

2.2.2 Genomic and cDNA database screening

Screening of genomic and cDNA database was performed by T-BLASTN searches from the data base www.sdsc.edu/mpr/dicty which contain the cDNA and genomic DNA sequence data information of *Dictyostelium*. The well characterised actin binding domain of filamin was used as bait in TBLASTN searches to identify the new proteins from the database that contain similar sequences as the bait. PhdB was identified as a new protein, which contained an actin binding domain similar to filamin. The PhdB actin binding domain has 40% identity with the first CH domain of filamin (Figure A)

```
Filamin_CH    MAAAPSGKTWIDVQKKTFTGWANNYLKERILKIEDLATSLEDGVLLINLLEIISSKKILK
PhdB_CH       -------------QIDSFTSWINQHLSERGLSVKDLSVDFQDGVLLLNLLEILSGKKIAR

Filamin_CH    YNKAPKIRMQKIENNNMAVNFIKSE-GLKLVGIGAEDIVDSQLKLILGLIWTLILRYQIQ
PhdB_CH       YVRSPKFLQHKIDNIMIAFNFMEKAFDIKVFGCNAKDIVDGNLKQTMGVIFLLIQK----
```

Figure A. Amino acids in red are identical, green and blue colors exhibit similarity.

2.2.3 CELL BIOLOGICAL METHODS

Growth and development of *Dictyostelium*

2.2.4 Growth in liquid nutrient medium

The procedure was adopted from Claviez *et al.,* (1982). *Dictyostelium discoideum* AX2 and the derived transformants were grown in liquid AX2 medium containing dihydrostreptomycin (40 µg/ml) and other appropriate selective antibiotics (depending upon the mutant) at 21°C either in a shaking-suspension in Erlenmeyer flasks with shaking at 160 rpm or the cells were grown on petri dishes. For all the cell biological work, the culture was harvested at a density of 3-5 x 10^6 cells/ml.

2.2.5 Growth on SM agar plates

In general, *Dictyostelium* cells were plated onto SM agar plates overlaid with *Klebsiella aerogenes* and incubated at 21ºC for 3-4 days until *Dictyostelium* plaques appeared on the

bacterial lawns. To obtain single clones of *Dictyostelium*, 50-200 cells were suspended in 100 µl Soerensen phosphate buffer and plated onto *Klebsiella*-overlaid SM agar plates. Single plaques obtained after incubation at 21ºC for 3-4 days were picked with sterile tooth-picks, transferred either to new *Klebsiella*-overlaid SM agar plates or to separate petri dishes in AX2 medium supplemented with dihydrostreptomycin (40µg/ml) and ampicillin (50 µg/ml) to get rid of the bacteria and any other appropriate selective antibiotic (depending upon the mutant).

2.2.6 Development of *Dictyostelium*

Development in *Dictyostelium* is induced by starvation. For analysis of development in suspension culture and on phosphate agar, cells grown to a density of 2-3 x 10^6 cells/ml were pelleted by centrifugation at 2,000 rpm (Sorvall RT7 centrifuge) for 2 min at 4ºC and were washed two times in an equal volume of cold Soerensen phosphate buffer in order to remove all the nutrients present in the AX2 media. After washing twice in Soerensen phosphate buffer, the cells were resuspended in Soerensen phosphate buffer at a density of 1 x 10^7 cells/ml and were shaken at 160 rpm at 21ºC for the desired time periods.

2.2.7 Aggregation competent cells

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 1x10^7 cells/ml in Soerensen buffer and incubated for 6 h at 21°C in shaking culture (160 rpm). The cells obtained by this method are referred as t6 cells or aggregation competent cells.
If the cells are harvested after 8 h of incubation in Soerensen buffer it is referred as t8 cells.

2.2.8 Aggregation analysis

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 (5x10^7cells/ml/well). At different time points during aggregation images was captured using an Olympus IX70 inverse microscope.

2.2.9 Chemotaxis analysis

Aggregation competent cells were washed and resuspended in Soerensen buffer. A small volume of cells was plated on a glass-bottomed chamber and allowed to adhere to the surface for ~20 min. A micropipette filled with cAMP (100 µM) was positioned to stimulate cells by

using a micromanipulator (Eppendorf-Netheler-Hinz GmbH), and the response and movement of cells was recorded by using a time-lapse video recorder and NIH Image software (1 image every 30 s). The movement of cells and changes of cell shape were analyzed with the DIAS program (Dynamic Image Analysis System; Wessels *et al.,* 1998).

2.2.10 Development on phosphate-buffered agar plates or water agar plates

Cells grown to a density of 2-3 x 10^6 cells/ml were washed twice with equal volumes of Soerensen phosphate buffer. 5 x 10^7 cells were then resuspended in 1 ml Soerensen phosphate buffer and evenly distributed onto phosphate-buffered agar plates (90 mm) or water agar plates (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 at indicated time points.

2.2.11 Preservation of *Dictyostelium* cells

To ensure proper maintenance of the *Dictyostelium* AX2 strain, mutants and the transformants, adequate stocks of frozen cultures and spores were prepared.

Dictyostelium cells were allowed to grow densely in AX2 medium to a concentration of 4-5 x 10^6 cells/ml. In a 15 ml Falcon tube, 9 ml of the densely grown culture was collected at 4°C and supplemented with 1 ml Horse serum and 1 ml DMSO on ice. The contents were mixed by gentle pipetting, followed by preparing aliquots of 1 ml in cryotubes (1 ml, Nunc). The aliquots were incubated on ice for 60 min, followed by incubation at –20ºC for at least 2 h. Finally; the aliquots were transferred to –80ºC for long term storage.

For reviving the frozen *Dictyostelium* cells, the aliquot was taken out from –80ºC and thawed immediately at 37ºC in a waterbath. In order to remove DMSO, the cells were transferred to a falcon tube containing 30 ml AX2 medium and centrifuged at 2,000 rpm (Sorvall RT7 centrifuge) for 2 min at 4ºC. The cell pellet was resuspended in 10 ml of AX2 medium and 200 µl of the cell suspension was plated onto SM agar plates overlaid with *Klebsiella*, while the remaining cell suspension was transferred into a 100-mm petri dish (Falcon) and appropriate antibiotics were added. Cells in the petri dish were allowed to recover overnight at 21ºC and the medium was changed the next day to remove the dead cells and the traces of DMSO, whereas, the SM agar plates coated with cell suspension and bacteria were incubated at 21ºC until plaques of *Dictyostelium* cells started to appear.

2.2.12 Preservation of *Dictyostelium* spores

Dictyostelium cells were harvested and plated onto 90 mm phosphate-buffered agar plates as described above (materials and methods, 2.2.6). The plates were incubated at 21°C till the mature fruiting bodies appeared. The spores were collected from the fruiting bodies, resuspended in Soerensen phosphate buffer to a density of 1 x 10^7 to 1 x 10^8 spores/ml and aliquoted 1 ml each in cryotubes (1.5 ml, Nunc). After immediately freezing the spores in liquid nitrogen, the aliquots were transferred to –80°C for long term storage.

For germination of the frozen spores, one of the aliquots was taken out of –80°C and thawed quickly to room temperature. The spore suspension was resuspended in 30 ml of AX2 medium in an Erlenmeyer flask and incubated at 21°C and 160 rpm.

2.2.13 Transformation of *Dictyostelium* cells by electroporation

The electroporation method for transformation of *Dictyostelium* cells described by de Hostos *et al.* (1993) was followed with little modifications. *Dictyostelium discoideum* AX2 or mutant cells were grown axenically in suspension culture to a density of 2-3 x 10^6 cells/ml. Cell suspension was incubated on ice for 20 min and centrifuged at 2,000 rpm (Sorvall RT7 centrifuge) for 2 min at 4ºC to collect the cells. The cells were then washed with an equal volume of ice-cold Soerensen phosphate buffer followed by an equal volume of ice-cold Electroporation-buffer. After washings, the cells were resuspended in Electroporation-buffer at a density of 1 x 10^8 cells/ml. For electroporation, 20-25 µg of the plasmid DNA was added to 500 µl (5 x 10^7 cells) of the above cell suspension and the cell-DNA mixture was transferred to a pre-chilled electroporation cuvette (2 mm electrode gap, Bio-Rad). Electroporation was performed with an electroporation unit (Gene Pulser, Bio-Rad) set at 0.9 kV and 3 µF without the pulse controller. After electroporation, the cells were immediately spread onto a 100-mm petri dish (Falcon) and were allowed to sit for 10 min at 21ºC. Thereafter, 1 ml of Healing-solution was added dropwise onto the cells and the petri dish was incubated at 21ºC on a shaking platform at 50 rpm for 15 min. After 15 min, 10 ml of AX2 medium was added into the petri dish and the cells were allowed to recover overnight. On the next day, the medium was changed to the selection medium containing appropriate antibiotic. To select stable transformants, selection medium was replaced after every 24 - 48 h until the control petri dish (containing cells electroporated without any DNA) was clear of live cells.

Electroporation-buffer:

100 ml 0.1 M potassium phosphate buffer

17.12 g sucrose

add distilled H_2O to make 1 litre and autoclave.

0.1 M Potassium phosphate buffer:

170 ml 0.1 M KH_2PO_4

30 ml 0.1 M K_2HPO_4

adjust to pH 6.1

Healing-solution:

150 µl 0.1 M $CaCl_2$

150 µl 0.1 M $MgCl_2$

10 ml electroporation-buffer

2.2.14 MOLECULAR BIOLOGICAL METHODS

2.2.15 Purification of plasmid DNA

Pure plasmid preparations in small and large scale were done by kits provided either by Macherey-Nagel (Nucleo-Spin kit for small scale plasmid preparations) or by Qiagen (Qiagen Midi- and Maxi-Prep kit for large scale plasmid preparations). These kits were used when the pure plasmid DNA was required for sequencing, PCR or transformation. Overnight culture of bacteria containing the plasmid is pelleted and the cells are lysed by alkaline lysis. The freed plasmid DNA is then adsorbed on a silica matrix, washed with ethanol, and then eluted into TE, pH 8.0. This method avoids the requirement of caesium chloride or phenol-chloroform steps during purification.

2.2.16 Isolation of *Dictyostelium* genomic DNA

Genomic DNA from *Dictyostelium* was prepared according to the method described by Nellen *et al.* (1987), with slight modifications. *Dictyostelium* cells were allowed to grow on *Klebsiella*-covered SM plates at 21ºC. After 2-3 days, when the plates were covered with densely grown *Dictyostelium*, cells were collected in 15 ml ice-cold water, pelleted and washed twice with ice-cold water to get rid of *Klebsiella.* Alternatively, the pellet of 1 x 10^8 *Dictyostelium* cells grown in shaking suspension was washed twice with ice-cold Soerensen phosphate buffer. The pellet of *Dictyostelium* cells was finally resuspended in 5 ml cold Nucleolysis buffer. The nuclei fraction was obtained by centrifugation at 3,000 rpm (Sorvall RT7 centrifuge) for 10 min. The nuclear pellet obtained was carefully resuspended in 1 ml TE, pH 8.0, with 0.5% SDS and 0.1 mg/ml proteinase K and incubated at 37ºC for 3-5 h. The genomic DNA was extracted twice with phenol/chloroform (1:1 v/v), precipitated by adding 2.5 vol. 96% ethanol and 1/10 vol. 3 M sodium acetate, pH 5.2. The DNA precipitate was carefully spooled with a Pasteur pipette, washed with 96% ethanol, air-dried and dissolved in the desired volume of TE, pH 8.0.

Nucleolysis buffer:

10 mM magnesium acetate

10 mM NaCl

30 mM HEPES, pH 7.5

10% sucrose

2% Nonidet P40

Estimation of DNA concentration:

1 O.D at 260 nm = 50 µg DNA

2.2.17 DNA agarose gel electrophoresis

Agarose gel electrophoresis was performed according to the method described by Sambrook *et al.* (1989) to resolve and purify the DNA fragments.

Agarose gel preparation:

Horizontal submarine gel electrophoresis was performed with 0.7% (w/v) agarose in 1x TAE buffer. Only for resolving fragments less than 1,000 bp, 1% (w/v) agarose gels in 1x TAE buffer were used. Briefly, the agarose was solubilized in 1x TAE buffer by boiling, cooled to ~60ºC, ethidium bromide was added to a final concentration of 0.5 µg/ml and mixed thoroughly. The agarose solution was then poured into a sealed gel-casting chamber of the desired size and the well-forming-comb was placed. After the gel was completely set, it was submerged in 1x TAE buffer in an electrophoresis tank, DNA samples were loaded and electrophoresis was performed at 1-5 V/cm. DNA-size marker (Life technologies) was always loaded along with the DNA samples in order to estimate the size of the resolved DNA fragments in the samples. The gel was run until the bromophenol blue dye present in the DNA-loading buffer had migrated the appropriate distance through the gel. The gel was examined under UV light at 302 nm and was photographed using a gel-documentation system (MWG-Biotech)

Sample preparation for electrophoresis:

To the DNA solution, 0.2 vol. of the DNA-loading buffer (10 mM Tris/HCl, pH 8.0, 0.1 mM EDTA, 40% sucrose, 0.5% SDS, 0.25% bromophenol blue) was added and the mixture was loaded into the gel.

2.2.18 Southern blotting

Southern blotting (Southern, 1975) is a technique used to transfer DNA from its position in an agarose gel to a nitrocellulose/nylon membrane. After transfer, the membrane can be hybridised with a radiolabelled probe to identify specific fragments.

The ethidium bromide stained agarose gel was photographed using a scale under UV light to document migration of DNA fragments with respect to the DNA-size marker. DNA was depurinated by incubating the gel in 2 vol. of 0.25 M HCl for 20 min at room temperature with gentle shaking. The gel was rinsed in deionised H_2O to remove excess HCl and was then incubated in 2 vol. of denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 30 min in order to denature the DNA. Now the transfer was performed by capillary transfer technique. Briefly, the gel was transferred directly from the denaturation solution to a buffer reservoir containing a supporting wick (made up of Whatman 3MM paper) and 20x SSC. A dry nylon membrane (Biodyne B membrane, Pall) of the same size as the gel was then directly placed on the alkaline gel. Three pieces of Whatman 3MM paper followed by blotting pads, all cut to the same size as the gel, were placed on top of the nylon membrane. A glass plate supporting approximately 500 g weight was finally kept on top of the stack and transfer of DNA to the membrane was allowed to proceed for overnight. Next day, position of the wells as well as the orientation of the membrane was marked before removing the membrane from the gel surface. The transferred DNA was then immobilized onto the membrane by baking at 80ºC for 1 h. Thereafter, the membrane was used for hybridisation with a desired radiolabelled probe.

2.2.19 Isolation of total RNA from *Dictyostelium* cells

The pellet of 1 x 10^8 cells (harvested at growth or different stages of development) was washed with ice-cold DEPC-H_2O (0.1% DEPC, mixed by stirring for 5-6 h, autoclaved) and resuspended in 10 ml 50 mM Hepes buffer, pH 7.5. To the cell suspension, 100 µl DEPC and 1 ml 10% SDS was added, mixed briefly, followed by immediately adding 1 vol. of phenol saturated with Hepes buffer, pH 7.5. The sample was then vortexed strongly and centrifuged at 3,000 rpm (Sorvall RT7 centrifuge) for 20 min at 4ºC. The upper aqueous phase was collected carefully and was extracted with an equal volume of phenol/chloroform (1:1 v/v), till no interphase was visible. This was followed by an extraction with an equal volume of chloroform and finally the RNA present in the upper aqueous phase was precipitated by adding 2 vol. ethanol and 1/10 vol. 2 M sodium acetate, pH 5.2 and incubating the samples overnight at –20ºC. Next day, the RNA was pelleted, washed with 70% ethanol, air-dried and dissolved in the desired volume of DEPC-H_2O. The concentration of RNA was determined by measuring the $O.D_{260}$ of the solution using a spectrophotometer. The RNA samples were stored at –80ºC.

DEPC-H_2O:

0.1% DEPC in H_20

mixed by stirring for 5-6 h

autoclaved

Estimation of RNA concentration

1 O.D at 260 nm = 40 µg RNA

2.2.20 RNA formaldehyde-agarose gel electrophoresis

The formaldehyde-agarose denaturing electrophoresis (Lehrach *et al.*, 1977) is used for separation and resolution of single stranded RNA.

Sample preparation for electrophoresis:

In general, 30 µg of purified total RNA was mixed with an equal volume of RNA-sample buffer and denatured by heating at 65ºC for 10 min. After denaturation, the sample was immediately transferred to ice and 1/10 vol. of RNA-loading buffer was added. Thereafter, the RNA samples were loaded onto a denaturing formaldehyde-agarose gel.

Formaldehyde-agarose gel preparation:

For a total gel volume of 150 ml, 1.8 g agarose (final concentration 1.2%) was initially boiled with 111 ml DEPC-H_2O in an Erlenmeyer flask, cooled to 60ºC and then 15 ml of RNA-gel-casting buffer, pH 8.0 and 24 ml of 36% formaldehyde solution were added. The agarose solution was mixed by swirling and poured into a sealed gel-casting chamber of the desired size. After the gel was completely set, denatured RNA samples were loaded and the gel was run in 1x RNA-gel-running buffer, pH 7.0, at 100 V until the bromophenol blue dye had migrated the appropriate distance through the gel.

A test gel was sometimes run with 5 µg of total RNA to check the quality of the RNA samples. In such a case, 10 µg/ml ethidium bromide was added to the RNA-sample buffer during sample preparation and after electrophoresis the gel was examined under UV light at 302 nm and was photographed using the gel-documentation system.

10x RNA-gel-casting buffer (pH 8.0):

200 mM MOPS

50 mM sodium acetate

10 mM EDTA

adjust pH 8.0 with NaOH

autoclaved

10x RNA-gel-running buffer (pH 7.0):

200 mM MOPS

50 mM sodium acetate

10 mM EDTA

adjust pH 7.0 with NaOH

autoclaved

RNA-sample buffer:

RNA-loading buffer:

50% formamide
6% formaldehyde
in 1x RNA-gel-casting buffer, pH 8.0

50% sucrose, RNase free
0.25% bromophenol blue
in DEPC-H_2O

Internal RNA-size standard:
26S rRNA (4.1 kb)
17S rRNA (1.9 kb)
5S rRNA (0.1kb)

2.2.21 Northern blotting

After electrophoresis, the RNA formaldehyde agarose gel was rinsed in sufficient amount of deionised H_2O for 5 min and then equilibrated in 10x SSC for 25 min. The resolved RNA was then transferred from the gel to the nylon membrane (Biodyne B membrane, Pall) using the transfer setup as described for Southern blotting (see materials and methods 2.2.18). After overnight transfer with 20x SSC, the transferred RNA was immobilised by baking the membrane in an oven at 80ºC for 1 h.

2.2.22 Hybridisation of Southern or northern blots with a radiolabelled DNA probe

Southern- or northern-blots were rinsed briefly with 2x SSC and incubated in a heat-sealable hybridisation-bag (Life technologies) in 15-20 ml of pre-hybridisation buffer for 1h at 37ºC on a shaking platform. After pre-hybridisation, the denatured radiolabelled DNA probe was added directly to the pre-hybridisation-buffer in the hybridisation-bag and the hybridisation was performed by incubating the blot overnight at 37ºC. After hybridisation, the blot was washed twice with 2x SSC/0.1% SDS for 10 min each at room temperature with gentle shaking followed by two washings with wash buffer for 30 min each at 37ºC with gentle shaking. The blot was then wrapped in a plastic wrap and autoradiography was performed by exposing the blot to X-ray film at –70ºC for the desired time.

Pre-hybridisation/Hybridisation buffer:
50% formamide
1% sodium lauryl sarcosinate
0.2% SDS
2 mM EDTA, pH 7.2, adjusted with NaOH
0.12 M phosphate buffer, pH 6.8:
2x SSC

4x Denhardt's reagent

2% bovine serum albumin

Wash buffer:

Same contents as Pre-hybridisation / hybridisation buffer without 4x Denhardt's reagent

100x Denhardt's reagent

2% ficoll 400

2% polyvinylpyrolidone

2.2.23 PCR-mediated screening of *Dictyostelium* transformants

A PCR approach was used for screening of the PhdB$^-$ mutant. AX2 cells were transformed with the PhdB gene replacement vector (see materials and methods, 2.2.28) and the transformants were selected for resistance to blasticidin (5 µg/ml). Single cell transformants were then obtained by spreader dilution of the whole pool of transformants onto SM plates overlaid with *Klebsiella*. Thereafter, the single transformants were picked up and grown in separate wells in a 24 well tissue culture plate. The selection medium contained streptomycin (40 µg/ml) and ampicillin (50 µg/ml) to get rid of the bacteria.

Preparation of DNA for PCR reaction:

After the cells had grown to confluency in the wells, cells were suspended in the medium present in the wells and transferred to a 1.5 ml microcentrifuge tube. The cells were then pelleted by centrifugation in a microcentrifuge at maximum speed for 15 s. The cells were washed twice with 1 ml of ice-cold H_2O and resuspended in 100 µl of lysis buffer. The cells were then incubated at 56ºC for 45 min followed by incubation at 95ºC for 10 min to liberate the genomic DNA.

PCR conditions:

For PCR, 15 µl of the processed cell suspension containing the liberated genomic DNA was used as a template. Reaction programme and composition of the reaction-mix are indicated below:

Lysis buffer:

0.5% Nonidet P-40

0.05 mg/ml proteinase K

in 1x PCR buffer

Reaction-mix (50µl final volume):

15 µl template

2.5 µl kofp 5' primer (2 pmol/µl)

2.5 µl kobsrrp 3' primer (2 pmol/µl)

1.0 µl dNTP-mix (10 mM each)

5.0 µl 10x PCR buffer

1.0 µl Taq polymerase (1 U/µl)

23 µl H_2O

10x PCR buffer:
100 mM Tris/HCl
500 mM KCl
15 mM $MgCl_2$
adjust to pH 8.3

Reaction programme:
1-step 92ºC for 3 min
2-step 35 cycles of-
92ºC for 1 min
54ºC for 1 min
72ºC for 3 min
3-step 72ºC for 10 min
4-step 4ºC till end.

2.2.24 Construction of vectors

2.2.25 Construction of vector for expressing different functional domains of PhdB as GFP fusion proteins

Functional domains of PhdB, the single actin binding domain at the N-terminus, the C-terminal ArfGAP/PH domain and the C- terminal PH domain were cloned separately into the *Dictyostelium* GFP fusion vector pBsrGFP.

The C-terminal region of PhdB comprising the ArfGAP - PH domain was PCR amplified with primer dh16 and dh8, forward and reverse primers respectively. The PCR product was cloned into pGEM-T Easy vector. The cloned insert was verified by sequencing. The cloned fragment was released from pGEM-PhdBCT plasmid as *EcoRI* fragment and subcloned into the *D. discoideum* expression vector pBsrGFP (Figure. B) as an *EcoRI* fragment. Expression of the GFP fusion protein was under the control of the actin 15 promoter and actin 8 terminator.

The C-terminal PH domain separately was also cloned into the pBsrGFP vector as an *EcoRI* fragment as was the single actin binding (CH) domain.

Initially the N-terminal CH and PH containing fragments were obtained by RT-PCR using the following primers.

N-terminal CH domain; dh6 and dh7 as forward and reverse primers, respectively.

N-terminal PH domain; dh29 and dh30 as forward and reverse primers, respectively.

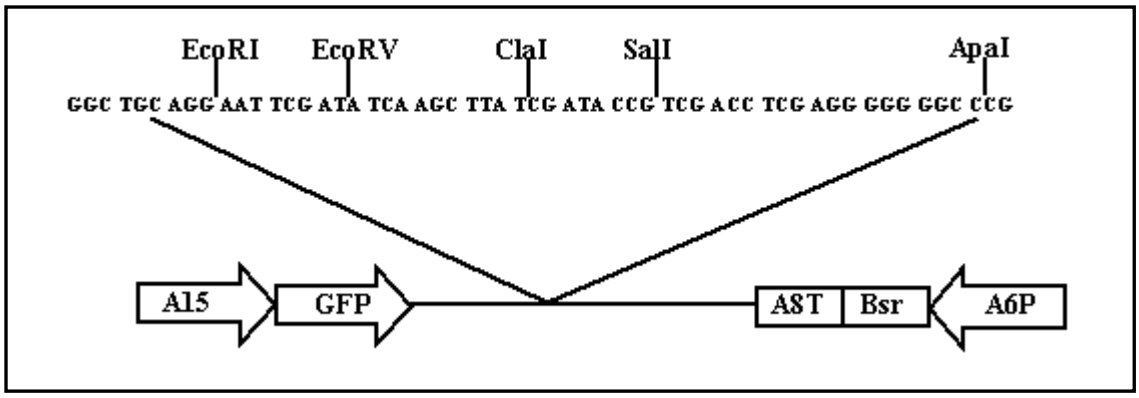


Figure B. Multiple cloning site of the pBsrGFP vector as well as location of the control regions and the blasticidin resistance gene

pBsr-GFP vector

A15P- Actin-15-Promoter
A6P- Actin-6-Promoter
A8T- Tandem termination sequence
GFP- Green Fluorescent Protein
Bsr-Blasticidin resistance

2.2.26 Construction of a vector for expression of the C-terminal peptide (ArfGAP/PH) as a GST-fusion protein

For expression of C-terminal peptide as a GST-fusion protein, pGEX-4T3, a GST expression vector, was used. The C-terminal fragment of PhdB was obtained from pGEM-PhdBCT plasmid as a *NotI* fragment. The fragment was purified from agarose gels and subcloned in frame at the *NotI* site of expression vector pGEX-4T3 and the cloned inserts were verified by restriction digestion. The obtained pGEX-PhdBCT expression vector was electroporated into *E. coli* DH5α cells and used for expression of GST-PhdBCT fusion protein.

2.2.27 Construction of a vector for expression of the C-terminal peptide (ArfGAP/PH) without any additional tag.

To immunise mice with the C-terminal peptide lacking any additional tag, we used a pIMS vector expression system, which contained all elements for regulated expression of the gene flanked by *EcoRI* sites. It lacked any additional tag normally used for protein purification.

The C-terminal cDNA fragment of PhdB was obtained from pGEM-PhdBCT plasmid as an *EcoRI* fragment. The fragment was purified from the gel and subcloned in frame at the *EcoRI* site of expression vector pIMS. Correct cloning and was verified by restriction enzyme digestion. The obtained pIMS-PhdBCT expression vector was electroporated into *E. coli* XL1 blue cells and used for expression of the C-terminal peptide

2.2.28 Construction of a PhdB gene replacement vector

For disruption of the PhdB gene in the wild type AX2 cells, a PhdB gene replacement vector was constructed using the plasmid pBSR△Bam. The vector contained a 1.4 kb blasticidin S resistance cassette fragment cloned at the multiple cloning site. Sequence analysis of the pBSR△Bam plasmid reveals the presence of single *EcoRI, EcoRV, and HindIII* restriction sites. For construction of the PhdB gene replacement vector, the 5' region of the PhdB gene (1.1 kb fragment) was PCR amplified using the forward primer (dh6) and reverse primer (Gefrp), the PCR product obtained was cloned in pGEM-T Easy cloning vector. The cloned fragment was released as *ApaI / SalI* fragment from pGEM-T Easy and cloned into pBSR△Bam at the *ApaI / SalI* sites. The 3' end DNA (1.0 kb) was PCR amplified using forward primer (dh8) and reverse primer (dh16). The PCR product obtained was cloned in pGEM-T Easy cloning vector. The 3' gene fragment was released as *NotI* fragment from the pGEM-T Easy vector and cloned in pBSR△Bam at the *NotI* site. The gene replacement vector now containing both 5' and 3' homologous regions flanked the 1.4 kb blasticidine resistance cassette. The replacement vector pBSR△BamPhdB was verified by partially digesting with *ApaI* and *BciVI,* which released the expected fragments of 4200, 1700 and 700 bp. The PhdB gene replacement construct obtained was further used to transform into *Dictyostelium* wild type AX2 cells.

2.2.29 Lambda ZAP II cDNA library screening

2.2.30 Preparation of plating bacteria

A single bacterial colony of *E. coli* XL1-blue cells was inoculated into 50 ml of LB medium supplemented with 0.2% maltose and 10 mM $MgSO_4$. The bacteria were allowed to grow overnight at 37ºC on a shaker.

2.2.31 Plating a lambda ZAP bacteriophage cDNA library

The stock of the recombinant bacteriophage (lambda ZAP library) was appropriately diluted in SM, mixed with 0.3 ml of XL1-blue cells and allowed to adsorb for 20 minutes at 37°C. Carefully mixed with 9 ml of melted top agarose and spread onto prewarmed 12 x 12 cm LB agar plates. The plates were allowed to stand for 5 min at room temperature to allow the top agarose to harden. The plates were then incubated at 37ºC for overnight or until the plaques reach a diameter of ~ 1.5 mm. The plates should not show confluent lysis. The plates were then transferred to 4ºC for at least an hour

SM (medium for phage storage and dilution):

100 mM NaCl

10 mM $MgSO_4$ x 7 H_2O

50 mM Tris/HCl, pH 8.0

0.01% gelatin

The solution was sterilized by autoclaving and stored at 4ºC

Top agarose

To LB medium add 0.7% (w/v) agarose, autoclaved

2.2.32 Screening bacteriophage plaques by hybridisation

The DNA from the bacteriophage plaques was transferred to nitrocellulose filter (BA85, Ø82 mm, Schleicher and Schuell) by overlaying the plates with the nitrocellulose filter for approximately 30 seconds, the position of the filters on the plate was marked with a needle. The filters were then transferred for 1 minute to a solution of 0.5 M NaOH containing 1.5 M NaCl for denaturation of the DNA. Neutralisation was in 0.5 M Tris/HCl, pH 8.0, 1.5 M NaCl for 3 min. The filters were then transferred to 2x SSC for 5 min and dried. The filters were hybridised to a ^{32}P labelled partial PhdB cDNA probe encompassing nucleotides 3697-4740 of the PhdB gene.

The plaques giving positive hybridisation signals were picked using a Pasteur pipette equipped with a rubber bulb. The hard agar beneath the selected plaque was stabbed by the Pasteur pipette and by applying mild suction the plaque, together with the underlying agar, was drawn into the pipette. The fragments of agar were transferred into 500 µl of SM in a micro centrifuge tube and incubated at room temperature for at least 2 h (or overnight at 4ºC) to allow the bacteriophage particles to diffuse out of the agar. An aliquot of the bacteriophages that were eluted from the agar was replated and rescreened by hybridisation until a single, well isolated, positive plaque was obtained.

2.2.33 Phage purification

1 µl and 10 µl of the bacteriophage suspension was mixed with 100 µl of the plating bacteria and incubated in a water-bath at 37ºC for 20 min. Now each aliquot of the infected bacteria was mixed with 4 ml of melted top agarose (at 50ºC) in a test tube and spread evenly onto pre-warmed 90 mm LB plates (at 37ºC). The plates were allowed to stand for 5 min at room temperature to allow the top agarose to harden. The plates were then incubated at 37ºC for overnight or until the plaques reach a diameter of ~ 1.5 mm. The plates should not show confluent lysis. The plates were then transferred to 4ºC for at least an hour. 5ml of SM buffer were added to the plate and the phage allowed to diffuse into the buffer.

2.2.34 Excision of plasmid containing the cDNA from bacteriophages

The bacteriophage containing the DNA clone of interest was infected again on XL1-blue cells, also ExAssist helper phage was added to excise the pBluescript phagemid from the lambda ZAPII vector. The lambda ZAPII vector (Stratagene) is designed to allow simple in vivo excision of any cloned insert contained within the lambda vector to form a phagemid containing the cloned insert.

The plaque of interest was picked from the agar plate and transferred to a sterile micro centrifuge tube containing 500 µl of SM buffer and 20 µl of chloroform. Vortexing of the microfuge tube was done to release the phage particles into the SM buffer. The micro centrifuge tube was incubated for 1-2 h at room temperature

Overnight culture of XL1-blue cells in LB broth, supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ were sedimented and the cells resuspended at an OD_{600} of 1.0 in 10 mM $MgSO_4$. To 200 µl of the above XL1-blue cells 250µl of phage stock (containing > $1x10^5$ phage particles) were added and 1 µl of Ex Assist helper phage (Falcon 2059 polypropylene tube). Incubation was at 37°C for 15 min. 3 ml of LB broth were add and incubation was for 2.5-3 h at 37°C with shaking. The Falcon tube was heated at 65°C for 20 min and then spun at 1000xg for 15 min. The supernatant which contains the excised pBluescript phagemid packed as filamentous phage particles was decanted.

2.2.35 Plating excised phagemid

To 200 µl of freshly grown cells (OD_{600}=1) 100 µl of phage supernatant obtained from the above step were added, the microfuge tube was incubated at 37°C for 15 min, 200 µl of the cell mixture from the microfuge tube were plated on LB ampicillin agar plates (50µl/ml) and the plates incubated overnight at 37°C. Colonies appearing on the plate contain the pBluescript double stranded phagemid with the cloned DNA insert.

Isolation of a partical cDNA of PhdB from the ZAPII cDNA library screening

To recover a full-length PhdB cDNA we screened the Lambda ZAP II cDNA library of *Dictyostelium* with a 1043 bp C-terminal PhdB probe (materials and methods 2.2.29). The C-terminal probe was obtained by a RT-PCR approach. We isolated a partial cDNA sequence spanning 3780 bp of the PhdB gene, corresponding to positions 750 to 4530 of the PhdB cDNA. At the N-terminus we are still missing approximately 700 bp and at the C-terminus 200 bp. We plan to use an RT-PCR approach to isolate the remaining coding sequence and to complete the full-length cDNA.

2.2.36 Transformation of *E. coli*

2.2.37 Preparation of competent *E. coli* cells by the $CaCl_2$ method

An overnight grown culture of *E. coli* (0.5 ml) was inoculated into 50 ml LB medium and incubated at 37ºC, 250 rpm, until an OD_{600} of 0.4-0.6 was obtained. The bacteria were then pelleted at 4ºC for 10 min at 4,000 rpm (Beckman Avanti J25, rotor JA-25.50) and the bacterial pellet was resuspended in 20 ml of ice-cold 0.1 M $CaCl_2$ and incubated on ice for 15 min. The bacterial cells were again pelleted and resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$. These $CaCl_2$-competent cells were stored at 4ºC for up to 1 weak. Alternatively, the pellet of $CaCl_2$-competent *E. coli* cells was resuspended in 0.1 M $CaCl_2$/20% glycerol and then aliquoted (200 µl / tube). The aliquots were then quickly frozen in a dry ice/ethanol bath and immediately stored at –80ºC.

2.2.38 Transformation of $CaCl_2$ competent *E. coli* cells

Plasmid DNA (~50-100 ng of a ligase reaction product or ~10 ng of a supercoiled plasmid) was mixed with 100-200 µl of $CaCl_2$-competent *E.coli* cells and incubated on ice for 30 min. The cells were then heat-shocked at 42ºC for 45 sec and immediately transferred on ice to cool for 2 minutes. The cells were then mixed with 1 ml of pre-warmed (at 37ºC) SOC medium and incubated at 37ºC with shaking at ~150 rpm for 45 min. Finally, 100-200 µl of the transformation mix, or an appropriate dilution, was plated onto selection plates and the transformants were allowed to grow overnight at 37ºC.

2.2.39 BIOCHEMICAL METHODS

2.2.40 Preparation of total protein from *Dictyostelium*

1 x 10^7 to 5 x 10^8 *Dictyostelium* cells obtained from growth as well as different stages of development were washed once in Soerensen phosphate buffer. Total protein was prepared by lysing the pellet of cells in 500 µl 1x SDS sample buffer. For detection of the protein expression in different cell lines, equal amount of protein (equivalent to 2 x 10^5 cells/lane to 1 x 10^7 cells/lane) was loaded onto a discontinuous SDS-polyacrylamide gel to allow for a quantitative comparison.

2.2.41 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed using the discontinuous buffer system of Laemmli (1970). Discontinuous polyacrylamide gel (10-15% resolving gel, 5% stacking

gel) was prepared using glass-plates of 10 cm x 7.5 cm dimensions and spacers of 0.5 cm thickness.

Protein solutions were mixed with suitable volumes of 2x SDS sample buffer, whereas protein pellets were resuspended in a suitable volume of 1x SDS sample buffer. The samples were denatured by heating at 95ºC for 5 min and loaded into the wells in the stacking gel. A molecular weight marker, which was run simultaneously on the same gel in an adjacent well, was used as a standard to establish the apparent molecular weights of proteins resolved on SDS-polyacrylamide gels. The molecular weight markers were prepared according to the manufacturer's specifications. After loading the samples onto the gel, electrophoresis was performed in 1x gel-running buffer at a constant voltage of 100-150 V until the bromophenol blue dye front had reached the bottom edge of the gel or had just run out of the gel. After the electrophoresis, the resolved proteins in the gel were either observed by Coomassie blue staining or transferred onto a nitrocellulose membrane.

SDS-sample buffer:

1x

50 mM Tris/HCl, pH 6.8

2 (% v/v) SDS

10 (% v/v) glycerine

0.1 (% v/v) bromophenol blue

2 (% v/v) β-mercaptoethanol

10x Gel-running buffer:

1.9 M glycine

0.25 M Tris/HCl, pH 8.8

1% SDS

Molecular weight markers:

LMW-Marker (Pharmacia) - 94, 67, 43, 30, 20.1, 14.4 kDa

See Blue pre-stained marker (Novex)- 250, 98, 64, 50, 36, 30, 16, 6, 4 kDa

Pre-stained marker (Bio-Rad)- 208, 115, 79.5, 49.5, 34.8, 28.3, 20.4, 7.2 kDa

2.2.42 Western blotting using the semi-dry method

The proteins resolved by SDS-polyacrylamide gel electrophoresis (SDS-PA gels) were electrophoretically transferred from the gel to a nitrocellulose membrane by using the method described by Towbin *et al.* (1979) with little modifications. The transfer was performed using Towbin's buffer in a semi-dry blot apparatus (Bio-Rad) at a constant voltage of 10 V for 35-45 min. The instructions provided along with the semi-dry apparatus were followed in order to set up the transfer.

Towbin's buffer (transfer buffer):

39 mM glycine

48 mM Tris/HCl, pH 8.3

0.0375% SDS

20% methanol or ethanol

2.2.43 Immunodetection of membrane-bound proteins

The western blot was immersed in blocking buffer (1x NCP) and the blocking was performed with gentle agitation either overnight at room temperature or for 2-3 h at room temperature with several changes of 1x NCP. After blocking, the blot was incubated at room temperature with gentle agitation with either commercially available primary antibodies at a proper dilution (in 1x NCP) for 1-2 h, or hybridoma-supernatant for overnight. After incubation with primary antibody, the blot was washed 5-6 times with 1x NCP at room temperature for 5 min each with repeated agitation. Following washings, the blot was incubated for 1 h at room temperature with a proper dilution (in 1x NCP) of enzyme conjugated secondary antibody directed against the primary antibody. The secondary antibody was conjugated with either Horse radish peroxidase (HRP) or alkaline phosphatase (AP). After incubation with secondary antibody, the blot was washed as described above. After washings, substrate reaction was carried out depending upon the enzyme coupled to the secondary antibody. Enzymatic chemi-luminescence (ECL) detection system (see materials & methods 2.2.44) was used for blots incubated with HRP-conjugated secondary antibody, whereas, the BCIP/NBT colour development substrate reaction was used for blots incubated with AP-conjugated secondary antibody (see Materials & Methods 2.2.45).

2.2.44 Enzymatic chemi-luminescence (ECL) detection system

The blot was incubated in ECL-detection-solution for 1-2 min and then wrapped in a saran wrap after removing the excess ECL-detection-solution. Now an X-ray film was exposed to the wrapped membrane for 1-30 min and the film was developed to observe the immunolabelled protein.

ECL-detection-solution:

2 ml 1 M Tris/HCl, pH 8.0

200 µl 250 mM 3-aminonaphthylhydrazide in DMSO

89 µl 90 mM p-Coumaric acid in DMSO

18 ml deionised H_2O

6.1 µl 30% H_2O_2 (added just before using)

2.2.45 BCIP/NBT colour development substrate reaction

The blot was developed using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as a substrate and nitro blue tetrazolium (NBT) as a colour indicator. The blot was incubated in 10 ml of BCIP/NBT substrate solution at room temperature with gentle agitation for 5 min or until sufficient colour development has occurred. The reaction was stopped by washing the membrane several times with deionised water and the membrane was allowed to dry on a piece of blotting paper.

BCIP/NBT substrate solution:

66 µl 50mg/ml NBT (Promega)

33 µl 50mg/ml BCIP (Promega)

10 ml 0.1M Na_2CO_3, pH 10.0

2.2.46 Purification of GST-CTPhdB fusion protein by sarkosyl solubilisation

The procedure was adopted from John and Benjamin, (1993). The C-terminal peptide of PhdB representing the ArfGAP/PH domain was cloned into the pGEX-4T3 vector and transformed into *E. coli* DH5α cells (materials and methods 2.2.26). Protein expression was induced by addition of 0.1 mM IPTG to a 500ml culture (OD_{600} ~ 5.0) and incubation for 3 h at 37°C, 250 rpm. The cells were pelleted by spinning for 15 min. at 5,000 xg, 4°C. The pellet was resuspended in STE buffer. To the cells was added lysozyme at 100 µg/ml and incubation was on ice for 15min. Later were added 5 mM of DTT along with 1.5% sarkosyl and 2% Triton X-100. The cells were mixed well and sonicated, 5 pulses (50 W, 20 kHz) while incubated on ice. The lysate obtained was cleared by spinning for 5 min at 11,000 xg at 4°C. To the supernatent Triton X-100 (2%) was added and mixed well. Then GST beads were added to the supernatent and incubation was over night at 4°C. The GST beads were pelleted at 5,000 xg and washed with ice cold PBS. The C-terminal recombinant protein bound to the beads was analysed by SDS-PA gel by staining with coomassie blue. The interaction between the recombinant protein and the GST beads was very strong and we were unable to release the peptide by equilibrating with Glutathione. Hence pure protein was obtained by separating the GST tag by cleaving the GST-fusion protein with thrombin.

STE Buffer:

10 mM Tris/HCl, pH 8.0

150 mM Nacl

1 mM EDTA

2.2.47 Purification of the C-terminal peptide of PhdB fusion protein expressed without additional tag.

The C-terminal sequence of PhdB encoding the ArfGAP-PH domain was cloned into the pIMS vector (materials and methods 2.2.27; Simon *et al.,* 1998) and transformed into *E. coli* XL1 blue cells. A 500 ml culture was grown at 37ºC in Luria broth containing 100 µg/ml ampicillin until the optical density at 600nm was 0.6. Isopropyl β-D-thiogalactoside (500 µM) was added and the cells were cultured for a further 5 h at 30ºC. The cells were then harvested by centrifugation and resuspended in 25 ml of ice cold buffer A (50 mM TrisHCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 20% sucrose). The cells were incubated on ice for 10 min and pelleted by spinning at 11,000 rpm for 5 min at 4ºC. The pellet obtained was resuspended in buffer P (PBS, 5 mM EDTA). Lysis was by one round of freeze thawing, the lysate was sonicated (3 pulses of 10 sec), keeping the tube immersed on ice. Sonication was followed by homogenisation using a Dounce homogeniser for 2-3 min in order to ensure complete and efficient cell lysis. The inclusion bodies were harvested by centrifuging at 11,000 rpm for 30 min at 4ºC, washed with buffer D [50 mM Tris/HCl, pH 8.0, 5 mM EDTA, 2 M urea and 5 mM DTT] and centrifuged at 25,000 rpm for 5 min at 4ºC. The pellet was repeatedly washed with Buffer D by gradual increase in the urea concentration (3-6 M). Protein from inclusion bodies was extracted with 8 M urea in buffer D. The protein sample was dialysed against 20 mM Tris/HCl, pH 8.0.

2.2.48 Quantification of protein

The colorimetric method described by Lowry *et al.* (1951) was used for quantification of protein. Protein samples (5-50 µl) and different concentrations of BSA (1-25 µg) were taken in separate 1.5 ml tubes and diluted with solution I to bring the final volume to 1 ml in each tube. After incubating the tubes at room temperature for 10 min, 100 µl of solution II was added to each tube, the contents in the tubes were immediately mixed by brief vortexing and the tubes were allowed to stand at room temperature for 20 min. Thereafter, the OD of the sample was measured at 660 nm and the concentration of protein in the sample was estimated from a standard curve obtained by plotting the OD_{660} values of 1-25 µg BSA.

Solution I:

0.2 ml 2% $CuSO_4.5H_2O$

0.2 ml 4% potassium sodium tartrate

9.6 ml 3% Na_2CO_3 in 0.1 N NaOH

make fresh at the time of use

Solution II:

1 vol. Folin-Ciocalteu reagent

2 vol. distilled H_2O

make fresh at the time of use

2.2.49 Subcellular fractionation

Wild type AX2 and GFP fusion protein expressing cells were grown to a density of 2-3 x 10^6 cells/ml, washed in Soerensen phosphate buffer and resuspended at a density of 2 x 10^8 cells/ml in MOPS buffer (20 mM 2-[N-morpholino] ethane sulfonic acid, 1 mM EDTA, 250 mM sucrose, pH 6.5) supplemented with protease inhibitor mixture; leupeptin (50 µg/ml), pepstatin A (10 µg/ml), benzamidine (2 mM), PMSF (1 mM). Cells were lysed by sonication (3 pulses at 50W, 20 kHz). Membrane and supernatant fractions were separated by centrifugation (100,000 xg for 30 min at 4°C), and subjected to immunoblot detection.

Soerensen phosphate buffer, pH 6.0: (Malchow *et al*., 1972)

2 mM Na_2HPO_4
14.6 mM KH_2PO_4

2.2.50 Triton X-100 extraction of *Dictyostelium* cells

The procedure was adopted from Prassler *et al.* (1997). Growth phase AX2 and GFP fusion protein expressing *Dictyostelium* cells were washed and resuspended in two volumes of lysis buffer (80 mM PIPES, pH 6.8, 30% glycerol, 0.5 mM DTT, 5 mM EGTA, 5 mM $MgCl_2$, 1 mM PMSF, Protease inhibitor cocktail; 10 µg/ml of each inhibitor). The cells were lysed by adding 1% Triton X-100 and incubated for 5 min at room temperature. Cytoskeleton associated proteins that are largely insoluble were pelleted by centrifugation at 15,000 xg at 4ºC for 5 min. The supernatant was carefully collected and the pellet was washed twice with lysis buffer without detergent and finally resuspended in the original volume of lysis buffer without detergent. Proteins of the Triton X-100-soluble and insoluble fractions were extracted in 2x SDS sample buffer.

2.2.51 IMMUNOLOGICAL TECHNIQUES

2.2.52 Generation of monoclonal antibodies

IMMUNIZATION OF MICE:

The overexpressed protein for antibody production as mentioned above (materials and methods 2.2.47), was partially purified by gel permeation chromatography (GPC), this sample was again resolved on a 12% SDS PA gels, the gel was copper stained and the protein of interest was excised from the gel and electroeluted from the gel slice using Biorad Model 422 electro eluter.

Female BALB/c, 6-7 weeks old mice were immunised intraperitoneally with 50 µg of protein suspended in Freund's complete adjuvant (FCA) or with 50 µg of protein suspended in Immun Easy Adjuvant (Qiagen) adjuvant, and immunised intramuscularly. The mice were boosted three weeks after the first immunization with the freshly prepared antigen. Two weeks later and three days before the fusion, the mice were again boosted by injecting the freshly prepared antigen.

2.2.53 Preparation of mouse feeder cells for fusion and cloning

One day prior to seeding of hybridomas from cell fusion or cloning procedures, mouse feeder cells (macrophages and other cells) were isolated. In general, 3-5 BALB/c mice per fusion were sacrificed for the isolation of peritoneal feeder cells. The mouse was sacrificed by cervical dislocation, disinfected with 70% ethanol and then laid on a dissecting board. Snipping its skin at diaphragm level and pulling the skin back exposed the peritoneal cavity of the mouse. Now 10 ml of cold Normal medium (NM) was injected into the peritoneal cavity using a 10 ml syringe and an 18 G needle and the peritoneal feeder cells were harvested by withdrawing as much solution as possible into the syringe. This step was repeated two more times and the feeder cells were collected in a pre-cooled 50 ml centrifuge tube. The feeder cells were then pelleted by centrifugation at 1,000 rpm for 5 min at 4°C (Beckman CS-6R) and the pellet was washed twice with 25 ml NM. The feeder cells were finally resuspended in an appropriate volume of NM or 1x HT medium.

2.2.54 Fusion

Three days after the last immunization, spleen cells were harvested from the immunised mouse and fused with myeloma cells. Two different myeloma cell lines, PAIB3Ag81 (PAI) and X63-Ag8.653 (Ag8) were used for fusion. On the day of fusion, both the myeloma cell lines were harvested in their log phase of growth and collected in two separate 50 ml sterile centrifuge tubes. The cells were pelleted by centrifugation at 1,500 rpm for 5 min (Beckman CS-6R) and washed twice with 25 ml RPMI 1640 medium (w/o HEPES, w/o glutamine). After washings, both types of myeloma cells were resuspended separately at a density of 2.5 x 10^6 cells/ml in 10 ml of RPMI 1640 (w/o HEPES, w/o glutamine).

Before isolation of spleen cells from the immunised mice, both the mice immunised using FCA and Immune Easy adjuvants was checked for the serum titre against the immunised protein by western blot analysis. As both the mice immunised with two different adjuvant showed good titre, both the mice were sacrificed to isolate spleed cells for fusion.

The mice was sacrificed by cervical dislocation and disinfected by immersing in 70% ethanol. The spleen was then aseptically removed according to standard protocols and transferred into a 60 mm petri dish containing 5 ml of cold RPMI 1640 (w/o HEPES, w/o glutamine). Now the spleen in the petri dish was taken to a sterile hood. The surface fats and other tissues adhering to the spleen were carefully removed using a sterile forceps and scissors and during this process, the spleen was transferred 2-3 times into a fresh 60 mm petri dish containing 5 ml of cold RPMI 1640 (w/o HEPES, w/o glutamine). After complete removal of adipose tissues and other adhering tissues, the spleen was transferred to a fresh 60 mm petri dish containing 5 ml of cold RPMI 1640 (w/o HEPES, w/o glutamine). The spleen was cut into small pieces with sterile scissors, followed by shearing the small pieces with sterile forceps. Now the cell suspension was collected, leaving the larger spleen pieces in the petri dish, and filtered through a sterile cotton-plugged syringe (10 ml). The leftover larger spleen pieces were again resuspended in 5 ml of cold RPMI 1640 (w/o HEPES, w/o glutamine) and were gently homogenised in a sterile homogeniser. The homogenised suspension was then filtered through the sterile cotton-plugged syringe and the filtrate containing the spleen cells was collected in a 50 ml centrifuge tube. The spleen cells were then pelleted by centrifugation at 1,500 rpm for 5 min (Beckman CS-6R) and the supernatant was aspirated with a sterile Pasteur pipette. They were then washed twice with 25 ml of RPMI 1640 (w/o HEPES, w/o glutamine) and finally resuspended in 10 ml of RPMI 1640 (w/o HEPES, w/o glutamine) at a density of ~1 x 10^7 cells/ml.

For fusion, the spleen cells were divided into two halves and mixed with the two different myeloma cells (spleen cells: myeloma = 2:1), this was done for both the animals which received antigen with different adjuvant (FCA and Immun Easy) in separate tubes and centrifuged at 1,500 rpm for 5 min (Beckman CS-6R). The supernatant was carefully aspirated and the pellet was loosened by gentle tapping and then mixed with a heat-closed Pasteur pipette. To the pellet 0.5 ml of pre-warmed (37°C) PEG 4,000 solution (Sigma) was added and the tube was incubated at 37°C in a water bath with gentle shaking for exactly 1 min. Thereafter, the tube was incubated at room temperature for 1 min and over the next 5 min, 10 ml of RPMI 1640 (w/o HEPES, w/o glutamine) was slowly added to the tube with gentle shaking at 37°C in a waterbath. Now the tube was incubated on ice for 10-15 min in order to stabilise the fused cells. The fused cells were then pelleted by centrifugation at 1,500 rpm for 5 min (Beckman CS-6R) and the pellet was resuspended in 125 ml NM. Now the fused cells were added (0.5 ml/well) to 24-well tissue culture plates that have been coated with mouse feeder cells 24 h prior to fusion. The plates were incubated in a CO_2 incubator at

37°C with 5% CO_2 and 95% relative humidity. The next day, selection of hybridoma cells was started by adding 0.5 ml of 3 x HAT medium to each well and the plates were incubated for another 2 days. Thereafter, the medium was changed every alternate day with 1x HAT medium (1 ml/well) and the hybrids were grown in 1x HAT medium for 2 weeks after fusion. After 2 weeks, the medium was changed to HT medium and the hybrids were grown in HT medium until the completion of cloning procedures.

2.2.55 Screening of hybridoma clones

When the hybridoma cells had covered 10% to 50% of the surface areas of the wells, the hybridoma-supernatants were collected and screened for PhdB specific antibody production. Screening of hybridoma clones obtained from spleen cells of the mouse immunised with the C-terminal PhdB protein was performed initially by indirect enzyme linked immunosorbent assay (indirect ELISA) and those clones which gave a positive reaction in the ELISA were further confirmed by immunoblotting.

2.2.56 Indirect ELISA for screening of hybridoma clones

Purified protein used to immunise mice was used to coat the ELISA plates (Nunc) with antigen (200ng/100µl). The plate was incubated overnight at 4°C. The next day, unbound antigen was washed out by inverting the plate and flicking the wells, followed by 3 washings with wash buffer for 10 min each. Blocking buffer was added to each well and the plate incubated for 1h at RT. After blocking, the plate was washed 3 times with wash buffer for 10 min each and after each washing, the residual liquid in the plate was removed by gently flicking the plate face down onto several paper towels lying on the benchtop. Now the hybridoma-supernatants to be analysed was added to the respective wells on the plate and incubated for 1hr at room temperature. The incubation with hybridoma-supernatants was followed by 4 washings with wash buffer as described above. Phosphatase conjugated goat anti-mouse IgG (diluted 1:10,000 in blocking buffer) was added to each well and the plate was again incubated at room temperature for 1 h. Following the incubation with secondary antibody, the plate was again washed 4 times with wash buffer. As mentioned above Para-nitrophenyl phosphate (pNPP, Sigma) solution was added to each well and the plate was incubated in the dark for 30 min to 1 h at room temperature. Hydrolysis of the pNPP substrate was detected by the appearance of a yellow colour. It was quantitatively monitored with an ELISA-plate reader using a 405 nm filter.

Blocking buffer:

5% bovine serum albumin

0.05% Triton X-100

0.02% NaN_3 in 1x PBS, pH 7.4

Carbonate buffer, pH 9.4:

1.59 g Na_2CO_3

0.2 g NaN_3

2.93 g $NaHCO_3$ adjust to pH 9.4 and add deionised H_2O to make 1 liter

Wash buffer:

0.05% Triton X-100

0.02% NaN_3 in 1x PBS, pH 7.4

pNPP substrate solution:

Sigma Fast tablet sets for 5 ml or 20 ml solution were used to the manufacturer's specifications.

2.2.57 Stripe test for screening of hybridoma clones

Whole cell homogenate of the *E. coli* cells expressing the C-terminal PhdB fusion protein was loaded onto a 12% SDS-polyacrylamide gel and after resolving, the proteins were transferred onto a nitrocellulose membrane. After overnight blocking in 1x NCP buffer. The membrane containing the transferred protein was cut into several small stripes (~30 stripes/mini-gel) and then each stripe was separately incubated with the hybridoma-supernatants at room temperature for overnight. The next day, stripes were washed 4 times with 1x NCP for 5 min each and then incubated with alkaline phosphatase conjugated goat anti-mouse IgG (1:5,000 in 1x NCP buffer). After usual washings, BCIP/NBT colour development substrate reaction was carried out.

2.2.58 Cloning of hybridoma cells

Hybridoma cells that were positive by ELISA and immunoblotting were selected for cloning in order to establish a single hybridoma cell line. The day before cloning, mouse feeder cells were isolated and checked for possible contamination by incubating 100µl of macrophage preparation in the incubator at 37°C with 5% CO_2 and 95% relative humidity. For cloning, 96 well microtiter plate was prepared by adding 3-4 drops of 1x HT medium or NM (depending upon the type of medium in which the hybridoma cells to be cloned are present). Hybridoma cells to be cloned were grown to log phase in 24 well plates and were resuspended in 1 ml fresh 1x HT medium or NM. A drop of the hybridoma cell suspension was then added to the

A1 well of the microtiter plate using a sterile cotton-plugged Pasteur pipette, mixed well by gentle pipetting and then transferred a drop from the well A1 to A2. Likewise, serial dilutions of the hybridoma cells were prepared in the wells A1 to A4 and then observed under the inverted microscope. The well showing 20-25 cells/microscopic field was selected for cloning. Using a sterile cotton-plugged Pasteur pipette, a drop of the cell suspension was carefully placed in the centre of each well of the microtiter plate (maximum 2-3 rows at a time to avoid drying of the drops) and the wells containing the drop were immediately observed under the inverted microscope. The wells that exhibited only one hybridoma cell/well (or per drop) were marked and filled with mouse feeder cell suspension. This procedure of cloning was repeated till at least 20 single-cells per hybridoma cell line were cloned. The microtiter plates were then covered with saran wrap and incubated in a CO_2 incubator (37°C, 5% CO_2, 95% relative humidity). After 1.5 to 2 weeks, the wells exhibiting 10-50% confluent cell growth were assayed for specific antibody production by checking their
hybridoma supernatants by immunoblotting and fresh medium (NM or 1x HT medium) were added to the wells. The positive subclones from each clone were expanded by transferring into a 24 well plate (2-3 subclones/24 well plate to avoid cross-contamination) and the plates were incubated in a CO_2 incubator (37°C, 5% CO2, 95% relative humidity). Medium in the 24 well plate was changed every second day and when the cell-growth in the well was >50% confluent, cells were either frozen in a cryotube or transferred to a 25 cm2 tissue culture flask (in 5 ml NM). From 25 cm2 tissue culture flask, the cells were transferred to a 75 cm2 tissue culture flask (in 10 ml NM) and then to a 175 cm2 tissue culture flask (in 50 ml NM), each time when the cell-growth in the flask was >50% confluent. Every alternate day, the hybridoma supernatant was collected and fresh medium was added.

2.2.59 Freezing and recovery of hybridoma cell lines

Hybridoma cells to be frozen were, in general, harvested in the log phase of growth. When the cell-growth in a 24 well plate or a 25-cm2 tissue culture flask was >50% confluent, the hybridoma supernatant was replaced with the fresh medium (1 ml/well or 5 ml/flask) in order to remove the dead cells. Cells were then resuspended in the medium; collected in a sterile 15 ml centrifuge tube and incubated on ice. After 30 min of incubation on ice, the cells were pelleted by centrifugation at 1,200 rpm (Beckman CS-6R) for 5 min at 4°C. The supernatant was aspirated and the pellet was resuspended in an equal volume of cold freezing medium. Aliquots of 1 ml/cryotube (Nunc) were prepared and incubated overnight at –80°C in a

thermal box to freeze the cells slowly (~2°C/min). The next day, the frozen aliquots were transferred to liquid nitrogen for long-term storage.

2.2.60 Recovery of frozen cell lines

The cryotubes were taken out of the liquid nitrogen and immediately thawed at 37°C in a waterbath. In order to remove DMSO, the cells were transferred to a 15 ml centrifuge tube containing 12 ml of NM and centrifuged at 1,200 rpm for 5 min at 4°C. The supernatant was aspirated and the cell pellet was resuspended in 1 ml NM and transferred to a well in 24 well plates. The plate was incubated overnight at 37°C in a CO_2 incubator with 5% CO_2 and 95% relative humidity. The next day, dead cells and traces of DMSO were removed by changing the medium in the well.

2.2.61 Generation of polyclonal antibodies against PhdB

IMMUNISATION OF RABBITS:

For generating polyclonal antisera against PhdB, rabbits were immunised with pure peptide used to generate monoclonal antibodies (C-terminal of PhdB). The recombinantly expressed C-terminal peptide was separated on 12% SDS-PA gels. The proteins separated were stained by Copper Chloride and the protein of interest was excised from the gel. The protein from the stained SDS-PA gel was eluted using Bio-Rad Model 422 Electro-Eluter (according to the supplier manual).

The pure protein obtained was used to immunise two female white New Zealand rabbits (100 µg/animal; Pineda Antikörper-Service, Berlin, Germany), followed by two boosts of 100 µg each at two weeks intervals.

Affinity purification of PhdB specific antibody.

We utilised the property of strong interaction of the C-terminal GST fusion protein with GST beads when solubilised using sarkosyl. The C-terminal protein used to immunise mice was expressed as GST fusion protein. The GST fusion protein was purified by using the sarkosyl detergent methods (materials and methods 2.2.46) The GST beads bound protein was incubated with 1:2 diluted polyclonal antibody for 1h at 4°C. The beads were separated by spinning for 2 min at 2,000 xg, 4°C and washed three times with ice cold PBS. The antibody bound to the beads by virtue of its interaction with the C-terminal peptide was eluted using elution buffer. The eluted antibodies were neutralised immediately with Tris-HCL pH 8.0.

Elution Buffer.

100 mM Glycine-HCL, pH3.0

2.2.62 Preparation of *Dictyostelium* cells for immunofluorescence analysis

2.2.63 Methanol fixation

After the cells have attached to the cover slip, the supernatant was aspirated and the cover slip was dipped instantaneously into the pre-chilled (-20ºC) methanol in a petri dish and incubated at –20ºC for 10 min. The cover slip was then taken out from methanol and placed on the para film in the humid-box with the cell-surface facing upwards, followed by 3 washings (each with 500 µl of PBG, pH 7.4, for 5 min at room temperature) the coverslip with cells was mounted using gelvatol, to visualise the GFP fusion protein.

2.2.64 Picric acid-paraformaldehyde fixation

After the cells have attached to the glass coverslips, the supernatant was gently aspirated from the edge of the coverslip and 200 µl of freshly prepared picric acid-paraformaldehyde solution was directly added onto the cell-surface of the coverslip and incubated at room temperature for 30 min. After incubation, the picric acid-paraformaldehyde solution was aspirated and the coverslip was washed once with Soerensen phosphate buffer for 5 min at room temperature. The coverslip was then picked up with a fine forceps and swirled in 10 mM PIPES buffer, pH 6.0, followed by blotting off the excess solution from the coverslip with a tissue paper. Now the coverslip was swirled in PBS/glycine and placed on a parafilm-covered glass-plate resting in a moist chamber with the cell-surface facing upwards. The coverslip was then washed with 500 µl PBS/glycine for 5 min followed by post-fixation with 500 µl 70% ethanol for 10 min. This was followed by 2 washings with 500 µl PBS/glycine for 5 min each followed by 2 washings with 500 µl of PBG for 15 min each. After washings, the cells were immunolabelled as described in materials and methods (2.2.65).

PBS/glycine:

500 ml PBS

3.75 g glycine

filter sterilized

20 mM PIPES buffer, pH 6.0:

0.605 g PIPES in 100 ml distilled H_2O, adjust to pH 6.0, filter sterilise and store at –20°C

Picric acid-paraformaldehyde solution:

0.4 g paraformaldehyde was dissolved in 5 ml ddH_2O by stirring at 40°C and adding 3-4 drops of 2M NaOH. After dissolving, the volume was adjusted to 7 ml with ddH_2O. To this

paraformaldehyde solution, 10 ml of 20 mM PIPES buffer, pH 6.0, and 3 ml of saturated picric acid was added and the pH was finally adjusted to 6.5.

2.2.65 Immunolabelling of fixed cells

The coverslip containing the fixed cells were 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 unattached antibody was removed by washing the coverslip 6 times with PBG for 5 min each. Now the coverslip was incubated for 1 h with 400 µl of a proper dilution (in PBG) of Cy3-conjugated secondary antibody. Following the incubation with secondary antibody, two washings with PBG for 5 min each followed by three washings with PBS for 5 min each were performed. After washings, the coverslip was mounted onto a glass slide (see materials and methods, 2.2.66)

2.2.66 Mounting of cover slips

After fixing the cells, the coverslip was swirled once in deionised water and the extra water was soaked off on a soft tissue paper. Now a drop of gelvatol was placed to the middle of a clean glass slide and the cover slip was mounted (with the cell-surface facing downwards) onto the drop of gelvatol taking care not to trap any air-bubble between the cover slip and the glass slide. Mounted slides were then stored in the dark at 4ºC for overnight. Thereafter, the mounted slides were observed under a fluorescence microscope or confocal laser scan microscope.

Gelvatol:

2.4 g of polyvinyl alcohol (Mw 30,000-70,000; Sigma) was added to 6 g of glycerol in a 50 ml centrifuge tube and mixed by stirring. To the mixture, 6 ml of distilled H2O was added and the mixture was incubated at room temperature. After several hours of incubation at room temperature, 12 ml of 0.2 M Tris/HCl, pH 8.5, was added and the mixture was heated to 50°C for 10 min with occasional mixing to completely dissolve polyvinyl alcohol. The solution was centrifuged at 5,100 rpm for 15 min. After centrifugation, 2.5% of diazobicyclo octane (DABCO), an anti-oxidant agent was added to reduce the bleaching of the fluorescence. The solution was aliquoted in small volumes in 1.5 ml microcentrifuge tubes and stored at –20°C.

PBG (pH 7.4)

0.5 % bovine serum albumin

0.1% gelatin (cold-water fish skin) in 1x PBS, pH 7.4

2.2.67 DAPI and phalloidin staining of fixed cells

DAPI staining of *Dictyostelium* nuclei and phalloidin staining of *Dictyostelium* F-actin was performed simultaneously. Staining of F-actin with phalloidin demarcated the cell-boundary, which facilitated in determining the number of DAPI stained nuclei within a particular cell.
Cells were harvested and the coverslips coated with cells were prepared as explained in materials and methods (2.2.64). Cells were then fixed onto the coverslip by picric acid-paraformaldehyde fixation method as discussed in materials and methods (2.2.64). After fixation and usual washings, coverslips were incubated for 30 min with 400 µl of PBG containing DAPI (1:1,000 dil.) and TRITC-phalloidin (1:200 dil.). Thereafter, the coverslip was washed twice with 400 µl of PBG for 5 min each followed by three washings with 400 µl of PBS for 5 min each. After washings, the coverslips were mounted onto the glass slides (see materials and methods, 2.2.66) for observation under a fluorescence microscope or confocal laser scan microscope.

2.2.68 MICROSCOPY

For conventional immunofluorescence microscopy, DAPI staining and phalloidin staining, the cells were observed under a fluorescence microscope (Leica DNR) equipped with a 100x Neofluar objective. The images were captured by a cooled charge-coupled device (CCD) camera (SensiCam, PCO). Various stages of development of *Dictyostelium* cells were also visualized using the fluorescence microscope (Leica DNR) equipped with a 2.5x or 5.0x objective and images were captured by a cooled CCD camera (SensiCam, PCO). Visual inspection of GFP-PhdBCT expressing *Dictyostelium* cells was performed using an inverted fluorescence microscope (Olympus 1X70). For studying the localization of GFP-PhdBCT fusion protein in live cells during cell locomotion an inverted confocal laser scanning microscope (Leica DM/IRBE) equipped with a 40x PL Fluotar 1.25 oil immersion objective or a 63x PL Fluotar 1.32 oil immersion objective was used. The 488-nm band of an argon-ion laser was used for excitation, and a 510-525-nm band-pass filter was used for emission. Confocal images of immunolabelled specimens were obtained with confocal laser scanning microscope (Leica DM/IRBE) equipped with 488-nm argon-ion laser for excitation of GFP fluorescence and a 568-nm krypton-ion laser for excitation of Cy3 fluorescence. For simultaneous acquisition of GFP and Cy3 fluorescence, the green and red contributions to the emission signal were separated by using a band-pass filter of 510-525-nm and a long-pass filter of 570-nm, respectively. The images from green and red channels were independently

attributed with colour codes and then superimposed using the accompanying software and converted to the TIFF format after the experiment

2.2.69 Computer analysis

Analysis of the sequences and homology searches were performed using the 'University of Wisconsin' GCG software package and different gene bank databases (Devereux *et al.*, 1984) and *Dictyostelium discoideum* gene databases. Structural predictions and multiple alignment of the protein sequences were made using Expasy Tools software, accessible on the world-wide web. For processing images, Corel Draw version 8, Corel Photopaint, Adobe Illustrator, Adobe Photoshop and Microsoft Power point softwares were used. Graphs and histograms were prepared using the Microsoft Excel software.

3. RESULTS

3.1 Screening of genomic and cDNA data bases sequences of *Dictyostelium*

Dictyostelium cells exhibit several physiological processes like chemotaxis, phagocytosis, pinocytosis, and phototaxis during the life cycle. Actin reorganisation regulates most of these processes (Noegel and Schleicher, 2000). Hence *Dictyostelium* offers a powerful system to study actin dynamics. To characterise novel actin binding proteins in *Dictyostelium*, we examined the cDNA and genomic DNA databases of *Dictyostelium*. The actin binding domain of filamin was employed to screen the genomic and cDNA database. Filamin is a well characterised actin binding protein, possessing a single actin binding domain. The actin binding domain was mapped to the amino terminal residues 105 to 120 and 360 to 372 and was shown to be responsible for actin binding (Bresnick *et al.,* 1990). Therefore by using the filamin actin binding domain as query for TBLASTN searches of the *Dictyostelium* EST and genomic database, we identified a novel gene assembled in the contig JC2e45e10 (set of overlapping segments of DNA) on chromosome 3 of *Dictyostelium*. The gene encoded a 1577 amino acid open reading frame. The calculated molecular mass of the protein was 175 kDa. Domain architecture analysis of the amino acid sequence of the new protein predicts a single CH (actin binding) domain towards the N-terminal end. The CH domain of the new protein has 40% identity (materials and methods 2.2.2) with the first CH domain of filamin which has two CH domains. Other than a CH domain towards the N-terminal end the new protein also contains a RhoGEF and an ArfGAP domain at its C-terminus each followed by a PH domain (Figure 1). Proteins carrying PH domains (Lemmon *et al.,* 2000) are either involved in signal transduction or they are a part of the cytoskeleton. Since the new protein possesses PH domains, we referred to the new protein as PhdB (pleckstrin homology domain containing protein B; protein B is because a recently characterised protein with a PH domain was named PhdA (Funamoto *et al.,* 2001).

Functional domains like the CH domain, N-terminal PH domain, and the C-terminal ArfGAP/PH domains were obtained by RT-PCR and the PCR product were cloned into a *Dictyostelium* GFP expression vector and expressed as GFP fusion proteins (materials and methods 2.2.25).

Figure 1. Domain architecture of the PhdB protein. The amino acid sequence of PhdB was analysed by SMART (Simple Modular Architecture Research Tool). Grey boxes display the functional domains present in PhdB.

To date a limited number (~100) of PH domain containing proteins have been characterised. No PH domain has been detected in plants or bacteria.

The sequence comparison of the N-terminal and C-terminal PH domains present in PhdB with the PH domains present in CRAC (cytosolic regulator of adenylyl cyclase) and PhdA was performed by clustalW (protein sequence alignment programme). Figure 1.2 depicts the sequence similarity of the N-terminal and C-terminal PH domains of PhdB with the PH domain of CRAC and PhdA; and the similarity was approximately 20%. In general the PH domains are very poorly conserved and typical pair wise identities between different PH domains present in different proteins are in the range of 10–20% and similarity is in the range of 20-30%. At the three-dimensional structure level PH domains are strikingly very similar (Lemmon *et al.,* 2002).

```
PhdA_PH       -KVIYSTVMKKAGGNKKGFLSRLFVLYKGFVIYYKT-KTLLTSPEK--PQGYIDLRECDP
CRAC_PH       ---SYSSIMKKAGGNGKGFLDRYFALHRNYILYYKLGKSSLKPDDKQEPQGYINLMDCNP
PhdB_NT_PH    ----------------SWRSKKFYLKNTHLYYHRY-SSTESPKEP-TKIKCINLILCSV
PhdB_CT_PH    HQGYLFKTSSPTSNNSSDWKKYLFVYKNDVLTYYKV-SKKNKRKEK----GIIDLFHSVK

PhdA_PH       SKVKTILDDTDMTFQIVHRAGRTFLIKGEEPKPFRRFFEICKCV----
CRAC_PH       DDTKEIAP--LMEFQISHKH-RTYIVKAKDESSMKQFLTLLIARIRSL
PhdB_NT_PH    KLAQVVDH--PHCFQLITPS-RIYFFSCEDSTVLFQWISLIRLSIKK-
PhdB_CT_PH    QESRPKQK---YSFTLVASQ-RLYFLASETEEEMKIWLDVLSSHT---
```

Figure 1.2. Multiple sequence alignment of PH domains. Sequence similarity of PH domains of PhdB with CRAC and PhdA was analysed by clustalW. The results obtained exhibited approximately 20% similarity among the PH domains analysed (amino acids in red are identical, in green and blue exhibit similarity).

3.1.1 Generation of monoclonal and polyclonal antibodies against PhdB

To identify the localisation and interaction of PhdB with other proteins, we generated monoclonal antibodies against PhdB. A C-terminal peptide consisting of the ArfGAP followed by the PH domain of PhdB was selected as the antigenic peptide to immunise mice. The C-terminal peptide was expressed without any tag in *E. coli* using the pIMS expression vector (materials and methods 2.2.27). The obtained pure protein (Figure 2A) was used to immunise mice. Primary screening of hybridoma supernatants for PhdB specific antibodies was performed by indirect ELISA (materials and methods 2.2.56). The hybridoma supernatants that proved to be positive by ELISA screening were further verified by stripe tests (materials and methods 2.2.57; Figure 2B) using whole cell homogenate of *E. coli* cells expressing the recombinant C-terminal peptide of PhdB. We identified a single clone secreting PhdB specific antibodies (K48-122). The K48-122 hybridoma clone was further subcloned in order to establish a single hybridoma cell line and three independent single cell clones (K48-122-1-3) were established. The supernatant of K48-122-1 was analysed by immunoblotting. In whole cell homogenate from AX2 cells (Figure 2D) the mAb K48-112-1 recognised the 175 kDa PhdB protein. The same antibody also recognised a GFP fusion protein of the C-terminal peptide originally used to immunise mice (Figure 2C).

Further we planned to generate polyclonal antibodies (materials and methods 2.2.61) against PhdB in addition to the existing monoclonal antibody using the same C-terminal peptide used to generate the monoclonal antibody. Two rabbits were immunised with the C-terminal peptide and the antibody produced was screened by western blot analysis. One of the animal sera recognised the 175 kDa PhdB protein in *Dictyostelium* cell extracts (Figure 4). The other animal serum also recognised the PhdB protein in *Dictyostelium* but in addition gave few additional non specific signals. The polyclonal antibody was of higher affinity when compared to the monoclonal antibody specific to PhdB. The monoclonal antibody K48-122-1 did not detect PhdB by immunofluorescence analysis. Therefore the polyclonal antibody was used in further experiments to characterise PhdB.

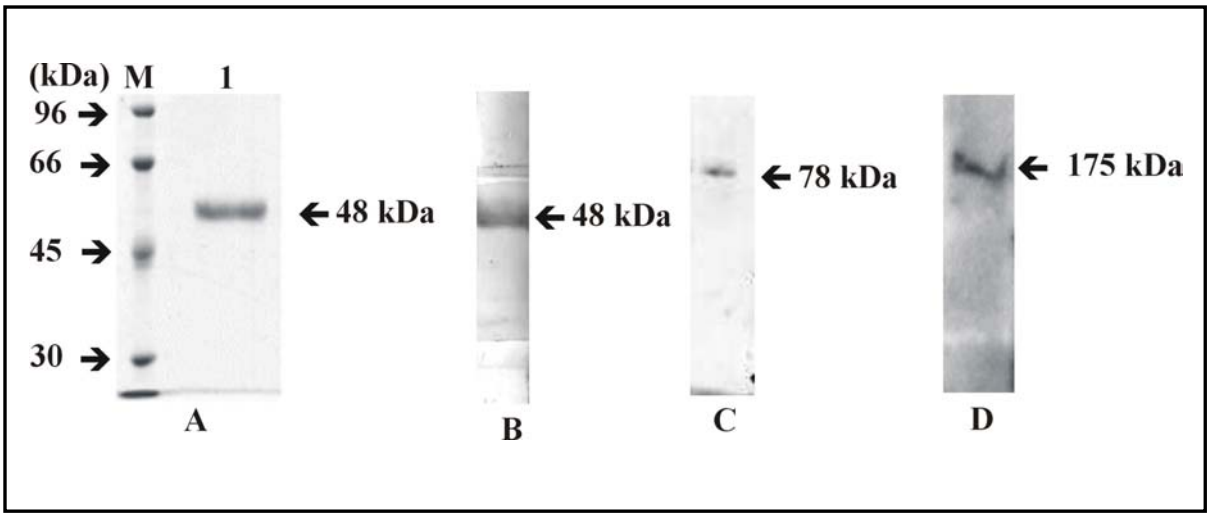


Figure 2. A) Coomassie blue stained gel showing the recombinant protein used for antibody production. The recombinant polypeptide expressed in *E.coli* XL1 blue was extracted with 8M urea buffer and resolved on 12% SDS-PA gel. The peptide was excised and purified from the gel by electroelution. The purified peptide obtained after electroelution was analysed by Coomassie blue staining and used to immunise the mice. Lane 1 represents the pure protein. The positions of the molecular mass marker proteins are indicated on the left (M).

B) Western blot analysis of C-terminal recombinant protein (stripe test) expressed in *E. coli.* Cells overexpressing the C-terminal peptide (used to immunise mice) were harvested and lysed in 1x sample buffer. The cell lysate was separated on 12% SDS-PA gel. Potein samples were immunoblotted and the blotted membrane was cut into small stripes and incubated with the monoclonal antibody K48-122-1 to detect the recombinant protein. The secondary antibody used was anti mouse IgG coupled to alkaline phosphatase.

C) Western blot analysis of C-terminal GFP fusion protein expressed in *Dictyostelium.* The C-terminal peptide used to immunise mice was expressed in AX2 cells as a GFP fusion protein, these cells were harvested and lysed in 1x sample lysis buffer. The cell lysate was separated by 12% SDS-PA gel. Protein samples were immunoblotted and the C-terminal GFP fusion protein was detected by monoclonal antibody (K48-122-1) at 78 kDa.

D) Western blot analysis of PhdB in *Dictyostelium*. Developing *Dictyostelium* cells (t6) were harvested and lysed in 1x sample buffer. The cell lysate was separated on 8% SDS-PA gel. Protein samples were immunoblotted and PhdB was detected by using monoclonal antibody (K48-122-1) at 175 kDa.

3.1.2 PhdB is expressed throughout the development of *Dictyostelium*

Dictyostelium cells grow as unicellular amoebae when nutrients are available. Depletion of nutrients triggers, a developmental programme leading to the formation of a multicellular fruiting body within 24 hours. This transition of cells from the growth phase to the developmental phase is a consequence of stage specific gene expression that involves activation of certain genes and repression of others. Therefore, a northern blot analysis was performed to study the PhdB gene expression profile during *Dictyostelium* development.
Hybridisation under stringent conditions with a probe (1043 bp from the 3' end of PhdB cDNA) revealed that the PhdB transcript is approximately 4.7 kb in size (Figure 3) and present through out the *D. discoideum* development.

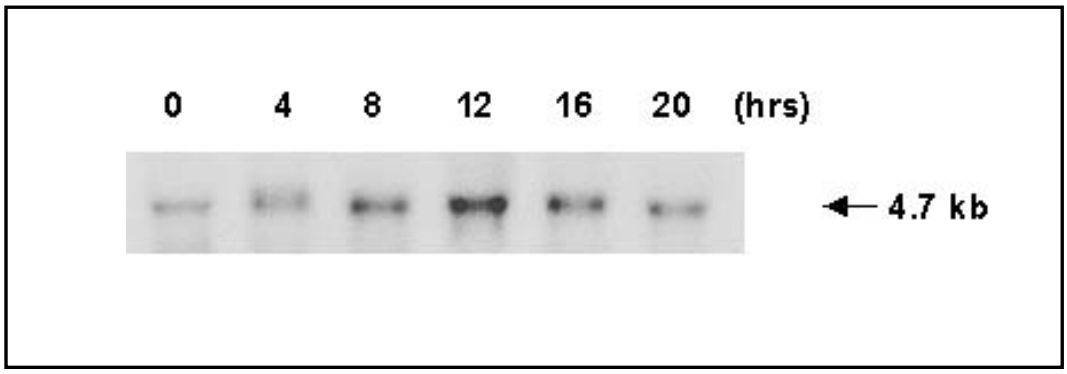


Figure 3. Northern blot showing the PhdB transcript pattern. Total RNA (30 μg) isolated from *Dictyostelium* cells at different time points of development was loaded per lane and separated on a 1.2 % agarose gel under denaturing conditions. RNA samples were transferred on to the nylon membrane followed by hybridisation with a probe derived from the 3' end of the PhdB cDNA (nucleotides 3697-4740). The probe recognised a 4.7 kb transcript, which represents the PhdB mRNA.

The presence of the PhdB protein at different development stages was demonstrated by western blot analysis using the polyclonal antibody pAbCT (Figure 4).

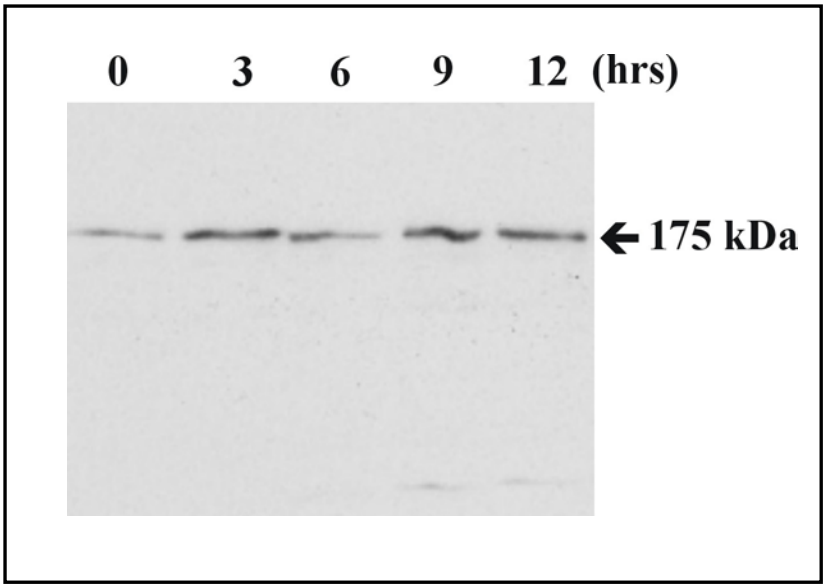


Figure 4. Western blot analysis of PhdB at different developmental stage. *Dictyostelium* cells harvested at different developmental time points were lysed with 1x sample buffer. The cell lysates were separated on 8% SDS-PA gel and analysed by western blotting using the polyclonal antiserum (unpurified serum) at 1:10,000 dilution, which recognised PhdB.

3.1.3 PhdB$^-$ mutant generation by homologous recombination

To define the function of the PhdB protein, we attempted to generate mutants which lack PhdB. Therefore a gene disruption vector was designed (materials and methods 2.2.28).

In brief, to construct a PhdB gene disruption vector, a 1.1 kb fragment of genomic DNA containing the first exon (nucleotides 1 to 1100 bp) of the PhdB gene was cloned into *ApaI* / *Sal* I digested pBluescript II KS+△Bambsr vector. The vector also contained a 1.4 kb blasticidin S resistance (bsr) cassette which confers resistance to blasticidin S. The bsr gene was driven by the *Dictyostelium* actin 15 promoter and contained the actin 8 terminator for expression in *Dictyostelium* cells.

Towards the 3' end of the drug resistance cassette a 1.0 kb homologous DNA of the PhdB gene was inserted. This fragment corresponds to residues 4310 to 5353 bp of the PhdB gene cloned into the *NotI* site of the pBluescript II KS+△Bambsr (Figure 5B). The organisation of the PhdB gene and of the gene disruption vector is depicted in Figure 5A and B respectively, as well as the gene after homologous recombination with the vector in Figure 5C.

The gene replacement vector constructed was linearised by digestion with PvuII. The 3.9 kb fragment obtained, which has a bsr cassette flanked by 5' and 3' side homologous DNA was transformed into strain AX2 by electroporation. The bsr resistant transformants were selected in 96 well plates in AX2 medium containing 5 μg of blasticidin / ml. Independent transformants were screened for a disrupted PhdB gene by PCR, Southern blot and western blot analysis.

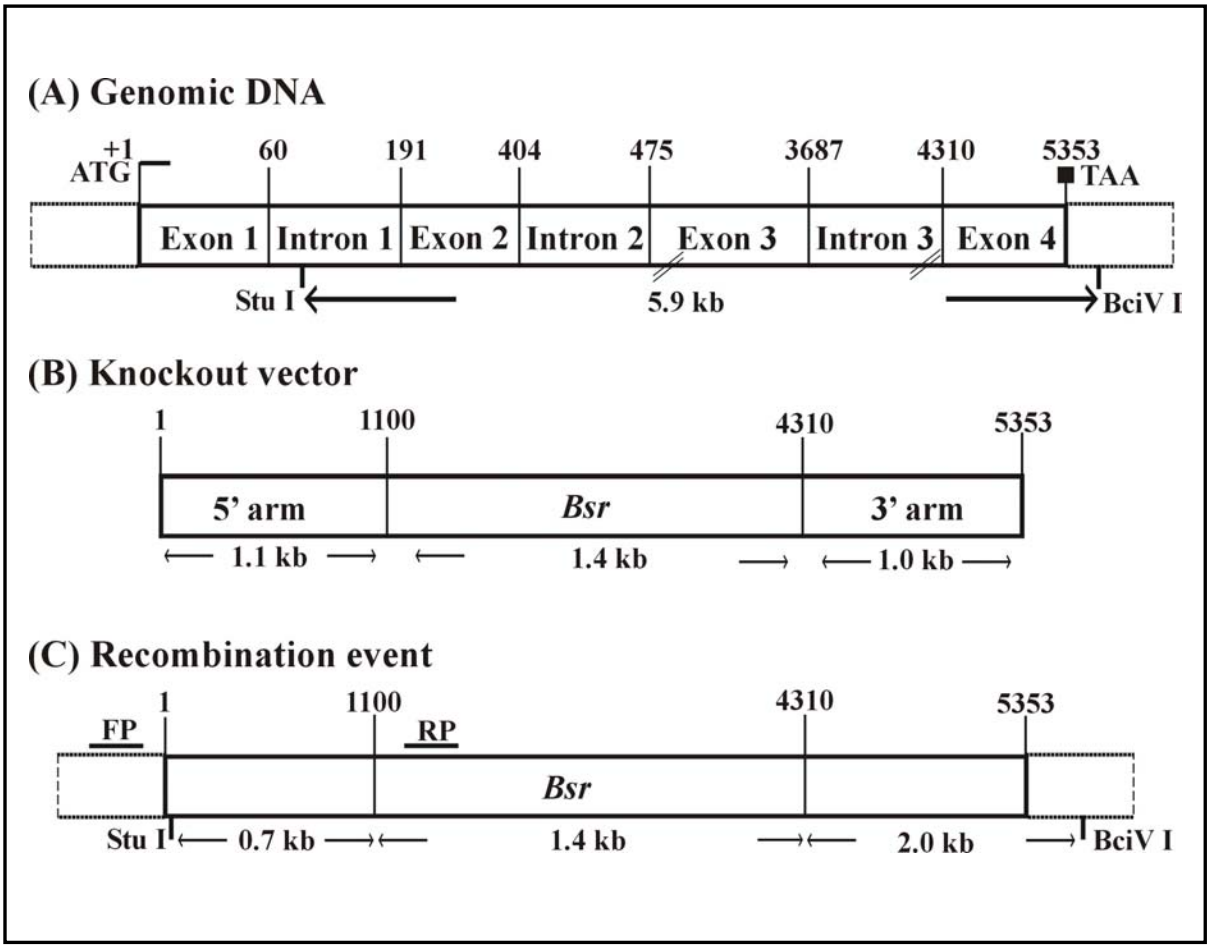


Figure 5. Organisation of the PhdB gene and the disruption vector. A) Genomic DNA organisation showing the homologous region. **B)** The gene disruption vector consisted of 5' and 3' sequences of the PhdB gene which flanked the blasticidin (bsr) resistance cassette. The 5' arm was cloned at the *ApaI* / *SalI* site of pBluescript II KS+△Bambsr and the 3' arm was cloned at the *NotI* site of pBluescript II KS+△Bambsr which flank the 1.4 kb blasticidin resistance cassette respectively. **C)** Genomic DNA organisation after the recombination event, FP and RP represents the location of forward and reverse primers respectively, used to screen the transformants by PCR.

3.1.4 PCR analysis

PCR analysis was performed to characterise the recombination event in the transformants obtained. Genomic DNA was isolated from the 50 transformants obtained and was used as the template for PCR (materials and methods 2.2.23). The forward primer (FP; kofp) was located upstream of the 5' arm, and the reverse primer (RP; kobsrrp) was located in the blasticidin resistance cassette (Figure 5C). PCR amplification using these primers generated a 1.6 kb fragment from 10 transformants (Figure 6A, displays two such positive clones), which indicates that the recombination event occurred between the genomic DNA and the replacement vector. No PCR product was obtained with wildtype cell genomic DNA.

3.1.5 Southern blot analysis

Southern blot analysis was performed to verify the recombination event at the right site in the transformants obtained. Genomic DNA of wild type and transformants was isolated and digested with *BciVI* and *StuI*. Both enzymes do not have any internal restriction site in the blasticidin resistance cassette, *BciVI* cuts the DNA downstream of the 3' end of the inserted DNA and *StuI* cuts within the 5' arm (Figure 5C). The digested DNA was resolved on an agarose gel, transferred onto a nylon membrane and the blot was probed with a ^{32}P labelled probe, which was a 1000 bp fragment corresponding to nucleotides 1 to 1044 bp of the gene. Southern blot analysis was performed on ten positive clones that were identified by PCR analysis. We observed that a gene replacement event has occurred at the PhdB locus, as the insertion of the 1.4 kb bsr cassette causes a shift of the 5.9 kb *BciVI / StuI* fragment to a 4.1 kb fragment in these transformants (Figure 6B, shows three such transformants).

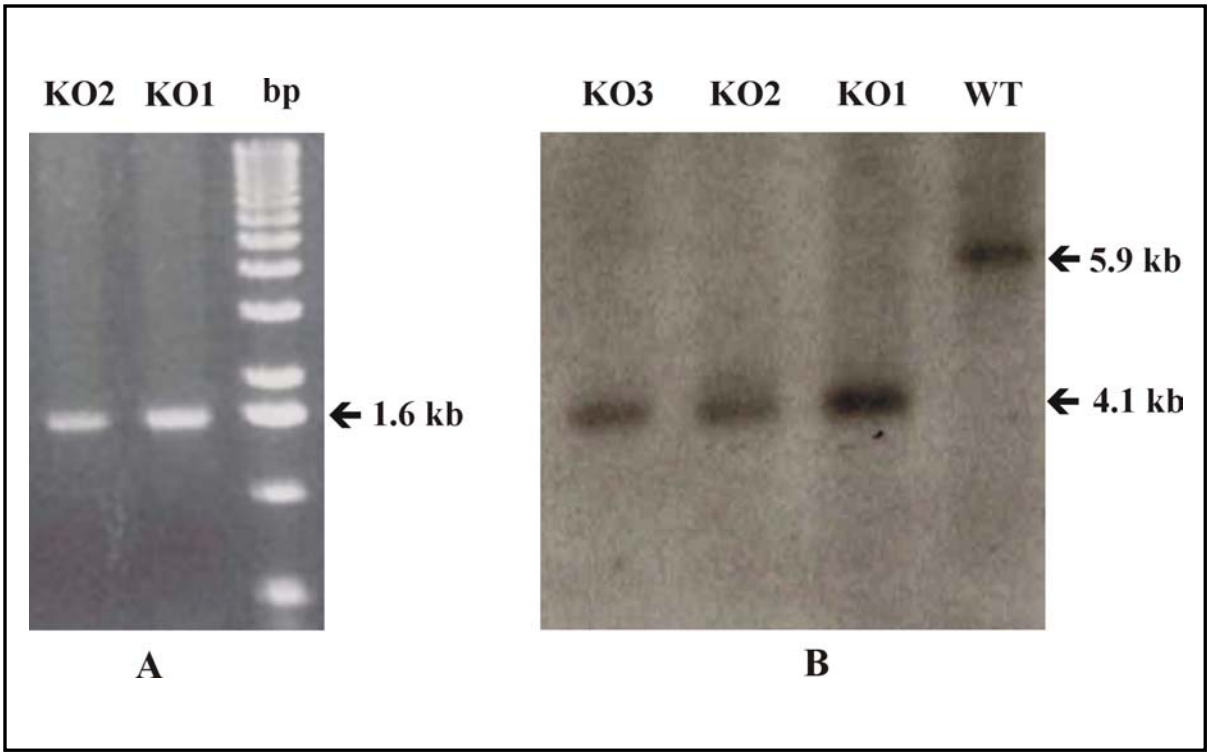


Figure 6. PCR and Southern blot analysis of transformants. A) PCR analysis was done using the genomic DNA isolated from the transformants. With the forward primer (kofp) and reverse primer (kobsrrp) a 1.6 kb gene product was amplified which indicates the event of recombination in the transformants. **B)** Disruption of the PhdB gene in AX2 cells by homologous recombination was confirmed by Southern blot analysis. *BciVI/ StuI* restricted genomic DNA of AX2 and mutant cells indicates that a gene replacement event has occurred, as the replacement of the gene sequence by the knockout construct causes the shift of a 5.9 kb band to a 4.1 kb band in the mutants.

3.1.6 Western blot analysis

The absence of the PhdB protein in PhdB$^-$ cells was verified by immunoblot analysis. Wild type and PhdB$^-$ cells were allowed to develop in shaking culture. Total cellular proteins (equivalent to 4 x 10^5 cells/lane) of each cell-type were extracted and separated by 8% SDS-PA gel. The separated proteins were transferred onto a nitrocellulose membrane and the blot was subsequently incubated with the PhdB specific polyclonal antibody followed by immunolabelling with peroxidase-conjugated anti-rabbit IgG. The antibody recognised a strong band at ~ 175 kDa (Figure 7A) in AX2 cells (lane WT), which corresponds to the molecular weight of the endogenous PhdB protein. This ~ 175 kDa band is absent in case of PhdB$^-$ cells (lanes KO1 and KO2). The same western blot (of figure 7A) was used to probe for actin, which is a constitutively expressed protein to confirm equal loading. Monoclonal antibody act1 was used to probe the blot. Act1 specifically recognised actin at 43 kDa (Figure 7B) in all the lanes (WT, KO1, KO2). The amount of actin detected indicates equal loading.

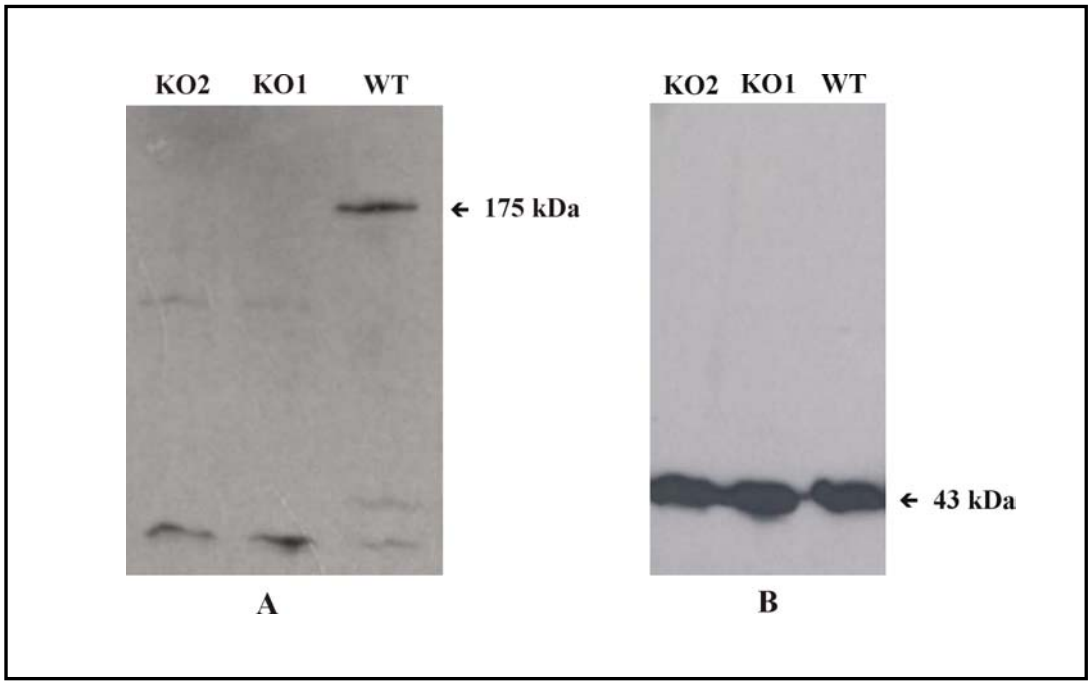


Figure 7. Western blot analysis. A. Western blot analysis of cell homogenates derived from AX2 and PhdB$^-$ cells. Total cellular proteins of AX2 (lane WT) and PhdB$^-$cells (lanes KO1 and KO2) were extracted in 1x sample buffer. The extracted proteins were resolved (equivalent to 4 x 10^5 cells/lane) on an 8% SDS-polyacrylamide gel, blotted onto a nitrocellulose membrane and immunolabelled with anti PhdB polyclonal antibody (pAbCT). The arrowhead on the right indicates the endogenous PhdB protein, which is visible only in AX2 cells (lane WT). The PhdB protein is absent in PhdB$^-$ cells (lanes KO1 and KO2). The immunoblots were processed after incubation with HRP-conjugated anti-rabbit IgG by ECL-detection system for visualization of the specific immunolabelled bands. **B) Internal control**. The membrane of the previous western blot (Figure 7A) was probed for actin using mAb act1, specific to actin. The arrowhead towards the right in figure B indicates actin, which shows equal loading of protein samples in both AX2 cells (lane WT) and PhdB$^-$ cells (lane KO1 and KO2).

3.1.7 PhdB$^-$ cells exhibit an aggregation defect

PhdB$^-$ cells grow in axenic medium at a rate compared to wild type strains when grown axenically. When no food source is available amoebae aggregate and develop into a fruiting body. To study the aggregation phase, PhdB$^-$ cells were developed under buffer and plated as monolayer cultures in Soerensen buffer in a petri dish. A major difference was observed when PhdB$^-$ cells were allowed to develop in Soerensen buffer. Wild type cells began to stream within 6h and formed large elaborately branched streams by 8h and completed aggregation by approximately 13 h, with a strong aggregation centre. Wild type cells were able to sense the starvation condition and initiated the developmental programme accordingly.

In contrast, PhdB$^-$ cells did not initiate aggregation till 8h and when streaming began the cells formed weak large territories without exhibiting appreciable branching. As aggregation progressed, the large territories broke into several smaller regions and formed weak small aggregation centres, very often depositing clumps of cells along the way (Figure 8).

It appears that PhdB$^-$ cells are delayed in sensing the starvation under submerged conditions, once sensed the development programme is switched on. The streaming efficiency, which is a characteristic feature exhibited by *Dictyostelium* during aggregation (chemotaxis) is altered. On the solid surface aggregates formed by PhdB$^-$ cells were capable of developing into a multicellular fruiting body when transferred on to a solid surface. These observations suggest that PhdB$^-$ cells have an abnormal aggregation behaviour.

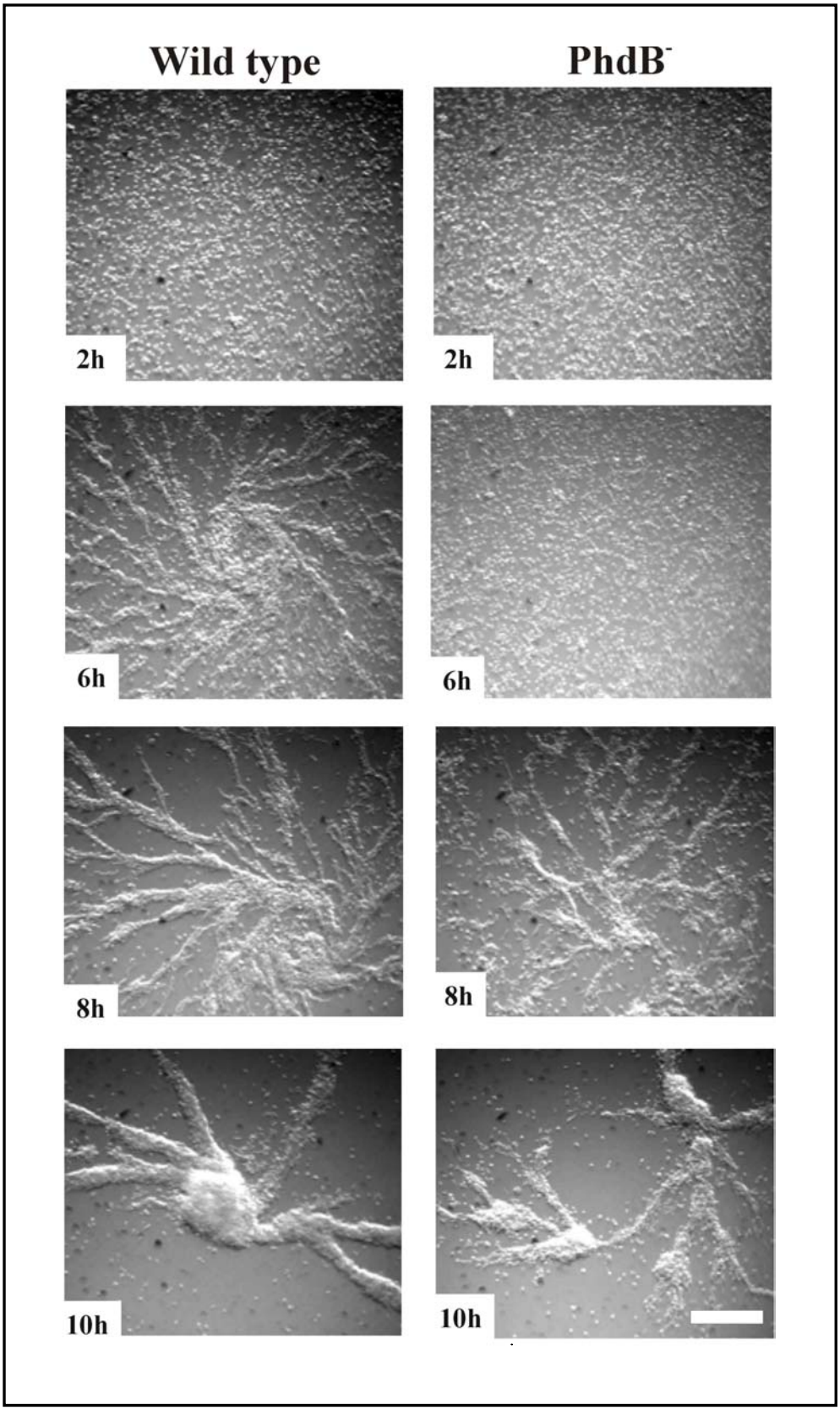


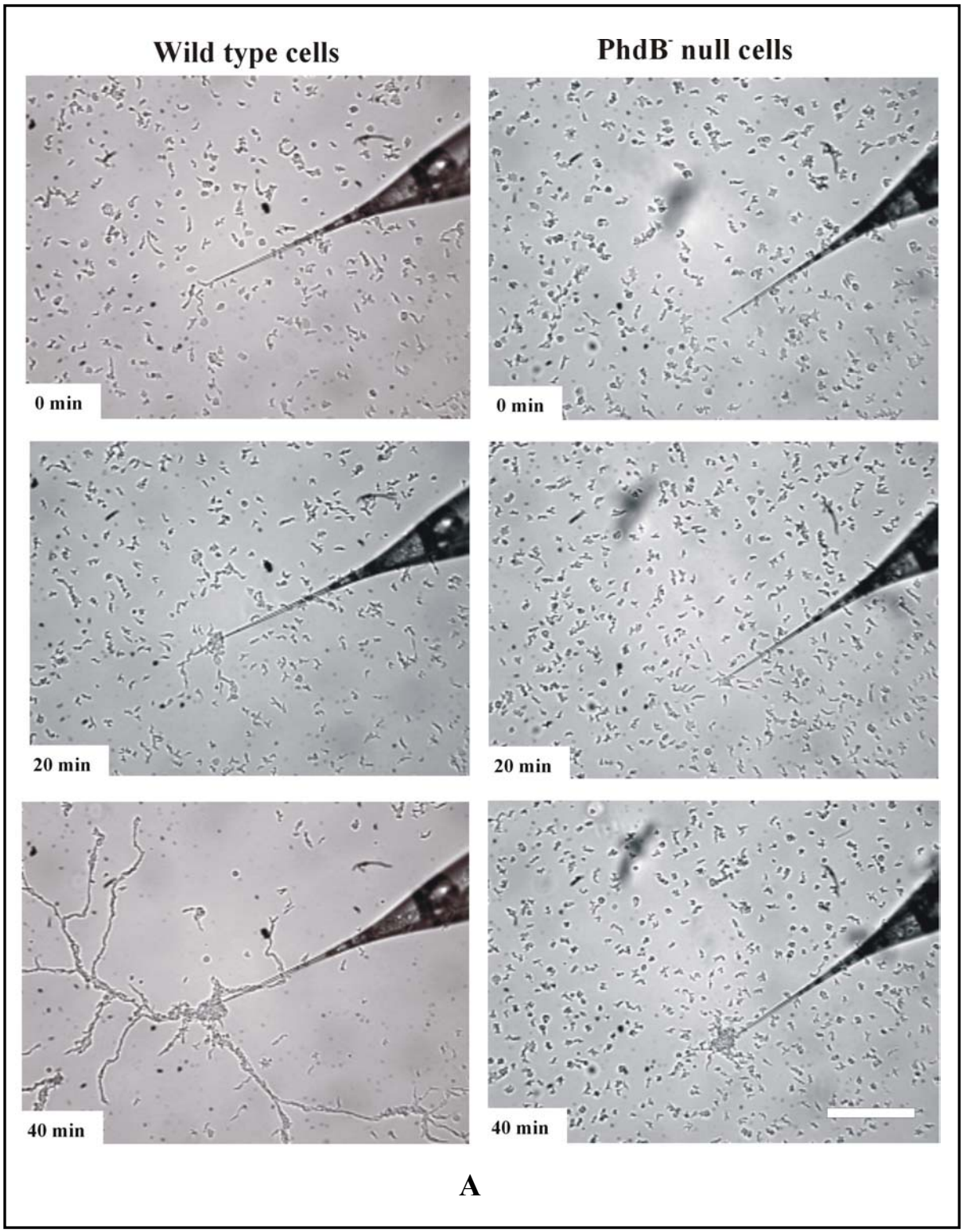
Figure 8. Aggregation analysis. *Dictyostelium* cultures were plated in monolayers with a density of $5x10^6$ cells/ml, submerged under starvation buffer in plastic petridishes. The cultures were incubated for development at 21°C and photographed every 2h. Wild-type AX2 cells form streams at 6h and the streams coalesce into strong aggregates within 13h. In contrast mutant cells show a delay of 2h in generating streams, the streams formed were of weak large territories which later coalesce into smaller aggregates. Bar, 300 μm.

3.1.8 PhdB$^-$ cells exhibit a defect in streaming during *Dictyostelium* chemotaxis

As PhdB$^-$ cells exhibited a defect in aggregation, which is a characteristic feature of *Dictyostelium* chemotaxis, we subsequently planned to analyse the process of chemotaxis.

For this purpose the movement of PhdB$^-$ cells towards a chemoattractant source was traced and compared with wild type cells. A micropipette filled with cAMP was the source of chemoattractant. When the wild type cells are challenged with the chemoattractant within seconds after activating the micropipette the polarised wild type cells rapidly sense the gradient and exhibit chemotaxis (Figure 9A).

As wild type cells chemotax towards a point source of cAMP, they eventually organise into streams. The streams are composed of cells migrating in a head to tail fashion. The streams come together and form a star like pattern. In contrast, PhdB$^-$ cells do not form streams when they move towards the cAMP source. Instead they migrated independently as single cells towards the cAMP source. This defect is observed even after a prolonged time in the chemoattractant gradient. Also, PhdB$^-$ cells do not recognise the gradient immediately, instead they show a short lag and then respond efficiently to move towards the tip of the micropipette. PhdB$^-$ aggregation competent cells (materials and method 2.2.7) at two different time points (t6 and t8) were used in the study, assuming that PhdB$^-$ cells may adopt to intra and extracellular signalling in later stage of aggregation. PhdB$^-$ cells even at the later time point did not form streams during chemotaxis (Figure 9B).


Wild type cells
PhdB⁻ null cells
0 min
0 min
20 min
20 min
40 min
40 min
A

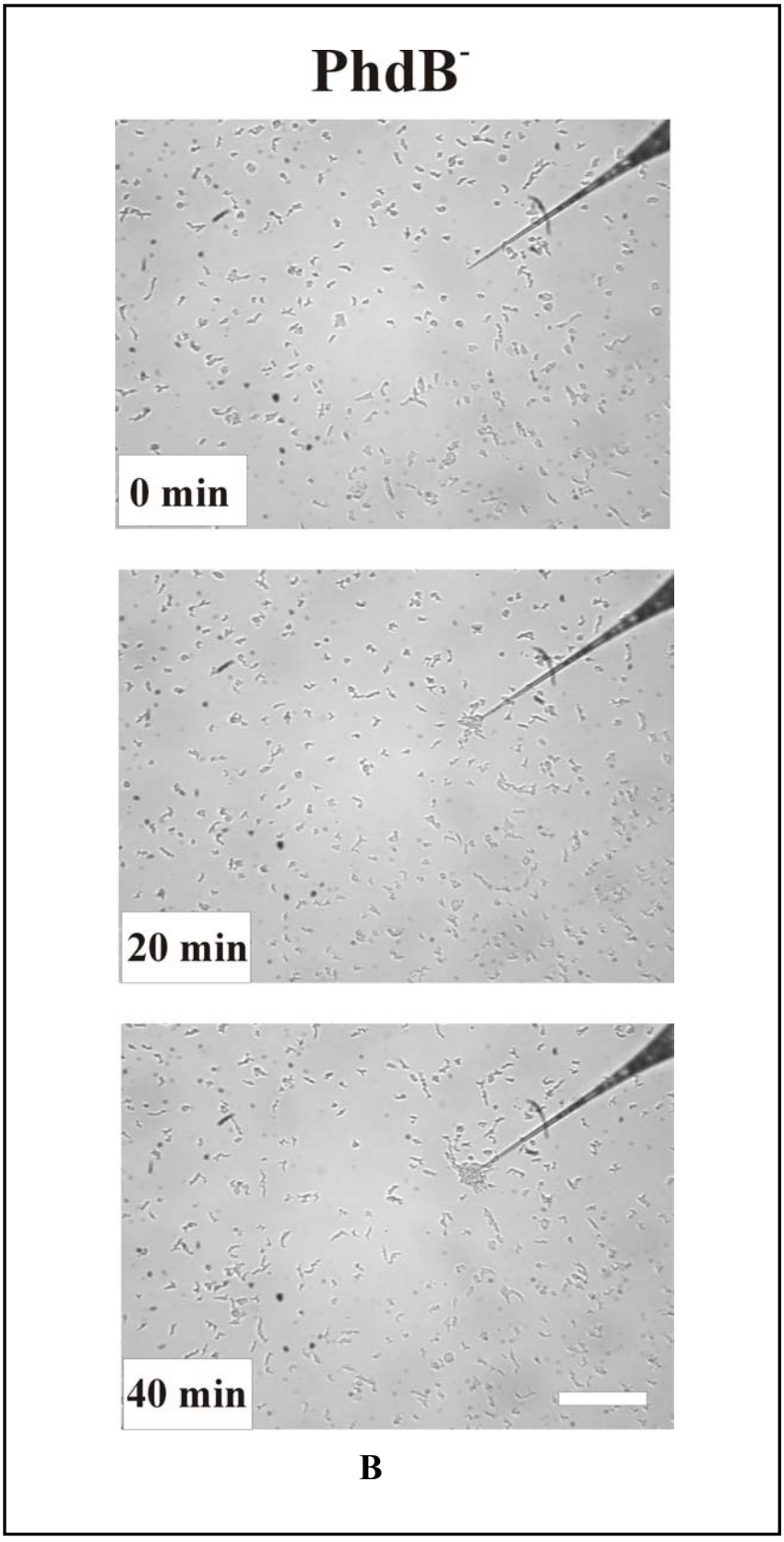


Figure 9. Chemotaxis analysis. A) Images of wild type and PhdB$^-$ cells taken as the cells move towards the cAMP filled micropipette. Aggregation competent cells (t6) were used to assay for chemotaxis. Bar, 400 μm. **B)** Images of PhdB$^-$ cells taken as the cells move towards the cAMP filled micropipette. Aggregation competent cells (t8) were used to assay for chemotaxis. Bar, 300 μm.

3.1.9 PhdB$^-$ cells leading edge is equally sensitive to cAMP as the one of wild type cells

As PhdB$^-$ cells exhibited a defect in the streaming process during chemotaxis, we analysed the sensing ability of PhdB$^-$ cells when they migrated directionally. Directional sensing refers to the ability of the cells to detect an asymmetric extracellular cue and generate an internal amplified response (Devreotes and Zigmond. 1988). When cells are exposed to gradients of chemoattractant concentration, signalling molecules accumulate at the membrane adjacent to the higher concentration and initiate downstream responses locally. A directional sensing response does not require the cell to be polarised. Unpolarised cells can also detect gradients with a similar degree of signal amplification (Van Es and Devreotes, 1999)

Polarisation defines the ability of the cell to assume an asymmetric shape with a defined anterior and posterior end. In polarised cells the anterior surface / leading edge is more sensitive to chemoattractants than other regions. When the direction of the chemoattractant gradient is changed, a polarised cell generally turns towards the new highest concentration and maintains its original anterior end. A very steep gradient in an opposite direction can sometimes override this asymmetry and generate a new axis in the new direction (Weiner *et al.,* 1999).

When we analysed this mechanism of chemotaxis, PhdB$^-$ cells were capable to extend pseudopods from their original migratory front and turn in response to abrupt changes in chemoattractant gradients like wild type cells (Figure 10). Therefore, PhdB$^-$ cells are able to maintain their leading edge sensitivity similar to that of wild type cells.

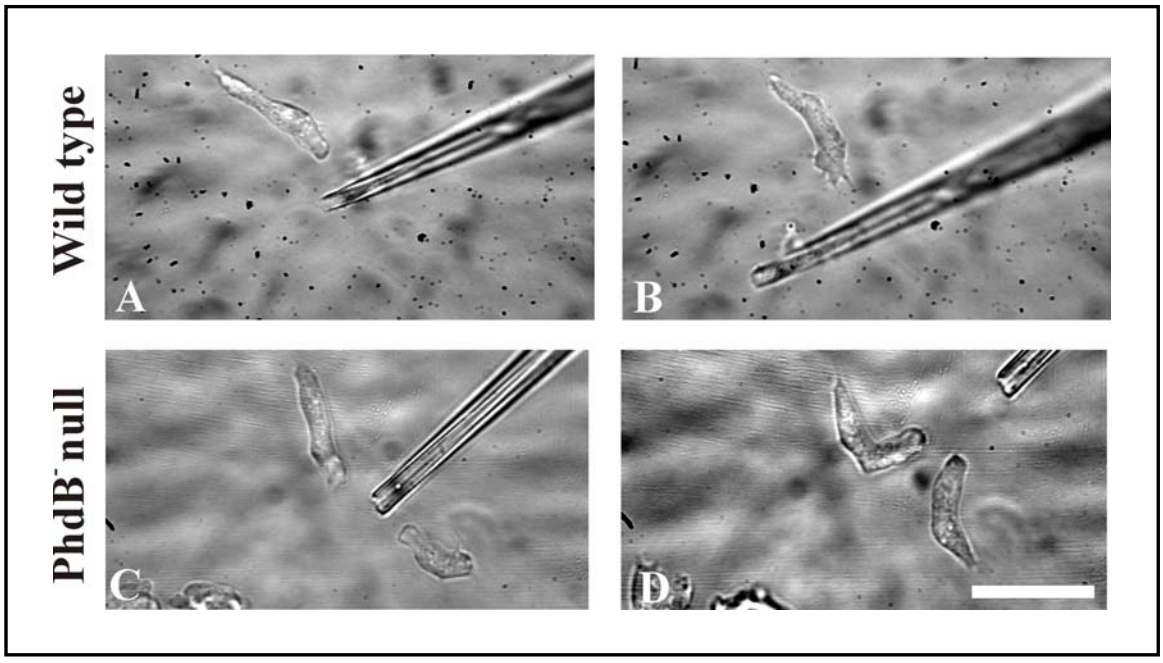


Figure 10. Response to random changes of the chemoattractant source.
Aggregation competent cells were overlaid with starvation buffer and the response to a random change in chemoattractant source was traced. A and B are images of wild type cells moving towards the tip releasing cAMP, the cell in frame B turns towards the tip when the tip position is changed, compared to frame A. C and D are images of PhdB$^-$ cells moving towards the tip releasing cAMP, the cells in frame D turn towards the tip when the tip position is changed as compared to frame C. Bar, 17 μm.

3.1.10 Analysis of cell motility

As PhdB$^-$ cells did not exhibit streams during the micropipette based chemotaxis assay, this defect led us to analyse their motility behaviour, hence their speed towards the cAMP releasing tip and maintenance of directionality and shape was analysed using DIAS (Dynamic Image Analysis System) (Soll, 1999). Wild type and PhdB$^-$ cells were starved for 6 h in Soerensen buffer and seeded on to a cover slip chamber and the rate of cell translocation was determined from time lapse video microscopic images using DIAS. PhdB$^-$ cells showed no difference in their speed during crawling towards the cAMP source when compared with wild type cells (Table 1). PhdB$^-$ cells moved with an average speed of 6.9 μm / min (+/- 2.5) towards the pipette which is similar to the one of wild type cells 6.4 μm / min (+/- 2.2) (Noegel *et al.,* 2004).

Also they maintained the directionality (persistence), when they moved towards the chemoattractant source. The shape, which describes the polarity of the cells, was similar (50%) in both mutant and wild type cells, which indicates that 50% of cells were polarised and moved at a given time. The shape was recorded by manually drawing the out line of the cell.

	PhdB$^-$	**Wild type**
Speed	6.9 μm / min (+/-2.5)	6.4 μm / min (+/-2.2)
Persistence	2.6 μm / min-deg (+/-1.3)	2.2 μm / min-deg (+/-1.3)
Roundness	50.9 % (+/- 7.5)	46.2 % (+/- 4.0)

Table 1. Analysis of cell motility. Time lapse image series were captured on a computer hard drive at 30 s intervals. The DIAS software was used to trace individual cells along the image series and calculate the motility parameters. Persistence is an estimation of movement in the direction of the path. Values are mean ± standard deviation of 30 AX2 and 30 PhdB$^-$ cells from at least three independent experiments. Both strains did not differ statistically in their motility behaviour.

3.1.11 PhdB$^-$ cells exhibit a defect in chemoattractant mediated actin polymerisation

Dictyostelium offers the unique opportunity to follow the changes in actin polymerisation upon exposure to a chemotactic stimulus. Characteristically, about 5 sec after stimulation a first peak of actin polymerisation takes place and a 1.6 to 2 fold increase in the amount of F-actin is observed. The F-actin is then depolymerized and after 15 to 20 sec a second long lasting peak of F-actin accumulation occurs which parallels the extension of the pseudopods. PhdB$^-$ cells showed a lower increase in the F-actin peak both at 5 seconds and 30 seconds after cAMP stimulation relative to AX2 in aggregation competent (t6) cells (Figure 11).

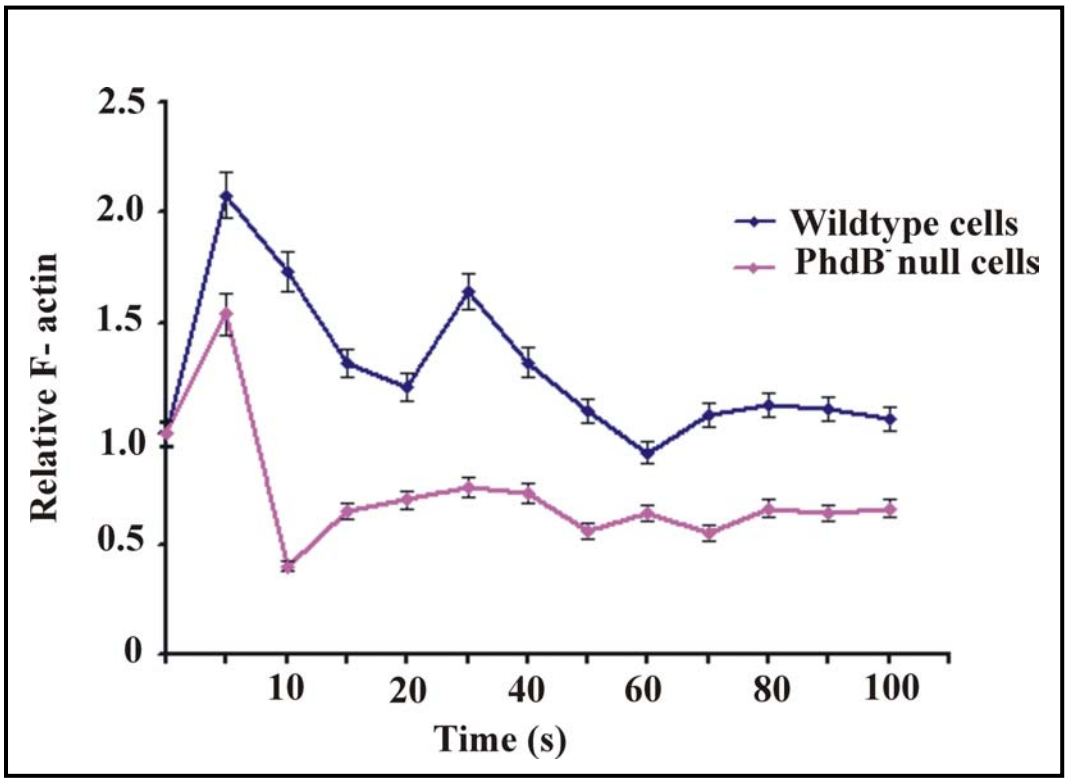


Figure 11. Actin polymerisation response to cAMP stimulation. The relative F-actin content was determined by TRITC-phalloidin staining of t6 cells fixed at the indicated time points after stimulation with 1µM cAMP. All values are the average ± standard deviaton of at least three independent experiments.

3.1.12 PhdB$^-$ cells express normal levels of csA

The driving force behind *Dictyostelium* aggregation is chemotaxis. In the beginning amoeba move as individual cells towards the cAMP signal. However when they are near the source, cells coalesce into multicellular streams. These streams move co-ordinatedly towards the signalling centre to form a mass of up 10^5 cells.

It has long been known that cells within streams adhere to each other and several proteins that mediate cell-cell adhesion were identified (Bozzaro and Ponte, 1995). Since PhdB$^-$ cells exhibit aberrant aggregation we were interested to analyse the expression pattern of contact site A (csA) during aggregation in PhdB$^-$ cells (Figure 12). Contact site A (also known as gp80) is one of the adhesion molecules expressed during the later stage of aggregation in

Dictyostelium. Contact site A has a mass of 54 kDa and its expression is induced by the cAMP pulses that mediate chemotaxis (Faix *et al.,* 1992). It is a globular protein of the immunoglobulin super family; it is heavily glycosylated, and hence it has an apparent molecular weight of 80 kDa on SDS-PA gels. Immunoblot analysis of csA in PhdB$^-$ cells showed no difference in the expression pattern during aggregation when compared to wild type cells. As can be seen in the figure 12A and B, csA is expressed only during later stages of aggregation as the cells progress to form strong aggregates. Individual cells in the initial stages of starvation express very low levels of csA.

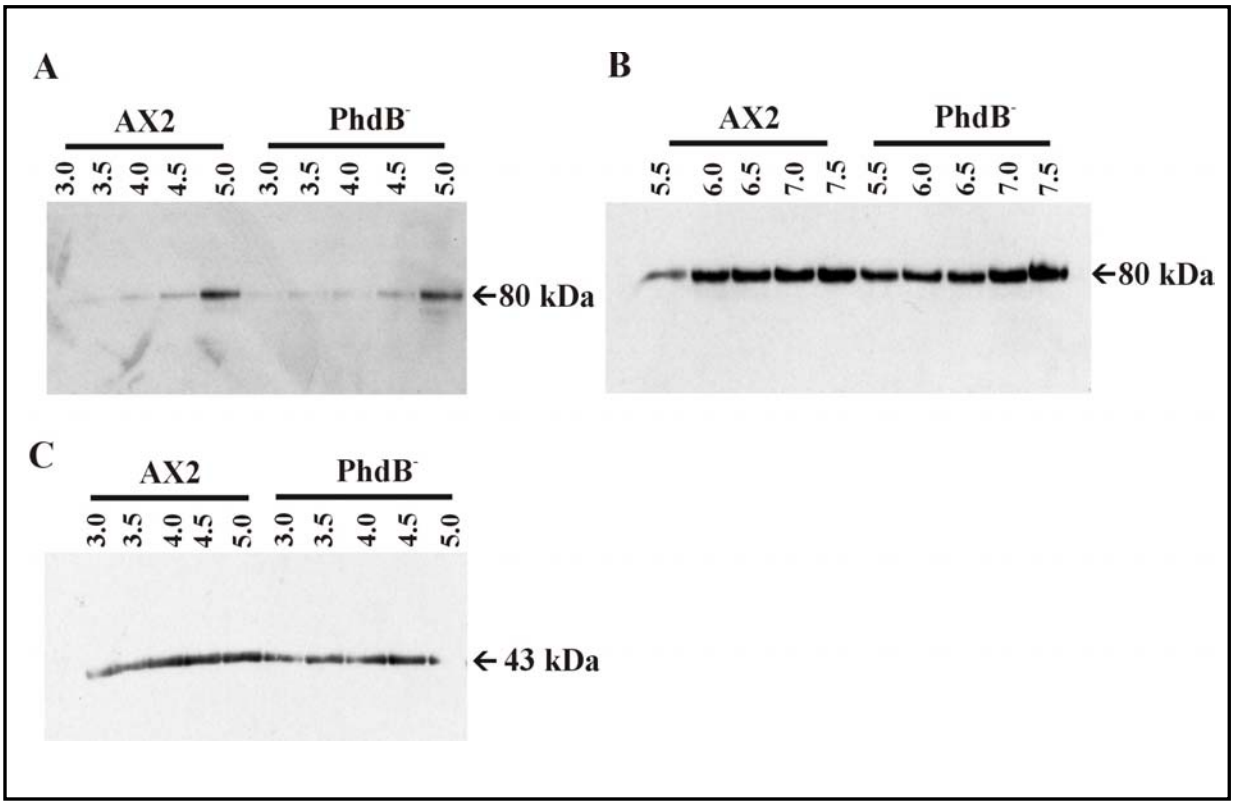


Figure 12. Western blot analysis of csA protein. A, B. Wild type and PhdB$^-$ cells growing under starvation condition at different time points were harvested and lysed with 1x sample buffer. The cell lysates were separated by 10% SDS-PA gels and the protein samples were immunoblotted. The level of csA expression in both wild type and PhdB$^-$ samples was identified by probing with mAb 33-294. The antibody recognises specifically csA at 80 kDa.
C. Internal control for equal loading of protein samples. The level of actin was identified by probing the blot (representing figure 12A) with mAb act1, which specifically recognises actin at 43 kDa.

As any laboratory condition under which aggregation is studied does not represent the exact stringent conditions experienced by *Dictyostelium* in their natural habitat (soil), we further analysed the expression of csA during aggregation under different physical conditions.

We analysed the expression of csA during aggregation of cells in liquid suspension in the shaking culture. Vegetative cells grown to a density of $3x10^6$ cells/ml were harvested and washed twice with ice cold Soerensen buffer. The cells were again resuspended in Soerensen buffer at a density of $1x10^7$ cells/ml and incubated in shaking culture. At different time points during aggregation samples were harvested and analysed for csA expression by western blot analysis in both AX2 and PhdB$^-$ cells. The expression of csA protein in PhdB$^-$ cells during aggregation in shaking culture was very much similar to that of wild type cells (Figure 13A).

As the agar plate may represent a solid substratum as it exists in the natural habitat during *Dictyosteium* development, we analysed the expression of csA during aggregation of cells on agar plates (solid substratum). The expression of csA during aggregation on agar plates was examined at both protein and mRNA level. Cells at different stages of development were collected for RNA and protein analysis. The expression of csA protein in PhdB$^-$ cells during aggregation on agar plate was similar to that of wild type cells (Figure 13B). The cDNA probe hybridised with the csA mRNA of ~ 1.9 kb in northern blot analysis. The pattern of csA mRNA expression in PhdB$^-$ cells was similar to wildtype cells (Figure 13C).

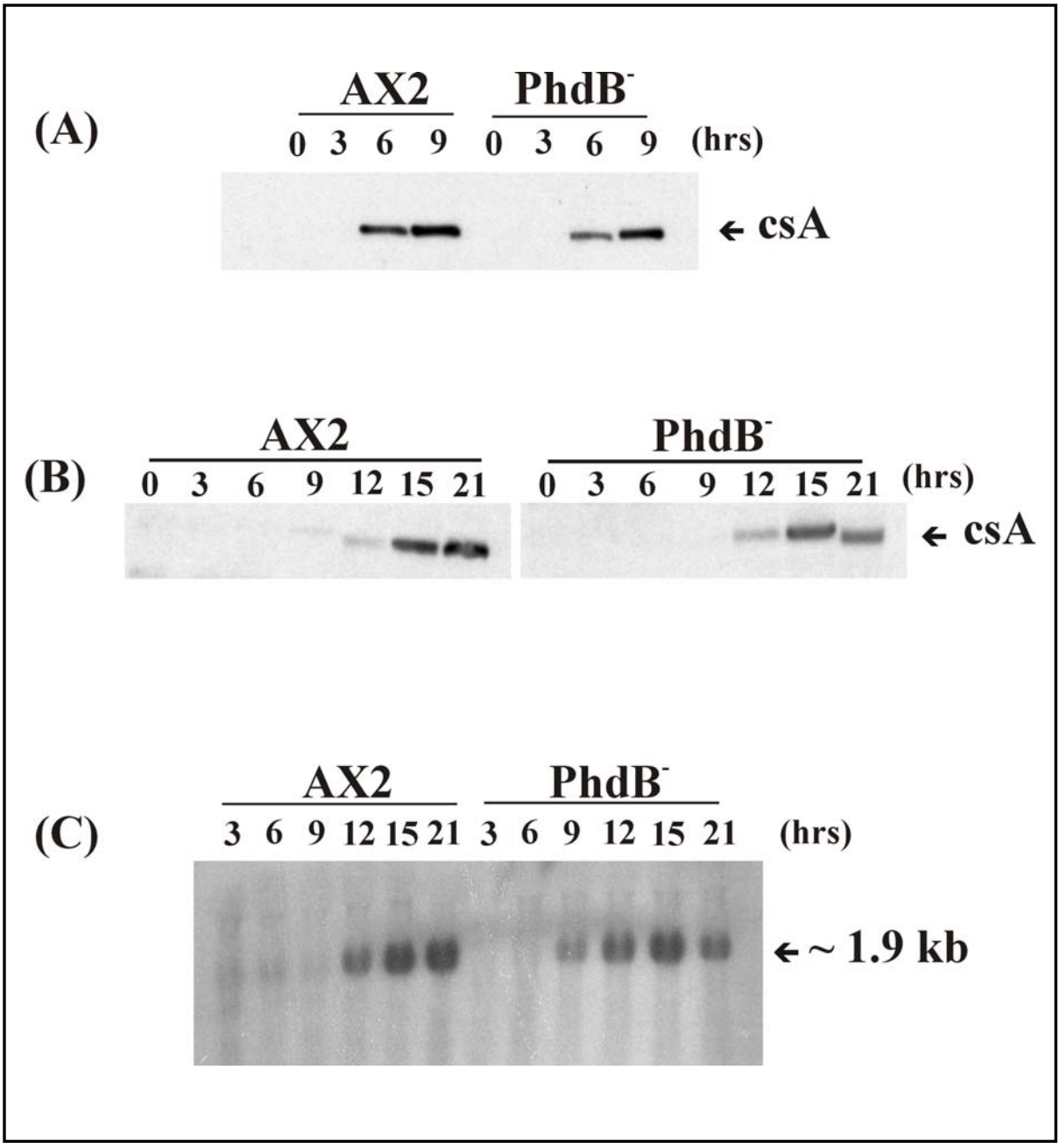


Figure 13. Expression of csA at different aggregation condition: A) Western blot analysis of csA. Both AX2 and PhdB$^-$ cells were starved in Soerensen buffer and allowed to develop in shaking culture. At different time points cells were harvested and lysed in 1x sample buffer for protein analysis. Samples extracted from 0, 3, 6, 9 hours of development was analysed by 10% SDS-PA gel. Proteins were transferred on to a nitrocellulose membrane and the protein detected with anti csA monoclonal antibody (mAb 33-294).
B) Western blot analysis csA. Both AX2 and PhdB$^-$ cells were starved and allowed to develop on phosphate agar plates. Samples extracted from 0, 3, 6, 9, 12, 15, 21 hours of development was analysed by 10% SDS-PA gel. Proteins were transferred on to a nitrocellulose membrane and the protein detected with anti csA monoclonal antibody (mAb 33-294). **C) Northern blot analysis of csA.** Both AX2 and PhdB$^-$ cells were allowed to develop on phosphate agar plates. At different time points cells were harvested for extraction of RNA samples. Samples of RNA extraction derived from the cells harvested at 0, 3, 6, 9, 12, 15, 21 hours were seperated on agarose formaldehyde gel, transferred to a nylon membrane and hybridized with a ^{32}P labelled csA cDNA probe.

Even though PhdB$^-$ cells exhibit a delay in aggregation, these cells expressed normal levels of csA at different laboratory conditions. As PhdB$^-$ cells formed small and weak aggregates which may represent a poor cell-cell contact, we were interested in determining if cell-cell contact plays a role in csA expression in PhdB$^-$ cells. Before csA is expressed in developing cells, EDTA sensitive binding sites mediate cell-cell adhesion. To accesses the role of cell-cell contact in regulating csA expression EDTA (10 mM) was added to cell cultures during different time points of development in order to block EDTA sensitive adhesion. Cell adhesion was determined after treating cells at different time points during their development with EDTA for 1h. In the initial stage EDTA when added appeared to regulate the expression of csA and in the later stages of development EDTA had no effect on csA expression in both AX2 and PhdB$^-$ cells.

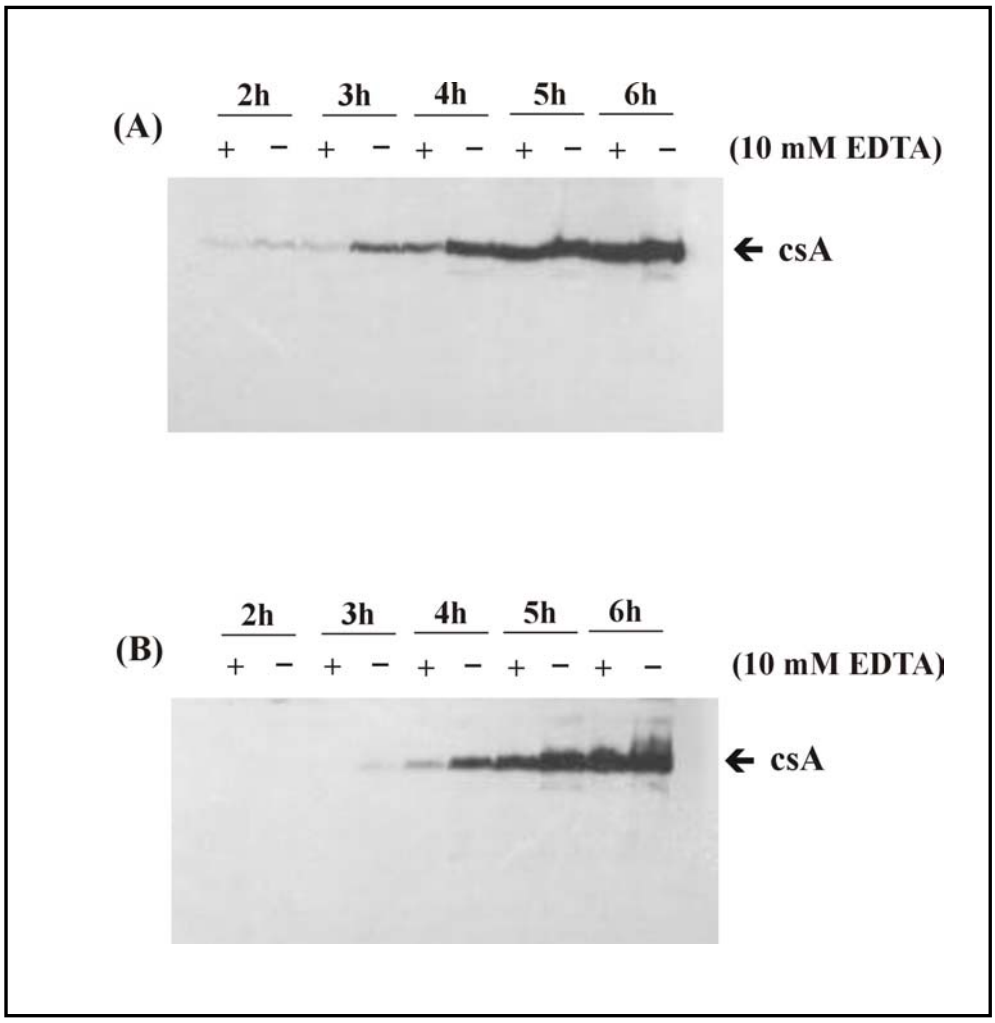


Figure 14. Effect of EDTA on csA expression during different time points of development. AX2 and PhdB$^-$ cells were harvested and washed twice with Soerensen buffer and resuspended at 1x10^7 cells/ml in Soerensen buffer. Development was carried out in liquid culture. Cell cultures were rotated at 160 rpm on a platform shaker at 21°C. **A and B**

represents AX2 and PhdB$^-$ cells respectively, at different time points of development 2, 3, 4, 5, 6 h, at which 10 mM of EDTA (final concentration) was added and incubated for additional hour and the cells were harvested. The protein samples were extracted from the harvested cells by lysing them in 1x sample buffer and analysed by 10 % SDS-PA gel. Proteins were transferred to a nitrocellulose membrane and the protein detected with anti csA monoclonal antibody (mAb 33-294).

We also looked into the cell association during the aggregation of cells in presence and absence of EDTA in both AX2 and PhdB$^-$ cells. During aggregation in the presence and absence of EDTA at different time points, the aggregates formed was quantitated by scoring the absorbance at 540 nm. The absorbance measured is inversely proportional to the aggregates formed.

Vegetative cells grown at a density of $3x10^6$ cells / ml was harvested and washed twice with Soerensen buffer and resuspended again in Soerensen buffer at a density of $1x10^7$ cells / ml. The cells were allowed to aggregate on a platform shaker rotating at 160 rpm at 21°C.

In the absence of EDTA, AX2 cells aggregation was efficient and reached a maximum by 6 h whereas PhdB$^-$ cells did not exhibit efficient aggregation (Figure 15A). This observation is also in accordance with the defect in aggregation, which we observed earlier when cells were allowed to aggregate on petridish in submerged cultures and on agar plates.

In the presence of EDTA, AX2 cells are able to form stronger and EDTA resistant aggregates by 6 h, also PhdB$^-$ cells formed weak and small aggregates but not as efficiently as AX2 cells, however PhdB$^-$ cells exhibit a similar process of aggregation even in the absence of EDTA when compared to AX2 cells (Figure 15B).

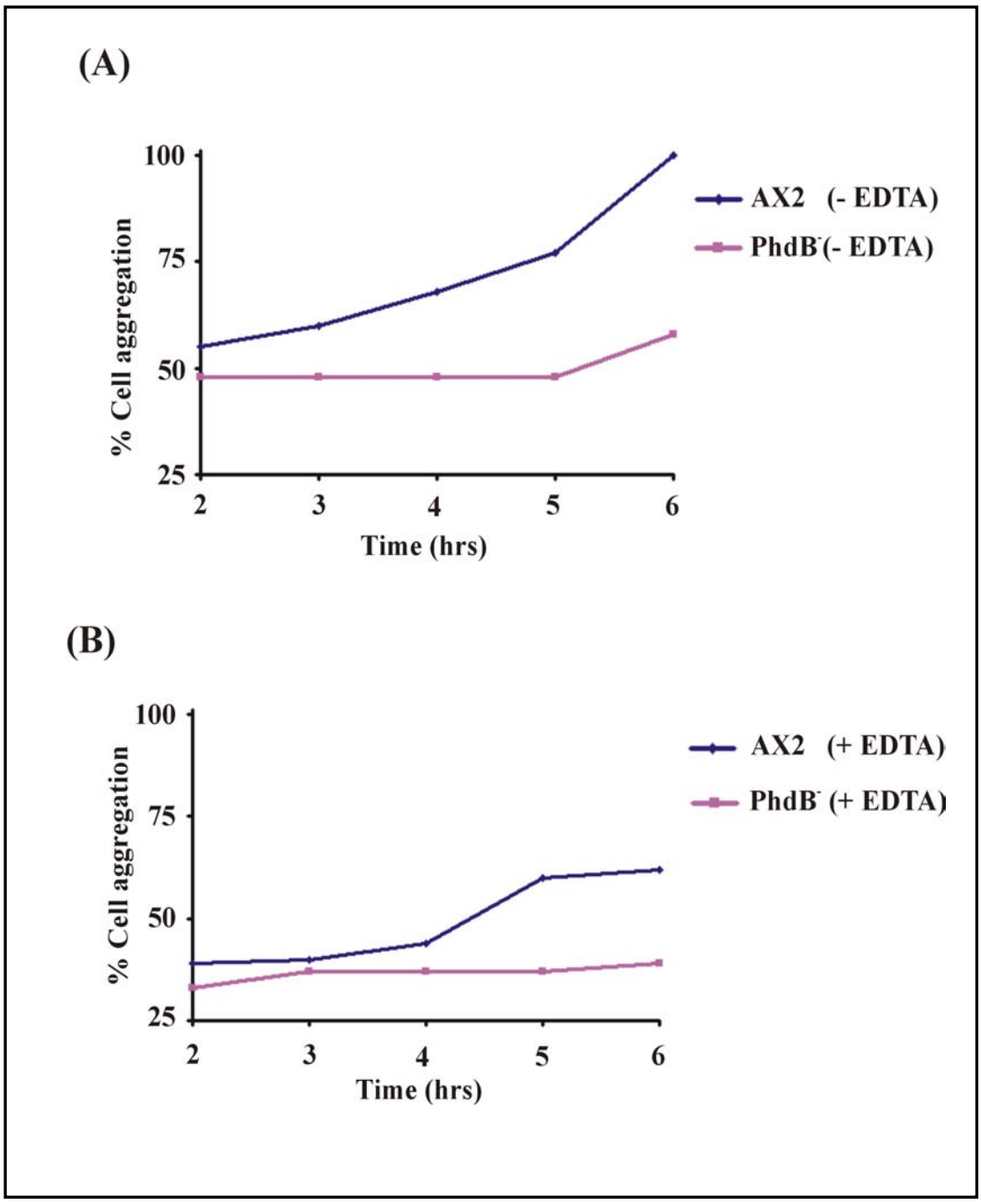


Figure 15. A) Aggregates formed by both AX2 and PhdB$^-$ cells in the absence of EDTA. In the absence of EDTA, AX2 cells formed strong compact aggregates by 6 h, where as PhdB$^-$ cells did not form efficient aggregates. Both AX2 and PhdB$^-$ cells were allowed to aggregate in Soerensen buffer. Samples at different time points (2. 3. 4. 5. 6 h) during aggregation was harvested and the aggregates formed was scored by measuring the absorbance of the harvested culture at 540 nm.
B) Inhibition of EDTA resistant cell adhesion by EDTA (10 mM). AX2 and PhdB$^-$ cells were allowed to aggregate in Soerensen buffer on a platform shaker rotating at 160rpm at 21°C. During different time points (2, 3, 4, 5, 6 h) of aggregation, EDTA (10 mM final concentration) was added and further cells were allowed to aggregate in the presence of EDTA for another hour. The effect of EDTA on cell aggregates formed was scored by measuring the absorbance at 540 nm.

3.1.13 PhdB$^-$ cells exhibit normal growth in axenic medium

Cell growth is the result of a interplay between varieties of cellular processes involving rearrangements of the actin cytoskeleton. The growth rates of PhdB$^-$ were compared with that of wild type cells. The growth patterns of AX2 and mutants were investigated at 160 rpm at 21°C with starting cell densities of 1 x 10^6 cells/ml. No significant difference was observed in the growth patterns of wild-type AX2 and the mutant strain (Figure 14). AX2 cells attained maximum cell densities of 8.0 x 10^6 cells/ml. While the PhdB$^-$ mutant attained maximum cell densities of 9.0 x 10^6 cells/ml. AX2 cells and mutant cells grow with the same generation (doubling) time of 11 h to 13 h in the cultures. This suggests that PhdB is not essential for growth under optimal conditions.

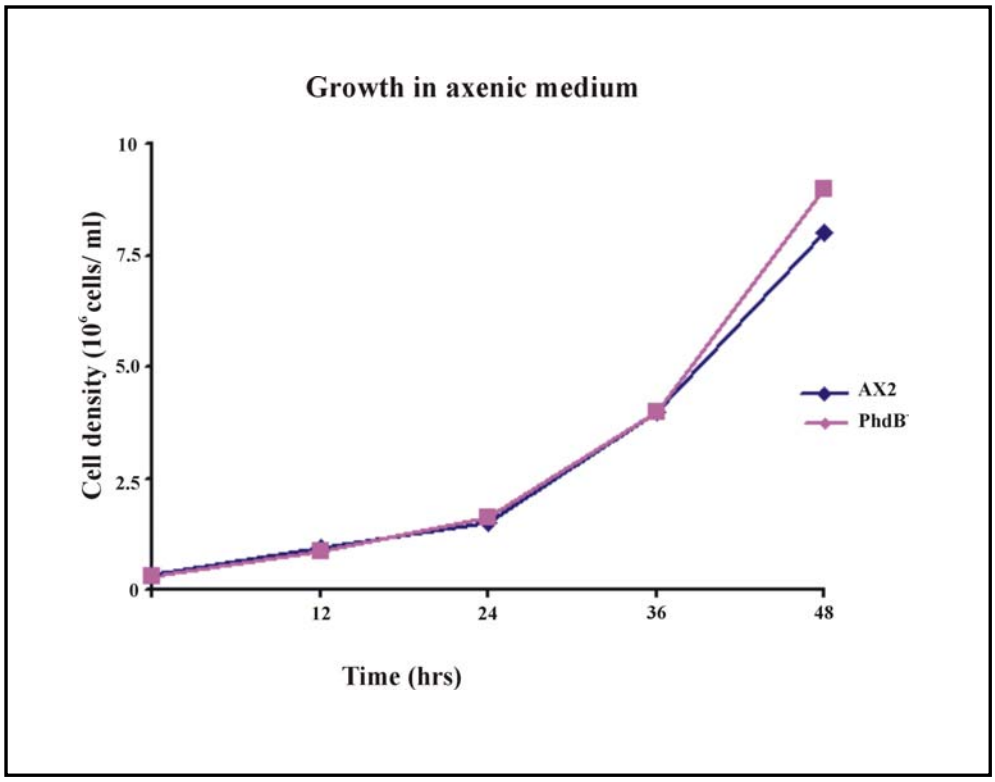


Figure 14. Growth in axenic medium. Wild type and PhdB$^-$ cells were cultured (inoculated at 1x10^6 cells/ ml) and grown at 21ºC with shaking at 160 rpm. The cells were counted at the indicated time points. Growth of PhdB$^-$ cells was normal and comparable to wild type cells. The data plotted here are from a single experiment, but similar results were obtained from three independent experiments.

3.1.14 PhdB$^-$ cells growth on agar plates with bacteria as food source

Dictyostelium cells develop when grown on agar along with bacteria, once the bacteria have been ingested. We also tested growth of AX2 and PhdB$^-$ cells on SM agar plates in the presence of *Klebsiella aerogenes* as their food source. The cells were inoculated in the centre of SM agar plates and overlaid with *Klebsiella aerogenes* (materials and methods 2.2.5). The increase in the colony diameter was taken as a relative measure of growth. The colonies of PhdB$^-$ cells have growth rates that correlate with the measured rates for AX2 cells (Figure 16). PhdB$^-$ cells, though exhibit a slightly decreased growth rate.

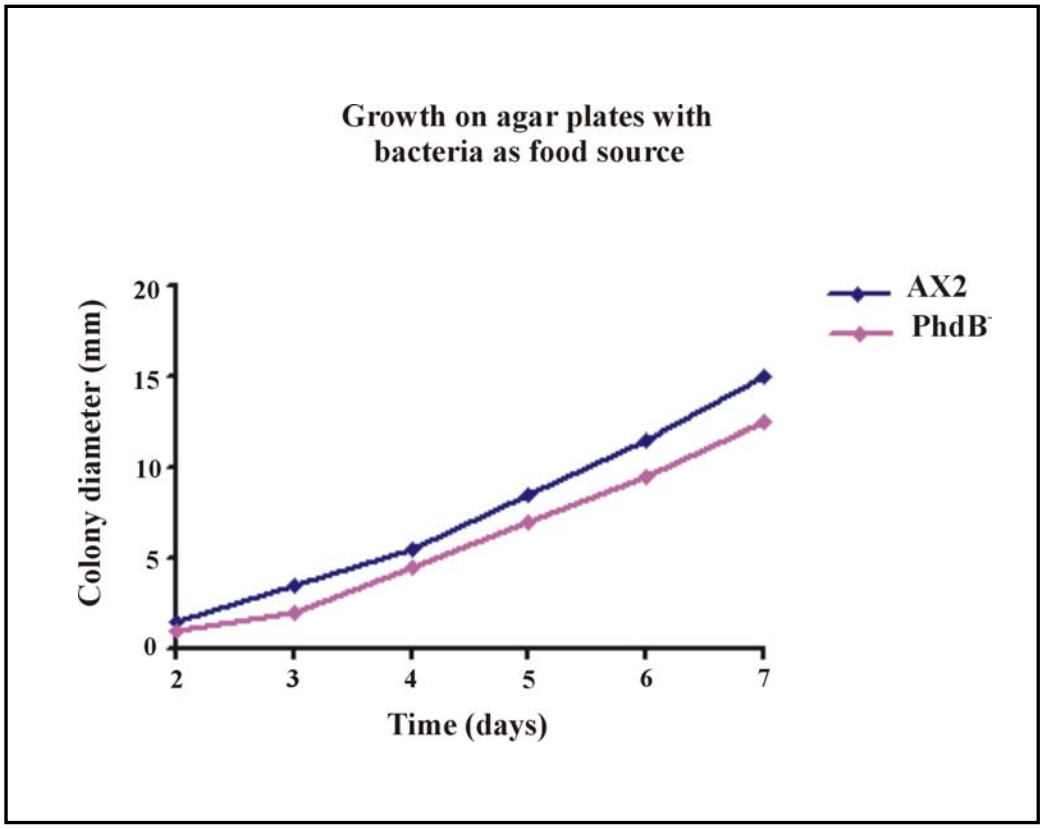


Figure 16. Growth on SM agar plates with *Klebsiella aerogenes.* Wild type and PhdB$^-$ cells (5000 in number) were spotted on two different SM agar plates and overlayed with *K. aerogenes*. Increase in colony diameter was recorded as a measure of growth rate. The growth rate of PhdB$^-$ cells is slightly reduced. The curves shown are representative of three independent experiments.

3.1.15 PhdB$^-$ cells exhibit normal pinocytosis

In axenic culture, *Dictyostelium* cells derive their nutrients solely by means of engulfing media via pinocytosis (Maniak *et al.,* 2001). To measure the kinetics of this process, which is constitutive in axenic strains of *Dictyostelium*, we used FITC-dextran, which has previously been shown to be an appropriate fluid phase pinocytic marker in *Dictyostelium* (Hacker *et al.,* 1997). The results obtained for AX2 and PhdB$^-$ cells show the accumulation of FITC-dextran in an essentially linear fashion over the first ~ 60 min. The kinetics (Figure 17) of accumulation of FITC-dextran for both AX2 and PhdB$^-$ cells exhibit no major difference in the fluid phase uptake.

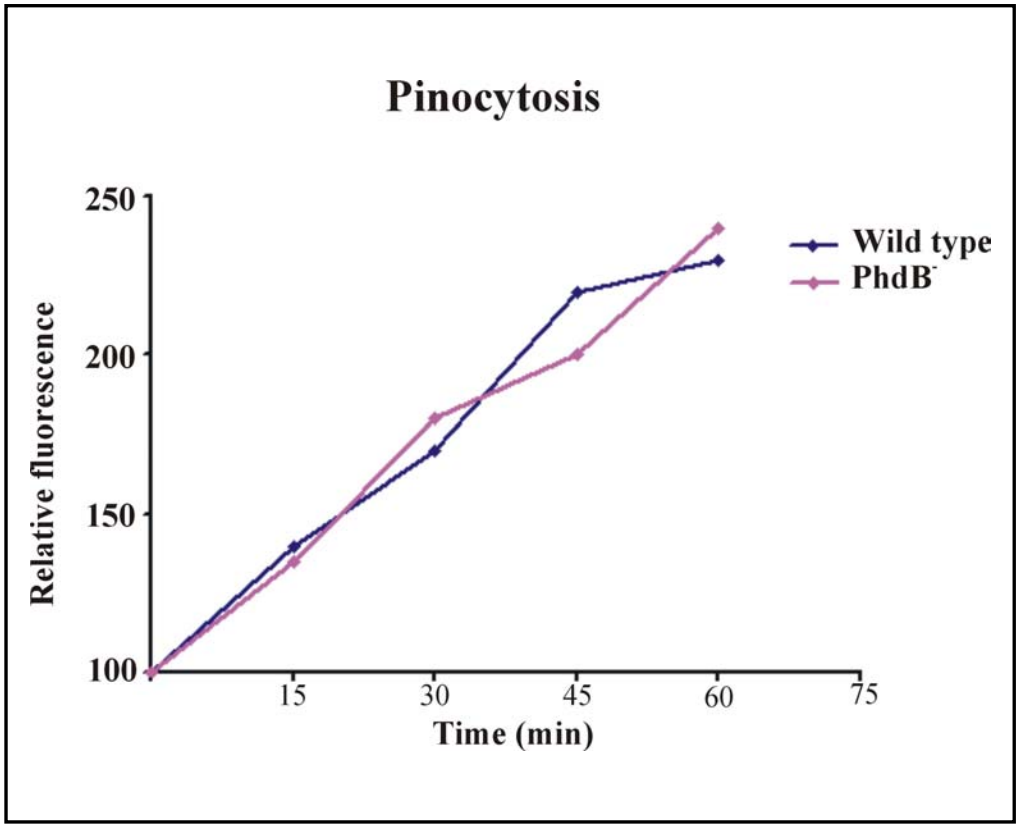


Figure 17. Pinocytosis of AX2 and PhdB$^-$ cells. Cells were resuspended in fresh axenic medium at 5x10^6 cells/ml in the presence of 2 mg/ml FITC-dextran. Fluorescence from the internalised marker was measured at selected time points.

3.1.16 PhdB$^-$ cells exhibit normal phagocytosis

Phagocytosis is an actin dependent process primarily performed by cells like neutrophils and macrophages. *Dictyostelium* is a professional phagocyte (Cardelli *et al.,* 2001) which can internalise bacteria as a source of food during growth. As we previously observed a slightly reduced rate in growth of PhdB$^-$ cells when allowed to grow in conjunction with bacteria on SM agar plates, we were interested to analyse the growth of PhdB$^-$ cells given bacteria as food source in liquid phase. The rate of phagocytosis was scored by an increase in cell number of *Dictyostelium* as they grow by feeding on bacteria in liquid culture.

When *Dictyostelium* ($1x10^6$ cells) grows on *E. coli* ($3x10^9$) in shaking suspension, the cell density of *Dictyostelium* doubles every 3 h. We observed that the doubling time of PhdB$^-$ cells was very much similar to the one of AX2 cells (Figure 18). This indicates that PhdB$^-$ cells perform phagocytosis normally in liquid culture unlike on solid substratum.

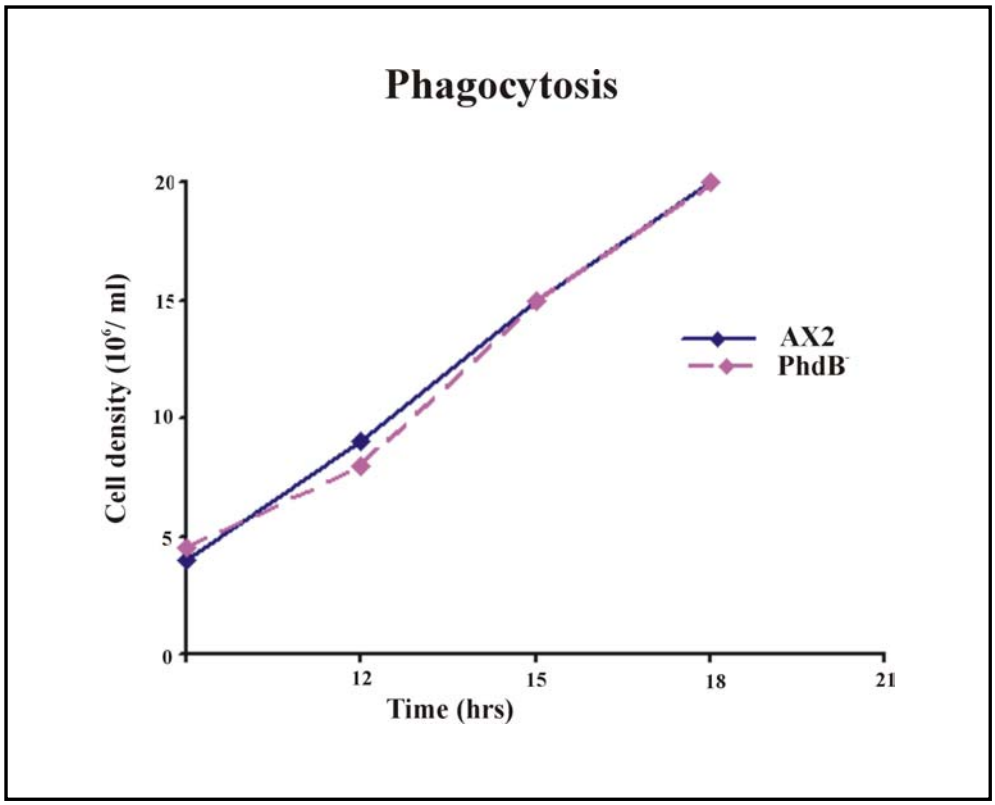


Figure 18 Phagocytosis of AX2 and PhdB$^-$ cells. *Dictyostelium* cells ($1x10^6$ cells) were cultured in conjunction with *E. coli* ($3x10^9$ cells / ml) over a period of time. The *Dictyoselium* cell density doubled every 3 h. Counting *Dictyostelium* cells at the indicated time points scored growth of wild type and PhdB$^-$ cells. The doubling time of PhdB$^-$ cells were normal and comparable to the one of AX2. The data plotted here are from a single experiment, but similar results were obtained from three independent experiments.

3.1.17 Developmental analysis

Upon starvation *Dictyostelium* cells undergo a developmental cycle in which single amoeba aggregate to from a multicellular fruiting body. This involves differentiation of *Dictyostelium* cells into spore-cells and stalk-cells and requires the sequential expression of developmentally regulated genes. Therefore we investigated the consequence of the absence of PhdB.

The cells can aggregate in starvation buffer under submerged conditions, while post-aggregation development and fruiting require a solid substratum and the commonly used substratum to study the development is agar. Therefore the developmental pattern of mutant strains was also assessed on an agar surface. Both in AX2 and PhdB$^-$ cells development of multicellular fruiting body was not disturbed. Nevertheless, there was a delay of 3-4 h in the development of the mutant to attain a fully developed fruiting body with spore heads (Figure 19). As observed in submerged condition, the delay in aggregation is also carried on to the developmental stage on phosphate agar plates. At 24 h PhdB$^-$ cells show no spore heads (only culminants can be seen) when compared to AX2 cells, which has completely formed fruiting body with spore heads. Later at 28 h PhdB$^-$ cells do completely develop into fruiting body with mature spore heads.

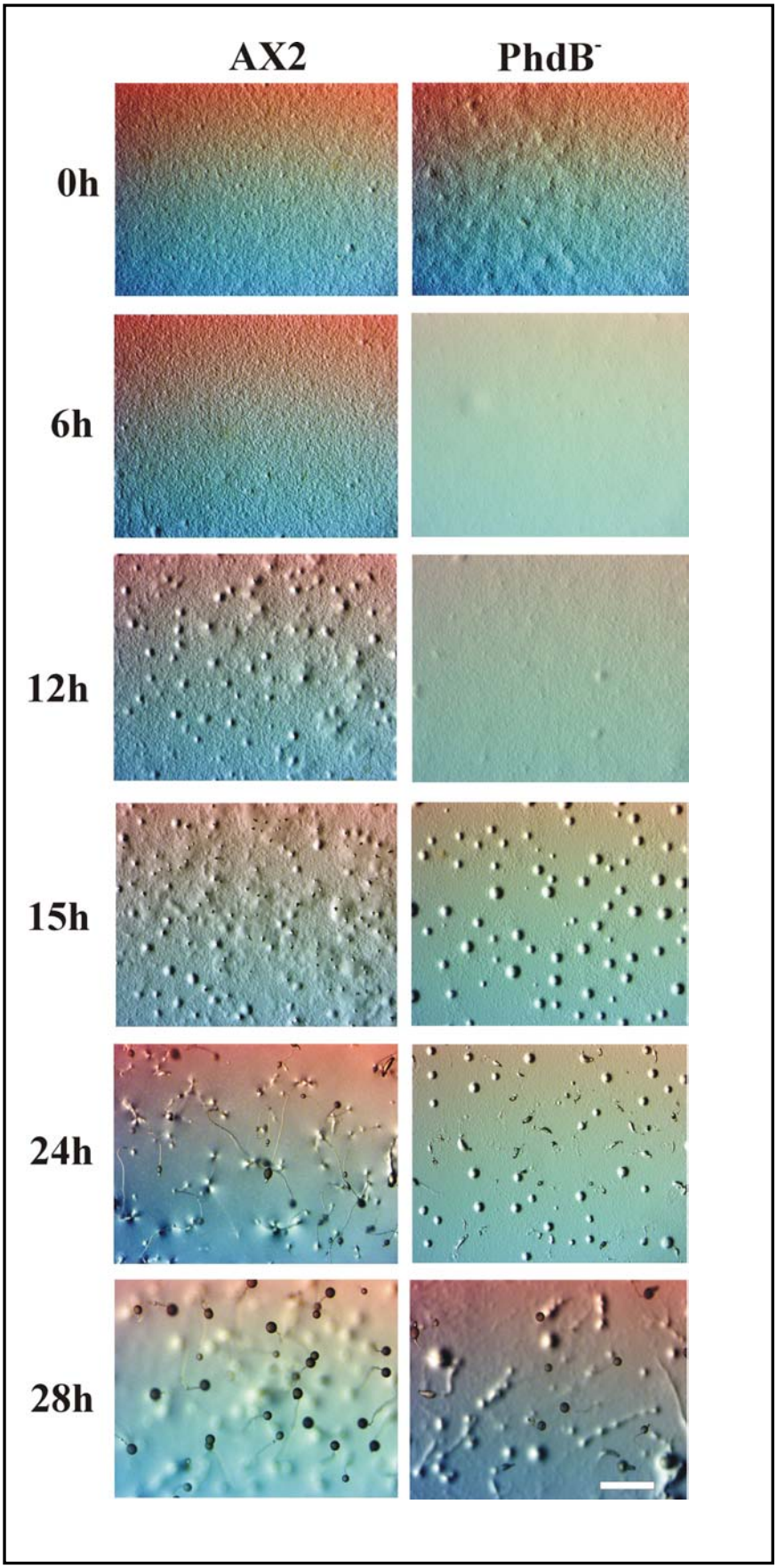


Figure.19 Development analysis. Wild type and PhdB$^-$ cells ($5x10^6$cells/ml) were resuspended in Soerensen buffer and plated on phosphate agar plates. During different time

points of development, images were acquired. Wild type and PhdB$^-$ cells exhibited normal development. AX2 cells developed by 24h, PhdB$^-$ cell were delayed by 3-4h. Bar, 1 mm.

3.1.18 Localisation of actin, CAP and filamin in PhdB$^-$ cells

PhdB$^-$ cells showed a defect in aggregation and streaming chemotaxis. Both processes are regulated by actin dynamics leading us to investigate the distribution of several F-actin regulating proteins like filamin, cyclase associated protein (CAP) and actin itself. Immunofluorescence analysis was performed and there was no significant difference in the localisation of these proteins (Figure 20). Both AX2 and PhdB$^-$ cells showed smooth cortical staining of actin with enrichment at membrane protrusions. CAP was distributed between plasma membrane and cytoplasm. Filamin also stained actin rich protrusions.

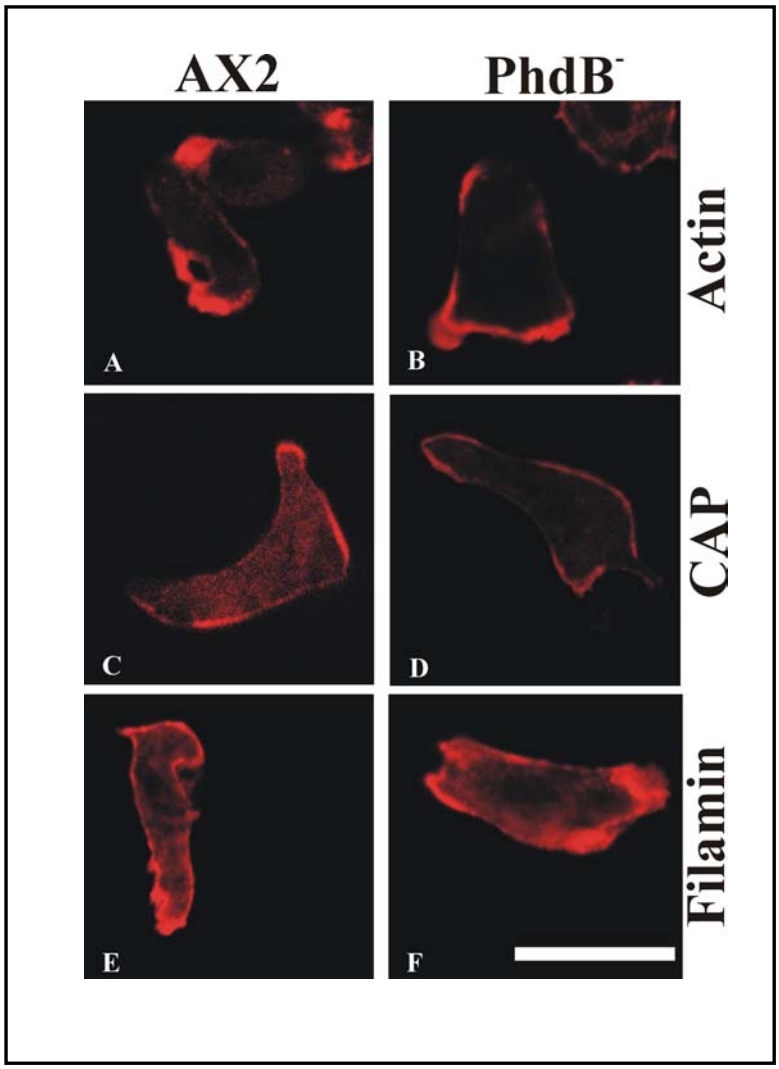


Figure 20. Localisation of F-actin regulating proteins. Aggregation competent cells were grown on cover slips and fixed with cold methanol. Monoclonal antibodies were used to stain actin (mAb act1), CAP (mAb 231-18) and filamin (mAb 82-382-8). A and B are cells stained for actin. C and D for CAP, E and F was filamin stained. The binding of the primary

antibodies was detected using a Cy3-labelled secondary antibody. Cells were observed under a confocal laser scan microscopy. Bar, 16 μm.

3.1.19 PhdB is associated with the *Dictyostelium* Triton X-100 insoluble cytoskeleton

Presence of functional domains like CH (actin binding) domain, Rho GEF, Arf GAP and two PH domains in PhdB suggested a possible function of PhdB in the actin cytoskeleton. To analyse whether PhdB is associated with actin cytoskeleton complex, the protein interaction with Triton X-100 insoluble cytoskeleton was analysed. Aggregation competent cells were harvested and lysed with 1% Triton X-100. The lysate obtained was fractionated into supernatant and pellet by a low speed spin (Figure 21C). Western blot analysis of both the fractions showed the protein to be present in both pellet and supernatant. This observation suggests that the protein is associated with the Triton X-100 insoluble actin cytoskeletal (TIC) and also present in the cytosol (Figure 21A). We detected PhdB at a lower size in western blot analysis many times during the experiment with Triton X-100 incubation, indicating that the protein is degraded in the presence of Triton X-100.

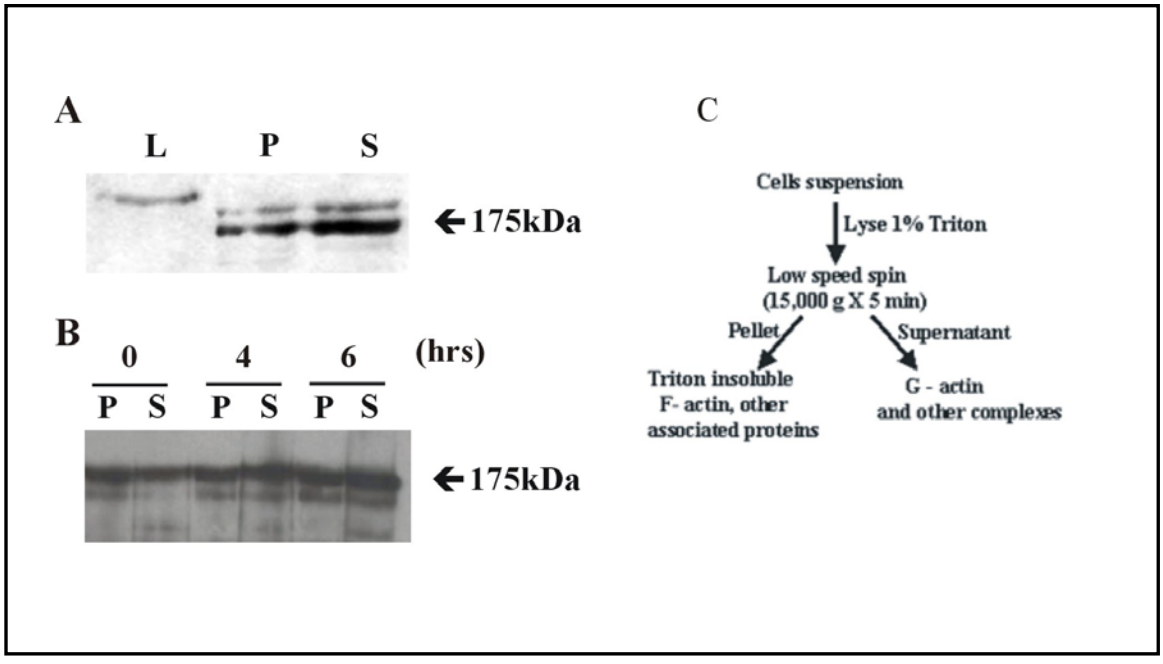


Figure 21. Western blot analysis of PhdB associated with membrane fractions and the Triton X-100 insoluble cytoskeleton compartment. A. Immunoblot analysis of PhdB associated with the Triton insoluble actin cytoskeleton component. Aggregation competent cells were harvested and lysed in 1% Triton X-100 and the lysate was separated into pellet and supernatant fraction by low spin (15,000g x 5min). Pellet (P) represents the Triton X-100 insoluble actin cytoskeleton and the supernatant represents the Triton X-100 soluble fraction. Pellet and supernatant fraction were separated by 8% SDS-PA gel and analysed by western blotting using pAbCT. The protein was found in both the fractions. **B.** AX2 cells grown in starvation buffer were harvested at different time points (0, 4, 6 h) of development. The cells

were lysed by sonication (3 pulses at 50W, 20 kHz) and separated into membrane fraction (P) and cytosol fraction (S). Both the membrane and cytosol fraction were separated by 8% SDS-PA gel and immunoblotted. PhdB was identified by pAbCT, and was found to be distributed equally in both membrane and cytosol fractions during different time points of aggregation. **C.** Flow chart showing the TIC isolation procedure.

3.1.20 PhdB is associated with membrane fractions in *Dictyostelium*

The PH domain characterised in CRAC and PhdA was shown to be responsible for anchoring the protein to the membrane *in vivo* (*Dormann et al.,* 2002). Therefore we tested the membrane association of PhdB in lysates of cells derived from different time points during the aggregation of *Dictyostelium* cells. Immunoblot analysis showed that the protein is equally distributed between membrane and cytosol during different time points of aggregation (Figure 21B).

3.1.21 The C- terminal peptide of PhdB binds to the F-actin cytoskeleton

Actin was identified as a ligand for few PH domains (Btk, PH domain) other than phosphoinositides. Yao *et al.,* (1999) showed the interaction of the PH domain of Bruton's tyrosine kinase (Btk) with actin. The amino acids responsible in Btks PH domain to bind to F-actin were mapped to many positively charged lysine residues. We were also interested to analyse the interaction of the C-terminal peptide of PhdB with actin, as the C-terminal PH domain possessed several positively charged lysine residues. The C-terminal peptide (CT-ArfGAP/PH) expressed as GFP fusion protein exhibited a strong association with the Triton X-100 insoluble actin cytoskeleton complex (Figure 22).

Also, cells expressing the CT-ArfGAP/PH peptide as GFP fusion protein were tested for the localisation of the GFP fusion protein in the cells by immunofluorescence staining. The GFP fusion protein was found to co-localise with F-actin at the pseudopods (Figure 23a). In addition, in vitro actin binding analysis was done with a CT-ArfGAP/PH-GST fusion protein. The fusion protein bound to GST beads was used to check the interaction with actin. The beads were incubated with AX2 cell lysate at 4°C for 1 h; the GST beads were separated by centrifugation and washed with cold PBS. Actin binding to the ArfGAP/PH peptide was analysed by western blot analysis using mAb act1, which recognised actin (Figure 23b). For control beads that contain only GST were used which did not bind actin.

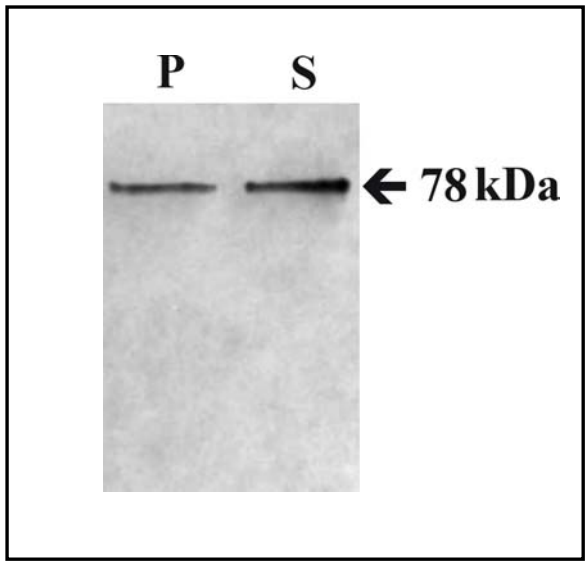


Figure 22. Immunoblot analysis showing CT-ArfGAP/PH associated with the Triton X-100 insoluble actin cytoskeleton. Aggregation competent cells expressing CT-ArfGAP/PH as GFP fusion protein were harvested and lysed in 1% Triton X-100 and the lysate was separated into pellet and supernatant fraction by low speed spin (15,000g x 5min). Pellet (P) represents the Triton X-100 insoluble actin cytoskeleton complex and supernatant (S) represents the Triton X-100 soluble fraction. The pellet and supernatant fractions were separated by 8% SDS-PA gel and analysed by western blotting using pAbCT. The GFP fusion protein was distributed between the Triton X-100 insoluble cytoskeleton and the soluble fraction.

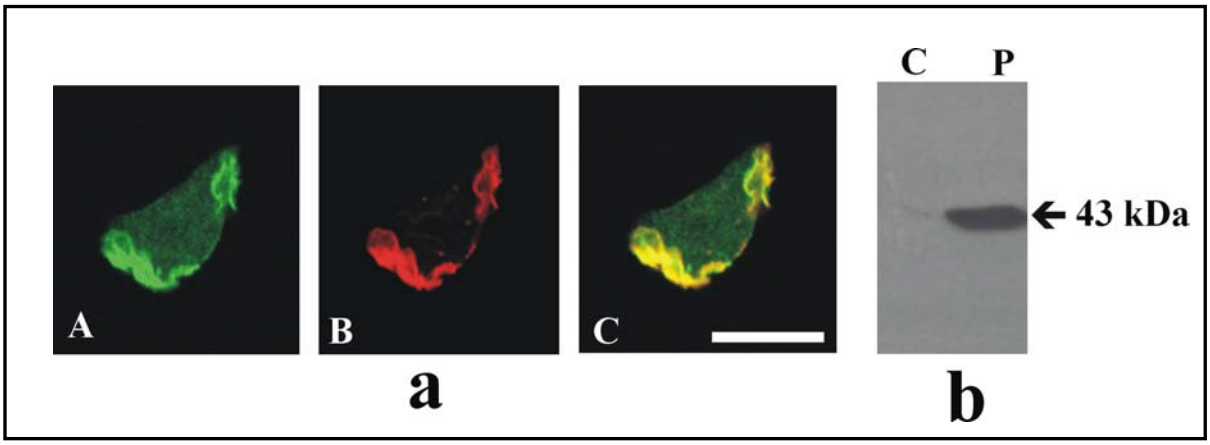


Figure 23. Immunofluorescence staining and in vitro actin binding assay. a-A) CT-GFP expressing cells **(a-B)**, CT-GFP expressing cells stained for F-actin by TRITC phalloidin, **(a-C)**overlay. **(b).** In vitro actin binding assay. The CT-GST fusion protein bound to GST beads was incubated with wild type (AX2) cell lysate at 4°C for one hour. The beads were washed with cold PBS and actin bound to the fusion protein was analysed by western blot analysis using mAb act1, which recognises actin. P represents the CT-GST peptide bound to actin. C, represents the control, where only GST protein bound to GST beads was incubated with AX2 lysates. Bar, 15 μm.

3.1.22 The C- terminal peptide of PhdB interacts with phosphoinositides

The PH domains present in CRAC and PhdA are shown to bind to phosphoinositides (Sun and Firtel 2003, Funamoto *et al.,* 2001). We used a lipid overlay assay to check the interaction of the C-terminal peptide of PhdB with different phosphoinositides like phosphatidylinositol 3,5 diphosphate (PtdIns (3,5) P_2), phosphatidylinositol 4,5 diphosphate (PtdIns (4,5) P_2) and phosphatidylinositol 3,4,5 triphosphates (PtdIns (3,4,5) P_3).

The C-terminal polypeptide encompassing ArfGAP/PH domain was expressed in *E. coli* as GST fusion protein. As the peptide was not soluble, we extracted the protein by detergent solubilisation, using sarkosyl as the detergent (materials and method 2.2.46). The detergent solubilised protein was incubated with GST beads.

The protein bound to GST beads was washed and cleaved with thrombin to obtain the peptide (Figure 24 A), which was then used to perform the overlay assay. A PVDF membrane, which carried spots of 200 pmoles of different phosphoinositides was incubated with the peptide and the protein bound to the membrane by virtue of its interaction with the phosphoinositides was detected by treating the membrane with pAbCT which recognised the ArfGAP/PH peptide (Figure 24B).

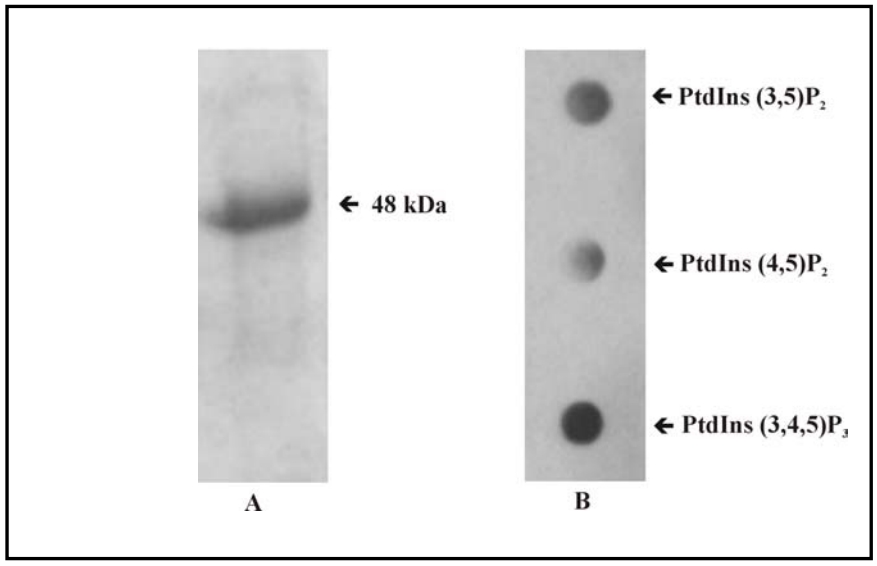


Figure 24. A) The CT-terminal polypeptide bound to GST beads was cleaved with thrombin to release the pure peptide of 48 kDa from the GST tag. **B)** The protein (200ng/ml) obtained was used for incubation of a PVDF membrane which carried different phosphoinositides (PtdIns (3,5)P_2, PtdIns (4,5)P_2, PtdIns (3,4,5)P_3 / 200 pmoles each). The protein bound to the membrane by its interaction with phosphoinositides was recognised by polyclonal PhdB specific antibody.

4. DISCUSSION

4.1 Identification of PhdB, a 175 kDa protein

We have identified a novel Pleckstrin homology domain containing protein (PhdB) by searching cDNA and genomic DNA databases of *Dictyostelium* for novel actin binding proteins and elucidated its role in *Dictyostelium* cell aggregation.

Domain architecture analysis of the primary structure of the new protein revealed the presence of a single CH domain, a domain which characterises various actin binding proteins, at the N-terminus. This single actin binding domain had 40% identity with the first CH domain of the filamin actin binding domain. Filamin is a well characterised actin binding protein and its actin binding domain is known to bind F-actin (Bresnick *et al.,* 1990).

In addition to the N-terminal CH domain the new protein also possesses a RhoGEF and an ArfGAP domain both of which are followed by single PH domains. PH domains are best known for their ability to bind phosphoinositides and to be targeted to cellular membranes (Ferguson *et al.,* 2000). Based on the occurrence of PH domains we assigned the name as PhdB (pleckstrin homology domain containing protein B). It is suffixed as protein B, because recently a new protein with a PH domain was identified in *Dictyostelium*, which was characterised and named as PhdA (Funamoto *et al.,* 2001). Amino acid sequence analysis of PH domains among PhdB, CRAC and PhdA showed very low sequence identities. In general the average sequence identity of PH domains is only 17 % (Bateman *et al.,* 2002) but all the three-dimensional structures determined to date are similar having seven β strands that form two perpendicular antiparellel β-sheet and one C-terminal alpha helix (Hurley and Misra, 2000). PhdB specific monoclonal and polyclonal antibodies generated recognised a protein at all stages of development at 175 kDa.

4.1.1 PhdB$^-$ null cells exhibit abnormal aggregation

To investigate the function of PhdB we have disrupted the PhdB gene of AX2 cells by homologous recombination using a blasticidin S resistance cassette (Sutoh K. 1993). PhdB$^-$ cells exhibited a defect in aggregation when seeded under starvation buffer. Free amoebae initiate a developmental programme upon starvation utilising cAMP as an intercellular and extracellular signal (Schaap and Wang, 1984). Aggregation is the first phase exhibited by individual cells to survive the harsh environment (no food source) and it is mediated by chemotaxis (Soderbom and Loomis *et al.,* 1998).

The aggregates formed by PhdB$^-$ cells in starvation buffer on a plastic surface were weak and small when compared to the ones of wild type cells. Also there was a delay in the formation of aggregates. Wild type cells begin to aggregate at ~ 6h and form tight aggregation centres within ~ 10 –12 h, which on a solid surface develop into multicellular fruiting bodies (Kay, 1992). PhdB$^-$ cells begin to aggregate at ~8h, which is a delay by 2h in initiation of aggregation when compared to AX2 cells, and upon the process of completion they break down into smaller aggregates. When PhdB$^-$ cells were starved on agar (solid platform) PhdB$^-$ cells formed normal fruiting body but they exhibited a 3-4 h delay in the formation of fruiting bodies. This observation is in accordance with the delay in aggregation, which we observed when cells were starved in starvation buffer. These aggregates develop into normal multicellular fruiting bodies on a solid platform. Nevertheless, the delay in aggregation was also carried on to the developmental stage. Hence PhdB$^-$ cells exhibited a delay also in development on the solid substratum.

4.1.2 PhdB$^-$ cells exhibit normal chemotaxis

When cells efficiently migrate towards the aggregation centre they may contribute to effective aggregation. As PhdB$^-$ cells exhibited a defect in aggregation we looked into the migration behaviour of PhdB$^-$ cells by assaying their chemotaxis towards the chemoattractant cAMP. When PhdB mutant cells were challenged with an external source of cAMP their migration towards the generated gradient was normal. PhdB mutants efficiently migrated up towards the cAMP gradient generated by a capillary tip (filled with 10^{-4}M cAMP). We also found that aggregation competent PhdB$^-$ cells move towards the source of cAMP with an average speed of 6.9 μm/min (+/-2.5), which is comparable with the wild type cell average speed, 6.4 μm/min (+/-2.2) (Noegel *et al.,* 2004). Moreover, we could observe visually the endogenous cAMP waves during live imaging which is evident because of co-ordinated contraction and elongation of the cells upon cAMP pulses.

Chemotactic migration involves cells to fix a sign post and move (Chung *et al.,* 2001); persistence is the measure of the ratio of speed and the direction change of a migrating cell, which defines the maintenance of direction by cells moving towards a source (Zhang *et al.,* 2001). PhdB$^-$ cells maintained their persistence like AX2 cells. PhdB$^-$ cells showed a rate of 2.6 μm/min-deg (+/-1.3) and AX2 cells showed a rate of 2.2 μm/min-deg (+/-1.3). Cell motility is also characterised by another parameter, the polarity. Cells moving towards the cAMP source become highly polarised and migrate. Polarity was measured by scoring the shape of the cells. We analysed the shape of both PhdB$^-$ and AX2 cells when migrating

towards an external source of cAMP and scored polarity as roundness. The higher the percentage of roundness lesser the cells are polarised. Both PhdB$^-$ and AX2 cells exhibited a roundness of ~ 50% which indicates a similar type of behaviour in the morphology of the cell when they are moving towards cAMP source. Also, the leading edge formed by polarised mutant cells when they migrated towards cAMP was equally sensitive as the one of AX2 cells. The mutant was capable of sensing drastic changes in the position of the cAMP source and responded by reorientation and extension of a new pseudopod. This property is achieved by the refined localised signalling happening at the leading edge of a cell (Postma *et al.,* 2004). It seems therefore, that chemotaxis is efficient and is not the cause of a change in the aggregation behaviour. A further property of cells that is important for cell aggregation is cell-cell adhesion mediated by cAMP signalling.

4.1.3 PhdB$^-$ cells aggregation is not mediated by csA

Dictyostelium cells aggregate to form a multicellular organism by cAMP driven chemotaxis and cell-cell adhesion (Loomis *et al.,* 1985). Aggregation in *Dictyostelium* possesses two distinct adhesion systems, one being EDTA-sensitive and the other EDTA-resistant. The molecules that mediate these adhesions have been known as contact sites. Contact site A mediates EDTA-resistant adhesion and contact site B mediates EDTA-sensitive adhesion. Also there exists a further molecule, which mediates EDTA-sensitive adhesion system called contact site C. As PhdB$^-$ cells exhibit a delay in aggregation we looked into the expression of the aggregation specific protein csA (contact site A). Contact site A is a glycoprotein that starts accumulating in the preaggregation phase and is highly expressed during aggregation. Contact site A minus mutants fail to form EDTA stable contacts during aggregation (Harloff *et al.,* 1989). It was also shown that constitutive expression of csA induces formation of EDTA resistant aggregation (Faix *et al.,* 1990).

Surprisingly the pattern and expression level of csA protein was unaltered in PhdB$^-$ mutant cells when they were allowed to aggregate in starvation buffer on a petriplate in comparison with AX2. This observation was not in accordance with the studies reported earlier (Desbarats *et al.,* 1994), which describe that csA expression is mediated by cell-cell adhesion. We expected that the csA expression level or pattern would be decreased or delayed in PhdB$^-$ cells. In laboratory conditions commonly used substrata for studying aggregation are cells seeded on polystyrene dishes, cells grown in shaking culture and cells allowed to develop on agar. Since we did not observe any change in csA expression in PhdB$^-$ when seeded on polystyrene plates, we assumed that the condition for aggregation may appear stringent for

PhdB$^-$ cells and hence they express csA like wild type cells to survive efficiently on petriplates. Consequently the expression of csA was analysed under two other different laboratory conditions. When PhdB$^-$ cells were allowed to starve in shaking culture at 160rpm, again the expression of csA was not altered, the pattern and level were similar to that of wild type cells. Also we checked the expression of csA at both protein and mRNA level when cells were allowed to aggregate and develop on solid agar substratum (solid substratum) and found no difference in the expression pattern of csA both at the protein and mRNA level when they were starved and allowed to develop on agar substratum. Also, csA mutants were shown to be able to grow normal under laboratory condition (which are less stringent) with no apparent difference in the timing of aggregation or any developmental defect (Harloff *et al.,* 1989). As the expression of csA does not rescue the delay in aggregation in PhdB$^-$ cells we assume that the delay in aggregation in PhdB$^-$ cells are not mediated by csA. Hence the delay in aggregation may be mediated by csB or csC or PhdB may participate in yet unidentified aggregation mechanism.

When cells are allowed to starve, in the first phase a low basal level of csA is expressed. The second phase results in high level of csA expression. As in the earlier or first phase of aggregation where EDTA sensitive molecules csB (gp24) mediate aggregation, the disruption of EDTA sensitive cell adhesion resulted in reduced levels of csA expression. EDTA probably has pleiotropic effects on *Dictyostelium* development. The inhibition of the initial phase of csA expression may result from the blockage of an earlier developmental event.

PhdB$^-$ cells exhibit the same pattern of csA expression, where during early stage of development csA expression was reduced in the presence of EDTA, but not during later development. Our results suggest that the expression of csA in PhdB$^-$ cells was very similar to wild type cells in the presence of EDTA during development. In addition we also observed that EDTA sensitive cell-cell adhesion which occurs prior to csA mediated aggregation also regulated csA expression.

Our results obtained from the cell association assays show that wild type AX2 cells were able to form strong EDTA resistant aggregates during the process of development. In contrast, PhdB$^-$ cells did not form strong EDTA resistant aggregates; also in control experiments PhdB$^-$ cells were unable to form strong aggregates even in the absence of EDTA. This observation again infers that the aggregation defect in PhdB$^-$ is independent of csA mediated cell-cell adhesion. Another possibility for delay and inefficient aggregation in PhdB$^-$ cells could be attributed to the csA expressed being non-functional. This remains to be investigated.

The phospholipid anchored adhesion molecule csA was found to form trans-homophilic binding mediated cell-cell adhesion (Sadeghi *et al.,* 1988; Stadler *et al.,* 1989). The contact site A molecule was shown to partition preferentially into raft-like membranes (Ingalls *et al.,* 1986). Harris *et al.* (2001) showed that csA oligomerises in raft-like domains in *Dictyostelium*, and these raft-like domains may provide the optimal micro-environment for the assembly of csA cis-oligomers and formation of adhesion complexes. Though csA expression was detected at cell-cell contact sites by immunofluorescence (data not shown) in PhdB$^-$ cells, these cells could have a defect in the formation of raft-like domains, which is suitable for csA function where the rafts may be able to recruit specific proteins. Another possibility is that the interactions between csA molecules at the rafts are very weak.

Similar defects as the ones observed for the PhdB$^-$ cells were described for the pkbA null cells of *Dictyostelium*. Ruedi *et al.* (1999) showed that pkbA null cells exhibited an aggregation defect. Also the pkbA null cells produced only few stable aggregates, furthermore the formation of aggregates was delayed. Aggregation centres appeared but were rapidly lost, with new centres appearing and disappearing. pkbA null cells showed a defect in chemotaxis and polarisation in cAMP gradients. Like PhdB$^-$ cells, pkbA cells expressed normal levels of csA despite their defect in aggregation. The reason for the pkbA null cells aggregation phenotype was proposed to be that Akt/PKB might affect the adaptation of the aggregation signalling pathways.
The protein kinase Akt/PKB in mammalian cells regulates various processes like inhibition of apoptosis, inhibition of GSK3 activity and regulation of cellular metabolism and protein synthesis (Bellacosa *et al.,* 1991; Coffer and Woodget, 1991). Akt/PKB is activated by both receptor kinase and G-protein coupled receptors (Burgering and Coffer, 1995; Sommer *et al.,* 2002). The *Dictyostelium* homolog of Akt/PKB has an N-terminal PH domain and homologous kinase and C-terminal regulatory domains. Akt/PKB in *Dictyosetlium* is activated by G-protein coupled cAMP receptor cAR1.

The finding that aggregation specific cell adhesion molecule csA is expressed similarly in PhdB$^-$ cells and wildtype cells suggests that the components required for aggregation other than PhdB are not limited in PhdB$^-$ cells. We also cannot exclude that some other genes and gene products required for chemotaxis signalling are not fully expressed or functional in PhdB$^-$ cells.

4.1.4 PhdB$^-$ cells exhibit a streaming defect during chemotaxis

When *Dictyostelium* cells were seeded in starvation buffer, they aggregate in a process driven by chemotaxis towards cAMP (Devreotes, 1989). *Dictyostelium* initially move as a single cell but later migrate as stream of cells attached in a head to tail fashion (Meili and Firtel. 2003). The streaming process in wild type begins at ~ 6h and is complete by ~ 12 h, which leads to the formation of tight aggregates containing 10^5 cells. Unlike wild type cells PhdB$^-$ cells moving towards a gradient generated by an external source of cAMP (10^{-4}M) do not form streams. Under laboratory conditions when allowed to grow in starvation buffer PhdB$^-$ cells take longer time to initiate streaming (inefficient). Once they begin the process they form weak streams that were very often break into small aggregates. We observed this behaviour in cells consistently during aggregation (t6 through t8, assuming that the cells adaptation to intra and extracellular cAMP signalling is stabilised at a later stage of aggregation). At 6 and 8 hours of starvation PhdB$^-$ cells did not form streams where as they can chemotax towards an external source of cAMP (10^{-4}M).

Aggregation specific adenylyl cyclase (ACA) is highly enriched at the uropod (rear end of chemotaxing cells) and is essential for cells to align in a head to tail fashion to form streams (Kriebel *et al.,* 2003). ACA at the rear mediates streaming by secreting cAMP at the back of the chemotaxing cells. The restricted cellular distribution of ACA in polarized cells explains the spatial regulation of signalling in *Dictyostelium* chemotaxis. ACA minus cells chemotax efficiently as wild type cells, but do not form streams. It is now clear that localisation of ACA at the rear end of chemotaxing *Dictyostelium* is necessary for streaming.

Apart from the localisation of ACA the auto catalysis mechanism of ACA seems to be necessary for the streaming process, because of a high concentration of cAMP is required to activate ACA, by which it produces sufficient amount of cAMP to communicate with the cell at the back which may contribute to the process of streaming.

Jung *et al.,* (1996) have described the role of classic myosin I isoforms in chemotaxis and streaming property of *Dictyostelium*. Myosin I isoforms include myoB, myoC, and myoD. MyoB$^-$ cells showed a reduction in streaming efficiency and reduced cell migration. MyoC$^-$ and myoD$^-$ are very similar to wild type cells in their efficiency to form streams. Suppression of the myoC protein level in myoB/myoD double mutant background led to a greater effect on streaming behaviour but did not alter the speed of whole cell migration. Streaming behaviour and chemotaxis therefore appear to be two independent processes in *Dictyostleium*.

This is supported by findings of Barclay and Henderson (1986), who showed an altered cAMP receptor activity in a chemosensory mutant of *Dictyostelium* which aggregated without stream formation.

The G protein coupled receptors are the key components in the detection of cAMP that control chemotactic cell movement. The cAMP chemoattractant receptor family in *Dictyostelium* is composed of four receptors cAR1, cAR2, cAR3 and cAR4. The four receptors differ in their affinity for cAMP. They achieve different functions by possessing different affinity, timing and pattern of expression. The high affinity receptor cAR1 is the first to be expressed during early aggregation and it is the primary receptor responsible for aggregation (Sun and Devreotes, 1991). The receptor cAR1 continues to be expressed in later development in all the cells. During later aggregation cAR3 receptors are expressed. The receptor cAR2 is expressed at the mound stage (Jhonson *et al.,* 1993) and cAR4 is expressed in a prestalk specific manner (Saxe *et al.,* 1993).

When cells receive the cAMP signal through the specific receptor, cAMP binding activates the aggregation specific adenylyl cyclase (Pitt *et al.,* 1992) that produces and secretes additional cAMP needed for the streaming movement of cells and for the completion of multicellular development. At this stage we speculate that PhdB$^-$ cells may exhibit a difference in cAMP sensitivity or a defect in the ability to produce necessary amount of cAMP, which may be required to bring back ACA into its autocatalytic regime.

Some of the physiological changes that may contribute to the PhdB$^-$ mutant phenotype are expression of higher levels of high affinity cAMP receptors, which may be sequestering limited cAMP without being able to transduce signals efficiently to activate ACA. Higher levels of low affinity cAMP receptor, which may require high concentrations of cAMP (produced by ACA) or may take longer time before ACA can raise the extracellular cAMP levels where it comes to the regime of autocatalysis again.

We also observed that the cAMP stimulated F-actin polymerisation event (Condeelis *et al.,* 1988) of aggregation competent (t6) PhdB$^-$ cells was reduced when compared to wild type cells, which showed a one fold raise in actin polymerisation after 5 sec of cAMP stimulation. After 15 to 20 sec a second long lasting peak of F-actin accumulation occured which parallels the extension of the pseudopods. This observation may also point to insufficient sensing and processing of cAMP signals.

Aggregation of individual *Dictyostelium* cells is also controlled by propagating waves of the chemoattractant cAMP (Parent and Devreotes, 1996). The cAMP waves are periodically initiated by cells in the aggregation centre and relayed by surrounding cells, which results in

outward propagating waves of cAMP. This outward propagating waves direct cells to move inward towards the aggregation centre (Siegert andWeijer, 1995). Any defects in the rhythm of cAMP pulses could also contribute to inefficient aggregation.

Based on our study we speculate that the new protein PhdB is a component during aggregation signalling, and may function by coupling with any of the four cAMP receptors.

Defect in activation of ACA which results in a defect in production of cAMP, which is required to activate PKA and release of cAMP into the extracellular space could be the reason for the defect in aggregation what we observed in PhdB$^-$ cells. Because the secreted extracellular cAMP relays the chemotactic signal for efficient streaming and aggregation during *Dictyostelium* development, any alteration in the production and secretion process could contribute to the defect observed in PhdB$^-$ cells. Therefore in PhdB$^-$ mutants, signalling of a specific receptor that couples PhdB for efficient aggregation may be switched off and other proteins and receptors in an attempt to overcome the aggregation defect, may lead to a delay and inefficient aggregation.

PhdB$^-$ mutants failed to stream but they migrated towards an external gradient generated by cAMP, which indicates that there is a fine tuning between cell to cell signalling during aggregation which it is not balanced in PhdB$^-$ mutants.

4.1.5 PhdB$^-$ cells exhibit normal pinocytosis, phagocytosis and development.

The major defect of PhdB$^-$ cells was in aggregation. Other properties of the cells analysed were not affected. PhdB$^-$ cells grow with similar doubling point like wild type cells. Growth with liquid medium as food source under standard lab condition was normal. This observation also supports the rate of pinocytosis whereby the ability of cells to internalise the fluid phase marker FITC-dextran was similar. Growth rates on nutrient agar plates with bacteria as food source were also similar in both AX2 and PhdB$^-$ cells, indicating further that phagocytosis is not significantly impaired in the PhdB$^-$ cells.

4.1.6 The PhdB protein may have a function at the membrane in association with the cytoskeleton

Subcellular fractionation of AX2 cells reveals that the PhdB protein is associated with the plasma membrane. Analysis of the Triton X-100 insoluble cytoskeletons reveals that the protein is also associated with the Triton X-100 insoluble cytoskeleton. The interaction of PhdB at the membrane and in the Triton X-100 insoluble cytoskeleton is most probably the

result of the affinity of the PH domain for membrane phospholipids and F-actin. Our studies with the C-terminal peptide of PhdB, which contains a PH domain, also show interactions with different phosphoinositides and F-actin *in vitro*. We speculate that the PH domain present in the C-terminal peptide could be responsible for the interaction with both ligands.

The ligands for a large number of PH domains are membrane bound inositol phospholipids. Harlan *et al.,* (1994) provided the first direct evidence for PH domain lipid interaction. The N-terminal PH domain of pleckstrin bound specifically to vesicles containing PtdIns(4,5)P_2. In vivo data are now emerging in support for the role of PH domains as membrane anchors. In cells, pleckstrin is associated with membranes by its N-terminal PH domain (Ma *et al.,* 1997). In Dbl the PH domain is required for its membrane association and oncogenic activity (Zheng *et al.,* 1996). The guanine nucleotide exchange factors of the small G-protein ARF1 also bind to membrane and phosphoinositides vesicles with their PH domains (Chardin *et al.,* 1996). The most often mentioned protein ligands for PH domains other than actin are the βγ subunit of trimeric G-proteins. In β-adrenergic receptor kinase (β-ARK) the PH domain is shown to interact with the βγ subunit (Koch *et al.,* 1993). The PhdB sequence also harbours a single Calponin homology (CH) domain, a RhoGEF and an Arf GAP domain. The presence of both RhoGEF and an ArfGAP domain predicts a possible dual function (Xia *et al,* 2002) of the PhdB protein in regulating GTPases as both being a GTPase exchange factor or a GTPase activating protein at the membrane.

PhdA like PKB localises to the leading edge of chemotaxing cells. This is dependent on PI3K. The phenotype of PhdA null cells show no stream formation during aggregation and exhibit reduced chemotaxis. PhdA null cells were also defective in kinetics and the level of actin polymerisation. Some of the features like absence of stream formation and the reduced response in actin polymerisation can be compared to PhdB$^-$ cells. PhdA was proposed to function as adoptor protein at the leading edge during chemotaxis. PI3K transduces the signals downstream by recruiting PhdA as one of the effector. Our observation that the leading edge in PhdB$^-$ cells was equally sensitive to cAMP like wild type cells may exclude a function of PhdB in chemotaxis at the leading edge. However, PhdB may still be a component of parallel cell-cell signalling pathways downstream of PI3K which may regulate chemotaxis under natural conditions. PhdA translocates from the cytosol to the plasma membrane in response to cAMP stimulation. This translocation property was attributed to its PH domain, which could bind to the PI3K products at the leading edge. As PhdA translocates during chemotaxis to the leading edge, its function was proposed to serve as docking sites for other

cellular proteins that must assemble at the leading edge in response to chemoattractant signals.

Similarly, PhdB could regulate the process of aggregation by having a function at the membrane and in association with the F-actin complex. The PH domains present may help in anchoring the protein to the membrane or associate it with the F-actin complex. A direct interaction of PH domains with F-actin has been shown in general.

Our results show that the expression of csA in PhdB$^-$ cells which exhibit a delay in aggregation is independent of the cause i.e. the loss of PhdB, as the delay in aggregation may be due to inefficient signalling between cell to cell which may be due to the defect in secreted cAMP or a defect in cell-cell contact formation. It also cannot be ruled out that csA expression in PhdB$^-$ cells may be independent of both cAMP and cell-cell contact mediated processes, and other gene regulation pathway may contribute to the observed delay in aggregation in PhdB$^-$ cells.

5. SUMMARY / ZUSAMMENFASSUNG

Summary

Proteins with pleckstrin homology (PH) domains have been shown to be mostly involved in cytoskeletal signalling. We have identified a new protein in *Dictyostelium discoideum*, whose primary structure analysis reveals the presence of two PH domains along with additional functional domains like CH, RhoGEF and ArfGAP domains. Henceforth the protein is referred to as PhdB (**P**leckstrin **h**omology **d**omain containing protein **B**). Monoclonal and polyclonal antibodies generated against PhdB recognise a 175 kDa protein throughout the developmental differentiation programme of *D. discoideum*. A GFP fusion protein containing the C-terminal PH domain of PhdB colocalises with F-actin at the pseudopods and is associated with the Triton X-100 insoluble F-actin cytoskeleton. The recombinant C-terminal domain of PhdB also binds to phosphoinositides and actin in vitro.

To define the function of the protein we generated null cells lacking the PhdB protein by homologous recombination. The phenotype of the null cells reveals that the protein is necessary for normal cell aggregation during development as PhdB$^-$ cells showed a developmental delay and failed to form streams unlike wild type cells during chemotaxis upon a challenge with an external source of cAMP.

During further analysis of the developmental defect we observed that the expression of the cell-cell adhesion molecule contact site A (csA) occurred independent of the formation of cell-cell contacts in PhdB$^-$ cells. The csA protein is a cAMP induced glycoprotein mediating EDTA-stable contacts. Furthermore, its expresion followed a time course similar to the one observed in wild type and normal levels of csA were reached. However, the presence of the csA did not allow the formation of strong aggregates by PhdB$^-$ cells. It therefore appears that the normal expression of csA which we observed during aggregation in PhdB$^-$ cells is independent of cell-cell adhesion.

Taken together, the abnormal aggregation and the defect in streaming of PhdB$^-$ cells indicate that the protein is a component of cell-cell signalling during *Dictyostelium* aggregation.

Zusammenfassung

In dieser Arbeit ist ein neues PH-Domänen Protein, PhdB, aus *Dictyostelium discoideum* charakterisiert worden. PH-Domänen werden häufig in Proteinen gefunden, die an Zytoskelett-assoziierten Signaltransduktionsprozessen beteiligt sind. PhdB ist ein 175 kDa Protein mit zwei PH-Domänen, einer CH-Domäne wie sie in Aktin-bindenden Proteinen vorkommt, einer RhoGEF und einer ArfGAP Domäne. Mono- und polyklonale Antikörper erkennen ein 175 kDa Protein in Westernblots, das während Wachstum und Entwicklung nachweisbar ist. Ein GFP-Fusionsprotein, das die C-terminale PH-Domäne enthält, ist im Aktinkortex der *Dictyostelium* Zelle lokalisiert, in vitro bindet diese Domäne an Aktin und an Phosphoinositide.

Um die Funktion des Proteins zu analysieren, wurden Mutanten geschaffen, in denen das Gen durch homologe Rekombination inaktiviert wurde. Diesen Mutanten fehlt das Protein vollständig. Die Untersuchung der PhdB$^-$ Zellen zeigte eine Veränderung in der frühen Entwicklung, in der die Zellen chemotaktisch auf cAMP zuwandern und Zellströme bilden. Während die chemotaktische Reaktion normal war, waren die Zellen nicht in der Lage zu adhärieren und im Zellverbund auf die cAMP-Quelle zuzuwandern. Normalerweise ist dieses Verhalten auf die korrekte Expression von Zell-Zell-Adhäsionsproteinen zurückzuführen. Erstaunlicherweise konnte das csA-Protein, ein wichtiges Zelladhäsionsprotein, in normalen Mengen und mit seinem korrekten zeitlichen Expressionsmuster nachgewiesen werden, ohne dass dadurch die Bildung eines stabilen Zellverbundes stattfand.

Zusammenfassend hat die Mutantenanalyse gezeigt, dass PhdB eine Funktion in der frühen Entwicklungsphase von *Dictyostelium* besitzt und in den geordneten Ablauf der Zelladhäsion involviert ist.

6. Bibliography

Allen, W.E., Zicha, D. Ridley, A.J. and Jones, G.E. 1998. A role for Cdc42 in macrophage chemotaxis. *J Cell Biol*. 141:1147-57.

Barclay, S., and Henderson, E.J. 1986. Altered cyclic-AMP receptor activity and morphogenesis in a chemosensory mutant of *Dictyostelium discoideum*. *Differentiation*. 33:111-20.

Bateman, A., Birney, E. Cerruti, L. Durbin, R. Etwiller, L. Eddy, S.R. Griffiths-Jones, S. Howe, K.L. Marshall, M. and Sonnhammer, E.L. 2002. The Pfam protein families database. *Nucleic Acids Res*. 30:276-80.

Bellacosa, A., Testa, J.R. Staal, S.P. and Tsichlis, P.N. 1991. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science*. 254:274-7.

Bertholdt, G., Stadler, J. Bozzaro, S. Fichtner, B. and Gerisch, G. 1985. Carbohydrate and other epitopes of the contact site A glycoprotein of *Dictyostelium discoideum* as characterized by monoclonal antibodies. *Cell Differ*. 16:187-202.

Bonner, J.T. 1947. Evidence for the formation of cell aggregates by chemotaxis in development of the slime mold *Dictyostelium discoideum*. *J Exp Zool*. 106:1-26.

Bozzaro, S., and Ponte, E. 1995. Cell adhesion in the life cycle of *Dictyostelium*. *Experientia*. 51:1175-88.

Bresnick, A.R., Warren, V. and Condeelis, J. 1990. Identification of a short sequence essential for actin binding by *Dictyostelium* ABP-120. *J Biol Chem*. 265:9236-40.

Brink, M., Gerisch, G. Isenberg, G. Noegel, A. Segall, J.E. Wallraff, E. and Schleicher, M. A. 1990. *Dictyostelium* mutant lacking an F-actin crosslinking protein, the 120 kD gelation factor. *J Cell Biol*. 111:1477-1489.

Bullock, W.O., Fernandez, J.M. and Short, J.M. 1987. XL1-blue: A high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. *BioTechniques*. 5:376-8.

Burgering, B.M., and Coffer, P.J. 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature*. 376:599-602.

Cardelli, J. 2001. Phagocytosis and macropinocytosis in *Dictyostelium*: phosphoinositide-based processes, biochemically distinct. *Traffic*. 2:311-20.

Chardin, P., Paris, S. Antonny, B. Robineau, S. Beraud-Dufour, S. Jackson, C.L. and Chabre, M. 1996. A human exchange factor for ARF contains Sec7- and pleckstrin-homology domains. *Nature*. 384:481-4.

Chen, M.Y., Insall, R.H. and Devreotes, P.N. 1996. Signaling through chemoattractant receptors in *Dictyostelium*. *Trends Genet*. 12:52-7.

Chen, M.Y., Long, Y. and Devreotes, P.N. 1997. A novel cytosolic regulator, Pianissimo, is required for chemoattractant receptor and G protein-mediated activation of the 12 transmembrane domain adenylyl cyclase in *Dictyostelium*. *Genes Dev*. 11:3218-31.

Chung, C.Y., Funamoto, S. and Firtel, R.A. 2001. Signaling pathways controlling cell polarity and chemotaxis. *Trends Biochem Sci*. 26:557-66.

Claviez, M., Pagh, K. Maruta, H. Baltes, W. Fisher, P. and Gerisch, G. 1982. Electron microscopic mapping of monoclonal antibodies on the tail region of *Dictyostelium* myosin. *EMBO J*. 1:1017-22.

Coates, J.C., and Harwood, A.J. 2001. Cell-cell adhesion and signal transduction during *Dictyostelium* development. *J Cell Sci*. 114:4349-58.

Coffer, P.J., and Woodgett, J.R. 1991. Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. *Eur J Biochem*. 201:475-81.

Condeelis, J., Hall, A. Bresnick, A. Warren, V. Hock, R. Bennett, H. and Ogihara, S. 1988. Actin polymerization and pseudopod extension during amoeboid chemotaxis. *Cell Motil Cytoskeleton*. 10:77-90.

Cox, E.C., Vocke, C.D. Walter, S. Gregg, K.Y. and Bain, E.S. 1990. Electrophoretic karyotype for *Dictyostelium discoideum*. *Proc Natl Acad Sci U S A*. 87:8247-51.

Daniels, R., Zenke, F.T. and Bokoch, G.M. 1999. alphaPix stimulates p21-activated kinase activity through exchange factor-dependent and -independent mechanisms. *J Biol Chem*. 274:6047-50.

de Hostos, E.L., Rehfuess, C. Bradtke, B. Waddell, D.R. Albrecht, R. Murphy, J. and Gerisch, G. 1993. *Dictyostelium* mutants lacking the cytoskeletal protein coronin are defective in cytokinesis and cell motility. *J Cell Biol*. 120:163-73.

Desbarats, L., Brar, S.K. and Siu, C.H. 1994. Involvement of cell-cell adhesion in the expression of the cell cohesion molecule gp80 in *Dictyostelium discoideum*. *J Cell Sci*. 107:1705-12.

Devereux, J., Haeberli, P. and Smithies, O. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res*. 12:387-95.

Devreotes, P. 1989. Cell-cell interactions in *Dictyostelium* development. *Trends Genet*. 5:242-5.

Devreotes, P.N., and Zigmond, S.H. 1988. Chemotaxis in eukaryotic cells: a focus on leukocytes and *Dictyostelium*. *Annu Rev Cell Biol*. 4:649-86.

Dormann, D., Weijer, G. Parent, C.A. Devreotes, P.N. and Weijer, C.J. 2002. Visualizing PI3 kinase-mediated cell-cell signaling during *Dictyostelium* development. *Curr Biol*. 12:1178-88.

Eichinger, L., and Noegel, A.A. 2003. Crawling into a new era-the *Dictyostelium* genome project. *EMBO J*. 22:1941-6.

Faix, J., Gerisch, G. and Noegel, A.A. 1990. Constitutive overexpression of the contact site A glycoprotein enables growth-phase cells of *Dictyostelium discoideum* to aggregate. *EMBO J*. 9:2709-16.

Faix, J., Gerisch, G. and Noegel, A.A. 1992. Overexpression of the csA cell adhesion molecule under its own cAMP-regulated promoter impairs morphogenesis in *Dictyostelium*. *J Cell Sci*. 102:203-14.

Ferguson, K.M., Kavran, J.M. Sankaran, V.G. Fournier, E. Isakoff, S.J. Skolnik, E.Y. and Lemmon, M.A. 2000. Structural basis for discrimination of 3-phosphoinositides by pleckstrin homology domains. *Mol Cell*. 6:373-84.

Firtel, R.A. 1996. Interacting signaling pathways controlling multicellular development in *Dictyostelium*. *Curr Opin Genet Dev*. 6:545-54.

Firtel, R.A., and Chung, C.Y. 2000. The molecular genetics of chemotaxis: sensing and responding to chemoattractant gradients. *Bioessays*. 22:603-15.

Funamoto, S., Meili, R. Lee, S. Parry, L. and Firtel, R.A. 2002. Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. *Cell*. 109:611-23.

Funamoto, S., Milan, K. Meili, R. and Firtel, R.A. 2001. Role of phosphatidylinositol 3' kinase and a downstream pleckstrin homology domain-containing protein in controlling chemotaxis in *Dictyostelium*. *J Cell Biol*. 153:795-810.

Gerisch, G., Weinhart, U. Bertholdt, G. Claviez, M. and Stadler, J. 1985. Incomplete contact site A glycoprotein in HL220, a modB mutant of *Dictyostelium discoideum*. *J Cell Sci*. 73:49-68.

Gottwald, U., Brokamp, R. Karakesisoglou, I. Schleicher, M. and Noegel AA. 1996. Identification of a cyclase-associated protein (CAP) homologue in *Dictyostelium discoideum* and characterization of its interaction with actin. *Mol Biol Cell*. 7:261-72.

Hacker, U., Albrecht, R. and Maniak, M. 1997. Fluid-phase uptake by macropinocytosis in *Dictyostelium*. *J Cell Sci*. 110:105-12.

Hall, A.L., Warren, V. and Condeelis, J. 1989. Transduction of the chemotactic signal to the actin cytoskeleton of *Dictyostelium discoideum*. *Dev Biol*. 136:517-25.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol*. 166:557-80.

Harlan, J.E., Hajduk, P.J. Yoon, H.S. and Fesik, S.W. 1994. Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate. *Nature*. 371:168-70.

Harloff, C., Gerisch, G. and Noegel, A.A. 1989. Selective elimination of the contact site A protein of *Dictyostelium discoideum* by gene disruption. *Genes Dev*. 3:2011-9.

Harris, T.J., Ravandi, A. and Siu, C.H. 2001. Assembly of glycoprotein-80 adhesion complexes in *Dictyostelium*. Receptor compartmentalization and oligomerization in membrane rafts. *J Biol Chem*. 276:48764-74.

Hurley, J.H., and Misra, S. 2000. Signaling and subcellular targeting by membrane-binding domains. *Annu Rev Biophys Biomol Struct*. 29:49-79.

Ingalls, H.M., Goodloe-Holland, C.M. and Luna, E.J. 1986. Junctional plasma membrane domains isolated from aggregating *Dictyostelium discoideum* amebae. *Proc Natl Acad Sci U S A*. 83:4779-83.

Insall, R., Kuspa, A. Lilly, P.J. Shaulsky, G. Levin, L.R. Loomis, W.F. and Devreotes, P. 1994. CRAC, a cytosolic protein containing a pleckstrin homology domain, is required for receptor and G protein-mediated activation of adenylyl cyclase in *Dictyostelium*. *J Cell Biol*. 126:1537-45.

Janetopoulos, C., Jin, T. and Devreotes, P. 2001. Receptor-mediated activation of heterotrimeric G-proteins in living cells. *Science*. 291:2408-11.

John, V., Frangioni, and Benjamin, G. Neel. 1993. Solubilizatoin and Purification of Enzymaticaly Active Glutathione S- Transferase (pGEX) Fusion protein. *Analytical Biochemistry*. 210:179-87.

Johnson, R., Saxe, C.L. 3rd. Gollop, R. Kimmel, A.R. and Devreotes, P.N. 1993. Identification and targeted gene disruption of cAR3, a cAMP receptor subtype expressed during multicellular stages of *Dictyostelium* development. *Genes Dev*. 7:273-82.

Jung, G., Wu, X. and Hammer, J.A. 3rd. 1996. *Dictyostelium* mutants lacking multiple classic myosin I isoforms reveal combinations of shared and distinct functions. *J Cell Biol*. 133:305-323.

Kay, R.R. 1992. Cell differentiation and patterning in *Dictyostelium*. *Curr Opin Cell Biol*. 4:934-8.

Kessin, R.H. 2003. Cell motility: Making streams. *Nature*. 422:481-2.

Klein, P., Sun, T.J. Saxe, C.L .3rd. Kimmel, A.R. Johnson, R.L. and Devreotes, P.N. 1988. A chemoattractant receptor controls development in *Dictyostelium discoideum*. *Science*. 241:1467-72.

Knecht, D.A., Fuller, D.L. and Loomis, W.F. 1987. Surface glycoprotein, gp24, involved in early adhesion of *Dictyostelium discoideum*. *Dev Biol*. 121:277-83.

Koch, W.J., Inglese, J. Stone, W.C. and Lefkowitz, R.J. 1993. The binding site for the beta gamma subunits of heterotrimeric G proteins on the beta-adrenergic receptor kinase. *J Biol Chem*. 268:8256-60.

Kriebel, P.W., Barr, V.A. and Parent, C.A. 2003. Adenylyl cyclase localization regulates streaming during chemotaxis. *Cell*. 112:549-60.

Kuspa, A., and Loomis, W.F. 1996. Ordered yeast artificial chromosome clones representing the *Dictyostelium discoideum* genome. *Proc Natl Acad Sci U S A*. 93:5562-6.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227:680-5.

Lee, J.O., Yang, H. Georgescu, M.M. Di Cristofano, A. Maehama, T. Shi, Y. Dixon, J.E. Pandolfi, P. and Pavletich, N.P. 1999. Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. *Cell*. 99:323-34.

Leevers, S.J., Vanhaesebroeck, B. and Waterfield, M.D. 1999. Signalling through phosphoinositide 3-kinases: the lipids take centre stage. *Curr Opin Cell Biol*. 11:219-25.

Lehrach, H., Diamond, D. Wozney, J.M. and Boedtker, H. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry*. 16:4743-51.

Lemmon, M.A., and Ferguson, K.M. 2000. Signal-dependent membrane targeting by pleckstrin homology (PH) domains. *Biochem J*. 350:1-18.

Lemmon, M.A., Ferguson, K.M. and Abrams, C.S. 2002. Pleckstrin homology domains and the cytoskeleton. *FEBS Lett*. 513:71-6.

Li. Z., H., M. Mo, Z. Liu, B. Lu, W. Wu, Y. Smrcka, A.V. Wu, G. Li, L. Liu, M. Huang, C.K. and Wu, D. 2003. Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42. *Cell*. 114:215-27.

Lilly, P.J., and Devreotes, P.N. 1995. Chemoattractant and GTP gamma S-mediated stimulation of adenylyl cyclase in *Dictyostelium* requires translocation of CRAC to membranes. *J Cell Biol*. 129:1659-65.

Loomis, W.F., Wheeler, S.A. Springer, W.R. and Barondes, S.H. 1985. Adhesion mutants of *Dictyostelium discoideum* lacking the saccharide determinant recognized by two adhesion-blocking monoclonal antibodies. *Dev Biol*. 109:111-7.

Lowry, O.H., Rosebrough, N.J. Farr, A.L. and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 193:265-75.

Ma, A.D., Brass, L.F. and Abrams, C.S. 1997. Pleckstrin associates with plasma membranes and induces the formation of membrane projections: requirements for phosphorylation and the NH2-terminal PH domain. *J Cell Biol*. 136:1071-9.

Ma, P.C., and Siu, C.H. 1990. A pharmacologically distinct cyclic AMP receptor is responsible for the regulation of gp80 expression in *Dictyostelium discoideum*. *Mol Cell Biol*. 10:3297-306.

Malchow, D., Nagele, B. Schwarz, H. and Gerisch, G. 1972. Membrane-bound cyclic AMP phosphodiesterase in chemotactically responding cells of *Dictyostelium discoideum*. *Eur J Biochem*. 28:136-42.

Maniak, M. 2001. Fluid-phase uptake and transit in axenic *Dictyostelium* cells. *Biochim Biophys Acta*. 1525:197-204.

Meili, R., and Firtel, R.A. 2003. Two poles and a compass. *Cell*. 144:153-6.

Muller, K., and Gerisch, G. 1978. A specific glycoprotein as the target site of adhesion blocking Fab in aggregating *Dictyostelium* cells. *Nature*. 274:445-9.

Nellen, W., Datta, S. Reymond, C. Sivertsen, A. Mann, S. Crowley, T. and Firtel, R.A. 1987. Molecular biology in *Dictyostelium*: tools and applications. *Methods Cell Biol*. 28:67-100.

Newell, P.C., Europe-Finner, G.N. Small, N.V. and Liu, G. 1988. Inositol phosphates, G-proteins and ras genes involved in chemotactic signal transduction of *Dictyostelium*. *J Cell Sci*. 89:123-7.

Noegel, A.A., and Schleicher, M. 2000. The actin cytoskeleton of *Dictyostelium*: a story told by mutants. *J Cell Sci*. 113:759-66.

Noegel, A.A., Blau-Wasser, R. Sultana, H. Muller, R. Israel, L. Schleicher, M. Patel, H. and Weijer C.J. 2004. The cyclase-associated protein CAP as regulator of cell polarity and cAMP signaling in *Dictyostelium*. *Mol Biol Cell*. 15:934-45.

Parent, C.A., and Devreotes, P.N. 1996. Constitutively active adenylyl cyclase mutant requires neither G proteins nor cytosolic regulators. *J Biol Chem*. 271:18333-6.

Parent, C.A., Blacklock, B.J. Froehlich, W.M. Murphy, D.B. and Devreotes, P.N. 1998. G protein signaling events are activated at the leading edge of chemotactic cells. *Cell*. 95:81-91.

Peck, J., Douglas, G, 4 th. Wu, C.H. and Burbelo, P.D. 2002. Human RhoGAP domain-containing proteins: structure, function and evolutionary relationships. *FEBS Lett*. 528:27-34.

Pitt, G.S., Milona, N. Borleis, J. Lin, K.C. Reed, R.R. and Devreotes, P.N. 1992. Structurally distinct and stage-specific adenylyl cyclase genes play different roles in *Dictyostelium* development. *Cell*. 69:305-15.

Postma, M., Bosgraaf, L. Loovers, H.M. and Van Haastert, P.J. 2004. Chemotaxis: signalling modules join hands at front and tail. *EMBO Rep*. 5:35-40.

Postma, M., Roelofs, J. Goedhart, J. Gadella, T.W. Visser, A.J. and Van Haastert, P.J. 2003. Uniform cAMP stimulation of *Dictyostelium* cells induces localized patches of signal transduction and pseudopodia. *Mol Biol Cell*. 14:5019-27.

Prassler, J., Murr, A. Stocker, S. Faix, J.Murphy, J. and Marriott, G. 1998. DdLIM is a cytoskeleton-associated protein involved in the protrusion of lamellipodia in *Dictyostelium*. *Mol Biol Cell*. 9:545-59.

Pupillo, M., Kumagai, A. Pitt, G.S. Firtel, R.A. and Devreotes, P.N. 1989. Multiple alpha subunits of guanine nucleotide-binding proteins in *Dictyostelium*. *Proc Natl Acad Sci U S A*. 86:4892-6.

Raper, K.B. 1935. *Dictyostelium discoideum*, a new species of slime mould from decaying forest leaves. *J Agr Res*. 50:135-147.

Rivero, F., Dislich, H. Glockner, G. and Noegel, A.A. 2001. The *Dictyostelium discoideum* family of Rho-related proteins. *Nucleic Acids Res*. 29:1068-79.

Ruedi, M., Ellsworth, C. Lee, S. Reddy, T.B. Ma, H. and Firtel, R.A. 1999. Chemoattractant-mediated transient activation and membrane localization of Akt/PKB is required for efficient chemotaxis to cAMP in *Dictyostelium*. *EMBO J*. 18:2092-105.

Sadeghi, H., da Silva, A.M. Klein, C. Sadeghi, H. da Silva, A.M and Klein, C. 1988. Evidence that a glycolipid tail anchors antigen 117 to the plasma membrane of *Dictyostelium discoideum* cells. *Proc Natl Acad Sci U S A*. 85:5512-5.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning. A Laboratory Manual. 2nd ed. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York*.

Saxe, C.L. 3rd., Ginsburg, G.T. Louis, J.M. Johnson ,R. Devreotes, P.N. and Kimmel, A.R. 1993. CAR2, a prestalk cAMP receptor required for normal tip formation and late development of *Dictyostelium discoideum*. *Genes Dev*. 7:262-72.

Schaap, P., and Wang, M. 1984. The possible involvement of oscillatory cAMP signaling in multicellular morphogenesis of the cellular slime molds. *Dev Biol*. 105:470-8.

Seastone, D., Zhang, L. Buczynski, G. Rebstein, P. Weeks, G. Spiegelman, G. and Cardelli, J. 1999. The small Mr Ras-like GTPase Rap1 and the phospholipase C pathway act to regulate phagocytosis in *Dictyostelium discoideum*. *Mol Biol Cell*. 10:393-406.

Servant, G., Weiner, O.D. Neptune, E.R. Sedat, J.W. and Bourne, H.R. 1999. Dynamics of a chemoattractant receptor in living neutrophils during chemotaxis. *Mol Biol Cell*. 10:1163-78.

Shaulsky, G., Fuller, D. and Loomis, W.F. 1998. A cAMP-phosphodiesterase controls PKA-dependent differentiation. *Development*. 125:691-9.

Siegert, F., and Weijer, C.J. 1995. Spiral and concentric waves organize multicellular *Dictyostelium* mounds. *Curr Biol*. 5:937-43.

Simon, M.N., Mutzel, R. Mutzel, H. and Veron, M. 1988. Vectors for expression of truncated coding sequences in Escherichia coli. *Plasmid*. 19:94-102.

Simpson, P.A., Spudich, J.A. and Parham, P. 1984. Monoclonal antibodies prepared against *Dictyostelium* actin: characterization and interactions with actin. *J Cell Biol*. 99:287-95.

Siu, C.H., and Kamboj, R.K. 1990. Cell-cell adhesion and morphogenesis in *Dictyostelium discoideum*. *Dev Genet*. 11:377-87.

Snaar-Jagalska, B.E., and Van Haastert, P.J. 1988. *Dictyostelium discoideum* mutant synag 7 with altered G-protein-adenylate cyclase interaction. *J Cell Sci*. 91:287-94.

Soderbom, F., and Loomis, W.F. 1998. Cell-cell signaling during *Dictyostelium* development. *Trends Microbiol*. 6:402-6.

Soll, D.R. 1999. Computer-assisted three-dimensional reconstruction and motion analysis of living, crawling cells. *Comput Med Imaging Graph*. 23:3-14.

Sommer, F., Klotz, T. Steinritz, D. Schmidt, A. Addicks, K. Engelmann, U. and Bloch, W. 2002. MAP kinase 1/2 (Erk 1/2) and serine/threonine specific protein kinase Akt/PKB expression and activity in the human corpus cavernosum. *Int J Impot Res*. 14:217-25.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol*. 98:503-17.

Stadler, J., Keenan, T.W. Bauer, G. and Gerisch, G. 1989. The contact site A glycoprotein of *Dictyostelium discoideum* carries a phospholipid anchor of a novel type. *EMBO J*. 8:371-7.

Studier, F.W., and Moffatt, B.A. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol*. 189:113-30.

Sun, B., and Firtel, R.A. 2003. A regulator of G protein signaling-containing kinase is important for chemotaxis and multicellular development in *Dictyostelium*. *Mol Biol Cell*. 14:1727-43.

Sun, T.J., and Devreotes, P.N. 1991. Gene targeting of the aggregation stage cAMP receptor cAR1 in *Dictyostelium*. *Genes Dev*. 5:572-82.

Sussman, M. 1951. The origin of cellular heterogeneity in the slime molds, Dictyosteliaceae. *J Exp Zool*. 118:407-17.

Sutoh, K. 1993. A transformation vector for *Dictyostelium discoideum* with a new selectable marker bsr. *Plasmid*. 30:150-4.

Tilton, B., Andjelkovic, M. Didichenko, S.A. Hemmings, B.A. and Thelen, M. 1997. G-Protein-coupled receptors and Fcgamma-receptors mediate activation of Akt/protein kinase B in human phagocytes. *J Biol Chem*. 272:28096-101.

Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A*. 76:4350-4.

Van Es, S., and Devreotes, P.N. 1999. Molecular basis of localized responses during chemotaxis in amoebae and leukocytes. *Cell Mol Life Sci*. 55:1341-51.

Van Haastert, P.J., Janssens, P.M. and Erneux, C. 1991. Sensory transduction in eukaryotes. A comparison between *Dictyostelium* and vertebrate cells. *Eur J Biochem*. 195:289-303.

Vieira, J., and Messing, J. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Genes Dev*. 19:259-68.

Wang, F., Herzmark, P. Weiner, O.D. Srinivasan, S. Servant, G. and Bourne, H.R. 2002. Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils. *Nat Cell Biol*. 4:513-8.

Weiner, O.D., Neilsen, P.O. Prestwich, G.D. Kirschner, M.W. Cantley, L.C. and Bourne, H.R. 2002. A PtdInsP(3)- and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity. *Nat Cell Biol*. 4:509-13.

Weiner, O.D., Servant, G. Welch, M.D. Mitchison, T.J. Sedat, J.W. and Bourne, H.R. 1999. Spatial control of actin polymerization during neutrophil chemotaxis. *Nat Cell Biol*. 1:75-81.

Wertman, K.F., Wyman, A.R. and Botstein, D. 1986. Host/vector interactions which affect the viability of recombinant phage lambda clones. *Gene*. 49:253-62.

Wessels, D., Voss, E. Von Bergen, N. Burns, R. Stites, J. and Soll, D.R. 1998. A computer-assisted system for reconstructing and interpreting the dynamic three-dimensional relationships of the outer surface, nucleus and pseudopods of crawling cells. *Cell Motil Cytoskeleton*. 41:225-46.

Williams, K.L., and Newell, P.C. 1976. A genetic study of aggregation in the cellular slime mould *Dictyostelium discoideum* using complementation analysis. *Genetics*. 82:287-307.

Wu, L., Hansen, D. Franke, J. Kessin, R.H. and Podgorski, G.J. 1995. Regulation of *Dictyostelium* early development genes in signal transduction mutants. *Dev Biol*. 171:149-58.

Xia, C., Ma, W. Stafford, L.J. Liu, C. Gong, L. Martin, J.F. and Liu, M. 2003. GGAPs, a new family of bifunctional GTP-binding and GTPase-activating proteins. *Mol Cell Biol*. 23:2476-88.

Xiao, Z., Zhang, N. Murphy, D.B. and Devreotes, P.N. 1997. Dynamic distribution of chemoattractant receptors in living cells during chemotaxis and persistent stimulation. *J Cell Biol*. 139:365-74.

Yao, L., Janmey, P. Frigeri, L.G. Han. W. Fujita, J. Kawakami, Y. Apgar, J.R. and Kawakami, T. 1999. Pleckstrin homology domains interact with filamentous actin. *J Biol Chem*. 274:19752-61.

Zhang, N., Long, Y. and Devreotes, P.N. 2001. Ggamma in *Dictyostelium*: its role in localization of gbetagamma to the membrane is required for chemotaxis in shallow gradients. *Mol Biol Cell*. 12:3204-13.

Zheng, Y., Zangrilli, D. Cerione, R.A. and Eva, A. 1996. The pleckstrin homology domain mediates transformation by oncogenic dbl through specific intracellular targeting. *J Biol Chem*. 271:19017-20.

Zhou, K., Pandol, S. Bokoch, G. and Traynor Kaplan, A.E. 1998. Disruption of *Dictyostelium* PI3K genes reduces [32P] phosphatidylinositol 3,4 bisphosphate and [32P]phosphatidylinositol trisphosphate levels, alters F-actin distribution and impairs pinocytosis. *J Cell Sci*. 111:283-94.

Zhou, K., Takegawa, K. Emr, S.D. and Firtel, R.A. 1995. A phosphatidylinositol (PI) kinase gene family in *Dictyostelium discoideum*: biological roles of putative mammalian p110 and yeast Vps34p PI 3-kinase homologs during growth and development. *Mol. Cell Biol*. 15:5645-56.

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 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 den

LEBENSLAUF

Name:	DHAMODHARAN.N
Adresse:	Luxemburger strasse, 124-136 Unicenter, Et-Zi: 25-26 50939, Köln, Germany.
(Heimat adresse)	1273/3 B&C Block, Dr. Ambedkar Nagar, DJ Halli, Bangalore560 045, India. Email: dhamuiisc@yahoo.co.in
Geburtsdatum:	02.03.1975
Staatsangehörigkeit:	Indisch

AKADEMISCHE QUALIFIKATION

Schulausbildung: (1989-1991)	RBBPAM High School, Bangalore, India.
Studium:	
Bachelors Degree: (1994-1997)	B.Sc. (Chemistry, Botany, Zoology) St. Joseph's College, Bangalore University. Bangalore, India.
Masters Degree: (1997-1999)	M.Sc. (Biochemistry) Central College, Bangalore University Bangalore, India.
Promotions studium: (06.2001 -07.2004)	Mathematisch-Naturwissenschaftliche Fakultat Universität zü Köln Betreuerin: Prof. Dr. Angelika A. Noegel Institut für Biochemie I, Medizinische Fakultät der Universität zü Köln.

CURRICULUM VITAE

Name:	DHAMODHARAN.N
Address:	Luxemburger strasse, 124-136 Unicenter, Et-Zi: 25-26 50939, Köln, Germany.
(Permanent)	1273/3 B&C Block Dr. Ambedkar Nagar DJ Halli, Bangalore 560 045 India. Email: dhamuiisc@yahoo.co.in
Date of birth:	02/03/1975
Nationality	Indian

ACADEMIC QUALIFICATIONS

High School: (1989-1991)	RBBPAM High School, Bangalore, India.
Bachelors Degree: (1994-1997)	B.Sc. (Chemistry, Botany, Zoology) St. Joseph's College, Bangalore University Bangalore, India.
Masters Degree: (1997-1999)	M.Sc. (Biochemistry) Central College, Bangalore University Bangalore, India.
Post graduation Studies: (2001-2004)	Mathematics and Natural Science faculty, University of Cologne Germany. Doctoral work was done under the supervision of Prof. Dr. Angelika A. Noegel in the Institute for Biochemistry I, Medical Faculty, Cologne University, Germany.