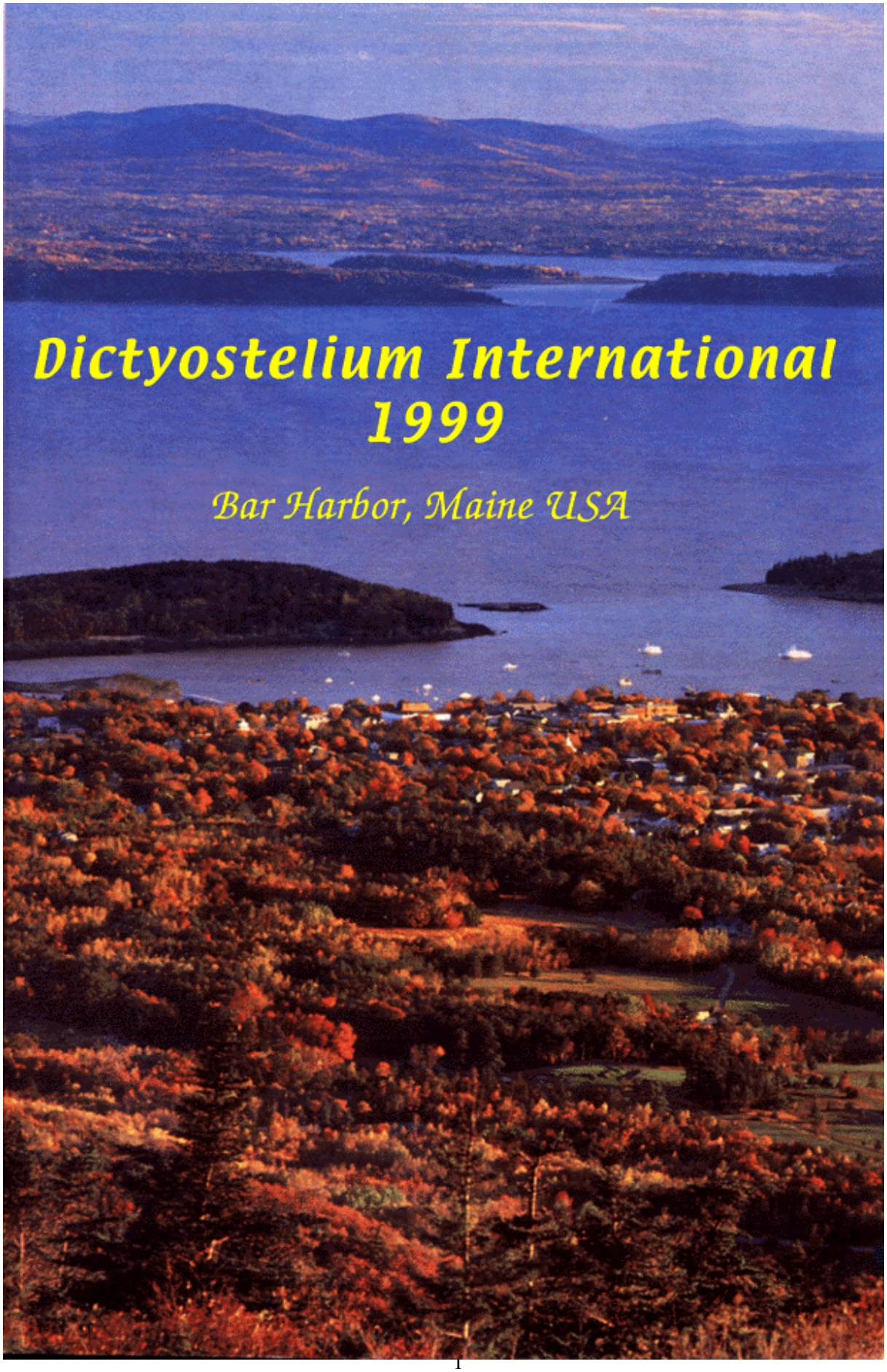
Dictyostelium International
1999
Bar Harbor, Maine USA

1999 INTERNATIONAL *DICTYOSTELIUM* CONFERENCE

BAR HARBOR, MAINE, U.S.A.
COLLEGE OF THE ATLANTIC
AUGUST 14TH- 19TH, 1999

Robert Gundersen[1] and David Knecht[2], Organizers

[1]Department of Biochemistry, Microbiology, and Molecular Biology, University of Maine
[2]Department of Molecular and Cell Biology, University of Connecticut

Acknowledgements

The organizers would like to thank the National Science Foundation, Developmental Mechanisms Program and the Department of Biochemistry, Microbiology, and Molecular Biology at the University of Maine for their generous support of this meeting.

Program

SATURDAY, August 14th

WELCOME DINNER & RECEPTION **5:30 pm**
OPENING COMMENTS AND INTRODUCTION 7:45 pm
PLENARY LECTURE 8:00 pm
"A Hundred Years of Cellular Slime Molds" by Dr. John Bonner

SUNDAY, August 15th

Breakfast: 7:00 – 8:30 AM
SESSION I – CELL DIFFERENTIATION (8:30 – 11:40 am)
Lunch and Afternoon Break: Noon - 3:30 pm
POSTER SESSION I 3:30 - 5:30 pm
Dinner: A, 5:30-6:30 & B, 6:30-7:30
SESSION II - SIGNALING 7:30 - 10:40 pm

MONDAY, August 16th

Breakfast 7:00 - 8:30 am
SESSION III: SIGNALING continued 8:30 - 11:50 am
Lunch and Afternoon Recreation Noon - 3:00 pm
WORKSHOP DISCUSSION: *DICTYOSTELIUM* GENOME 3:00 - 5:00 pm
Dinner: A, 5:30-6:30 & B, 6:30-7:30 pm
SESSION IV - GENE EXPRESSION 7:30 - 10:40 pm

TUESDAY, August 17th

Breakfast: 7:00 – 8:30 AM
SESSION V - Cytoskeleton and Vesicle Trafficing 8:30 - 11:40 am
Lunch and Recreation Noon - 3:30 pm
Afternoon Whale Watch (You must sign up)
POSTER SESSION II 3:30 - 5:30 pm
Dinner: A, 5:30-6:30 pm & B, 6:30-7:30 pm
SESSION VI: CELL ADHESION 7:30 - 10:20 pm

WEDNESDAY, August 18th

Breakfast: 7:00 – 8:30 am
SESSION VII: MORE CYTOSLKELETON 8:30 - 11:40 am
Lunch & Afternoon Recreation Noon - 3:00 pm
SESSION VIII: DEVELOPMENT 3:00 - 5:50 pm
LOBSTER COOKOUT 6:00 - 9:00 pm

THURSDAY, August 19th

Breakfast: 7:00 – 8:30 am
Discussion of NIH Non-mammalian Models 9am

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Chair: Harry MacWilliams

Cell-cycle regulation of the ribonucleotide reductase B gene *of Dictyostelium* discoideum.
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POSTER SESSION I 3:30 - 5:30 pm

Dinner: A, 5:30-6:30 & B, 6:30-7:30

SESSION II - SIGNALING 7:30 - 10:40 pm
Chair: Peter Devreotes

BREAK 8:40 - 9:00 pm

MONDAY, August 16th

Breakfast 7:00 - 8:30 am

SESSION III: SIGNALING continued 8:30 - 11:50 am
Chair: Aline M. da Silva

BREAK 10:10 - 10:30 am

Lunch and Afternoon Recreation Noon - 3:00 pm

WORKSHOP DISCUSSION: *DICTYOSTELIUM* GENOME 3:00 - 5:00 pm
Chair: Gad Shaulsky

Dinner: A, 5:30-6:30 & B, 6:30-7:30 pm

SESSION IV - GENE EXPRESSION 7:30 - 10:40 pm
Chair: Jeff Williams

BREAK 9:00 - 9:20 pm

TUESDAY, August 17th

Breakfast: 7:00 – 8:30 AM

SESSION V - CYTOSKETON & VESICLE TRAFFICKING 8:30 - 11:40 am
Chair: James Cardelli

BREAK 10:00 - 10:20 am

Lunch and Recreation Noon - 3:30 pm
Afternoon Whale Watch (You must sign up)

POSTER SESSION II 3:30 - 5:30 pm

Dinner: A, 5:30-6:30 pm & B, 6:30-7:30 pm

SESSION VI: CELL ADHESION 7:30 - 10:20 pm
Chair: Richard Gomer

BREAK 9:00 - 9:20 pm

WEDNESDAY, August 18th

Breakfast: 7:00 – 8:30 am

SESSION VII: MORE CYTOSLKELETON 8:30 - 11:40 am
Chair: Robert Insall

BREAK 10:00 - 10:20 am

Lunch & Afternoon Recreation Noon - 3:00 pm

SESSION VIII: DEVELOPMENT 3:00 - 5:50 pm
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THURSDAY, August 19th

Breakfast: 7:00 – 8:30 am

Morning free for checkout and sight seeing.

Have a safe trip home.

POSTER SESSION I : Sunday 3:30 - 5:30 pm

POSTER SESSION II : Tuesday 3:30 - 5:30 pm

Cell-cycle regulation of the ribonucleotide reductase B gene of Dictyostelium discoideum.

Harry MacWilliams[1], Pascale Gaudet[2], Claire Bonfils[2], and Adrian Tsang[2]

[1]Zoologisches Institut, Ludwig-Maximilians-Universität, Munich and
[2]Department of Chemistry and Biology, Concordia University, Montreal

The cell cycle is of central importance in the control of differentiation pathway choice in Dictyostelium development. Molecular biological characterization of the Dictyostelium cell cycle has only begun, however; and few cell Dictyostelium cell cycle markers are available. Ribonucleotide reductase is essential for DNA synthesis, and is thus required by all cells, particularly during the S-phase. The enzyme consists of two subunits, and in most organisms the transcription of one or the other genes is regulated by the cell cycle. The gene for the Dictyostelium small subunit and its promoter have been described (Tsang et al, BBA 1309, 100-108 (1996); Bonfils et al, JBC, in press). During development, RNRb promotor activity correlates in space and time with classical descriptions of of cell cycle activity (Bonner and Frascella, J Exp Zool 121, 561 (1952); Durston and Vork, Exp Cell Res. 115, 454.457 (1978).

We find that in untransformed Ax2 cells, cell-cycle synchronized by cold release, the RNRb message rises to a peak at about one hour before S-phase, and thereafter falls to about one fifth of the peak value. When the same experiment is carried out using transformants carrying a destabilized β-galactosidase reporter driven by the 400-bp RNRb promoter, the endogenous RNRb message, the gal message, and the gal activity all show the pattern seen in the endogenous message in untransformed cells. Rather different results are obtained, however, in two other experiments with synchronized cells. If RNRb-destabilized-gal transformants are synchronized by stationary phase release, the reporter activity is maximal 3 hours after S-phase. In cold-synchronization experiments using a destabilized GFP reporter and a low-flourescence growth medium, the reporter fluorescence appears maximal several hours before S-phase.

One might thus be moved to suspect that the "cell cycle" regulation of RNRb is simply an artefact of the synchronization procedure. However, using double-staining for the destabilized gal reporter and for BrdU, one can measure cell cycle regulation without synchronization. Such experiments show that in non-synchronized cells growing in HL-5, RNRb is strongly regulated by the cell cycle and expressed one to several hours before the S-phase.

In mammalian cells, RNR belongs to a class of genes which are induced at or around the G1/S („Start“) restriction point. In Dictyostelium, where G1 is believed to be absent and S-phase is very short, our data suggest that the promoter is turned on in late G2. RNRb promoter activity in Dictyostelium may be a marker of the G2/M transition.

Identification of pathways regulated by YakA that control growth, development and stress responses in *Dictyostelium* cells.

Alexandre Taminato, Douglas Robinson*, Marcos Navarro, Rodrigo Romanhole, Guokai Chen#, James Spudich*, Adam Kuspa# and Glaucia Mendes Souza.

Instituto de Quimica - Dep. Bioquimica - Universidade de Sao Paulo - Brazil
*Stanford University – Dep. Biochemistry - USA
#Baylor College of Medicine - Dep. Biochemistry - USA

YakA is a protein kinase involved in the regulation of the growth to development transition in *Dictyostelium discoideum*. YakA controls the cell cycle by regulating the interval in between cell divisions and upon nutrient starvation inhibits expression of PufA, a negative regulator of PKA-C mRNA translation. YakA's inhibition of growth and vegetative gene expression allows an increase in PKA-C levels that triggers development. In this work we will present evidence that YakA may be signalling growth arrest and entry into development by regulating the Ras pathway. High-copy suppression of *yakA* null cells revealed several members of this pathway, including *gefA*, a Ras GTP-exchange factor, *papA*, a PDGF-associated protein and *stiA*, a hsp90 co-chaperonin as genes regulated by YakA. A role for YakA in the regulation of stress responses has also been determined. *yakA* null cells are highly sensitive to oxidative stress. Several transcription factors were isolated as second-site suppressors of this phenotype indicating that YakA may be a regulator of detoxifying and general stress responses. These include *msnA*, a gene similar to the general stress response transcription factor Msn2 from yeast, and *keaA*, similar to Keap1, an inhibitor of the transcription factor Nrf2 that regulates the expression of genes encoding detoxifying enzymes in humans. Another suppressor, *omsA*, is similar to the omnipotent suppressor from yeast S5, that regulates translational fidelity. This was indicative of a higher rate of mutations in *yakA* null cells that was confirmed by measuring spontaneous rate of mutations in WT and *yakA* null cells. These results indicate a broad role for YakA in the regulation of gene expression in several responses of *Dictyostelium* cells which include growth conditions, nutrient and oxidative stress and development.

Structure and function of a novel gene (*dia1*) specifically expressed during transition from growth to differentiation in *Dictyostelium* cells

Shigenori Hirose, Yuji Inazu, Soo-Cheon Chae* and Yasuo Maeda

Biological Institute, Graduate School of Science, Tohoku University, Aoba, Sendai 980-8578, Japan
*Present address: Center for Molecular Genetics, Department of Biology, University of California at San Diego, La Jolla, CA92093, USA

In *Dictyostelium discoideum* Ax2, there is a specific check-point (PS-point) from which cells exit the cell cycle to differentiate under starvation conditions. Using synchronized cells by temperature-shift method and differential display method, we isolated a novel gene, *dia1* (differentiaion-associated gene 1), as one of genes specifically expressed in cells differentiating from the PS-point. The *dia1* mRNA has an open reading flame of 1,368bp, and codes for 48.6kDa protein (DIA1) consisting of 455 amino acids. The DIA1 protein is highly serine-rich (21.3% of the total amino acid residues), and the serine residues are predominentaly located in the C-terminal region (350-440a.a). After PSORT, the protein is predicted either to be secreted to extracellular space or to be integrated in cell membrane with at least two transmembrane domains. Unexpectedly, the overexpression of *dia1* mRNA was found to impair cell differentiation. That is, the *dia1*-overexpressing cells exhibited delayed aggregation on ager surface after starvation, and stopped their development at the migrating slug stage. Under submerged conditions, the starved cells initiated to aggregate with 1-2 hr delay, as compared with parental Ax2 cells. Surprisingly, however, the once-formed aggregates were dispersed to become round-shaped single cells during further incubation. In contrast to the *dia1*-overexpression, antisense-mediated gene inactivation of *dia1* enhanced the progress of cell differentiation. Although the results obtained seem to be somewhat strange, the biological significance is discussed, with reference to the *dia1*-function in growth/differentiation transition.

Altered Prestarvation Response in Two Dictyostelium Mutants

Marc E. Colosimo and Eugene R. Katz

Department of Molecular Genetics and Microbiology, SUNY Stony Brook, Stony Brook, NY 11794

As wild type amoebae grow, they secrete an autocrine factor, prestarvation factor (PSF), that allows them to measure the amount of food bacteria compared to their cell density. As the cells grow and consume the bacteria, the ratio of PSF to bacteria reaches a threshold, signaling the cells to prepare for eventual starvation. This prestarvation response (PSR) usually occurs three to four generations before the end of exponential growth leading to the accumulation of several aggregation genes, such as discoidin and α-mannosidase. Neither the mechanism by which the cells sense this signal nor the relationship of the prestarvation response to subsequent development is well understood. We have attempted to gain a further insight into the response by studying two related mutants.

The mutant HK19 (nysA201) appears to show an increased sensitivity to PSF. HK19, in the presence of bacteria, expresses discoidin and α-mannosidase during mid-log growth, three generations earlier than the wild type control. Nonetheless, HK19 is able able to grow to normal densities. Discoidin production in HK19 is repressible by folate. Quite interestingly, when mid-log vegetatively growing (with bacteria) HK19 cells are exposed to pulses of either cAMP or folate, clumping of cells and a greatly decreased growth rate are seen. HK19 forms normal fruiting bodies about an hour earlier than wild type.

The mutant HK320 (nysA201,nocA320) shows a complex response to PSF. Growing amoebae appear to constitutively express PSR genes, although amoebae newly germinated from spores require several generations of growth before beginning to express these genes. Unlike HK19, discoidin expression in HK320 is not repressed by folate. In fact, discoidin expression continues at a high level through most of development. CAMP pulses during vegetative growth do not result in cell clumping.

F-box Proteins and Mutants That Cheat

Herbert L. Ennis, Dee N. Dao, Stefan U. Pukatzki, and Richard H. Kessin

Department of Anatomy and Cell Biology, Columbia University
630 West 168th St., N.Y., N.Y. 10032 USA

Dictyostelium development is a cooperative event, but it is difficult to explain how it evolved. One of the problems, as pointed out by Buss and others, is that in the wild, parasitic forms *Dictyostelium* should arise that form only spore cells (Armstrong, 1984; Buss, 1982; Buss, 1999). Such parasites, not having to contribute 20% to the stalk cells, would increase in the population and eventually ruin the cooperativity that leads to fruiting body formation. There have been two reports that in freshly isolated populations of cells, variants exist that preferentially make spores in chimeras with wild-type cells (Filosa, 1962;Buss, 1982)

The mechanisms that these cells, which we call *cheaters,* use to avoid the stalk cell fate in a chimera could be based on manipulations of cell-cell communication. If such strains can be produced in the laboratory, the basis of the parasitism, and the communication among cells during normal development can be studied in a way that is independent of previous approaches.

Insertional mutagenesis methods allowed us to create and select a mutant that cheats and to recover the affected gene. When developed alone, the terminal phenotype of the *cheater* A (*chtA*) mutant is a long slug that does not culminate to form a fruiting body. The slugs of a *chtA* mutant have expanded prespore regions. In chimeras with wild- type, *chtA* cells form only spores. Cheating is not passive – the *chtA* cells prevent the wild-type cells from becoming spores. See the accompanying poster for details.

The *chtA* gene codes for a developmentally regulated transcript that has the characteristic motifs of F-box proteins, including an F-box and WD40 repeats. In other F-box proteins, this domain interacts with a specific complex that contains ubiquitinating enzymes, with WD40 domains interact with one or more target proteins, thus bringing them into contact with the proteolysis machinery. The role of F-box protein in other organisms is to mediate the destruction of specific phosphorylated proteins, consequently we speculate that ChtA is essential for the destruction of proteins that are involved in the decision to culminate. We present a model to explain the cheating behavior.

Armstrong, D. P. (1984). Why don't cellular slime molds cheat? *J. Theor. Biol.* **109**, 271-283.
Buss, L. W. (1982). Somatic cell parasitism and the evolution of somatic tissue compatibility. *Proc. Natl. Acad. Sci. USA* **79**, 5337-5341.
Buss, L.W. (1999). Slime molds, ascidians, and the utility of evolutionary theory. Commentary. *Proc. Natl. Acad. Sci. USA*, in press.
Filosa, M. F. (1962). Heterocytosis in cellular slime molds. *Am. Naturalist* **XCVI (no. 887)**, 79-92.

Nuclear translocation of the Dd-STATc protein in response to DIF

Masahi Fukuzawa, Tsuyoshi Araki and Jeff Williams
The University of Dundee, MSI/WTB Complex, Dow St, Dundee DD1 5EH

Dd-STATc was isolated using the SH2 domain of Dd-STATa as a probe and the two proteins are highly homologous in their terminal halves. However, Dd-STATa is nuclear localised in pstA cells in the slug tip while Dd-STATc is nuclear localised in the pstO cells and in ALC. Also, while Dd-STATa translocates to the nucleus in response to extracellular cAMP, Dd-STATc translocates to the nucleus in response to DIF. We have identified regions within Dd-STATc that direct its regulated movement to the nucleus and we have begun to identify proteins that interact with these sequences. These results will be discussed with respect to the mechanism of DIF action.

Development in the Absence of DIF-1

Christopher Thompson and Rob Kay
MRC Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, UK

The signaling molecule DIF-1 has been proposed to play a central role in the control of stalk and spore differentiation during *Dictyostelium* development. However, most evidence comes from studies in cell culture because genetic approaches to the study of DIF biosynthesis have not been wholly successful.

Two major questions thus remained unresolved: 1. Which cells make DIF and how is DIF biosynthesis regulated? 2. What is the effect of development in the absence of DIF?

To address these issues, we set out to gain a biochemical understanding of the DIF biosynthetic pathway with the ultimate goal thus being the cloning and disruption of genes required for DIF biosynthesis. We characterised enzyme activities implicated in DIF biosynthesis and cloned the gene for the final step in the pathway, the des-methyl-DIF-1 methyltransferase (*dmtB*). The product of the gene appears to be indistinguishable from the endogenous activity by all tested criteria and its developmental regulation closely mirrors DIF-1 biosynthesis. Furthermore there is no detectable des-methyl-DIF-1 methyltransferase activity in *dmtB* disruptants. The regulation of *dmtB* thus gives a good measure of DIF biosynthesis. We find that DdmtB activity is localised in prespore cells, induced by cAMP and repressed by DIF thus giving rise to model for cell fate choice and proportioning.

DIF-1 is undetectable in the *dmtB* knockout strain as assayed both by chlorine labelling and HPLC. However, examination of the mutant phenotype and expression of prestalk and prespore markers is somewhat surprising. Firstly, development is slow and very much perturbed, resulting in the formation of long, thin and twisted fingers/slugs. However, eventually, rather abnormal culminants do form with most of the stalk tube on the substratum and the spore head only slightly raised. Secondly, not only are prespore and DIF-induced prestalk genes expressed at comparable levels to the wild type, but also examination of the expression of specific markers of cell types (by *in situ* hybridisation or in lacZ transformants) reveals that patterning is unperturbed. These results cannot be explained by a build up of the DIF-1 precursor des-methyl-DIF-1 as we have determined that it has no stalk cell inducing bioactivity. However, stalk inducing bioactivity in extracts of the mutant is comparable to wild type. We have performed preliminary HPLC experiments to study the identity/nature of this activity which reveal it to be novel and absent (or at much lower levels) in the wild type. Furthermore, this novel activity drives expression of DIF responsive genes through the same minimal DNA sequences required for DIF action.

We have thus identified a novel activity which is upregulated in the DIFless mutant, the characterisation of which is ongoing. These results provide evidence that DIF function is critical for normal development and that compensatory mechanisms come into play in its absence. This goes some way to explain why an analysis of DIF biosynthesis has remained intractable to genetic approaches.

Tale of Two Transporters: Evidence for the involvement of TagA and RhT in cell-type proportioning.

J. Randall Good and Adam Kuspa
Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030 USA

We have identified a prespore-specific transporter, RhT, with properties of the MDR-class of ATP-binding cassette (ABC) transporters. The activity is defined by its ability to transport the fluorescent dye rhodamine 123 (Rh123) and its inhibition by low temperature, energy poisons and standard inhibitors of MDR transporters, such as verapamil. All vegetative cells have the capacity to transport Rh123. During development, however, prestalk cells appear to lose their RhT activity. Thus, prestalk regions become stained in developing structures after treatment with Rh123, while prespore cells appear unstained. By examining RhT activity in dissaggregated slug cells marked with GFP reporter genes, we found that cotB-expressing cells transport Rh123 while cotB-negative cells do not, consistent with the in vivo staining pattern. To investigate the potential role for RhT in development, we examined the effects of RhT inhibitors on cell differentiation. The RhT inhibitors were found to induce stalk cell formation in submerged culture with similar dose-response curves to their Rh123 transport inhibition activities. Cerulinin is an inhibitor of polyketide synthase which specifically inhibits DIF biosynthesis and stalk cell induction in submerged culture (1). Cerulinin abolished the induction of stalk cells by the RhT inhibitors, but this effect could be reversed by DIF. These results are consistent with the notion that RhT inhibitors induce stalk cell formation by blocking the export of endogenously synthesized DIF. We hypothesize that RhT is a prespore-specific DIF transporter which is involved in the maintenance of the prestalk/prespore ratio for the following reasons: 1) the RhT has the properties of an MDR subclass of ABC transporters which are known to transport small lipophilic compounds; 2) DIF is chemically similar to the RhT substrate and inhibitors; 3) DIF inhibits the transport activity of RhT, consistent with substrate competition; and 4) the RhT inhibitors induce stalk cell formation in submerged culture and this effect is blocked by a specific inhibitor of DIF biosynthesis.

We have also used PCR to identify genes that may encode MDR-like transporters. To date, we have identified over a dozen genes, including members of the MRP and Tag families that have been described previously (2,3). One of these genes, *tagA*, encodes a protein similar to TagB and TagC, the serine protease/membrane transporters thought to transport peptide signals important for cell differentiation (2, 4). TagA mRNA is detectable at 2 and 4 hours of development. TagA-null cells aggregate into relatively normal mounds, but form tips precociously. At high cell densities on filters, multiple tips form from a single aggregate. TagA-null cells produce 2.5-fold more *ecmA*-positive cells, compared with wild-type cells, and correspondingly fewer spores. Mosaic analyses suggest that the increased propensity of TagA-null cells to form prestalk cells is cell-autonomous.

1. R. R. Kay (1998). J. Biol. Chem. 273(5):2669-2675.
2. G. Shaulsky et al. (1995). Genes & Devel. 9:1111-1122.
3. R. Sucgang et al. (1999). *Dictyostelium* Meeting Abstract.
4. C. Anjard et al. (1998). Devel. Biol. 193:146-155.

Chemoattractant sensing in eukaryotic cells.

C.A. Parent, W. M. Froehlich, J. Borleis, and P.N. Devreotes
Department of Biological Chemistry, Johns Hopkins School of Medicine, Baltimore, MD 21205

Directional sensing is an essential physiological process displayed by many eukaryotic cells. In phagocytic cells such as leukocytes and the free-living amoebae *D. discoideum*, chemotaxis is mediated by heterotrimeric guanine nucleotide binding protein (G protein)-linked signaling pathways. By exploiting the properties of the green fluorescent protein (GFP) to visualize signaling components in live cells, we have previously shown that CRAC, a pleckstrin homology (PH) domain-containing protein, is recruited to the plasma membrane selectively at the stimulated edge of chemotaxing cells (C.A. Parent, et al. Cell 95: 81 (1998)). More recently, the PH domain of PKB was also shown to be selectively recruited to the plasma membrane (R. Meili, et al. EMBO J. 18: 2092 (1999)). We have now established that the highly dynamic nature of CRAC's recruitment to the plasma membrane is dependent upon its PH domain. Moreover, this recruitment is inhibited by caffeine. In order to identify the binding site for the PH domain, we developed an in vitro assay where the GTP_S-mediated association of the PH domain with the plasma membrane remains stable for up to 15 minutes. Using this assay we show that inhibitors of PI3 kinase, Wortmannin and LY294002, block the association of the PH domain with activated membranes. In order to assess if other PH domains behave like CRAC's PH domain, we fused GFP to the PH domain of the mammalian BTK (PHBTK-GFP) and GRP1 (PHGRP1-GFP) proteins, transformed these constructs in AX3 cells, and observed the cellular localization of the fusion proteins under basal and stimulated conditions. Under basal conditions, the PHBTK-GFP cells showed an even cytosolic signal. However, contrary to the PHCRAC-GFP cells, the fluorescent signal did not change following receptor stimulation. Conversely, the PHGRP1-GFP cells showed fluorescent signal on the plasma membrane in both unstimulated and stimulated cells. We conclude that (1) not all PH domain containing proteins are recruited to the plasma membrane following chemoattractant receptor stimulation and (2) phosphorylated inositol phospholipids are involved in the PH domain binding site.

Dynamic localization of a heterotrimeric G-protein _-subunit in living cells

Tian Jin*, Ning Zhang, Yu Long, Carol Parent, Peter Devreotes

Department of Biological Chemistry, Johns Hopkins University School of

Medicine, Baltimore, MD 21205

Chemotactic eukaryotic cells display different sensitivity to chemoattractants on their surface. Previous studies have shown that chemoattractant receptors are uniformly distributed on the surface of the cells. We find G-protein _-subunits are present both in the cytosol and on the plasma membrane. In highly polarized cells, chemotactic sensitivity and G-protein _-subunits associated with the cell surface are localized in a shallow anterior-posterior gradient. In addition, uniformly applied stimuli recruit PH-domains preferentially to the anterior. When a loss in polarity is induced by treatment with inhibitors of actin polymerization, the membrane-associated _-subunits and the sensitivity of PH-domain recruitment become evenly distributed. Our observations suggest that the local concentration of G-proteins determines the relative chemotactic sensitivity of polarized cells, which provides a simple explanation to on fundamental question of chemotaxis-why a chemotactic cell displays behavior polarity.

Study of G-protein _-subunit from *Dictyostelium discoideum*

Ning Zhang, and Peter N. Devreotes

G-protein mediated signal transduction pathways play a crucial role in the developmental program of the simple eukaryotic organism *Dictyostelium*. In *Dictyostelium*, there are eleven G_s and a single G_ identified. We report here the purification of the G_-subunit and isolation of its cDNA. A greater than 20,000 fold of purification yielded about 2 _g G_. Degenerate PCR primers, based on the sequence of NH_2-terminal 21 amino acids, were designed to isolate a DNA probe. The isolated probe was then used to isolate the full-length cDNA of G_. Its amino acid sequence consists of 68 residues. Like G_s from other species, it contains two and a half _-helix, which can form stable coiled-coil with NH_2-terminal of G_. There is a CXXL motif at COOH-terminal that can be modified by a lipid to target G__ to the cytoplasmic membrane. Northern analysis suggests that G_ and G_ are expressed in parallel throughout development. To study its function, we introduced CXXL-deleted G_(CDG) into *wt* to compete with endogenous G_. Overexpression of CXXL-deleted G_ shifts G__ to cytosol, which impairs ligand-induced actin polymerization and chemotaxis. However, G-protein mediated cAMP production and cGMP production are the same as *wt*. Our data suggest that cytosolic G__ is capable of transducing "global" signaling, such as cAMP and cGMP production. Membrane localization of G__ is required for cells to response to a gradient, or to give localized response.

Role of G-Protein carboxyl methylation in signal transduction

J. Stock, Y. Chen, and T. Cox.

All heterotrimeric G protein and most small molecular weight Ras-related G proteins are reversibly methyl esterified at carboxyl terminal prenylcysteine residues. In human neutrophils, increases in Rho/Rac modification have been associated with inflammatory responses and prenylcysteine methyltransferase inhibitors have been shown to be anti-inflammatory (Phillips et al., Science,1993, 259:977-980). A genetically tractable model system with neutrophil-like signaling systems was required to further investigate the role of G-protein methylation in signal transduction. *Dictyostelium* was the obvious choice. Results will be presented demonstrating cAMP-induced changes in G-protein methylation similar to those that have previously been observed in neutrophils in response to the chemotactic peptide fMetLeuPhe. The *Dictyostelium* G-protein methyltransferase has been cloned and sequenced. Expression of this gene increases dramatically during early development and is then repressed subsequent to aggregation. A deletion has been constructed, and the phenotype of a methylation-deficient strain has been characterized. The
results are interpreted in terms of previously proposed "targeting" models for the role of methylation in eukaryotic signal transduction.

CONTROL OF CHEMOTAXIS BY PI3 KINASE-REGULATED PATHWAYS

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Dictyostelium PI3 kinase is required for proper chemotaxis and rearrangements of the actin cytoskeleton. Previously, we described the phenotypes of a null mutation of the *Dictyostelium* homologue of mammalian Akt/PKB. These cells are unable to properly polarize in cAMP gradients and show abnormal chemotaxis. We demonstrated that the PH domain of the protein rapidly and transiently translocates to the plasma membrane in the direction of the cAMP gradient. We believe that this transient translocation is important for the activation of Akt and provides evidence for localized phospholipid binding sites at the plasma membrane being essential for activating localized response at the plasma membrane and controlling directional movement.

We have additional evidence that the activation of PI3 kinase, possibly via a Ras pathway, is essential for mediating Akt activation and controlling directional and localized responses within the plasma membrane. We show that point mutants in PI3 kinase that abrogate interaction with Ras impair the activation of PI3 kinase. We demonstrate that in PI3 kinase null mutants that Akt is unable to translocate to the plasma membrane, indicating an important role for PI3 kinase in mediating this response.

Further support for our findings and models derives from analysis of pdkA, a PH domain-containing protein that is required for proper activation of adenylyl cyclase and chemotaxis. pdkA, like Akt, translocates to the plasma membrane in response to cAMP signaling and is highly localized in the area and direction of the chemoattractant source. This protein does not translocate in the PI3K double knockout mutant. Point mutations in the PH domain that should abrogate its interaction with $PI(3,4,5)P_3$, the product of PI3 kinase, drastically affect translocate, arguing that activation of PI3K is required for this process.

We have analyzed an Akt/PKB-related gene, PKBR1. The kinase and C-terminal domains are highly related to those of Akt including conserved phosphorylation sites by an upstream kinase. However, instead of an N-terminal PH domain, PKBR1 has a putative myristoylation site. Tagged versions of the protein are localized in the plasma membrane, whereas tagged versions of protein that would block the ability of the protein to be myristolated are cytoplasmic. We demonstrate that, like Akt, the protein is rapidly activated in response to cAMP signaling. This gene is expressed early in development but shows an increase of expression at the mound stage, the time at which *bona fide* Akt protein disappears from cells. Null mutants of PDKR1 arrest at the mound stage. Interestingly, expression of Akt from a GBF promoter at the mound stage complements the PKBR1 null phenotype, suggesting they may have common downstream substrates. The relationship between activation by cAMP and PI3K will be discussed.

A double knockout of Akt and PKBR1 results in cells that grow very slowly. Most interestingly, these cells are unable to polarize or move in cAMP gradients. This finding suggests that the ability of *akt* null cells to move, although very poorly, may be due to partial complementary effects of PKBR1 that is expressed during the aggregation stage. These results further support the model that PKB and PKB-related genes are essential for controlling cell polarity and chemotaxis.

A p21-activated protein kinase, PAKa, is required for cytokinesis and the regulation of the cytoskeleton in *Dictyostelium* cells during chemotaxis

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We have identified a *Dictyostelium* gene encoding a serine/threonine kinase, PAKa, that is a member of the Ste20/PAK family of p21-(Rac/Cdc42)-activated protein kinase. In chemotaxis assays, *paka* null cells produce many random, lateral pseudopodia, chemotax very inefficiently, and have a much higher frequency of making wrong turns than wild-type cells. We find that PAKa co-localizes with myosin II to the cleavage furrow of cells undergoing cytokinesis and the posterior of polarized, chemotaxing cells, which is consistent with the defects of the null strain in cytokinesis and cell movement. PAKa appears to be required for the formation and/or maintenance of the cortical cap of myosin II at the posterior of cells, as this myosin cap is absent or significantly reduced in *paka* null cells. PAKa kinase activity is rapidly and transiently stimulated in response to cAMP, suggesting that PAKa activity is highly regulated. Our data suggest that PAKa regulates myosin II filament assembly in the posterior cortex in response to chemotattractant stimulation, which is important to maintain polarity and restrict the formation of random, lateral pseudopodia. As PAKa does not appear to phosphorylate myosin II, we suggest that PAKa functions, in part, to negatively regulate myosin II heavy chain kinase leading indirectly to an increase in myosin II assembly. Using a GFP fusion, we demonstrate that the N-terminal region lacking the CRIB and kinase domains is sufficient for the subcellular localization of PAKa in live, chemotaxing cells. In response to receptor-saturating concentrations of cAMP, which leads to a loss of cell polarization, PAKa delocalizes along the cortex of the whole cell. The activation of PAKa upon cAMP stimulation appears to be absent or delayed in *pkb* null cells. A PAKa mutant lacking the putative PKB phosphorylation site in the regulatory domain does not delocalize upon the loss of cell polarization, which suggests the regulation of PAKa localization via PKB signaling pathway. We suggest that the localization of PAKa in a subdomain of the cell is an essential part of the mechanism that controls polarization and chemotaxis of cells.

A temperature sensitive ACA mutant.

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Until recently only two adenylyl cyclases had been identified in *Dictyostelium*, one of which, ACA, has been shown to be essential for cAMP signalling during aggregation. ACA has the typical twelve membrane-spanning structure of mammalian forms of the enzyme and is activated by a G-protein dependent mechanism initiated by binding of extracellular cAMP to plasma membrane cAMP receptors. ACG has a single transmembrane-spanning domain and is thought to be an osmosensor expressed only during spore germination. In order to gain further insight into the developmental roles of ACA we have isolated a temperature sensitive mutant from a PCR-mutagenised library kindly provided by Carole Parent and Peter Devreotes. The mutant (ACA-ts2) was obtained by transforming ACA null cells with a library of the autonomously replicating expression vector pCP33 incorporating an ACA gene part of which had been amplified by error prone PCR and which was driven by the actin15 promoter. The mutant aggregates and fruits when plated at 22C and remains flat when developed at 26.8C. We have tried to determine how far into development ACA is required by shifting the ts mutant at various times from 22C to 26.8 degrees. We find that developing populations shifted to the non-permissive temperature at any time up to about 7 hours cease development and revert to a flat morphology. On the other hand populations shifted after 7-10 hours continue development, form normal migrating slugs and eventually perfectly normal fruiting bodies. We will report further observations on the mutant.

Wave propagation in *Dictyostelium discoideum* slugs

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Based on the observation of periodic movement of cells in the prespore zones of slugs we proposed that their movement was controlled by propagating waves of cAMP that originated in the tip. Up to now we were only able to visualise the associated optical density waves in Dictyostelium mucoroides slugs. We report the presence of optical density waves in slugs of *Dictyostelium discoideum*. A periodic signal is generated in the slug tip and then relayed along the prespore region. This was observed in a number of strains including AX2 and the wild-type strain NC4 but not in AX3 or DH1, possibly reflecting differences in the excitability of different strains. Wave velocity is slightly slower than in mounds, while wave period is considerably longer. However, we found that there is a very close correlation between wave propagation and periodic cell movement. That the signal waves are initiated in the tip was confirmed by experiments in which the tip was removed. Waves already present in the prespore region propagated to the end, however no further waves were initiated, since the pacemaker in the tip was missing. Isolated tips continued to migrate with optical density waves still visible. These results support the view that cell movement in slugs is controlled by the propagation of a chemotactic signal that originates in the slug tip. We will present further experimental evidence using a variety of mutants showing that this signal is almost certainly cAMP.

Light affects cAMP signaling and cell movement activity in *Dictyostelium discoideum*

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(Video sequences will be presented.)

The multicellular slug stage of the cellular slime mould *Dictyostelium discoideum* responds in a very sensitive manner to external stimuli such as temperature and light. Within the migrating slug the behavior of up to 100,000 individual amoeba is coordinated by cell-cell signaling and chemotaxis. Two different hypotheses have been proposed to explain phototactic turning. The differential speed hypothesis assumes that light locally speeds up cell movement in the tip thus leading to bending of the anterior zone towards the light source (Bonner, 1994). The tip activation/inhibition hypothesis assumes that light acts directly on cell-cell signaling by shifting the position of the organizing center in the tip (Fisher, 1997). To examine these hypotheses, we investigated the influence of light irradiation on cell-cell signaling and cell movement at different stages during multicellular development. Cell movement was observed using near infrared light that was inactive for phototaxis. We found that light acts directly on the cAMP signaling system. While pre-aggregation cells did not change their movement activity by light irradiation, aggregating cells changed their periodicity of cAMP signaling and slug tip cells released cAMP upon light irradiation. Concomitant changes in cell velocity occurred only in slug cells. Thus, the effect of light on cell movement activity seems to be dependent on a modulation of cell-cell signaling. These results suggest that the two previously proposed hypotheses on slug phototaxis should be merged into a single hypothesis, in which changes in both cell-cell signaling and cell movement orient a slug towards a light source.

Bonner, J.T. 1994. The migration stage of dictyostelium: Behavior without muscles or nerves. *Fems Microbiol Lett*. 120:1-2.

Fisher, P.R. 1997. Genetics of phototaxis in a model eukaryote, Dictyostelium discoideum. *Bioessays*. 19:397-407.

In vitro approaches to characterize and identify cAR1 kinase

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Like other G protein-coupled receptors, the aggregation-stage cAMP receptor of *Dictyostelium*, cAR1, undergoes reversible ligand-induced phosphorylation of C-terminal domain serine residues. Our understanding of the function of cAR1 phosphorylation derives largely from previous studies of phosphorylation-deficient alleles. These revealed an important role for phosphorylation in lowering the receptor's affinity for cAMP. In addition, our observation of a dominant mound-stage arrest when these alleles were over-expressed suggests a role for phosphorylation in inactivating cAR1 at this stage. Despite being highly correlated, the mutant alleles demonstrated that cAR1 phosphorylation is not essential for the adaptation of adenylyl cyclase activation and other transient cAR1-mediated responses.

Towards identifying the kinase responsible for cAR1 phosphorylation ("cAR1 kinase"), we are developing the means to detect and assay the cAR1 kinase in a cell-free system so that it may be purified. We have demonstrated an activity in crude lysates and in washed membranes which phosphorylates the endogenous receptor with the appropriate ligand-dependency and specificity and, furthermore, effects the electrophoretic shift seen *in vivo*. However, we have not yet found means to dissociate the activity from the receptor that would be suitable for a reconstitution assay. We have, therefore, explored alternative substrates. Neither a synthetic peptide, based on the major target of cAR1 phosphorylation, nor cAR1 expressed in insect cell membranes served as a phosphate acceptor when incubated with *Dictyostelium* lysates. In contrast, an enriched preparation of cAR1 (CHAPS-insoluble floating fraction or CHIFF) and a GST fusion protein containing the receptor's C-terminal domain were both phosphorylated upon reconstitution by a soluble activity.

In a separate effort, we have begun to characterize cAR1 kinase in terms of its sensitivity to various Ser/Thr kinase inhibitors. *In vivo* cAR1 phosphorylation, monitored as an electrophoretic shift on immunoblots, was found to be impaired by the cell-permeant inhibitor H89 in a dose-dependent manner (IC_{50}=65 μM), well above the reported dose required for PKA inhibition but within the range of a variety of other protein kinases. Staurosporine and K-252a, on the other hand, did not result in inhibition of *in vivo* phosphorylation. We are extending this inhibition profile for cAR1 kinase to better define which class of kinase it is and to assess the relevance of the *in vitro* activities described above.

Activated G_ subunits inhibit multiple signal transduction pathways.

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Eukaryotic cells respond to many different environment signals by way of G protein-mediated signal transduction pathways and some of these pathways have profound influences on cell fate. Mutant G_ subunits with impaired GTPase activity are thought to alter signal transduction presumably due the maintenance of an activated state. A mutation analogous to those found in "activated" G_ subunits was introduced to *Dictyostelium* G_4 subunit gene (Q200L substitution) to determine if an altered G_4 subunit affected signal transduction. Expression of the G_4-Q200L subunit from a high-copy-number vector is detrimental to cell viability but expression of the subunit from a low-copy-number vector partially inhibited G_4-mediated responses to folic acid, including the accumulation of cyclic nucleotides and chemotactic cell movement. In addition, the G_4-Q200L subunit severely inhibited responses to cAMP, including cyclic nucleotide accumulation, cAMP chemotaxis, and cellular aggregation. Analogous mutations in the G_2 subunit (Q208L substitution) and the G_5 subunit (Q199L) also were found to inhibit both folic acid and cAMP chemotactic responses and aggregation. All aggregation-defective G_ mutants were capable of multicellular development after a temporary incubation at 4^{o} C and this development was found to be dependent on wild-type G_4 function.

A Novel Secreted Protein Complex That Facilitates Receptor/G-Protein Mediated Chemotaxis

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Dictyostelium and mammalian cells share many complex signaling networks mediated by 7-transmembrane receptor/G-protein coupled pathways. The pulsatile release of cAMP during the early development of *Dictyostelium* directs the chemotatic migration of 10^5 cells into patterned multicellular structures. The extracellular cAMP signal is transduced through the membrane by specific receptor/G-protein pathways that transiently activate adenylyl cyclase (AC). This response rapidly adapts to a persistent cAMP signal. While G__ is implicated in AC activation, the mechanism for adaptation of the chemotatic response to cAMP is unknown. We identified a novel G_ (G_9) that is highly similar to G_ proteins belonging to the "inhibitory" G_ subclass, although G_9 lacks the carboxy-terminal ADP-ribosylation site found in most members of this class. Our data suggest that G_9 may participate in the adaptation pathway. Specifically, we created G_9-null strains and strains that express constitutively activated forms of G_9 (G_9^{G38V} or G_9^{Q196L}). G_9-null cells form significantly smaller mounds relative to wild-type cells and are also hyperactivated for the cAMP response. These data are consistent with the loss of an inhibitory function in G_9-nulls. In contrast, both G_9^{G38V} and G_9^{Q196L} cells display an opposite phenotype relative to G_9-nulls. When developed on agar or in monolayer, G_9^{G38V} and G_9^{Q196L} cells form extremely large aggregation territories, with streams stretching distances greater than two centimeters when developed at standard densities.

Dictyostelium is sensitive to a variety of secreted factors that regulate chemotaxis and development. Since the developmental defect of G_9-null strains occurs during early development, we were interested if G_9-nulls responded differently to factors secreted by wild-type cells. We identified a new factor secreted by developing *Dictyostelium* that potentiates the chemotatic response to cAMP. G_9-nulls are hypersensitive to this factor, responding more quickly and at cell densities 8 times lower than wild-type when developed in monolayer. These results again are consistent with the loss of an inhibitory response. Using this assay, we purified the factor to homogeneity. Western blot analysis (anti-CMF, kind gift of R. Gomer), as well as other physical data, indicate that this factor is distinct from CMF. This new factor is a glycosylated multimeric complex; two proteins have been individually isolated and are being sequenced. These intriguing observations suggest that G_9 is part of a complex signaling network that mediates cell movement under conditions of varied cell density. This network may provide a model in which to understand the complexities of chemotaxis and signaling in *Dictyostelium* and may be extended to neutrophil response and assembly of mammalian tissues and organs during metazoan development.

The Late Adenylyl Cyclase Gene *acrA* is Essential for Sporulation and Necessary for Slug formation in acaA$^-$ pkaC^{over} Cells

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By screening morphological mutants generated by Restriction Enzyme Mediated Integration (REMI) we discovered a novel adenylyl cyclase gene, *acrA*, that is expressed at low levels in growing cells and at more than 25 fold higher levels during development. Growth and development up to the slug stage are unaffected in *acrA*$^-$ mutant strains but the cells make almost no viable spores and produce unnaturally long stalks. A few percent of the prespore cells encapsulate into defective spores. Unlike wild type spores in which the galactose polysaccharide is sequestered from exogenously added ricin, *acrA*$^-$ spores bind ricin. The sporulation defect of *acrA*$^-$ is cell autonomous indicating that wild type cells expressing ACR do not secrete a factor that can rescue the mutant.

In wild type cells adenylyl cyclase activity increases during aggregation, dips during the slug stage and then increases considerably during terminal differentiation. The increase in activity following aggregation fails to occur in *acrA*$^-$ cells. Moreover, the basal level of adenylyl cyclase activity observable in vegetative cells is missing in *acrA*$^-$ cells. Unlike ACA which is activated by manganese and GTP_S, ACR is preferentially active in the presence of magnesium and unaffected by GTP_S. These properties of the *acrA* dependent enzyme activity are similar to those of the vegetative activity observed by Kim, et al. (1998).

We have found that some of the defects in terminal differentiation in *acrA*$^-$ strains can be overcome by introducing a high-copy vector in which actin 15 drives expression of a constitutively active form of *acaA*. The hundred fold increase in the number of viable spores in this transformed line indicates that the major role of ACR in wild type cells is to produce sufficient cAMP during culmination. However, spores formed in this transformed line fail to remain dormant indicating that the act15::acaA construct is not sufficient for spore maintenance.

Using homologous recombination of a disrupted form of *acrA* we generated a strain that lacks both ACA and ACR and overexpresses PKA-C from the *acaA*$^-$ *pkaC*^{over} strain of Wang and Kuspa (1997). Like the host strain, the double mutant strain grows well and can form mounds if plated at high cell density. However, further development is arrested and no spores are formed when both ACA and ACR are missing even if such cells are developed as chimeras with wild type cells. The mutant strain has no measurable adenylyl cyclase. It appears that either ACA or ACR is sufficient for the formation of migrating slugs, but when both are missing, cells arrest at the mound stage. Expression of cells type specific genes (*ecmA* and *cotB*) is markedly reduced in cells lacking both adenylyl cyclases even though *pkaC* is overexpressed. The possibility of a feedback loop connecting PKA-C and ACR is being explored.

Although ACR is necessary for sporulation, it does not appear to be sufficient. Strains lacking the SDF-2 receptor histidine kinase, DhkA, fail to sporulate efficiently and yet have as much ACR activity as wild type strains during culmination. It appears that cAMP generated by ACR is rapidly broken down by RegA unless the signal transduction pathway leading from SDF-2 to inhibition of this cytoplasmic phosphodiesterase is intact.

We will also describe the results of on-going experiments with a strain in which both *rdeA* and *acrA* are disrupted.

Mutant analysis of chemoattractant-induced $[Ca^{2+}]_i$ signals in response to folate and cAMP using the aequorin method.

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Recent evidence suggests that receptor-activated Ca^{2+} signals in response to cAMP proceed via multiple G-protein-dependent and -independent Ca^{2+} mobilization pathways, possibly involving depletion of IP_3-sensitive stores and/or increases in intracellular cGMP levels. Compared to the transduction of chemotactic cAMP signals, the pathway(s) regulating folate-induced Ca^{2+} responses have been neglected. The present study was designed to genetically dissect and compare the role of heterotrimeric G-proteins and the second messengers IP_3 and cGMP in regulating receptor-activated $[Ca^{2+}]_i$ signals in response to both folic acid and cAMP.

It was found that folic acid receptor-mediated changes in $[Ca^{2+}]_i$ require the presence of G_4__ protein, as shown by the inhibition of $[Ca^{2+}]_i$ increase in aequorin-expressing G_ (LW6/AEQ) and G_4 null mutants (g_4/AEQ) and the restoration with altered kinetics and temperature-sensitivity in G_ null mutants over-expressing wild-type (LW6/G_+AEQ) and temperature-sensitive G_ isoforms (LW6/G_ts+AEQ). This contrasts with the reported G-protein-independence of Ca^{2+} responses to cAMP in differentiated cells, which is confirmed by temperature-shift experiments in LW6/G_ts+AEQ mutants. Neither folate nor cAMP-induced $[Ca^{2+}]_i$ changes are significantly altered in a heteroautotrophic PLC null transformant (plc^-/AEQ), suggesting that IP_3-dependent Ca^{2+} release from intracellular stores and/or capacitative Ca^{2+} entry across the plasma membrane play little or no role in chemotactic signal transduction. In contrast, $[Ca^{2+}]_i$ changes elicited by both attractants are significantly prolonged in two *stm*F mutants lacking cGMP-specific phosphodiesterase activity (NP368/AEQ, NP377/AEQ). This confirms an important role of cGMP in regulating the Ca^{2+} uptake and/or extrusion system of *Dictyostelium* amoebae. In response to cAMP stimuli, this cGMP-dependent component of the Ca^{2+} response appears to be developmentally down-regulated.

We conclude that the folate-induced $[Ca^{2+}]_i$ response requires a G_4__-coupled receptor and is mediated by cGMP, whereas cAMP receptor-activated $[Ca^{2+}]_i$ changes appear regulated in a more complex manner by multiple pathways - one being cGMP-dependent (presumably G-protein dependent) and present at the early aggregation stage while the other is G-protein-independent and dominant once tight aggregates have formed.

An IP_3-Like Receptor is Essential for Receptor-Mediated Ca^{2+} Influx

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During *Dictyostelium* development, rapid transient elevations in free cytoplasmic Ca^{2+} levels $[Ca^{2+}]_i$ are associated with cell movement and aggregation whereas slower long-term increases in $[Ca^{2+}]_i$ may mediate changes in gene expression and prestalk cell differentiation. Elevation of $[Ca^{2+}]_i$ by the chemoattractants folate and cAMP is brought about by the activation of a single Ca^{2+} entry pathway which may involve the emptying of intracellular Ca^{2+} stores. In higher eukaryotes, the IP_3 receptor acts as an intracellular Ca^{2+} release channel mediating the emptying Ca^{2+} stores which, in turn, activates Ca^{2+} entry across the plasma membrane by the capacitative entry pathway.

Starting from a clone provided by the Japanese EST project, the entire 9.8kb coding region of a *Dictyostelium* IP_3-like receptor ($DdIP_3R$) was obtained and mapped to chromosome 6. Although database searches revealed the stongest homology to IP_3 receptors, the *Dictyostelium* gene also shared homology with ryanodine receptors which are another class of intracellular Ca^{2+} release channels. A $DdIP_3R$ null strain was generated by homologous recombination in Ax2.

The major biochemical phenotype of the $DdIP_3R$ null strain is that chemoattractant-mediated Ca^{2+} entry is abolished. Other agents such as arachidonic acid or calmidazolium, which activate Ca^{2+} entry in Ax2, also fail to stimulate Ca^{2+} entry in this mutant. However, cAMP signal transduction responses, multicellular development and prestalk-prespore cell gene expression are largely unaffected in the $DdIP_3R$ null strain.

In Ax2 cells, DIF-1 stimulates Ca^{2+} entry with similar kinetics to that of cAMP, although the magnitude of this response is smaller. This rapid DIF-1 induced Ca^{2+} influx is also abolished in the $DdIP_3R$ null mutant. Interestingly, DIF-1 mediated *in vitro* stalk cell formation is also impaired in the $DdIP_3R$ null strain but spore cell formation is unaffected.

These observations suggest that the $DdIP_3R$ is an essential component of both chemoattractant and DIF-1-mediated Ca^{2+} entry in *Dictyostelium*. Future studies will address whether the $DdIP_3R$ is a plasma membrane Ca^{2+} channel directly mediating Ca^{2+} entry or if it is located in discrete intracellular Ca^{2+} store(s), mediating Ca^{2+} influx indirectly via a mechanism similar to capacitative entry.

Prolyl Oligopeptidase Acitivity and IP_3 Signalling During Early Development

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Although the therapeutic properties of lithium ions (Li^+) are well known, with two major targets being GSK-3 and inositol signalling, the mechanism of its action remains to be determined. To define sights of lithium action, we have isolated Li^+ resistant mutants, one of which results in the loss of the *Dictyostelium* prolyl oligopeptidase gene (*dpoA*). We find no evidence for an interaction with GSK-3, but instead find that loss of *dpoA* causes an increased concentration of IP_3. This increase is necessary for aggregation in the presence of lithium and is consistent with the involvement of IP_3 during aggregation. The same increase in IP_3 is induced in wild type cells by a prolyl oligopeptidase inhibitor, which acts over a short time period to increase IP_3 concentrations via an unconventional mechanism that involves enhanced dephosphorylation of inositol (1,3,4,5,6) pentakisphosphate (IP_5). Preliminary data suggests that the mechanism of this is independent of G_ activity. Furthermore, two more mutants have been isolated in this pathway: LisD, a armadillo repeat containing protein, and LisE. I will present new results to further describe the pathway and mechanism of *dpoA* in IP_3 regulation during early development.

A Xestospongin C-sensitive Ca^{2+} store is required for cAMP oscillations in *Dictyostelium*

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Xestospongin C (XeC) is known to bind to the IP3-sensitive Ca^{2+} store in mammalian cells and to inhibit non-competitively IP3-induced Ca^{2+} release. We found that XeC reduced Ca^{2+} uptake into purified vesicle preparations and inhibited IP3- and Thapsigargin-induced Ca^{2+} release. XeC caused a time-dependent increase of the cytosolic Ca^{2+} concentration and inhibited receptor-mediated Ca^{2+} influx in single cells as well as in cell suspension. cAMP-oscillations and lightscattering spikes receded in the presence of XeC indicating that a functional IP3-sensitive store is required for pulsatile cAMP-signalling and Ca^{2+} influx.

Searching for the role of serine/threonine phosphatases in *Dictyostelium*.

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Previous work demonstrated that *Dictyostelium* extracts contain PP2A, PP2B and PP2C catalytic activities with biochemical properties similar to those of their mammalian and yeast counterparts. In contrast, no type 1 protein phosphatase (PP1) was detected during *Dictyostelium* life cycle even though PP1 activity has been shown to be present in all other organisms analyzed. Increasing effort are been devoted to the identification of *Dictyostelium* genes that code for protein phosphatases. Such genes should be important tools to better understand the role of these enzymes in *Dictyostelium* growth and development. Complete cDNA clones for PP2B, PP2A and PP6 catalytic subunits were isolated but the role played by these enzymes in *Dictyostelium* is still unclear. More recently a novel PP2C gene found in *Dictyostelium* was described as essential for its cell type differentiation.

Through the use of PCR with a pair of degenerated oligonucleotides primers derived from conserved residues in the catalytic core of the PPP family we have isolated genomic DNA sequences encoding putative PP catalytic subunits. These clones were used as probes to screen a cDNA library derived from *Dictyostelium* vegetative cells and we have isolated full length cDNAs for PP1 and PP4 catalytic subunits. The deduced aminoacid sequence from the cDNA that encodes PP1 reveals an average identity of 80% with other PP1 sequences. The main difference we have observed is the substitution of a conserved cysteine to a phenylalanine (Phe269). In all other known PP1 this cysteine is present in a motif of the catalytic domain that possibly interacts with enzyme inhibitors. *Dictyostelium* recombinant PP1 expressed in *E.coli* is active and shows biochemical properties similar to those of a typical PP1. However mutation of Phe269 to Cys renders the recombinant enzyme more active. PP1 activity seems to be essential for growth of *Dictyostelium* cells, since we have failed after many attempts to knock out the only *Dictyostelium* PP1 gene. Taken together, these observations contrast with the failure in detecting biochemically PP1 activity in *Dictyostelium* cell extracts. Hence the question is why is this enzyme activity not demonstrable in *Dictyostelium* cell extracts? One possibility is the existence of an inhibitory subunit tightly associated with the PP1 catalytic subunits in the cell extracts.

DNA microarrays for the detection of *Dictyostelium* gene expression

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The expression of thousands of genes can be detected simultaneously with DNA microarrays. In the basic form of the method, the target DNA is printed in a tight array on a glass slide (DNA chip). The DNA chip is hybridized with fluorescent probes that are cDNAs synthesized from cellular RNA. The fluorescence intensity is measured with a laser detector. The data is interpreted as the relative change in RNA accumulation between the experimental sample and a reference sample.

The *Dictyostelium* genome project at Baylor College of Medicine is producing sequence data as well as short genomic DNA fragments that are cloned in plasmids. We tested whether these genomic DNA clones can be used as targets for expression arrays that will account for the entire genome.

Starting with a small array of several hundred probes, we have adapted the yeast microarray method[a] to the detection of gene expression in *Dictyostelium*. We optimized the system for target DNA preparation, for probe labeling, and for quantitation. The sensitivity of the arrays was confirmed by comparing RNA samples from vegetative AX4 cells to RNA from developing cells. In addition, the array was used successfully to detect differences between wild type AX4 cells and several developmental mutants (e.g. *yakA, tagB, regA,* and *pkaC*). Results from these analyses will be presented.

The goal of establishing a microarray for the entire *Dictyostelium* genome is now within reach. The challenges are to make the technology accessible to the entire community and to develop tools for interpretation and management of the data[b].

a- Schena, M., Shalon, D., Davis, R.W., and Brown, P.O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science 270*, 467-470.

b- Sucgang, R., Shaulsky, G. and Kuspa, A. (1999). Toward the Functional Analysis of the *Dictyostelium discoideum* Genome. J. Euk. Micro. *in press*.

The German *Dictyostelium* Genome Project

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The first complete genome of a bacterial organism, *Haemophilus influenzae*, was published in 1995. Now, four years later, complete genomic sequences of 15 eubacteria, 4 archaea, and 2 eukaryotes are available to the scientific community. In addition, the determination of the genomic sequence of a large number of diverse organisms from simple bacteria to homo sapiens is in progress and powerful techniques for studying the transcriptional activities of complete genomes are being further developed.

The *Dictyostelium* genome has a size of 34 Mb dispersed over six chromosomes ranging from four to seven Mb. Sequencing of the genome is funded by three sources, the DFG, the NIH, and the EU. Consequently, the *Dictyostelium* genome project is an international collaboration between the Baylor College of Medicine, the Sanger Centre, and the Genome Sequencing Centre Jena together with the Institute of Biochemistry I in Cologne. It was decided to shotgun sequence and assemble reads from individual chromosomes that have been separated by pulsed field gel electrophoresis. Libraries of chromosomes I, II, IV/V, and VI have been prepared by the Sanger Centre in Hinxton. Currently chromosome II enriched libraries are being used for shotgun sequencing in Germany.

It is estimated that a 1x coverage of the genome will reveal sequence information for approximately 90 % of all genes. Thus in the early stage of the project a light shotgun sequencing of the entire genome was performed as part of the German project. This approach resulted in an early overview of the total gene content and gene family distribution of *D. discoideum*. Results demonstrating the value of this approach will be presented.

All shotgun reads as well as the corresponding BLAST results are publicly available on our websites (http://www.uni-koeln.de/dictyostelium/ and http://genome.imb-jena.de/dictyostelium/) and provide a valuable resource for the *Dictyostelium* community.

The *Dictyostelium* Genome Sequencing Project: Progress on Chromosome 6

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Sequencing of the *Dictyostelium* genome is proceeding apace, using a chromosome-by-chromosome strategy. Together with the Sangre Center, the Baylor team is performing the shotgun sequencing and assembly of the 4.2 Mb Chromosome 6. Plasmid-based libraries were constructed from DNA fractions enriched for the chromosome. Assembly will be assisted by sequencing sub-libraries constructed from YACs that form the minimum tiling set for Chromosome 6. The sequencing progress and the development of resources for the *Dictyostelium* research community will be presented.

Dictyostelium cDNA project: Summary of slug cDNAs and outline of the second phase sequencing

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The first phase of the cDNA project, the sequence analysis of slug stage cDNAs, has finished. During this phase of the project the cDNA inserts of almost 20000 clones were sequenced from their 3'-ends. The cDNA sequences were classified into non-redundant EST groups based on the 3' nucleotide sequences and representative clones, those with the longest insert cDNAs in a particular EST group, were sequenced from their 5'-ends. The results are as follows;

Total clones selected	18832
Total obtained sequences	14459
Number of EST groups	5030
Identical to known *Dictyostelium* genes	477
Homologous to known genes	1155

Those representative clones with internal gaps, i.e. those where 5' and 3' reads did not overlap, will now be sequenced using internal primers.

Two new activities will now be undertaken.

i) Large-scale expression analyses using the cloned cDNA in conjunction with micro- and/or macro-array technology are planned.

ii) To obtain further sequence information from *Dictyostelium* genes, we have started the second phase of the cDNA sequencing project. We plan to pick up 20000 cDNA clones prepared from growing cells and cells at 6 hours of development.

The current status of this second phase of the sequencing project will be reported.

Proteome studies of early *Dictyostelium discoideum* development

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Dictyostelium discoideum has been widely used to examine biological pattern formation and morphogenesis due to its simplified process of development and cell differentiation. Of particular interest, is how the signal molecule differentiation-inducing factor (DIF), which has previously been shown to induce stalk cell differentiation (1), regulates the global proportions of prestalk and prespore cells within the multicellular aggregate. A mutant *D. discoideum* strain, HM44, cannot produce DIF, and is unable to produce stalk cells. However, addition of exogenous DIF to HM44 allows stalk cell differentiation to occur.

The proteome is the full complement of proteins expressed in an organism or tissue (2), and represents the effective print out of the organism's genomic information. The approach taken here was to use proteome technology to examine the global response of cellular protein expression in early HM44 prestalk cell differentiation and development (3). A new DIF-less *D. discoideum* mutant (strain HM1030) will also be analysed against the parent strain (Ax2) for differences in global protein expression.

Proteome separation technology using 2D-PAGE has proven a valuable approach to analyse the global protein expression in a simple parallel process, providing reproducible and complete separation of complex protein mixtures. Differences between differential displays of a proteome on 2D gels represent key alterations in the amounts of individual proteins or in the activity of the proteins regulated by post-translational modifications, eg phosphorylation, glycosylation. Proteins potentially involved in response to DIF in HM44 and HM1030 can therefore be visualised and identified. This work has also studied, for the first time, the utility of the *Dictyostelium* expressed sequence tag (EST) database for protein identification. Proteome maps of *Dictyostelium* (mound stage) and the *Dictyostelium* extracellular matrix will also be presented.

Ref.

(1) Kay, R.R. & Jermyn, K.A. (1983) A possible morphogen controlling differentiation in *Dictyostelium*. *Nature* **303:**242-244
(2) Wilkins, M.R., Williams,K.L., Appel, R.D. & Hochstrasser, D.F. (Eds) Proteome Research: New Frontiers in Functional Genomics. Springer, Berlin 1997.
(3) McLure, L.E., Wilkins, M.R., Kay, R.R., Walsh, B.J. and Williams, K.L. Proteome Analysis Of Early *Dictyostelium Discoideum* Development: Identification Of Differentiation-Inducing Factor (DIF) Regulated Proteins. (in preparation)

DICTYDB: A DATABASE OF *DICTYOSTELIUM* INFORMATION

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The UCSD Dicty Web Site (www-biology.ucsd.edu/others/dsmith/dictydb/ html) now includes movies of *Dictyostelium* development, links to mitochonrial DNA, rDNA palindromes, and to the Dicty genome sequencing centers, and links to the Tsukuba cDNA program including the Groups of cDNA clones with statistical data. Chromosome Maps are presented using the GenomicView display developed for the Saccharomyces Genome Database. DictyDB is now available on the Web at the Cornell Genome Center both via the enhanced, faster WebAce2 and AcePerl/ AceBrowser ACeDB servers. The two servers present data in complementary fashion, and AceBrowser is particularly suited for presentation of enhanced biological information. Links are provided to GenBank, SwissProt, Pfam and other databases, as well as to clone sites in Tsukuba. An All Dicty BLAST server will soon be available at the San Diego Supercomputer Center that will permit rapid queries of the raw Dicty genome sequence data from the Baylor, Jena, and Sanger genome centers as well as all the Dicty sequences at Genbank.

DictyDB_2.5 is scheduled for release in August, 1999. In addition to standard updates through July, 1999, of Proteins, DNA and RNA sequences, Papers (at least through Franke EndNote library Version 10A and Update 15A), and Dicty investigators, DictyDB_2.5 contains some 16,000 Tsukuba cDNA clones as Clone and Sequence subclasses. Data on knockout mutant alleles of mapped genes are graphically presented. DictyDB is being extended to include BLAST data, with links to BLAST hits, and identification of overlapped DNA sequences, with presentation as subsequences. In addition to the Web ACeDB servers, DictyDB is available as a standalone Macintosh application using MacAce or as a Unix or Linex application using xace. xace has more features, including dot plots via Dotter and multiple sequence alignments via Blixem, than does either MacAce or the Web ACeDB servers.

The effective user interface of ACeDB permits graphic displays of maps, sequences, and other data, as well as text displays of text and numerical information. Primary data objects are loci, clones, DNA and protein sequences, references, and authors. The genetic map shows mapped loci, clones, and mutant alleles for a given chromosome, in kb units. The DNA sequence display views sequence features, including exons, sites, and GeneFinder analyses. Users can easily "browse" information via hypertext links. New genomic sequence data are readily incorporated using .ace files created by the standard sequencing programs Phred, Phrap, and Consed, and ACeDB has been used for large genome programs such as the *C. elegans* program.

DictyDB is intended to be a resource for examination and manipulation of *Dictyostelium* genetic and biological information. We intend to develop DictyDB into a sophisticated tool for prediction and representation of biochemical pathway and genetic network information of the *Dictyostelium* life cycle.

Dd-STATa and the regulation of ecmB and cudA gene transcription

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The STAT (Signal Transducer and Activator of Transcription) proteins lie at the end of many cytokine signalling pathways and are also important in mouse and fly development. Dd-STATa, the best characterised Dictyostelium STAT,translocates to the nucleus in response to extracellular cAMP and is localised in the nuclei of all cells at the tight aggregate stage. By the slug stage nuclear enrichment becomes confined to a subset of pstA cells located at the extreme slug tip. Analysis of a Dd-STATa null mutant, carried out in a collaboration with Rick Firtel's laboratory (Mohanty et al., 1999, Development in press), has shown that Dd-STATa is the previously predicted repressor protein that prevents premature commitment to stalk cell differentiation and that Dd-STATa is also required earlier in development for cells to become chemotactically responsive. Recent analyses of specific promoter regions in two different genes, ecmB and cudA further suggest that Dd-STATa has multiple functions: 1) as a direct competitive inhibitor of ecmB transcription and 2) as an activator of cudA.

1) In addition to acting as a long range repressor of the ecmB gene transcription, by binding to sites hundreds of nucleotides away from the activator region (Mohanty et al., 1999, Development in press), Dd-STATa may also reinforce this repressive action by directly competing for binding to the activator site. The evidence for this comes from sequence identification of the ecmB activator and characterisation of the protein that binds to this sequence. Band shift analysis shows that Dd-STATa binds avidly to the ecmB activator sequence in vitro but analysis of the Dd-STATa null shows that in vivo activation of ecmB must be via a different protein. Band shift analysis using nuclear extracts from the Dd-STATa null has identified a candidate for this activator. One attractive interpretation of our data is that Dd-STATa and the newly discovered, putative activitor compete for binding at the ecmB activator locus in vivo.

2) The cudA gene encodes a nuclear protein of unknown function and has the most complex pattern of expression thus far described for a Dictyostelium gene. At the slug stage cudA is expressed in the prespore cells and in the pstA* cells, a sub-set of the prestalk cells that comprise the slug tip. The cudA mRNA is also present in pstAB cells, a cone of prestalk cells that form the inner part of the basal disc at culmination. Cap-site distal promoter sequences (the psp domain) direct expression in prespore cells while proximal sequences (the pstA*/AB domain) direct expression in pstA* and pstAB cells. The pstA*/AB domain contains a binding site for Dd-STATa and in the Dd-STATa null mutant the pstA*/AB domain is not utilised. Dd-STATa may therefore serve as a direct activator of cudA transcription in pstA* cells and pstAB cells. The psp domain of cudA contains a sub-region that has the potential to direct expression in all cells within the slug (the general activator region) and a counteracting sub-region that prevents expression in the prestalk cells (the pst repressor region). A very similar form of prespore-specific regulation has recently been described in Adrian Tsang's laboratory for the rnrA gene (Bonfils et al., 1999J Biol Chem, 274, 29, 20384-20390) and the putative pst repressor regions of cudA and rnrA share considerable sequence homology.

Glycogen Synthase Kinase-3 (GSK-3) regulates the nuclear localisation of STATa

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GSK-3 is a highly conserved, multifunctional protein kinase that is required for both *Dictyostelium* and metazoan development. The STAT family of transcription factors which mediate cytokine responses in mammalian cells and are required during *Drosophila* development, are also conserved between *Dictyostelium* and animals. The predicted sequence of the *Dictyostelium* STATa protein contains a cluster of putative GSK-3 phosphorylation sites. In this report, we demonstrate that the *Dictyostelium* homologue of GSK-3, GskA, phosphorylates STATa and regulates its nuclear localisation.

Our observations from both *in vitro* and *in vivo* experiments indicate the presence of a cluster of overlapping GskA phosphorylation sites within the STATa protein. As seen for other GSK-3 substrates, these sites are generated by a prior phosphorylation event, either by a priming kinase or overlapping GSK-3 phosphorylation. This leads to the incorporation of up to 6 phosphate moieties into the STATa protein. We have generated mutants of STATa which either lack the GSK-3 phosphorylation sites or have acidic residue substitutions for phospho-serine. When cells are stimulated with cAMP, STATa becomes phosphorylated on a tyrosine residue at the C-terminus, and enters the nucleus. We find increased nuclear staining in cells expressing a STATa that is missing the GSK-3 phosphorylation sites. This same increase is seen for the endogenous STATa protein in GskA mutant cells. The opposite behaviour is seen for the STATa mutant containing acidic substitutions. In this case, very few nuclei contain STATa protein and the cytoplasm becomes brightly stained. To examine the mechanism by which GskA regulates the nuclear localisation of STATa, we treated cells with the nuclear export inhibitor leptomycin B (LMB). This treatment leads to the accumulation of STATa containing the acidic substitutions in the nucleus. Our observations indicate that GskA regulates the nuclear localisation of STATa by controlling its rate of nuclear export.

The Novel Tyrosine Kinase ZAK1 Activates GSK3 to Direct Cell Fate Specification

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The 7-TM cAMP receptors (CARs) regulate GSK3 protein kinase by parallel and antagonistic pathways to establish an anterior/posterior developmental axis. We had previously found that receptor cAR4 inhibits GSK3 and prespore pathways. Harwood and coworkers had further uncovered an activation pathway for GSK3, mediated by cAR3 that antagonizes cAR4 and promotes posterior cell formation. In this study, we have identified a novel tyrosine kinase ZAK1, with separate serine/threonine- and tyrosine-specific catalytic motifs, that functions downstream of cAMP receptor signaling and that is required for activation of GSK3 during development. To characterize the function of ZAK1, we generated zak1-nulls. zak1-nulls had reduced prespore expression but expanded ecmB-lacZ spatial staining pattern, similar to the phenotypes of car3- and gsk3-nulls. Over-expression of ZAK1 activated prespore gene expression above wild type levels, and ecmB expression was totally repressed. Over-expressed ZAK1 in gsk3-null cells had no effect, suggesting the ZAK1 mediated antagonistic regulation of prestalk and prespore pathways are GSK3 dependent. Consistent with these data, GSK3 kinase activity was reduced in zak1-nulls compared to wild type. zak1-nulls (and cAR3-nulls) were unable to activate GSK3 in response to cAMP. Furthermore, purified, recombinant ZAK1 specifically phosphorylated and activated GSK3 in vitro. We propose that ZAK1 lies downstream of cAMP/CAR3-signaling to activate GSK3 for cell pattern formation in Dictyostelium and speculate that similar mechanism may exist to activate GSK3 in other system. Experiments are in progress to identify the target phosphorylation site(s) in GSK3 and to determine the mechanism of ZAK1 mediated GSK3 activation.

Identification and characterization of *Dictyostelium* src homology-2 (SH2) containing protein tyrosine kinases.

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Src homology-2 (SH2) domains and their associated proteins constitute critical signal transduction components in metazoans. Such SH2 proteins are found in the regulation of many cellular processes, including growth, development, motility, metabolism, immune response, and gene transcription. The dynamic and reversible binding interactions of SH2 domain containing proteins with phosphotyrosines (pTyr) is co-ordinated by protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). The discovery of three *Dictyostelium discoideum* STATs suggests that SH2 domains play crucial roles in *Dictyostelium* signaling pathways. Recent work by the Williams and Firtel laboratories has demonstrated that, as in metazoans, regulated tyrosine phosphorylation of STAT proteins controls spatial patterning during development. To learn more about SH2 mediated signaling in *Dictyostelium*, we decided to identify and characterize tyrosine kinases that could function in these pathways.

We employed a broad approach to identify novel tyrosine kinases in *Dictyostelium*. We made use of the *Dictyostelium* cDNA database and identified two putative kinases, which by BLAST analysis are related to tyrosine kinases. A third was identified by PCR. The full length cDNAs for these genes (SHK1-3) were isolated. All three have an N-terminal kinase domain related to *Dictyostelium* tyrosine kinases. The C-terminal half of each protein has a SH2 domain. The SH2 domain of SHK1 is closely related to the SH2 domain of mammalian STAT Induced STAT Inhibitor-2. The SH2 domain of SHK2 is related to that of src-kinases and the SH2 domain of SHK3 shows little homology to any other known SH2 domains. The SH2 domains are followed by a potential site of tyrosine phosphorylation near the C-terminus. All three genes show similar patterns of expression, with transcripts significantly up-regulated during mound formation.

We have shown that these proteins function as tyrosine kinases. The kinase activity of SHK2 is constant throughout development but that of SHK1 increases at 12 hours and remains high throughout culmination. The kinases are found associated with the plasma membrane. This localization is dependant on the SH2 domain as determined by an Arg to Ala substitution in an essential part of this domain required for phosphotyrosine binding. SHK2 is cytoplasmic in vegetative cells but membrane associated in pulse cells, suggesting this response is developmentally regulated. Overexpression of SHK2 results in a severe aggregation defective phenotype. However, the SHK2 Arg to Ala substitution as well as kinase dead mutants do not exhibit this severe phenotype. SHK1 and 3 have been disrupted. SHK1 nulls form very small plaques on bacteria and small fruiting bodies. SHK3 nulls form larger than normal streams and mounds. Putative SHK1/3 double knockouts and do not aggregateform small plaques and develop poorly. Possible roles of these genes in chemotaxis and STAT function will be discussed.

DdMyb2 mediates YakA and PKA functions during early development of *Dictyostelium discoideum*

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Dictyostelium development is induced by starvation leading to the induction of the adenylyl cyclase ACA. Signaling with extracellular cAMP produced by ACA is essential for cell aggregation and gene expression during early development. Two kinases, YakA and cAMP-dependent protein kinase (PKA) have been reported as essential components for the induction of ACA expression. Previously we have shown that DdMyb2, a *Dictyostelium* homolog of the Myb family of transcription factors, is also necessary for ACA expression. We show here, using mutants with either deletion or ectopic expression of YakA, PKA catalytic subunit (PKA-C) and DdMyb2, that DdMyb2 acts downstream of YakA and PKA to induce ACA expression. ACA expression is dependent on DdMyb2 even in cells overexpressing PKA-C or YakA. Moreover, ectopic expression of DdMyb2 rescues ACA expression and cAMP relay response of *pkaC*$^-$ and *yakA*$^-$ null cells; *pkaC*$^-$ cells aggregate and *yakA*$^-$ cells can form fruiting bodies if DdMyb2 is ectopicaly expressed. We also found that expression of YakA and DdMyb2 is dependent on PKA. We propose that starvation signals are mediated by PKA to upregulate RNA levels of YakA and DdMyb2, and by YakA to activate DdMyb2.

The NF-_B pathway in Dictyostelium discoideum

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Using antibodies raised against components of the mammalian NF-_B pathway, we have demonstrated the presence in *Dictyostelium* cells extracts of proteins homologous to Rel/NF-_B transcription factors p50, p65 and p52/p100, to the I_B inhibitor I_B_ and to IKK kinases IIKK_ and IKK_. Using gel shift experiments, we have demonstrated the presence in nuclear extracts of developing *Dictyostelium* cells of proteins binding to bona fide _B oligonucleotides and to a GC-rich _B-like sequence, lying in the promoter of *cbpA*, a developmentally regulated *Dictyostelium* gene encoding the Ca^{2+}-binding protein CBP1. Using immunofluorescence, we have shown a specific nuclear translocation of the p65 and p50 homologues of the NF-_B transcription factors as vegetatively growing cells develop to the slug stage. Taken together, our results demonstrate the presence of a complete NF-_B signal transduction pathway in *Dictyostelium discoideum* that could be involved in the developmental process.

Traincard et al. JCS, in press

The CAR1 Transcription Factor CRTF Plays Multiple Roles through *Dictyostelium* Development

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Initiation of *Dictyostelium* development requires the induction of cAMP receptor 1 (CAR1) which relays the pulsatile extracellular cAMP signal to regulate chemotaxis and gene expression. Expression of the *CAR1* gene is itself regulated by cAMP pulses. We have previously identified an element and a factor (CRTF) that binds to the CAR1 early promoter. We have purified CRTF to homogeneity by DNA-affinity chromatography and isolated its cDNA. The CRTF cDNA has an open reading frame that encodes a protein of 876 amino acid residues with an estimated molecular weight of 100kDa and a novel Zn-finger DNA-binding motif. The CRTF mRNA is expressed throughout development and CRTF-null strains created by homologous recombination have multiple developmental defects. In the early stages of development, CRTF-null cells are defective in aggregation and expression of CAR1. Other early gene expression is also compromised. While CRTF-nulls will aggregate and develop when plated at high density, their development is very delayed and extremely inefficient. <50% of the CRTF-null aggregates appear to complete development after 48 hours, but they do not make mature spores. The defect of CRTF-nulls in spore maturation can not be recovered when developed in mixture with wild type cells or with 8-Bromo-cAMP in monolayer cell culture assay. To our surprise, treatment of CRTF-null cells with cAMP pulses can rescue CAR1 expression to wild type levels, indicating that there might be other factors involved in the regulation of CAR1 by cAMP. However, the cAMP pulses can not rescue the late stage developmental defects of CRTF-null cells. We have rescued CRTF-nulls by expression of HA-tagged CRTF driven the by actin 15 promoter. With these rescued strains, we are studying the mechanism of CRTF function by western blot and immunofluorescence staining techniques.

Genetic Pathways Mediating the Cellular Response to DNA Damage

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In order to protect themselves from genetic damage, cells have mechanisms for detecting the specific type of damage and mounting the appropriate biochemical response. Often, genes encoding DNA repair enzymes also appear to have a role in the development of the organism.

The signal transduction mechanisms which activate the repair processes are essentially unknown. Moreover, previous work from our lab and others indicates that unique pathways exists for each type of DNA damage although common elements are shared.

Dictyostelium discoideum is unusually resistant to DNA damage by UV light, gamma irradiation and chemicals. In addition, its relatively simple developmental program makes is ideal for detecting relationships between the pathways of DNA repair and morphogenesis. Earlier work from our laboratory identified genes homologous to those implicated in xeroderma pigmentosum and Cockayne=s syndrome in humans. The expression of these genes in *Dictyostelium* was shown to be modulated by the onset of development, UV light and the antitumor drug cisplatin. Each condition results in different pattern of gene expression supporting the idea that a unique signal transduction pathway is involved for each condition.

We have undertaken several parallel approaches to understanding these processes, two of which will be described.

1) Evidence exists that reactive oxygen species (ROS) may play a role in mediating the cellular response to UV light. We have examined the enzymes that modulate the level of ROS in cells. We have identified two catalase genes and a Cu^{++}/Zn^{++} superoxide dismutase gene, and studied their developmental expression and response to DNA damage. Genetic alteration of their activities will allow us to modulate the levels of ROS in the cells.

2) We have used REMI to isolate mutants with increased resistance to cisplatin. These mutants define several novel signaling pathways, and some of these mutants display alterations in development. This work should help define why tumor cells frequently become resistant to cisplatin during the course of therapy.

Overall, *Dictyostelium* provides a powerful system for investigations that have direct application to the biology of cancer and mutagenesis.

Macropinocytosis and Phagocytosis are Biochemically Distinct Processes.
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Macropinocytosis and phagocytosis are two morphologically similar processes that require the polymerization of F-actin to internalize large volumes of fluid and particles, respectively. Our laboratory is investigating the molecular mechanisms that regulate these two processes. Both processes require, in addition to F-actin, the activity of coronin, Rab7, myosin I, Scar, and the ATPase proton pump. We propose a model that predicts that phosphatidylinositol $(4,5)P_2$ is a key early substance required for both processes and that the activity of PI 3-kinase to generate PI $(3,4,5)P_3$ drives macropinocytosis while the activity of phospholipase C to form DAG and IP_3 drives phagocytosis. In addition, phagocytosis requires the activity of the small GTPases Rap1 and RacC; proteins that appear to negatively regulate macropinocytosis. In contrast, macropinocytosis requires the activity of PI 3-kinase and the G-actin sequestering protein profilin; proteins that appear to negatively regulate phagocytosis. Together, our studies support the hypothesis that although macropinocytosis and phagocytosis share common proteins that polymerize F-actin and recruit vesicles from the endo-lysosomal pathway, the signalling pathways that initiate these two processes are biochemically distinct and competing.

RtoA Contains a Serine-Rich domain with Vesicle-Fusing Properties

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Little is known about how a set of undifferentiated cells can break symmetry and differentiate into distinct cell types. In order to identify genes involved in this process in *Dictyostelium*, we employed shotgun antisense to identify mutants which are unable to properly proportion into prestalk and prespore cells (Wood et al., Development 122, 3677-3685, 1996). One of these mutants, *rtoA*, has an increased percentage of prestalk cells.

RtoA is a 40 kD protein with a possible transmembrane domain, a putative ATP/GTP binding domain, and 11 perfect repeats of a 10 amino acid-long serine-rich sequence which comprises 60% of the protein. This serine-rich domain has a predicted random coil structure, which we confirmed by circular dichroism and NMR. Light and electron microscopy, light scattering and fluorescence energy transfer all indicate that a recombinant protein containing this domain is able to catalyze the fusion of phospholipid vesicles, suggesting that RtoA functions to induce vesicle fusion.

Western blots of fractionated cells indicate that RtoA is associated with the cytosol as well as small subcellular structures, possibly vesicles. Using electron microscopy, we observe that cells lacking RtoA do not initiate the fusion of a specific subset of their vesicles. While the rate of pinocytosis of fluorescently labeled dextran is normal in *rtoA* cells, the subsequent excretion is markedly reduced. In addition, the pinocytic vesicles are over acidified. Our data thus suggest that RtoA directly causes vesicle fusion during processing of endosomal vesicles, and that the disruption of this process alters exocytosis. We hypothesize that RtoA affects initial cell type choice by regulating vegetative cell physiology. Gross *et al.* (Differentiation 38, 91-98, 1988) predicted that initial differentiation is regulated by the pH of specific vesicles, with acidification favoring development of prestalk cells, and alkalization promoting prespore differentiation. The fact that *rtoA* cells have a higher percentage of prestalk cells and have a class of vesicles with a lower average internal pH correlates beautifully with the Gross *et al.* prediction.

Infection of *Dictyostelium discoideum* with the Gram-negative bacterium *Legionella pneumophila*- a new model system for intracellular pathogenesis

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L. pneumophila is a bacterial pathogen that lives and replicates inside of eukaryotic cells. *L. pneumophila* enters cells by phagocytosis, but the bacterium-containing phagosome evades the endocytic pathway and the bacterium is not digested. Instead, *L. pneumophila* replicates within the phagosome until the cell is filled with bacteria and bursts, initiating another round of infection. *L. pneumophila* normally exists as an intracellular parasite of fresh-water amoebae, but if inhaled by a person can cause Legionnaire's disease by growing within the macrophages of the lung.

We discovered conditions in which *L. pneumophila* grows inside of *Dictyostelium* just as it does in amoebae and macrophages. During the infection bacterial titer increases over 150 fold in four days. Electron microscopy shows that the bacteria are growing inside of *Dictyostelium* in a membrane-bound compartment. Characteristic of *L. pneumophila* infections, the replicative phagosome is associated with rough endoplasmic reticulum. *L. pneumophila* mutants that are unable to grow in amoebae and macrophages are also unable to grow in *Dictyostelium*.

We are utilizing *Dictyostelium* genetics to identify host cell functions required to support growth of *L. pneumophila*. We are testing known *Dictyostelium* mutants for their effects on growth of *L. pneumophila*. G-beta mutants, myoA/B mutants, and cells that overexpress constitutively active rab7 have no dramatic effect on bacterial growth. We are also selecting REMI mutagenized *Dictyostelium* for resistance to infection by *L. pneumophila.*

F-actin associated proteins and their role in the formation of phagosomes and macropinosomes

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Axenic strains of Dictyostelium are equally competent to grow on bacteria or liquid medium. Particles are taken up by phagocytosis and liquid medium is mainly internalised by macropinocytosis. Both processes depend on the actin cytoskeleton for the protrusion of the plasma membrane. Less than one minute after the engulfment is complete the cytoskeleton dissociates from the membrane of the freshly formed vesicle. Then the lumen of the vesicle is acidified within seconds by the action of a vacuolar-type proton-ATPase and digestion of the content begins.

The actin-binding proteins coronin and DAip1, both belonging to the family of WD40-repeat proteins, specifically contribute to the internalisation step at the plasma membrane. GFP-fusions to coronin and DAip1 localise to the cortical cytoskeleton and are enriched at sites of protrusive activity, e.g. phagocytic cups and crown-like structures previously shown to be sites of macropinocytosis. In coronin mutants phagocytosis and macropinocytosis are strongly reduced and the phenotype of cells lacking DAip1 is very similar. CytochalasinA, a drug that caps the fast growing end of actin filaments and prevents actin polymerisation, also inhibits both the formation of fluid-filled or particle containing endosomes. On this basis, both DAip1 and coronin may be involved in the regulation of actin polymerisation.

However, coronin and DAip1 may act by different mechanisms in the cell. From in vitro studies of yeast coronin, this protein has been proposed to regulate the polymerisation at the barbed end of actin filaments. Yeast Aip1, on the other hand, interacts with cofilin, a protein that depolymerises actin from the pointed end. In Dictyostelium an actin depolymerising drug, latrunculinA, increases phagocytosis but not macropinocytosis at low concentrations. The same effect is observed in a cell-line overexpressing DAip1 20-fold. Therefore, it is conceivable that DAip1 acts as an actin depolymerising protein in vivo, most likely by stimulating the activity of Dictyostelium cofilin.

Protrusion formation induced by fusion of contractile vacuoles into the plasma membrane

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Mechanism of pseudopod extension of amoeboid cells has long been the subject of extensive studies, but there still remains much controversy. This is mainly due to the diversity of pseudopods, not only among different organisms but also within the same species. The difficulty of obtaining a homogenous population of pseudopods has hampered analytical studies of pseudopod extension. In this study, we investigated the formation and extension of the cylindrical protrusion that is initiated by fusion of a contractile vacuole into the plasma membrane in the presence of quinine. These protrusions, which had similarities with naturally occurring lobopodia, especially of cells at the slug stage, are experimentally inducible and can be identified without ambiguity by the presence of contractile vacuole markers.

These protrusions were typically cylindrical with a round head and extended at a high speed, often exceeding 1 μm/sec. Fluorescent particles attached to the cell surface remained stationary with respect to the substratum during locomotion. Immunofluorescence study revealed that markers of the contractile vacuole membrane (calmodulin and V-ATPase) stayed at the proximal end of the protrusions while they extended. When contractile vacuole membranes had been stained in vivo with a styryl dye RH795, the region of strong fluorescence remained restricted to a small region around the distal end of the extending protrusion. This observation rules out exocytotic insertion of intracellular vesicles into the leading edge as a major supply of the membrane lipid in this type of protrusion. The presence of up to 300 mM sorbitol did not suppress extension of the protrusions, suggesting that passive influx of water into the cell cannot account for the protrusive force.

In mhc- cells, fusion of the contractile vacuole into the plasma membrane occurred but no protrusion was formed. Observations with GFP-myosin cells showed that a cortex of myosin II, which was initially absent from the protrusion, gradually extended from the main body of the cell into the protrusion, eventually surrounding the entire protrusion, when it ceased to elongate. A layer of F-actin was observed underneath the plasma membrane of the protrusions. Addition of cytochalasin A nearly abolished the actin layer in the protrusion and resulted in the formation of spherical blebs rather than cylindrical protrusions. The rate of the flux of the cytoplasm from the original cell body into the blebs in the presence of cytochalasin A was four-fold higher than that into cylindrical protrusions without cytochalasin A.. These observations suggest that actin filaments play a role in maintaining the shape of protrusions, rather than providing protrusive force, in this type of protrusions.

We conclude that the protrusive force in this type of protrusions is generated by the contraction of the original cell body and transmitted by the resultant flow of the cytoplasm into the protrusion.

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Clathrin Is Essential For Suppressing Pseudopod Formation In The Posterior Half Of A Cell For The Maintenance Of Cell Polarity And For The Integrity Of The Uropod In Dictyostelium

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Although the traditional role of clathrin has been in vesicle trafficking and the internalization of receptors, a novel role in cytokinesis was recently revealed in an analysis of a clathrin minus Dictyostelium mutant. Mutant cells grown in suspension were demonstrated to be defective in assembling myosin II into a normal contractile ring (Niswonger and OíHalloran, 1997). To test whether this defect reflected a more general one of cytoskeletal dysfunction, we analyzed clathrin-minus cells for cell shape, cell polarity, pseudopod formation, cell locomotion, chemotaxis, distribution of actin, microtubule organization and vesicle movement. Clathrin-minus cells
crawled and chemotaxed. They also exhibited a normal one minute velocity cycle, normal localization of F-actin to pseudopods, normal organization of the microtubule organizer and associated microtubule arrays, and normal vesicle motility. However, they abnormally extended twice as many pseudopods form the posterior half of the cell body when compared to wild type or rescued cells. Clathrin minus cells also exhibited a decrease in the stability of both pseudopod and uropod morphology. These abnormalities resulted in a dramatic decrease in cellular polarity, a reduction in average velocity, an increase in the frequency of sharp turns and a reduction in chemotactic efficiency. Of all of the cytoskeletal and regulatory mutants of Dictyostelium so far motion analyzed with 2D-DIAS and 3D-DIAS, the motility defects of clathrin-minus mutants are some of the most extreme. These results are the first to suggest a role for clathrin in cell polarity, pseudopod formation and locomotion. The results demonstrate that clathrin, like myosin II, is necessary for the suppression of pseudopod formation in the posterior two thirds of a crawling cell, which is essential for efficient cell locomotion, efficient chemotaxis and the maintenance of cell polarity. The results also demonstrate that clathrin is necessary for the integrity of the uropod and the stability of pseudopod morphology.

Dynein is a Cortical Microtubule Anchor for Centrosome Positioning in *Dictyostelium*

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Dynein is a microtubule-based motor protein responsible for several aspects of organelle transport, cilia and flagellar bending, and mitotic spindle function. We show here that dynein also supports the interphase microtubule array and helps determine centrosome position. Overexpression of the dynein motor domain in *Dictyostelium* leads to a collapse of the interphase microtubule array, forming loose bundles of microtubules that often enwrap the nucleus. Using GFP-tubulin to visualize microtubules in live cells, we show that the collapsed arrays remain associated with the centrosomes and are highly motile. The centrosomes move at rates up to 2.5 _m/sec and frequently make complete rotations along the inner surface of the plasma membrane. The bulk of the microtubule array trails behind, giving the appearance of a comet tail. This is strikingly different from wild type cells where centrosomes show limited movement that is often constrained by tension on the microtubule array. Centrosome motility in the mutant cells appears to involve force-generating microtubule interactions at the cell cortex, with the rate and direction most consistent with a dynein-mediated mechanism. We have mapped the dominant-negative expression effect to the carboxy-terminal region of the dynein heavy chain. This suggests an interaction domain that regulates motor activity. We conclude that in *Dictyostelium*, dynein functions to stabilize the interphase microtubule array and acts to maintain centrosome position.

Dynein Intermediate Chain Mediated Dynein-Dynactin Interaction is required for centrosome replication and separation in Dictyostelium.

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The intermediate chain of cytoplasmic dynein (IC) is thought to regulate dynein function. In vitro binding studies suggested that IC mediates the direct interaction between dynein and dynactin complex. In order to determine the physiological role of IC and the significance of dynein-dynactin interaction, we inducibly expressed IC truncation mutants in wild type *Dictyostelium* AX3 cells and studied the phenotypes of these cell lines. IC_C, which lacks the C-terminal 373 amino acids, associated with dynactin, but was unable to associate with dynein heavy chain, while IC_N, lacking the N-terminal 47 or 106 residues, bound to dynein heavy chain but not dynactin complex. Both mutants interfered with wild type IC function by disrupting the interaction between dynein and dynactin complex. Both resulted in abnormal Golgi localization, consistent with previous findings that dynein is required for perinuclear Golgi distribution. In addition, IC mutant expression resulted in disorganized interphase microtubule (MT) networks including large microtubule bundles, multiple cytoplasmic asters and MTs unconnected to an organizing center. The MT disorganization is accompanied by abnormal DNA content, suggesting a defect in mitosis. Immunofluorescence analysis revealed abnormal centrosome size, location and number. Interestingly, these centrosomal defects correlate with microtubule abnormalities. Ultrastructural analysis of centrosomes in IC mutants showed accumulation of large centrosomes typical of prophase during interphase as well as unusual paired centrosomes suggesting defects in centrosome replication and separation. These results demonstrate that cytoplasmic dynein function is required for the proper organization of the interphase microtubule network and that this function requires dynein-dynactin interaction. In addition these studies suggest that cytoplasmic dynein is required for normal centrosome replication and separation in *Dictyostelium*.

Simulation of stream breakup during *Dictyostelium* aggregation

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Understanding the regulation of fruiting body size in *Dictyostelium* is of major interest to our lab. Under some conditions, aggregation streams containing too many cells to form a fruiting body of proper size will break up into smaller groups. We have found mutants with defects in the aggregation process. In these mutants, over- or under-secretion of a factor that regulates cell-cell adhesion causes changes in group size. This correlates with previous observations by other workers showing that in some cases alterations in cell-cell adhesion can cause alterations in group size.

To develop a quantitative understanding of how cell-cell adhesion might regulate group size, I wrote a simple computer simulation of an irregular ribbon of cells. Cells in the ribbon have a small random component in their motility, and are held in the stream by their adhesion to other cells. The adhesion increases with the number of other cells a cell is touching. In addition, I modeled the cells as having a chemoattraction to nearby cells. In the model, if the motility strength is greater than the adhesion strength, the cell moves; otherwise it stays put. Cells that are not touching another cell (such as a cell that used its random motility to move away from the stream) always chemotax towards nearby cells. The simulations indicate that if the adhesion is constantly high, the stream stays intact; if adhesion is constantly low the stream disperses, but if the adhesion is low for a while and then increases, the stream tends to break up into groups. There is a rough correlation between the amount of adhesion and group size. We are currently examining how the temporal regulation of adhesion strength affects group size.

Aardvark: a _-catenin–like protein is required for axis formation and terminal differentiation during *Dictyostelium* morphogenesis.

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In common to metazoan development, we have found that regulation of GSK-3 is required for correct *Dictyostelium* pattern formation. Metazoan GSK-3 acts within the Wnt signalling pathway and we are investigating whether other components of this pathway are required during *Dictyostelium* development. _-catenin is a downstream effector of GSK-3 and is required both for nuclear signalling and as a component of the adherens junction. We have isolated a *Dictyostelium* gene, Aardvark (aar), that encodes a protein with a related structure to __-catenin. We have identified the presence of cell-cell junctions within the fruiting body and these have an adherens junction morphology. Loss of aar disrupts these junctions. This is the first report of a non-metazoan adherens junction and indicates that Aar serves similar cytoskeletal functions to _-catenin.

The wild type fruiting body comprises of a spore head supported by a single stalk tube. Loss of *Aar* leads to the formation of super-nummary stalk tubes. The presence of these ectopic developmental axes suggests that Aar regulates an "organiser" of stalk tube formation. In addition, we find that a*ar*¯ cells have a reduced response to differentiation inducing extracellular signals. We conclude that as with metazoan _-catenin, Aar plays both a structural and signalling role during development.

Novel Roles of Cell Adhesion Molecules during Development of *Dictyostelium*

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Multicellularity in *Dictyostelium* is achieved by the expression of cell adhesion molecules. However, cells during chemotactic migration must constantly break and re-make intercellular contacts, suggesting a highly dynamic process of adhesive interactions among the cells. An analysis of contact regions and membrane rafts isolated from cell aggregates revealed that they both contained high levels of the GPI-anchored cell adhesion molecule gp80, but devoid of the other cell adhesion molecules, DdCAD-1 and gp150. In addition, several major proteins including comitin, porin and actin shared by both membrane fractions. Chemical cross-linking revealed that gp80 molecules were clustered in both rafts and contact regions. Significantly, the relative amount of membrane rafts was much reduced in gp80-null cells and detergent-resistant contact regions became undetectable. Thus, gp80 clustering may be necessary for the coalescence of rafts into cytoskeleton-associated membrane contact regions and constitute the basis for the dynamic adhesive interactions during cell streaming.

Immunofluorescence staining studies showed the co-localization of gp80 and gp150/LagC in the intercellular contact regions. Interestingly, a lower molecular form of gp150 was found in isolated sheath material, indicating that it is a component of the extracellular matrix. Furthermore, *lagC*-null cells failed to synthesize EcmA protein and cellulose and thus no sheath was formed, suggesting that gp150 might play a regulatory role in the formation of the extracellular matrix. In the absence of the slime sheath, gp80 was unable to maintain stable cell-cell contacts. Cell motility within these aggregates led to the shedding of gp80 complexes and the dissociation of cells. In chimeras of wildtype and *lagC*-null cells, wildtype cells expressing gp150 sorted to the periphery of the aggregate and synthesized both EcmA protein and cellulose, resulting in the deposition of the extracellular matrix and the rescue of morphogenesis. These results highlight the importance of the sheath in maintaining multicellularity and its role in the progression of developmental events.

The Cell Adhesion Molecule gp150 is Encoded by the *lagC* Gene and is Involved in the Formation of the Extracellular Matrix during *Dictyostelium* Development

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gp150 is a cell surface glycoprotein which mediates EDTA-resistant cell-cell adhesion in the post-aggregation stages of *Dictyostelium* development. An analysis of its tryptic fragments by MALDI-TOF mass spectrometry led to the identification of gp150 as the product of the *lagC* gene, which was previously cloned based on a gene-disruption mutant strain with a block at the loose aggregate stage. Antibodies raised against GST-LagC fusion proteins recognize specifically the gp150 protein, which is absent in *lagC*-null cells. gp150 is present in cell-cell contact regions, consistent with its role as a cell adhesion molecule. gp150 exists in two molecular weight forms, and subcellular fractionation indicates that the lower molecular weight form is a sheath component. Confocal microscopy revealed novel patterns of gp150 expression during development. Cells entering the loose aggregate last begin to express high levels of gp150 and they move to the periphery of the aggregate to form a one-cell layer of gp150-positive cells. These cells are also actively involved in the synthesis of extracellular matrix components, such as EcmA protein and cellulose. Then they move upward from the basolateral regions, forming the tip structure of the tight mound. At later stages, gp150 is synthesized in both anterior and posterior cells and become concentrated in the intercellular contact regions throughout the slug. Thus, the early expression of gp150 defines a novel layer of cells involved in the synthesis of the sheath, which is a prerequisite for the formation of tight mounds and further morphogenesis.

Dynamics of Rotation and Sorting in "Pancake" Aggregates of Dictyostelium

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The cellular dynamics of aggregation and sorting were followed in a novel set-up which constrained developing cells of Dictyostelium to the 2d plane between a cover-slip and an agarose layer. We used time lapse fluorescence microscopy of strains expressing GFP under the control of either a prespore (cotB) or a prestalk (ecmA) promoter to analyze relative movement of the cell types over periods of 10 hours. Under these conditions the cells aggregate into groups containing up to one thousand cells each within which the cell types were fully intermingled. Rapid coherent rotational motion of the cells within such round "pancake" structures was followed and quantitated. Subsequently, prestalk and prespore cells sorted out and formed discrete regions, typically leading to an elongated motile structure, the flattened equivalent of a migrating slug.

We have also observed rapid rotational movement in aggregated cells of the Wang-Kuspa strain that carries a null mutation in the aggregation adenylyl cyclase (*acaA*$^-$) and over expresses PKA-C (Rappel et al., 1999). Since these cells accumulate no measurable cAMP, it seems unlikely that chemotaxis to cAMP is necessary for rotational movement. Model calculations show that adhesive energies between the cells coupled with propulsive forces generated by the cytoskeleton can account for our observations, without the need for any chemical wave guidance.

Rappel, W-J., Nicol, A., Sarkissian, A., Levine, H. and Loomis, W.F. (1999) Self-organized vortex state in two-dimensional *Dictyostelium* dynamics. Phys. Rev. Letters (in press).

Regulation of Cell Adhesion and Cell-Type Proportioning by the *Dictyostelium* Protein AmpA.

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The *Dictyostelium* protein AmpA contains multiple repeats of cell adhesion regulatory domains found in the disintegrin and ornatin protein families. Although the two domain types are structurally distinct, both function as anti-coagulant moieties first described in pit viper venoms (the disintegrins) and in leech saliva (the ornatins). When starved *Dictyostelium* cells are plated for development, as much as 30% of the total AmpA protein is secreted. During morphogenesis, *ampA* reporter gene expression is restricted to the Anterior-Like Cell (ALC) population. ALCs play important roles in regulating tissue proportioning, and also undergo dramatic cell migration patterns that facilitate slug migration and morphogenesis of the final culminant.

We describe here the phenotypic characterization of an *ampA*$^-$ strain generated by targeted gene replacement. Axenically growing *ampA*$^-$ cells are smaller and tend to associate into clumps to a greater extent than wild type cells. The increase in *ampA*$^-$ cell-cell adhesion becomes more dramatic when cells are starved in non-nutrient buffer suspensions. Starved *ampA*$^-$ cells plated for development are also more adhesive to the underlying substrate compared to wild type cells. The increase in cell-cell and cell-substrate adhesion may be responsible for the delays in developmental progression we observe in *ampA*$^-$ structures. The severity of the delay depends on prior growth conditions: axenically grown cells are delayed in tip extension, 2 day bacteria-fed cells are delayed at aggregation and arrest development at the mound stage, and 3 day bacteria-fed cells fail to initiate development. *ampA*$^-$ slugs make numerous sharp turns and loops not observed in wild type slugs, and leave a larger number of cells in their slime trails. Finally, structures formed by axenically grown *ampA*$^-$ cells show early and increased expression of *ampA* (ALC) and *pspD* (prespore) reporter genes, and a delayed expression of the *ecmA* (prestalk) reporter gene. Reporter gene analysis further shows that prestalk cells are delayed in migration to form the tip in *ampA*$^-$ structures.

Based on sequence homology, evidence that AmpA is secreted, the *ampA*$^-$ mutant phenotypes, and reporter gene expression patterns, we propose that AmpA functions as an anti-adhesive protein to prevent a premature increase in cell-cell adhesion and to facilitate the movements of migratory cells during *Dictyostelium* morphogenesis.

3D *in vivo* analysis of *Dictyostelium* mounds reveals directional sorting of prestalk cells and defines a role for the myosin II regulatory light chain in prestalk cell sorting and tip protrusion

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Two models have been proposed for how cell sorting occurs in *Dictyostelium*: chemotaxis or differential adhesion. To distinguish between these possibilities, we have used time-lapse 3D microscopy to observe the motion of GFP-tagged prestalk and prespore cells. We found that one class of prestalk cells (ecmA-expressing cells) moved independently and directionally to form a cluster. Such motion supports a chemotaxis model in which a long-range signal attracts many of the prestalk cells to the site of cluster formation. Surprisingly, the ecmA prestalk cluster that we observed was initially found at a random location within the mound, defining an intermediate sorting stage not widely reported in *Dictyostelium*. The cluster then moved en masse to the top of the mound to produce the classic, apical pattern of ecmA prestalk cells. Migration of the cluster was also directional, suggesting the presence of another long-range guidance cue. Once at the mound apex, the cluster continued moving upward leading to protrusion of the mound's tip. To investigate the role of the cluster in tip protrusion, we examined ecmA prestalk-cell sorting in a myosin II regulatory light chain (RLC) null in which tips fail to form. In RLC null mounds, ecmA prestalk cells formed an initial cluster that began to move to the mound apex, but then arrested as a vertical column that extended from the mound's apex to its base. Mixing experiments with wild-type cells demonstrated that the RLC-null ecmA prestalk-cell defect is cell autonomous. These observations define a specific mechanism for myosin's function in tip formation, namely a mechanical role in the upward movement of the ecmA prestalk cluster.

Multiple Ras Pathways in *Dictyostelium* Movement and Endocytosis

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We have used gene disruptions to investigate the functions of several different *Dictyostelium* Ras proteins. The resulting phenotypes were unexpected, and in each case involved cell movement.

As we have reported previously, loss of RasG causes cells to lose polarity, move slowly, and fail in cytokinesis. RasS, which had previously been assumed to be involved with aggregation, is in fact required for normal fluid-phase endocytosis and phagocytosis. Vegetative *rasS* null cells also move unusually rapidly, which suggests that endocytosis and cell movement are reciprocally regulated. Lastly, and perhaps most surprisingly, loss of RasD caused almost no changes in normal development - the timing of aggregation and cell-type specific differentiation was the same as wild type, and cell-type proportions and patterning were also normal. Slugs from *rasD*$^-$ cells do, however, show a clear defect in phototaxis and thermotaxis.

We have also isolated a large family of RasGEFs, the proteins which activate Ras pathways. *Dictyostelium* contains at least eight unpublished RasGEFs, in addition to aimless, the first to be isolated. We have prepared knockout strains for several of these genes and will discuss their phenotypes at the meeting.

Function of small G protein racB and racC in *Dictyostelium.*

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Thus far 14 homologues of mammalian rac proteins have been identified in *Dictyostelium*. It is unclear whether each of these genes has a unique function and whether all play a role in actin cytoskeletal organization. One of the most well characterized rac genes is racE, which is involved in maintaining intact cytoskeletal structure and controlling cytokinesis[1]. RacC, on the other hand, has been shown to regulate polymerization of actin by inducing spherical actin containing protrusions called petalopods and to play a role in phagocytosis. We continued to study RacC and, in addition, have begun to examine the function of several other Rac genes

We have studied the effect of racC on the cytoskeleton using cell lines conditionally overexpressing wild type (WT racC) and the constitutive active form of racC (CA racC). Alterations in the cytoskeleton were monitored by time lapse movies of cells also expressing a probe which tracks dynamics of F-actin. This probe consists of the actin binding domain of ABP-120 fused to GFP (GFP-ABD. The ultra-structure of actin filaments and 3-dimensional cell shape was also analyzed by electron microscopy. In contrast with WT racC expressing cells, CA racC cells have an irregular cortex with almost no protrusions and a very flattened shape. Distictive vacuolar structures connected by tubular network were observed by CMFDA (chloromethyl fluorescein diamine) staining were greatly enlarged in CA racC expressing cells. Cells expressing CA racC also become multinucleated during prolonged induction.

RacB is highly homologous to RacC, however, cells expressing CA racB display a distinctive phenotype. Early in induction of CA racB, they contain numerous protrusions, become detached and start to form bleb-like protrusions resembling apoptotic membrane boiling. Later, the cells stop dividing and eventually die. On the other hand, WT racB expressing cells are extremely flat, displaying extensive cortical flow.

We have also cloned the rest of the RacA gene. This gene is quite distint from any other Rac gene yet described. The gene encodes a xx ORF encoding a protein of predicted size of xx kd. The 5' end of the gene is homologous to other Rac genes, however the 3' extension has no significant homology to other sequences in the database. Antibody localization and expression of the CA version of the protein are in progress to try to ascertain the function of this novel Rac gene.

From these data, we propose that each rac protein will have a different role in regulating cytoskeletal function and the shape of a cell.

Identification of a Ponticulin Gene Family.

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The major , high-affinity link between the cytoskeleton and the plasma membrane in vegetative ameoba is the small transmembrane protein ponticulin. Recently, three homologues of the ponticulin gene have been identified as members of expressed sequence tag libraries (Kobayashi,A., Shimizu,H. and Urushihara,H. - *Dictyostelium discoideum* Sexual cDNA library project, Yoshino,R., Morio,T. and Tanaka,Y. Developmental cDNA in Dictyostelium discoideum(slug stage) and Morio,T., Urushihara,H., Saito,T., Ugawa,Y., Mizuno,H., Yoshida,M., Yoshino,R., Mitra,B.N., Pi,M., Sato,T., Takemoto,K., Yasukawa,H., Williams,J., Maeda,M., Takeuchi,I., Ochiai,H. and Tanaka,Y, in The *Dictyostelium* developmental cDNA project: generation and analysis of expressed sequence tags from the first-finger stage of development, DNA Res. 5 (6), 335-340 (1998)). These ests have been generated to mRNA isolated from different stages in the *D. discoideum* life-cycle: 1. fusion competent *D. discoideum* cells; 2. Slug stage cells; 3. First finger stage cells. The genes were identified by sequence homology. The proteins encoded by these genes are all similar in size to ponticulin and are predicted to have many of the same characteristics as ponticulin: a cleaved signal sequence, a lipid anchor, transmembrane peptide domains and a -strand structure. In addition, these proteins are all predicted to have six cysteines, of these five are absolutely conserved. The sixth cysteine has been shifted by two amino acid residues. Because of the sequence similarity and their predicted conserved structures, these genes are all members of the ponticulin gene family. We propose that these genes be referred to as ponB, ponC, and ponD to reflect their homology to ponA the gene that encodes ponticulin. Currently we are investigating the relative abundance and expression profiles of these genes. [Supported by - NIH R55AR44721, R01AR44721, Research Excellence Program in Biotechnology, Oakland University to ALH, HHMI Undergraduate Biological Sciences Fellowship to LLA]

CHARACTERIZATION OF VILLIDIN, A VILLIN-LIKE PROTEIN FROM *DICTYOSTELIUM*

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A large number of actin-binding proteins regulates the actin cytoskeleton in motile non-muscle cells. Most of the filament fragmenting proteins share repetitive sequence motifs, of which severin contains three, whereas gelsolin and villin contain six domains. In addition villin harbors an extra C-terminal headpiece domain of 8.5 kDa which confers filament bundling activity. During the last *Dictyostelium* meeting we reported cloning and sequencing of a new gene which codes for a unique villin-like molecule of 1704 amino acids with a molecular mass of 190 kDa and a calculated pI of 6.9. Villidin is developmentally regulated; in early developmental stages it is only present in very small amounts, whereas the concentration increases at the beginning of the aggregation stage and reaches the maximum just before culmination. The protein seems to be present in the cytoplasm as well as at membranes.

The protein sequence shows four distinguishable elements: (1) The C-terminus exhibits 40% similarity to villin and protovillin[1]. The first of the six villin domains seems to be absent in villidin, but the typical headpiece is present. Sequence motifs known to bind F-actin can be found in the second domain as well as in the headpiece (KKEK-motif). (2) The N-terminus is novel among villin-like proteins and harbors WD-40 repeats. These two elements with homologies to other proteins are linked by (3) a PST-rich region and (4) a region without any obvious structural or functional significance. To investigate the contribution of each of these domains to the subcellular distribution of villidin, GFP fusions have been generated and analyzed in vivo using confocal laser scanning microscopy. GFP fusions of the N-terminal domain comprising the WD-40 repeats localize at macropinosomes and phagosomes, and dissociate shortly after the endosome is formed. Double staining with TRITC-phalloidin shows colocalization with actin at these regions. In aggregation competent cells this GFP fusion redistributes to newly formed pseudopods. In addition, in fixed cells the N-terminal GFP fusion appears to localize at membrane compartments. GFP fusions of the central region and, surprisingly, of the C-terminal domain, displayed no obvious enrichment, either during endocytosis or during pseudopod formation.

Vilidin-minus mutants have been generated using a replacement vector. Complete absence of villidin did not affect growth and development.

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DdABP1: A novel SH3 domain-containing actin-binding protein that binds *Dictyostelium* myosin I heavy chain kinase.

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The *D. discoideum* myosin I heavy chain kinase (MIHCK) phosphorylates and activates the small, single-headed myosin I isozymes. MIHCK is a member of the p21-activated kinase (PAK) family, and consists of a proline-rich N-terminal domain, a CDC42/Rac binding domain and a C-terminal kinase domain. GFP-MIHCK fusion proteins expressed in *D. discoideum* localize to the cell periphery, but only if the N-terminal domain is present. To identify proteins that might interact with the N-terminus of MIHCK, a fragment consisting of residues 1-228 of MIHCK was used as bait in a yeast two-hybrid screen of a *D. discoideum* library (gift of S. Lu, Baylor) . This screen identified a novel SH3 domain-containing protein that we have tentatively named DdABP1, since its overall structure is most similar to *S. cerevisiae* ABP1 (ScABP1) and mammalian SH3P7. DdABP1 consists of a 150 residue N-terminal actin binding domain that shares 28-35% sequence identity with the actin binding domains of ScABP1, SH3P7 and *D. discoideum* coactosin. The actin binding domain of DdABP1 is followed by six highly acidic 16-19 residue long repeats that have no counterpart in ScABP1 or SH3P7. The C-terminal SH3 domain of DdABP1 shares 48% sequence identity with the SH3 domain of ScABP1 and, interestingly, 58% sequence identity with the SH3 domains of *D. discoideum* myoC and myoD. GST-DdABP1 has been cloned and expressed in bacteria and used to generate a rabbit polyclonal antibody. When used to probe crude extracts of *D. discoideum* the antibody detects proteins of 55 kDa and 18 kDa (presumably DdABP1 and coactosin). The anti-DdABP1 antibody co-immunoprecipitates MIHCK, but not MIHCK lacking the N-terminus. Studies to determine if the SH3 domains of DdABP1, myoB, myoC (gift of Dr. J. Hammer, NIH) and myoD directly bind the N-terminus of MIHCK are underway (Funded by the MRC of Canada).

Evidence for a Functional Role of Myosin II in Rear Retraction

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To test the function of myosin II in rear retraction of a cell, we have been examining GFP fusions of two myosin II mutants.
Previous observations of a normal myosin heavy chain tagged with GFP (GFP-MHC) revealed a "C" to spot cycle, especially in cells moving in a mound (Clow and McNally (1999), Mol. Biol. of the Cell 10: 1309-1323). In directionally moving cells, a "C" forms at the rear cell cortex, possibly by myosin II polymerization. As the cell pulls in its rear, the "C" condenses into a spot, which subsequently disappears, possibly due to myosin II depolymerization.

To test the role of myosin polymerization (i.e. filament formation) in the "C" to spot and rear retraction, we examined a mutant with predominantly monomeric myosin (3xAsp). In GFP-3xAsp the "C" to spot transition still occurred in the mound, but the rear did not retract properly and remained elongated for an extended period, leaving a "dangling dot" of GFP-3xAsp myosin. Our interpretation is that in 3xAsp myosin the "C" to spot still occurs because myosin monomers (or short filaments) can still move over actin. However, this "C" to spot is not productive because thick filaments are needed to exert enough force for proper retraction. These observations provide the first direct functional evidence that myosin filaments are required for proper rear retraction of a moving cell.

After the cell rear is pulled in, myosin must be repositioned to start a new retraction cycle. To determine whether myosin depolymerization is required for this step, we examined a myosin that constitutively forms thick filaments (3xAla). In this mutant, the cortical myosin "C" resembled an "I", demonstrating that the cell posterior is much less curved during rear retraction. Moreover, a myosin spot often persisted near the middle of a then curved "C" yielding a "winged spot" pattern. The 3xAla myosin spot was always larger than normal and often failed to dissipate. These observations show first that myosin depolymerization is required for dissolution of the spot in the normal myosin "C" to spot cycle, and second that the level of myosin polymerization is important for the normal changes in shape of the cell posterior during retraction.

Based on these results, we propose the following working model: Filamentous myosin II binds to cortical actin at the cell rear. As it condenses from a "C" to a spot, myosin facilitates rear retraction by contracting the cortical actin meshwork. This contraction may help pull in the cell posterior and/or help break adhesion bonds embedded in the cortical actin meshwork. Myosin repositioning is than facilitated by myosin depolimerization.

Dynacortin, a genetic link between the global cortical tension generating system of RacE and coronin and the equatorial cortical tension generating system of cortexillin I and myosin II.

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In order to gain insights into the mechanisms of cytokinesis, we have conducted a chemical mutagenesis screen for cytokinesis deficient *Dictyostelium* strains. One strain, 11-5.1, has defects when grown on surfaces, has a highly penetrant cytokinesis defect when grown in suspension culture, and develops abnormally on *Klebsiella* lawns. Using library complementation and multi-copy suppression analysis, we recovered four genes as suppressors of 11-5.1. These genes include versions of cortexillin I, a novel protein named dynacortin, coronin and an ezrin-radixin-moesin family protein. Three clones of cortexillin I were recovered independently. Two were recovered by rescuing growth in suspension and one was recovered by rescuing development on *Klebsiella* lawns. All three clones are predicted to encode versions of cortexillin I that are missing the amino-terminal actin-binding domain. The mRNA for cortexillin I is absent in 11-5.1, strongly suggesting that the defect in 11-5.1 is due to loss of cortexillin I. This allows us to rename 11-5.1 as *cortI*$^{11\text{-}5.1}$. Constructs that encode versions of dynacortin were recovered two times independently by rescuing *cortI*$^{11\text{-}5.1}$'s growth in suspension defect. The full-length dynacortin gene encodes a novel 354 amino acid soluble phosphoprotein that is 18% serine. The *cortI*$^{11\text{-}5.1}$ rescuing clones of dynacortin encode only the carboxy-terminal 181 amino acids of dynacortin. We overexpressed the full-length dynacortin in cells and it induced a dominant cytokinesis defect in wild type, *cortI*$^{11\text{-}5.1}$, and myosin-II null cells, indicating that neither cortexillin I nor myosin-II is required for this dominant defect. In contrast, overexpression of dynacortin in RacE null cells failed to induce this dominant defect, suggesting that RacE might be required for a part of dynacortin's functions. Since the *cortI*$^{11\text{-}5.1}$ rescuing version of dynacortin encodes only the carboxy half of the protein, it suggests that this version might be antagonizing the normal dynacortin function. Antibodies raised against dynacortin revealed that dynacortin localizes to the cortex of the cell but is enriched in dynamic cortical domains including pseudopodia, lamellipodia, filopodia, and the phagocytic cup. A similar localization pattern was observed for a GFP dynacortin fusion protein in live cells verifying the localization. This pattern is remarkably reminiscent of the cortical localization of coronin and the regions of high filamentous actin content. Indeed, these proteins are "co-enriched" in the cortex. During cytokinesis, dynacortin localizes globally at the cell cortex similar to coronin and RacE. Intriguingly, the coronin construct that was recovered from the suppression experiment expresses an antisense version of coronin and was discovered in a cell that also contained a rescuing cortexillin I construct. This suggests that antagonism of coronin might offer a selective advantage to a cortexillin I impaired cell. Together, the data favor a model in which there is a global system for cell shape control and cortical tension generation that includes dynacortin, coronin, and RacE. During cytokinesis, the cortically localized cortexillin I and myosin-II become enriched in the cleavage furrow where they generate a regional increase in cortical tension that creates a tension gradient that leads to cell cleavage. If this system is impaired, as in the case of *cortI*$^{11\text{-}5.1}$, it can be compensated for if the global tension generating system is reduced to restore the tension gradient. Therefore, the cleavage furrow does not appear to be a discrete structure but is a function of spatially restricted and globally distributed tension generators in a continuous cortex.

A mathematical model for cell movement in multicellular systems: *Dictyostelium* aggregation and slug movement.

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A mathematical model for cell movement in multicellular systems has been developed that allows us to simulate and visualize, in three dimensions, individual cell movements in a number of multicellular systems. These include cell movement during, aggregation and slug stage of *Dictyostelium discoideum*, embryogenesis, limb formation and wound healing.

The model is quite adaptable to a number of systems, due to the way it is designed: The building blocks of the model are individual cells, and each cell has certain given properties, not necessarily the same for all cells. The basic properties are that a cell can deform under force (either stretch or compress), while conserving its volume, it also adheres to other cells and it can generate an active motive force. The response of a cell depends on its internal parameter state, and on the information it receives from its external environment, which includes neighbor cells, the extracellular matrix and chemical signals. The cell experiences forces from its surrounding and the net force on a cell is calculated by summing up all these forces. Each cell is then moved and deformed according to the given equations of motion, and the movement of all the cells represents the collective movement of the entire tissue.

Here I will introduce the model and and show examples of its applications and compare the results with experimental data. Among the simulations I will show is how different cell types sort out based solely on differences in adhesion. These results are compared to cell sorting experiments done by Steinberg *et al.* (1, 2) using values for adhesion within the range of the experimental values, and show that the model reproduces the experiments very well. I here show simulations of *Dictyostelium* aggregation, where cells are moving chemotactically in response to cAMP waves. In these simulations one can see the stream formation and how the mound arises due to the inward motion of the cells towards the signaling center. I also show simulations of 2-D slugs, and compare them to observations of 2-D slugs done by Bonner (3).

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Functional analysis of a prokaryotic antisense system in Dictyostelium

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In E. coli, copy number of RI plasmids is controlled by the copA/T antisense system. The short copA transcript as well as the 5' located copT segment in the recA coding mRNA are highly structured and it has been shown that these secondary structures are required for efficient sense - antisense interaction and thus for posttranscriptional recA silencing.
In a Dictyostelium expression vector with _-galactosidase driven by the actin 6 promoter, we fused the copT sequence in frame 5' to the _-gal region. Since the vector carried the Blasticidin resistance, low copy number (approx. 1 to 5 copies per genome) transformants were isolated. In a second step, copA, driven by the developmentally regulated discoidin promoter and integrated into a high copy number Neomycin resistance vector was introduced. Analysis of _-gal expression showed a significant decrease in cells where copA expression was induced. This clearly demonstrated that a 5' inserted copA/T system could efficiently cause antisense mediated gene silencing. We are now investigating the effect of mutations in the copA/T system which maintain complete complementarity but change secondary structure and have been shown to be approx. 100fold less efficient in E. coli. With the advent of RNAi (interfering RNA) we are also trying to express double stranded copA/T RNA to investigate it's effect on copT-_-gal fusions. PTGS (posttranscriptional gene silencing) involves upf/smg genes (for RNAi), RdRp (for quelling) and possibly RNaseIII homologs for antisense functions. We have so far cloned an RdRp and RNaseIII gene from Dictyostelium and are now investigating the effects of functional knock outs on antisense mediated gene silencing.

Characterization of the cellulose synthase gene in *Dictyostelium*

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Cellulose is a major component of the extracellular matrices formed during development of the social amoeba, *Dictyostelium discoideum*. Cellulose microfibrils are imbedded in the slime sheath that surrounds migrating slugs and are woven together to form the stalk tube of fruiting bodies. Moreover, thick cellulosic cell walls are laid down during terminal differentiation of both spores and stalk cells. We isolated two independent insertional mutants that fail to accumulate cellulose at any stage of development and showed that the same gene was disrupted in both strains. The predicted product of this gene, *dcsA*, shows significant similarity to cellulose synthases of *Acetobacter xylinum* and *Agrobacterium tumefaciens* as well as putative plant cellulose synthases. Cellulose synthase activity was completely missing in extracts of the mutant strains but was recovered in strains transformed with a construct expressing the intact *dcsA* gene that rescues the mutant phenotype. Although the 120kD protein synthesized from the *dcsA* gene was present in these rescued strains from the start of development, cellulose was not prematurely synthesized and only accumulated at the normal time after the mound stage. Thus, the cellulose synthase encoded by *dcsA* appears to function together with other developmentally regulated components to synthesize cellulose. It has long been noted that *Dictyostelium discoideum* holds particular promise in understanding the mechanisms of eukaryotic cellulose synthesis. Its promise derives from the inducibility of cellulose synthesis in *Dictyostelium*, the existence of a well-characterized *in vitro* assay system for the enzyme activity, and the applicability of a wide range of powerful molecular genetic methodologies. With the identification of the gene for the catalytic subunit this potential can at last be realized. To the advantages listed above can be added the presence of a single gene for the cellulose synthase, which will greatly facilitate the molecular genetic analysis of cellulose synthesis in this organism.

Contributions of Cellulose and a Cellulose-Binding Protein to Assembly of the Dictyostelium Spore Coat

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In response to an appropriate trigger, the spore coat forms at the surface of each prespore cell within 30 mins from 9 major proteins, a galuran, and cellulose. The proteins and galuran are delivered from prespore vesicles (PSVs) by exocytosis, whereas cellulose is extruded *de novo* across the plasma membrane. From the admixture of vesicle-derived molecules and nascent cellulose appears the trilaminar coat with cellulose fibrils comprising the central region.

SP85 is a multidomain coat protein whose C-terminal region binds cellulose *in vitro*. To map domains critical for targeting SP85 to the PSV and interacting with the coat *in vivo*, its N- and C-terminal regions, and a hybrid fusion of the N- and C-regions, were expressed in prespore cells with N-terminal c-myc epitope tags. Immunofluorescence showed that only the N-terminal region and the N/C-hybrid accumulated in PSVs, whereas only the C-terminal region and N/C-hybrid were incorporated into the coat after secretion. To determine if SP85 is important for the incorporation of other coat proteins, an SP85-null strain was created and found to mislocalize the coat protein SP65 to the interspore matrix. *In vitro* binding studies demonstrated that the SP85 C-terminal region bound SP65 and cellulose simultaneously, and SP65 incorporation was partially rescued *in vivo* by the C-terminal region. SP85-null spores showed increased latent permeability to a fluorescent lectin probe and increased germination efficiency, suggesting that coat barrier functions were compromised. Dominant negative reductions in barrier functions also resulted from expression of the SP85 terminal regions, suggesting that SP85 cross-linking activities were important in both the prespore vesicle and at the cell surface. Thus, separate domains of SP85 specify prespore vesicle compartmentalization and coat incorporation, and additional domains cross-link SP65 to the coat and interact with other binding partners which contribute to coat barrier functions.

The contribution of cellulose to the coat assembly process was investigated in strain DG1099, a cellulose synthetase-negative strain recovered from a REMI-screen. Imunolocalization revealed that DG1099 fails to exocytose proteins and galuran from the PSV. However, exocytosis was rescued by co-development with cellulose-normal cells. When co-developed with strains whose coat proteins lacked certain carbohydrate epitopes present on the DG1099 coat proteins, it was possible to track the fate of the secreted DG1099 proteins. DG1099-derived coat proteins, including SP85, became associated with the cell surfaces of both cellulose-negative and cellulose-positive cells. Association with future cellulose-positive cells appeared to precede cellulose deposition, which was seen by Calcofluor-induced fluorescence to start at a single point and subsequently spread to enclose the entire amoeba. The cell surface structures of the cellulose-negative cells were fragile and shortly after their formation contracted into small knots thereby expulsing the enclosed amoebae. In conclusion, cellulose has two roles in spore coat formation. First, it provides a signal for exocytosis of coat proteins and the galuran, presumably by an indirect, transcellular mechanism. Second, it stabilizes a precoat which is formed by the exocytosed proteins and galuran.

Ribonucleotide Reductase Expression During Development

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Prior to differentiation, prespore cells undergo a wave of DNA synthesis. The role of this developmentally programmed burst of DNA synthesis is unknown. The enzyme ribonucleotide reductase catalyses the reduction of ribonucleotides to deoxyribonucleotides, the precursors in DNA synthesis. Temporally and spatially correlated with this wave of DNA synthesis is the elevated expression of the gene encoding the small subunit of ribonucleotide reductase, *rnrB* (1). We have examined the promoter of the *rnrB* gene. Deletion analysis showed that expression of this gene in vegetative cells involves an A/T-rich element whereas its expression in prespore cells during development requires a region encompassing two G/C-rich elements, designated box A and box B (2).

We have constructed a mutant in which the *cis*-regulatory elements responsible for the prespore expression of *rnrB* have been replaced with a marker conferring resistance to blasticidin. We are in the process of analyzing the effect of this mutation on development. In addition, we are identifying factors that interact with the *cis*-regulatory elements.

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Macrocyst cycle in *Dictyostelium discoideum*: - A part of the genome is reserved for it.

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Cells of *Dictyostelium discoideum* proliferate by fission whenever food supply is sufficient. When starved, they set some of the population aside high on the ground as spores at the sacrifice of others. When submerged, however, spore formation is no longer beneficial, so that they switch the emergency mode to macrocyst formation for future possible germination. Inside the shelter of macrocyst walls, they occasionally exchange genetic materials, creating new types. This is the story of macrocyst cycle, a sexual process in *D. discoideum.* We take it as a model system for fertilization in higher organisms and are studying molecular mechanisms of sexual cell fusion in *D. discoideum.* Here I discuss about our results with an attention to relationships between sexual and asexual development.

Common pathways: We have several lines of evidence that suggests shared mechanisms between asexual and sexual developmental processes. First, we isolated chemical and REMI mutants that are defective in both fruiting body and macrocyst formation; One chemically-induced mutant, XMC2, is severely reduced in sexual fusion potency and is also aggregation-defective in fruiting body formation. A REMI-mutant TMC1 is fully potent for sexual cell fusion but defective in post-fusion development of macrocysts. It showed the aggregationless phenotype in asexual development, and likely to be interfered at somewhere in the cAMP-mediated signaling pathway. Second, zygote-specific gene *G9b* we isolated by mRNAdd is induced by starvation, and thus expressed during fruiting body formation. Thirdly, members of a clustered multigene family *GP138* (Hata *et al*. in this meeting), which is responsible for sexual cell fusion, are differentially expressed in asexual and sexual processes but are closely related.

Specific pathways: Some of other mutants are completely normal in fruiting body formation, while defective in macrocyst formation. Two chemically induced independent mutants, XMC7 and XMC17, and a REMI mutant MCF1 belong to this category. The relevant gene for defective phenotype in MCF1 was isolated and named *macA*. This gene is 6.3 Kb long and encodes a protein with two membrane localization signals at N- and C-termini. It is an absolutely novel gene, and we cannot find any homologous sequences in databases. Identification of the product is in progress.

Analysis of gamete cDNAs: We randomly sequenced gamete cDNAs and are analyzing their functions (Iijima *et al*. in this meeting). We succeeded in isolation of many genes specific to gametes, but those indispensables for macrocyst development have not been obtained yet. Since raising antisense mutants and gene disruptants are laborious, we may make better use of those mutants by establishing a collaboration system.

Membrane protein gp64 inhibits the whorl formation of Polysphondylium pallidum

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gp64 is a 36-cysteine containing and glycolipid (GPI) anchored membrane protein. Disruptants of gp64 gene show very normal development in the light, whereas they show an unusual phenotype in the dark: they cause extensive branching on a lying stalked cell mass (the cell mass remains in contact with the substratum, because of the lack of light). In the control cells, a cell mass shows a migratory slug without branching in the dark, because of the delay of development in the dark.

Assuming that gp64 functions as a repressor and is able to express permanently, then the multicellular mass of the transformant would continue to remain unmatured without branching. We linked the Dictyostelium ecmB promoter to the coding region of gp64, and introduced the construct into cells: in fact, the transformant synthesized gp64 continuously and constitutively, and showed a Dictyostelium fruiting body like phenotype without branching.

In chimeric organisms composed of only10% disruptants and 90% wild type cells, branched fruiting body comprised 40% of the multicellular body at 36 hr in the dark, whereas in wild type and the knockout cells, branched fruiting body comprised only 5% and 96%, respectively, under the above conditions, suggesting that a diffusible molecule induces branching.

These results strongly suggests that gp64 represses the whorl formation of Polysphondylium. Currently, we are also analyzing four Dictyostelium gp64's homologs which also are cysteine-rich and GPI anchored proteins and preparing overproducing and antisense constructs for these homologs.

Expression of human gonadotropins in *Dictyostelium*

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The gonadotropins human choriogonadotropin (hCG), luteinizing hormone (LH), and follicle stimulating hormone (FSH) regulate cellular and endocrine function of the reproductive organs. These complex glycoproteins consist of two subunits (_ and _). Each subunit contains several disulphide bonds, and N-linked glycosylation is required for folding and secretion of these hormones. Until recently, these hormones were mainly expressed in mammalian cells like Chinese Hamster Ovary (CHO) cells.
Dictyostelium is a host cell which provides simple genetics in combination with complex protein synthesis. We show that gonadotropins can be produced and secreted by this organism (1, 2). Furthermore, FSH and hCG produced by *Dictyostelium* bind to their human receptors and elicit a biological response comparable to the wild-type hormones.
We constructed variant genes which contain regions with codons that are preferably used in *Dictyostelium*. Results show that especially adaptation of the first 10 codons plays an important role in raising the expression level. Experiments mutagenizing the amino acids on position 94 and 95 in the _ subunit of hCG demonstrates that structure-function analysis using random mutagenesis and screening of recombinant glycoprotein hormones is feasible (2). Since *Dictyostelium* can be used in medium throughput growing protocols (1,000-10,000's), expression of gonadotropins in *Dictyostelium* opens the way to the engineering of potential new gonadotropin analogues. The results presented here indicate that *Dictyostelium* may be succesfully used as a heterologous expression host for screening purposes of complex human glycoproteins.

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Screening for *Dictyostelium* cell death mutants

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Development generally implies elimination of a number of cells by a process called programmed cell death (PCD). PCD involves a cascade of ordered complex molecular steps in the dying cell and has been described in a variety of eukaryotic and prokaryotic models. We have chosen *Dictyostelium* as a model to study cell death; indeed it appears that PCD plays a great part in its development during stalk cell formation. Previous studies in the laboratory have shown that stalk cells present hallmarks of death resembling those observed in other eukaryotes but PCD in *Dictyostelium* is somewhat different from apoptosis (1).
In order to identify genes involved in *Dictyostelium* cell death, a program of REMI electroporation and PCD resistant mutants screen was set up using HMX44 as recipient strain. Transfected cells were tested for resistance to death upon differentiation conditions. Surviving cells (cell death mutants) were recovered by their ability to regrow in rich medium.
As 10-20% of untranfected cells do not die, successive rounds of differentiation-regrowth are required to allow death resistant mutants to emerge from this background (2).
Six cell death resistant mutants have been isolated so far using this approach. Sequences flanking plasmid insertion have been recovered; their analysis is under way.

Attemps are also made to reduce the background of untransfected surviving cells; two approaches are undertaken :
* GSK-A inactivation : Harwood *et al*. (3) showed that knock out of *GskA* gene lead to the formation of an abnormal high number of stalk cells. Inactivation of this gene in HMX44 cells before REMI electroporation might improve the stalk cell formation and thus reduce background during regrowth.
* PKA overexpression : like GSK-A, PKA is a key enzyme in Dictyostelium development . Its activation can trigger both stalk and spore maturation. Overexpression of *Dd* PK2 under various promoters will be tested in HMX44 strain; the resulting strains might become our starting cells to study PCD.

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GTPase-binding homology (GBH) domain protein isolated in a yeast 2-hybrid screen.

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Elucidating the regulatory mechanisms of G protein-mediated signal transduction requires the identification of components that interact with G protein subunits. A yeast 2-hybrid system was used to search for proteins that interact with the *Dictyostelium* G_4 subunit. One of the genes, *gipA*, identified in this search encodes a protein with a GBH (GTPase-binding homology) domain near the carboxyl terminus. GBH domains have been found on several other proteins that interact with GTPases such as ras proteins or small G proteins. The sequence identity between gipA and other GBH domain containing proteins is limited primarily to the GBH domain suggesting the gipA protein might represent a different class of GBH-domain proteins. The entire open reading frame of the *gipA* gene has been isolated from the *Dictyostelium* genome. An insertional mutation within the gipA gene results in an aggregation defective phenotype. The *gipA* mutant cells cannot chemotax to cAMP but retain the chemotactic responsiveness to folic acid. These phenotypes suggest the gipA protein might function in cAMP responses by interacting with the G_2 subunit.

A Cell-Counting Factor Regulating Aggregate Size in *Dictyostelium discoideum*

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During development, *Dictyostelium* cells form large aggregation streams which break up into groups of roughly $2x10^4$ cells that then go on to form a fruiting body. The mechanism which determines this group size or number is mostly unknown. Using shotgun antisense to generate mutants which are unable to regulate aggregate size, we previously identified and cloned the *SmlA* gene (Brock *et al*., Development 122, 2569-2578, 1996).

smlA cells form very small fruiting bodies because they oversecrete an unknown factor which causes aggregation streams to break up groups of approximately $5x10^3$ cells. We have purified this counting factor and find that it behaves as a complex of polypeptides with an effective molecular weight of 450 kD. One of the polypeptides is a novel, 40 kD hydrophobic protein we have named countin. The *countin* gene is expressed in vegetative cells and throughout development.

In transformants with a disrupted *countin* gene, there is no detectable secretion of counting factor, and the aggregation streams do not break up, resulting in huge aggregates (up to $2x10^5$ cells) and fruiting bodies. The large aggregate size seen in *countin* cells can be reversed by mixing them with 10% Ax4 cells. In addition, these cells can be induced to form small aggregates by mixing them with 10% *smlA* cells. A similar effect can be observed when *countin* cells are developed on filter pads soaked with conditioned medium (CM) from Ax4 or *smlA* cells. Finally, removing countin from *smlA* CM by incubating the CM with antibodies to countin negates the ability of *smlA* CM to induce small aggregates.

These experiments support the following hypothesis. To sense the number of cells in an aggregation stream, *Dictyostelium* cells secrete counting factor which diffuses into the surrounding environment. The concentration of counting factor increases as the number of cells in the stream increases, and the cells are able to sense this. If the concentration goes above a threshold level, the stream breaks up into smaller mounds. Thus, *Dictyostelium* cells use a secreted signal to regulate mound size and hence fruiting body size.

Phosphothreonine and Phosphoserine Containing Proteins during Formation, Dormancy, and Germination of *Dictyostelium discoideum* spores.

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Previous studies have implicated PKA as a positive regulator of spore formation and maintenance of dormancy in spores of *Dictyostelium discoideum* (1, 2). In addition, we have previously demonstrated the role of PKA in the osmotic regulation of spore dormancy (3). The adenylyl cyclase, ACG acts as an osmosensor and modulates the production of cAMP. High osmotic pressure present in the mature sorocarp induces the highest ACG activity levels. The rapid increase in cAMP releases the regulatory subunit from the PKA complex stimulating activity (3,4). PKA thus catalyzes the phosphorylation of protein(s) to maintain dormancy. To investigate potential targets of a PKA signal transduction cascade, we have screened total cell lysates for phosphorylated serine and phosphorylated threonine containing proteins. Using previous methods to stimulate and deactivate the germination process, we have identified proteins that are modulated during spore germination. Heat-shock induced germination stimulates a change in the phosphorylation profile of spores. Immediately following the activation step, we have identified two proteins that demonstrate different levels of phosphorylation compared to dormant spores. Also through 5 hours of germination we have identified proteins that progressively change phosphorylation states. Wild type NC4 spores remain dormant until aged 14-21 days old and acquire the ability to spontaneously germinate when cleared of the sori matrix. We have identified two proteins in which the phosphorylation state is modified during the aging process. The phosphorylation state of these proteins may account for the ability of spores to activate in the absence of spore germination promoters. SG1, a mutant of wild type NC4, ages prematurely to acquire the ability to spontaneously germinate after one to two days. This mutant exhibits a phosphorylation pattern that differs from that of the wild type parent. The differences between the phosphorylation patterns may represent abnormal regulation that allows for accelerated development in the mutant.

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Regulation of cbpA Expression During Dictyostelium Development

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The cbpA gene encodes the protein CBP1, a member of the family of small, four EF-hand Ca2+-binding proteins in Dictyostelium discoideum. During vegetative growth, neither cbpA mRNA nor CBP1 are detectable in the cells. However, upon starvation, both appear just prior to cell aggregation, reach maximum levels in aggregates and remain at high levels throughout late development. Cell separation experiments indicate that CBP1 is expressed equally in prestalk and prespore cells.

When starved AX2 cells were exposed to cAMP pulses or to continuous cAMP in fast- and slow-shaken suspensions, and examined by Northern blot analysis for expression of different developmental genes, cbpA was found to exhibit an expression pattern similar to both early and primary late genes. To characterize the cbpA promoter, a 1337 bp fragment of genomic DNA upstream of the gene was cloned and sequenced. Within this sequence, the region between -250 and -550 bp contained an almost perfect 70 bp inverted repeat flanking a very GC-rich (GCR) 14 bp sequence that resembled a mammalian Rel/NF-kB-binding site. Furthermore, each half of the repeat sequence contained consensus GBF (GRE)- and DdSTAT (SRE)-response elements. Analysis of cbpA promoter deletions fused to lacZ indicated that the minimal promoter necessary for cbpA expression and correct temporal and spatial regulation resides between -252 and -443
bp (i.e., the GCR and cbpA proximal GRE and SRE sequences). Electrophoretic mobility shift assays (EMSAs) revealed that the GCR, GRE and SRE sequences each bind a protein(s) expressed only during development. However, competition EMSAs and Northern blot analysis of cbpA
expression in GBF-null and DdSTATa-null strains suggested that neither of these transcription factors is essential for normal cbpA regulation.

When the GCR sequence of the minimal promoter was deleted (leaving only the proximal GRE and SRE), lacZ expression was restricted spatially to prestalk and stalk cells. This finding indicates that the GCR-binding factor(s) might function as a positive regulator of cbpA expression in prespore cells and spores. (Supported by the NSERC of Canada)

An F-Box Protein and Terminal Differentiation

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In a selection for mutants that preferentially become spore cells, we isolated a gene which we call *chtA* (Cheater A). The mutation in *chtA* causes abnormal slug development and culmination, while the pre-culmination stages are unaffected. The slugs are longer than wild-type slugs, and initiation of culmination, which includes the arrest of slug migration and the movement of the posterior toward the anterior compartment of the slug, does not occur. The mutant does not form a fruiting body, but the terminal slug-like structure extends upward to the lid of the Petri dish. When the *chtA* mutant is developed in a chimera with wild-type cells, *chtA* mutant cells adopt the spore-cell fate while the wild-type cells differentiate into non-viable stalk cells.

The *chtA* mutant correctly expresses the pre-spore marker (*pspA*) during the first stages of development, which include the loose aggregate, tight aggregate, and tipped mound stages. *PspA* expression patterns reveal an expansion of the pre-spore compartment in the mutant. Although pre-spore cells form in the slug, they are unable to mature into spores. The pre-stalk marker, *pstA*, shows normal expression throughout pre-culmination stages, even at the slug stage when the GFP-marked pre-spore cells reach into the pre-stalk compartment. The *chtA* mutation does not block pre-stalk cell differentiation within the slug. However, the mutation prevented pre-stalk cell maturation within the terminal structure.

The developmentally regulated 4.2-kb *chtA* transcript is expressed from 4 hours of development onwards. The gene has an F-box domain and five WD40 domains, a pattern consistent with members of the family of F-box proteins. F-box proteins form part of a complex and cull specific proteins destined for proteosomal proteolysis. The details of F-box protein action will be presented. We postulate that ChtA is required for the ubiquitination and degradation of proteins that are involved in the decisions to culminate and to differentiate into mature spores and stalk. Our colleagues Margaret Nelson, Rick Firtel, and Jeffrey Williams have also cloned this gene.

Transient expression of a mitochondorial gene cluster (dia3) including rps4 is essential for the phase-shift of Dictyostelium cells from growth to differentiation

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Using synchronized Dictyostelium discoideum Ax-2 cells and the differential display method, a mitochondrial gene cluster (referred to as differentiation-associated gene 3; dia3) was isolated as one of genes expressed specifically during the transition of Ax-2 cells from growth to differentiation. The dia3 gene encodes for a mitochondrial protein cluster (NADH dehydrogenase (NAD) subunit 11, 5, ribosomal protein S4 (RPS4), RPS2 and NAD4L). Northern blot analysis using non-synchronized Ax-2 cells has shown that the dia3 RNA of about 8kb is scarcely expressed during the vegetative growth phase, and the maximal expression was attained at 2 hours after starvation. To analyze the gene function of dia3, we tried inactivation of rps4 by means of homologous recombination and obtained several transformed clones showing mitochondrial DNA heteroplasmy. The rps4-inactivated cells grew normally in nutrient medium, but many of them failed to differentiate after starvation, presumably by a reduced level of the cAMP receptor 1 (car1) expression. On the other hand, the overexpression of rps4 was found to enhance somewhat the progress of cell differentiation, thus suggesting importance of the rps4 expression in the phase-shift of Dictyostelium cells from growth to differentiation. Here it is of interest to note that the RPS4 protein has several nuclear localization signals after PSPORT2 Prediction.

A cellular basis for division of labour in *Dictyostelium discoideum*

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The dictyostelid amoebae, or cellular slime molds, are likely the most "primitive" eukaryotic organisms to display social behaviour with reproductive division of labour. Unicellular amoeabe of *Dictyostelium* aggregate to form a colony within which division of labour takes an extreme form, pre-stalk amoebae die and form a stalk; pre-spore amoebae form a ball of spores above the stalk. The overall shape of this 'fruiting body' evidently favours dispersal of the spores. The central problem in all this from the point of view of sociobiology is, What might be the evolutionary advantage that accrues to those amoebae that die and in so doing, apparently enhance the reproductive fitness of others but not themselves?

Cell fate is ultimately decided by a combination of intrinsic and extrinsic cues, the latter involving intercellular interactions. The intrinsic cues encode a cellular 'quality,' a variable related to fitness, and the extrinsic cues enable cells to assess relative qualities. In the present study we investigate a possible basis for the assessment of cellular quality in two strains of *Dictyostelium discoideum*, X22 and NP20. Our results show that the spore forming efficiency of NP20 is higher than that of X22, both when measured individually as well as in 1:1 chimeras. Consistent with this, the ratio of stalk length to sorus diameter is higher in X22. FACS analysis show that amoebae and spores of NP20 (or X22) are larger (or smaller) and display low (or high) auto-fluorescence. The concentration of DIF, a supposed pre-stalk inducer, was measured in both the strains. NP20 has a 1.25 fold higher concentration of DIF(on a per-cell basis) than X22. Interestingly, DIF bioassay of stalk cell differentiation in monolayer cultures shows that X22 amoebae are more sensitive to DIF than NP20. Thus, in chimaeric fruiting bodies NP20 increases its fitness by forming more spores than appropriate. These results also show that apart from intrinsic differences there is some extracellularly factors which may change the behaviour of cells in chimaeric mixtures. These findings will be discussed in the light of a proposed evolutionary model (Atzmony et al., 1997) based on the individual cell as the unit of selection in Dictyostelid slime molds.

AN ANALYSIS OF CELL DEATH IN Dictyostelium discoideum.

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Programmed cell death (PCD) is a universal mechanism by which cell number regulation and patterning is achieved by multicellular organisms during growth and development. 'Apoptosis' seems to be the principal means whereby most PCD takes place. We have been trying to look at the mechanism by which PCD occurs in the pathway leading to
stalk cell differentiation during D.discoideum development. Our results show that:
(i) There is no internucleosomal cleavage of DNA leading to DNA fragmentation. Correspondingly, there is no calcium-magnesium dependent endonucleolytic activity in nuclear extracts from various developmental stages. However, nuclear condensation and peripheralization does occur in the stalk cells, also repoted by Cornillon et al.
(ii) A gradual increase can be seen in the number of cells with exposed phosphatidyl serine residues as detected using Annexin V. This is an indicator of altered membrane permeability and asymmetry during development. Under low-density conditions and under conditions that promote calcium-induced stalk cell differentiation amoebae show
Propidium iodide uptake within a short time window, also indicating altered cell membrane permeability.
(iii) The number of cells showing Caspase-3 activity (as measured using the fluorescent synthetic peptide substrate Ac-DEVD-AMC) increases as development proceeds and then declines in the terminally differentiated fruiting body.
In summary, our results indicate that cell death in D.discoideum shows some of the features of apoptotic cell death recognized in other multicellular systems.

Developmental Analysis of the Dictyostelium LIM-only protein limB

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LIM family proteins are defined by a very conserved and specific zinc-finger sequence motif. The LIM domain appears to mediate protein-protein interaction and family members include transcription factors, kinases, and cytoskeletal proteins. We have identified limB, a 23 kDa protein comprised entirely of 3 LIM domains. limB mRNA is not expressed in growing cells, but is induced early during aggregation with maximal expression observed at 10-15 hr of development. limB-nulls grow normally, but are delayed in development and form extremely large aggregation territories and correspondingly large multicellular structures and fruiting bodies. No major defects are observed in prespore/pretalk gene expression patterns. Cells that overexpress limB also grow normally but exhibit defects in mound formation. Resulting fruiting bodies, however, appear normal. We are interested to define proteins that interact specifically with limB. We expressed and purified limB in fusion with FLAG and an HMK phosphorylation site. The limB fusion was labeled in vitro and used as a far western probe on Dictyostelium protein blots. limB does not interact with purified limB protein but does recognize a specific 32 kDa protein in wild-type and limB-null cells. The 32 kDa limB-interacting protein is present in detergent soluble and insoluble cellular compartments and is detected in growing cells and during development. cDNA expression libraries are being screened in an effort to identify the 32 kDa limB-interacting protein and to understand its function and that of limB during development.

Identification and characterization of a new filamin binding protein

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The filamin family of proteins is a group of ubiquitous F-actin crosslinking proteins in eucaryotic cells. Recent studies give evidence that this protein family not only stabilizes cortical cellular actin but links actin networks to membrane proteins and directs intracellular signaling reactions to the actin scaffold. Several membrane proteins and membrane receptors as well as intracellular signaling molecules have been proven to be direct binding partners of human non-muscle filamin. In order to find binding partners of the *Dictyostelium discoideum* filamin (120 kD gelation factor) we used a part of the filamin cDNA as bait in the yeast two-hybrid system. We identified a 2.7 kb fragment encoding the C-terminal part of a so far unknown protein as a potential filamin binding partner and subsequently cloned the full-length cDNA. Northern blot analysis reveals a single mRNA of approximately 7 kb which is expressed at moderate levels late in development. The deduced protein consists of 2046 aminoacids with a calculated molecular mass of 230,700. Computer program analysis predicts two leucine-zipper motifs, one at the very N-terminus and one at the C-terminus and a prolonged coiled-coil structure in the C-terminal part. Two-hybrid studies with shortened filamin constructs indicate the filamin rod-domain 3 as the binding region of the new protein. For localization studies in *Dictyostelium discoideum* a GFP fusion with the 2.7 kb C-terminal fragment has been generated. Analysis of the intracellular GFP distribution reveals a strong plasma membrane and some vesicular staining. Immunofluorescence studies indicate a colocalization with filamin as well as actin at the plasma membrane.

The Characterization of a Major Myosin II Phosphatase in *Dictyostelium discoideum*

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In *Dictyostelium* cells, myosin II has functions in cytokinesis, cell locomotion, and cortical tension maintenance. Phosphorylation of the myosin heavy chain at threonine residues 1823, 1833, 2029 is responsible for maintaining myosin in the non-assembled state, and subsequent dephosphorylation of these residues is a prerequisite for assembly into the cytoskeleton. Given the predicted central role of myosin heavy chain dephosphorylation in this system, we have biochemically characterized myosin heavy chain phosphatase activities in *Dictyostelium* utilizing myosin II phosphorylated by Myosin Heavy Chain Kinase A as a substrate.

The major MHC phosphatase activity was identified as protein phosphatase 2A (PP2A). The purified PP2A holoenzyme was composed of a 37 kDa catalytic subunit, a 55 kDa B-subunit and a 65-kDa A-subunit. The PP2A holoenzyme displays two orders of magnitude more activity towards myosin phosphorylated on the heavy chains than it does towards myosin phosphorylated on the regulatory light chains, consistent with a role in the control of filament assembly. The PP2A holoenzyme promotes bipolar filament assembly *in vitro* via dephosphorylation of the myosin heavy chain.

In order to improve understanding of PP2A function, we have characterized the genes encoding the *Dictyostelium* PP2A subunits, and have examined the immunolocalization of the PP2A subunits in *Dictyostelium.* The cDNAs encoding the B- and C-subunits were isolated from a *Dictyostelium* library and the deduced amino-acid sequences reveal strong conservation with the mammalian PP2A homologues. Southern blot analysis suggests that each of the PP2A subunit genes is present in a single copy. The PP2A subunits were localized mainly to the cytosol in *Dictyostelium* cells. However, immunofluorescence confocal microscopy demonstrates that the B-subunit of PP2A is highly enriched in centrosomes, suggesting a potential role for this PP2A regulatory subunit in centrosomal function.

Myosin Heavy chain kinases regulate Myosin filament assembly in *dictyostelium*.

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Myosin II assembly into the cytoskeleton in *Dictyostelium* is regulated by phosphorylation of sites in the tail portion of the myosin heavy chain (MHC). We are studying a family of novel protein kinases that participate in this process. The prototype for this family, Myosin Heavy Chain Kinase A (MHCK A), phosphorylates MHC to drive myosin filament disassembly. MHCK A (130 kDa) has a novel domain structure consisting of a helical coiled-coil domain, a catalytic domain unrelated to conventional protein kinases, and a WD-repeat domain. Biochemical and in vivo studies have demonstrated that the WD-repeat domain of MHCK A plays an important role in controlling the cycle of myosin assembly/disassembly by dramatically stimulating MHCK A activity towards myosin II filaments. Recent progress towards understanding the mechanism by which the WD-repeat domain confers the ability of MHCK A to phosphorylate myosin will be presented.

Myosin Heavy Chain Kinase B (MHCK B) is another member of this family of novel protein kinases in *Dictyostelium* which appears to play a role in controlling myosin II assembly. *Dictyostelium* cells that over-express MHCK B (MHCK B+ cells) are unable to grow to high density in suspension culture. This growth defect is rescued upon co-expression of a mutant myosin (3x ALA myosin) which cannot be phosphorylated in the mapped regulatory sites of the myosin heavy chain rod. For initial biochemical studies, native MHCK B (83 kDa) has been partially purified from MHCK B+/3x ALA *Dictyostelium* cells using cation exchange, hydroxyapatite, and Cibacron Blue dye-binding chromatography steps. Biochemical characterization of the semi-pure kinase has revealed that native MHCK B is a bona fide myosin heavy chain kinase that undergoes autophosphorylation. Continuing studies involving the identification of possible regulators of MHCK B activity will also be presented.

Characterization of *ras*C expression during growth and development.

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*ras*C encodes for one of a large group of *Dictyostelium* Ras subfamily proteins. The 189 amino acid protein is 65 % identical to the better characterized RasG and RasD proteins. We have recently generated antibodies specific to RasC and have shown that the RasC protein is present throughout growth and development. Peak protein levels were detected during late aggregation to early slug formation, correlating well with earlier published mRNA expression data. Using a PCR-based technique for chromosome walking, we have cloned a 0.6 kb fragment of DNA upstream of the *ras*C coding region encoding the putative promoter. Sequence analysis of the putative promoter reveals the presence of two possible transcription start sites. Whole mount *in-situ* staining for β-galactosidase activity of transformants carrying a *ras*C-*lac*Z fusion construct revealed enrichment of *ras*C expression in the terminal stalks and prestalk cell population with lower but detectable levels of expression in the prespore cell population. In efforts to understand the functional role/s of *ras*C in *Dictyostelium* growth and development, we are attempting to generate strains of *ras*C disruptants by creation of a null mutant by homologous exchange. An initial effort at an extensive screen for such knock out transformants was unsuccessful suggesting the possibility that the gene product may be essential for growth.

CELL CYCLE DEPENDENT REGULATION OF THE EXPRESSION OF EARLY DEVELOPMENTAL GENES

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Cell cycle phase in Dictyostelium is correlated with a preference for centre-initiating behaviour and initial cell type choice. G2-phase cells that are more efficient at initiating development exhibit a strong tendency to sort to the prespore region of aggregates, while S/M-phase cells sort to the prestalk region. It was previously been reported that G2 phase cells show an earlier induction of cAR1 gene expression than S/M phase cells. We show that increased levels of mRNAs encoding several genes induced in early development are observed in G2 relative to S/M phase cells after four hours of development. When the position of cells expressing high levels of a discIg::lac Z fusion was monitored in early aggregates, blue stained cells are found located preferentially in the centre. These experiments confirmed that G2 phase cells are prespore sorters is because they produce the machinery necessary for cAMP relay earlier than S phase cells, and are thus found at the centre of early aggregates.

Identification and characterization of ß-COP, a major component of COPI coated vesicles in *Dictyostelium discoideum*

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We have isolated a cDNA coding for ß-COP from *Dictyostelium discoideum* by polymerase chain reaction using degenerate primers derived from rat ß-COP. The complete cDNA clone has a size of 2.8 kb and encodes for 912 amino acids. As compared to the homologous proteins from rat, *Drosophila* and yeast, *Dictyostelium* ß-COP exhibits highest homology to the mammalian ß-COP, and it is considerably smaller than the known ß-COP proteins due to a shortened variable center part region that is thought to form a linker between the highly conserved N- and C-terminal domains. *Dictyostelium* ß-COP is encoded by a single gene which is transcribed at moderate levels into two RNAs which are present throughout development. For localization studies a GFP fusion with the full length ß-COP has been generated for expression in *Dictyostelium discoideum*. Analysis of the GFP distribution shows a diffuse vesicular staining as well as staining in the vicinity of the nucleus. Based on co-immunofluorescence studies with anti-comitin mAbs, an established Golgi marker, the stained compartment was identified as the Golgi apparatus. The effects of DMSO and Brefeldin A on the distribution of the ß-COP-GFP fusion protein were investigated.

Mutant *rac1B* expression in *Dictyostelium*: effects on growth, endocytosis and development.

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Rac is a small G-protein in the Ras superfamily that has been implicated in the control of cell growth, adhesion, and the actin-based cytoskeleton. Eight *rac*-related genes have been identified in *Dictyostelium*, three of which (*rac1A*, *rac1B* and *rac1C*) encode proteins 80% identical to the human *rac1* gene product. To investigate the role of one of these homologues, we have established *Dictyostelium* cell lines that conditionally express native or mutant Rac1B proteins. Epitope-tagged mutants were constructed by site-directed mutagenesis and inserted into the folate-inducible discoidin expression vector pDXD3C. Expression of either myc-tagged wild-type (WT), constitutively active (G12V and Q61L), or dominant inhibitory (T17N) Rac1B protein significantly reduced cellular growth rates in liquid suspension. When cultured on a solid substratum, "overexpressing" WT and constitutively active G12V and Q61L mutant cells appear larger than normal in size, multi-nucleate and often connected by a cytoplasmic bridge, indicating a defect in cytokinesis. Rac1B overexpressing mutants endocytose more FITC-dextran than do control cells, whereas regulatory mutants exhibited reduced rates of fluid-phase endocytosis. All mutants display normal rates of fluid-phase exocytosis. Developmental phenotypes range between arrest at the early mound stage (T17N, G12V, Q61L) and occasional formation of abnormal fruiting bodies (WT, T17N). Failure to complete development is not due to inhibition of chemotaxis, as *Dictyostelium* cells expressing mutant Rac1B proteins are still able to migrate up a chemotactic gradient in a dose-dependent manner. Ongoing work includes further characterization of the membrane trafficking phenotypes and biochemical identification of Rac1B-binding proteins. The ultimate goal is the understanding of Rac1 function using a cell type that is amenable to both biochemical and molecular genetic manipulations.

Human pyridoxal kinase gene complements *Dictyostelium* pyridoxal kinase knockout

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Pyridoxal kinase converts pyridoxal (vitamin B_6) to pyridoxal phosphate, an essential co-factor for amino acid transamination: a particularly important reaction for *Dictyostelium*, which depends on protein degradation for survival during development. The pyridoxal kinase gene (*PykA*) was isolated from *Dictyostelium discoideum* using insertional mutagenesis. The phenotype was agg-minus and this phenotype was also observed in a knockout strain produced in an AX2 background. Compared to AX2, the knockout mutant cells grew poorly in axenic medium with low yield but this was restored by the addition of exogenous pyridoxal phosphate. Sequencing of the genomic DNA and a cDNA clone indicated the presence of one intron in the gene, which codes for a predicted protein of 301 amino acids. The wild type gene was expressed in *E. coli* and the protein product was purified and shown to have pyridoxal kinase enzymatic activity. Transformation of the *Dictyostelium* knockout mutant with the wild type *Dictyostelium* pyridoxal kinase gene produced complementation of the agg-minus phenotype (with almost normal fruiting bodies being formed) and increased the growth yields in axenic medium to normal AX2 values. As the amino acid sequence of the protein shares 42% identity with the amino sequence of human pyridoxal kinase, transformation of the *Dictyostelium* knockout mutant was carried out with the human pyridoxal kinase gene. It was found to produce complementation of the mutant almost as well as that seen for the *Dictyostelium* gene, demonstrating a remarkable degree of cross-species conservation of structure and function.

Isolation of a novel gene, *227* , that regulates aggregative gene expression

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At the early stage of *Dictyostelium* development, a set of aggregative genes, such as *cAR1, gp80,* and *pde,* are turned on. The expression of these genes then carries out the cAMP signaling relay and chemotaxis, which leads to cell aggregation to form mounds. We isolated a Restriction-Enzyme-Mediated-Insertion mutant227 that is defective in aggregation. However, after supplied with cAMP pulse for five hours, it can go through the development at a similar pattern as *wt*. Biochemical analysis showed that the defect in generating cAMP results from low cAR1 expression. GP80 expression is also low in mutant227. So, *227* is required for G-protein mediated up-regulation of aggregative genes. The gene *227* encodes a novel cytosolic protein of 254 amino acids. There is a C2 motif in the gene. How it regulates aggregative gene expression is still under investigation.

UBIQUITIN-MEDIATED PROTEOLYSIS IS ESSENTIAL FOR DEVELOPMENT OF *DICTYOSTELIUM DISCOIDEUM*.

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In the social amoeba Dictyostelium discoideum, the ubiquitin system is activated upon stress and at the onset of development, which suggests that ubiquitin-mediated proteolysis is a hallmark of the developmental program of this organism. Dictyostelium undergoes simple development with clearly defined stages. A homogeneous population of cells aggregates and differentiates into two different cell types, which leads to the formation of fruiting bodies consisting of a spore mass on top of a thin stalk.
In a genetic screen to identify developmental genes, we have isolated the nosA gene (for no spores A). NosA is homologous to UFD2 from S. cerevisiae, a component of the UFD (ubiquitin-fusion-degradation) pathway. This pathway has been studied in yeast for its ability to degrade ubiquitin-_-galactosidase fusion proteins. We identified at least three proteins that are stabilized as ubiquitin-conjugates in the nosA mutant, suggesting that the role of NosA during development is to remove a specific set of proteins to ensure developmental progression.

We asked what other components act in the same pathway with NosA. We introduced second-site mutations into the genome and isolated those that suppress the nosA mutant phenotype. In this way, we have recovered a number of suppressors, many of which are in novel genes. One suppressor, which we call sonA (for suppressor of nosA), codes for a protein which contains a non-removable ubiquitin-like moiety at its N-terminus.
Pulse-chase and western blot analysis revealed that SonA is a stable protein that does not become ubiquitinated. There is also no difference in stability of SonA between wild type and the nosA mutant. This suggests that the ubiquitin-like domain of SonA confers an activity other than being the primary degradation signal that is recognized by NosA.
The isolation of nosA in D. discoideum links a component of the UFD pathway to cellular differentiation and defines a distinctive phenotype that can be employed for further biochemical and genetic studies.

snfA is a *Dictyostelium* Snf1/AMP kinase ortholog with a role in growth

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SNF1/AMP-activated protein kinases (SNF1/AMPKs) are a conserved group of eukaryotic serine/threonine protein kinases that mediate a variety of stress responses, including response to nutrient stress. This presentation describes the isolation of *snfA*, the gene encoding the *Dictyostelium discoideum* SNF1/AMPK ortholog, and an examination of *snfA* gene function. *snfA* is a single copy gene that encodes a predicted 80.3 kd polypeptide. A single 2.8 kb *snfA* transcript is present during vegetative growth and all stages of development. The predicted SnfA protein consists of three domains: an amino terminal kinase domain that is highly conserved in other SNF1/AMPK family members, a less-well conserved C-terminal domain that in budding yeast regulates kinase activity, and a unique aspargine-rich central domain encoded by a series of AAT trinucleotide repeats. A SNF1/AMPK activity (SnfA activity) assessed by phosphorylation of a specific peptide substrate (SAMS peptide) was detected in *Dictyostelium* cell lysates. Attempts to disrupt *snfA* were unsuccessful but SnfA activity was reduced to 35% of wild-type levels in transformants that expressed *snfA* antisense RNA. Since *Dictyostelium* development is a response to nutrient deprivation, the potential role of *snfA* in regulating the entry into development was investigated. No alteration in the ability to enter or progress through development was observed in cells with reduced SnfA activity. Growth in shaker culture, however, was inhibited in the antisense transformants.

Regulation of cell-cell adhesion by a cell-counting factor

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The ability of cells to determine the size of a biological structure plays an important role in organ development and regeneration. *Dictyostelium discoideum* uses such a mechanism during development. It must recruit enough cells to form a large, tall fruiting body, while restricting the number of cells to ensure that the fruiting body does not collapse under its own weight. We previously identified a mutant, *smlA*, that is defective in this process. *smlA* cells form extremely small fruiting bodies because they oversecrete a factor that causes the aggregation streams to break up prematurely, leading to small aggregates. In an attempt to understand this disruption, we have examined the cells' ability to adhere to each other in the presence of conditioned medium (CM) from both wild type and *smlA* cells. We find that after 8 hours of starvation, *smlA* cells are less adhesive than wild type cells. Thus the aberrant disruption of aggregation streams may be due to defects in adhesion in *smlA* cells. It has been demonstrated by others (Siu et al, 1985) that the protein gp80 is responsible for Ca^{++} independent adhesion at this time in development. Western blots stained with anti-gp80 show that at 8 hours of starvation, *smlA* cells have less gp80 than wild type cells do. During later development, gp80 expression increases in *smlA* cells. This suggests that gp80 expression or adhesion may be modified by *smlA* CM.
Computer simulations of cells in an aggregate stream show that high cell-cell adhesion causes the stream to remain intact. Decreasing the adhesion allows the stream to fragment, and a subsequent increase in adhesion causes the fragmented stream to re-condense into groups. The simulations show that a decreased initial adhesion results in smaller subsequent groups, in agreement with our observations of the decreased adhesion in *smlA* cells.

The action of lithium ions on glycogen synthase kinase-3 suggests its regulation by magnesium and ATP.

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Genetic studies suggest that Glycogen Synthase Kinase-3 (GSK-3) family members play a crucial role during both metazoan and *Dictyostelium* development (1, 2). GSK-3 is the only protein kinase known to be inhibited by Li^+ ions (3). To understand why GSK-3 is susceptible to Li^+ inhibition we investigated the biochemical effects of a number of GSK-3 inhibitors. We found that ADP and Be^{2+} competed for ATP binding to inhibit kinase activity and competed with each other for inhibition at the same site. In contrast, we found that Li^+ is a competitive inhibitor with respect to Mg^{2+} but not ATP and furthermore the mode of inhibition observed for Li^+ is independent of ADP or Be^{2+}. This suggests that a second Li^+-sensitive Mg^{2+} interaction at a site distinct from the Mg^{2+}/ATP-binding cleft is required for GSK-3 activity. As ATP chelates free Mg^{2+} ions, this interaction could act as part of a homeostatic mechanism in which the concentration of free Mg^{2+} acts as a "sensor" for the cellular ATP concentration. In support of this hypothesis we show that GSK-3 activity is inhibited at a high ATP: Mg^{2+} ratio, an effect not seen in the closely related protein kinase CDC2.

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Structures and molecular components of actin rods in dormant spores of *Dictyostelium discoideum*

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The organization of actin undergoes changes during sporulation of *Dictyostelium discoideum*; actin is tyrosine-phosphorylated (1,2), and a new type of actin rod appears in dormant spores (3). We have examined the ultrastructure of these rods by quick freeze methods and have determined the locations of actin-binding proteins and actin-related proteins. Tyrosine-phosphorylation of actin in these rods was also analyzed by cryoimmuno-transmission electron microscopy. At the middle culmination stage, rods appear in premature spores as bundles composed of tubular fibers averaging 12.6 nm in diameter, and hexagonally cross-linked before actin-tyrosine phosphorylation is detected. In mature spores, formed at the end of culmination, phosphorylation levels increase and rods elongate. Maximum phosphorylation levels are reached by 12 hours following the end of culmination, and phosphorylated actin is detected on these rods. Elongated nuclear rods push out the nuclear envelope causing the nucleus to acquire a lemon-like shape. The terminal end appears to make direct contact with the inner surface of the nuclear envelope. Immediately following the activation of spores for germination, actin is rapidly dephosphorylated, followed shortly thereafter with the disappearance of rods. During this disappearance, shortened tubular fibers once again become arranged in a hexagonal pattern. Strains lacking either _-actinin or the ABP-120 possess rods. Arp3, profilin and cofilin, but not EF-1_ are detected on these rods. Thus, it has been shown that actin can form tubular fibers, and Arp3 and profilin, as well as tyrosine-phosphorylated actin are constituents of actin rods *in vivo*.

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Spore aging affects the pathways for actin tyrosine dephosphorylation upon germination of *Dictyostelium discoideum*

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In the presence of germination signals, dormant spores of *Dictyostelium discoideum* rapidly recover a dynamic actin-based cytoskeleton required for spore swelling and the initiation of a new life cycle. Previously we have shown that half of the actin molecules in dormant spores are maintained in a tyrosine-phosphorylated state, and a decline of the actin phosphorylation levels is essential for spore swelling (Kishi et al., J. Cell Sci., 111: 2923-2932, 1998). We also have found that a trigger for actin dephosphorylation is D-glucose which is an ingredient of a nutrient germination medium. In this study, we found that both the trigger and pathway for actin dephosphorylation dramatically changed with spore aging in sori.

The trigger molecules for actin dephosphorylation in 1 to 3 day old spores were restricted to metabolizable hexoses. In 4 day old spores, non-metabolizable glucose and oligosaccharides as well as metabolizable hexoses exhibited dephosphorylation-stimulating activity. Treatment with the respiratory inhibitor, dinitrophenol, blocked actin dephosphorylation stimulated by all of the metabolizable hexoses, non-metabolizable hexoses and oligosaccharides in spores aged 1 to 3 and 4 days. Initiation of actin dephosphorylation in 1 to 3 day old spores may require uptake of hexoses and energy production through consumption of the hexoses. In 4 day old spores, the non-metabolizable hexoses and oligosacchrides may induce digestion of storage carbohydrates, such as trehalose or glycogen, and subsequent metabolism of derived glucose may lead to actin dephosphorylation. In contrast, incubation with non-nutrient phosphate buffer was sufficient for a decline in actin phosphorylation in spores more than 6 days old. This result suggests that in older spores, the removal of spore germination inhibitors from the spore surface is a trigger for actin dephosphorylation. Moreover, dinitrophenol never inhibited actin dephosphorylation, indicating that the dephosphorylation pathway in the older spores is independent of energy production. Working models for *Dictyostelium* spore germination processes will be shown.

mrpA: an ABC transporter involved in the starvation response of *Dictyostelium discoideum*

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A common motif of detoxification employed by many eukaryotes and prokaryotes is the expression of ABC transporters that hydrolyze ATP to actively export myriad substances out of the cell. Using the fluorescein derivative calcein as a candidate substrate, we have characterized two
major types of transporter activities in Dictyostelium cells. We report that Dictyostelium cells exhibit transporter activities that have pharmacological characteristics of MDR-type and MRP-type transporters. Both types of transporter activities are known to be sufficient to confer multidrug resistance to cancer cells in mammalian systems. We have also cloned mrpA, the Dictyostelium homolog of the mammalian MRP transporter, and have shown that it is responsible for prestalk specific calcein transport activity. Cells that are mutant for mrpA exhibit an increased
sensitivity to some nutrients during starvation-induced multicellular development.

The regulation of cAMP and cGMP signal transduction by a cell number-sensing factor in *Dictyostelium*

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The development of starved *Dictyostelium* cells includes aggregation into groups of roughly $2x10^4$ cells. *smlA* mutant cells (Brock et. al., Development 122, 2569-2578, 1996) form large numbers of small mounds and fruiting bodies. This is due to oversecretion of a cell number-sensing factor named counting factor (CF). CF is a complex of polypeptides, and one of these is a 40 kD protein called countin (Brock and Gomer, Genes&Development, in press). Disruption of the *countin* gene causes cells to form huge mounds and fruiting bodies.

We found that CF regulates cAMP signal transduction. *smlA* and *countin* cells have fewer cAMP receptors than Ax4 cells, suggesting that CF regulates cAMP receptor numbers in the Ax4 background. However, *countin* does not affect the cAMP receptor level in Ax2 cells, as these cells already have a decreased number of cAMP receptors compared to Ax4. Compared to wild-type cells, *countin* cells have a very weak cAMP signal relay while *smlA* cells have a much stronger relay. The cGMP signal transduction pathway is also regulated by countin. High levels of CF inhibit the cAMP-induced cGMP pulse, while low levels of CF prolong the cGMP pulse. CF may thus regulate mound size by regulating the response of cells to the chemotactic cAMP signal.

Effects of Chimeric-Actin Expression on Cell Motility and Development of *Dictyostelium discoideum*

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Tetrahymena actin has low sequence homology with typical actins (about 75%) and lacks binding to some actin binding proteins and phalloidin. It can, however, co-polymerize with typical actins and interact with myosins. To elucidate how actin, a multifunctional protein, works in various phases of cell motility and development, we constructed three chimeric actin genes from *Tetrahymena* and *Dictyostelium* actin sequences and expressed them in *Dictyostelium discoideum* Ax2 cells. The preliminary results of our earlier experiments on cell motility have been reported in Dicty '96 at Sendai. In this report, we present the detailed descriptions of the developmental regulation of cell motility and the morphogenesis of multicellular stages in these exogenous-actin-expressing cells.

T39D cells express the chimeric actin composed of residues 1-38 of *Tetrahymena* actin and residues 39-375 of *Dictyostelium* actin in addition to endogenous *Dictyostelium* actin. T84D cells express the chimeric actin composed of residues 1-83 of *Tetrahymena* actin and residues 84-375 of *Dictyostelium* actin. D84T cells express, residues 1-83 of *Dictyostelium* and residues 84-375 of *Tetrahymena*. The mTAc cells express the full-length *Tetrahymena* actin, the gene sequence of which is modified to be expressed in *Dictyostelium* cells.

All these transformants had normal morphology in vegetative growth phase and grew normally in suspension, although with some clone-dependent variation. D84T cells at pre-aggregation stage showed round morphology and decreased motility. T39D, T84D and mTAc cells showed motility at almost the same or slightly higher level than control cells. D84T cells delayed in aggregation and their morphogenesis arrested at the mound stage. T84D cells and mTAc cells made culmination, but the shapes of their final fruiting bodies were rather aberrant. T39D cells made normal fruiting bodies.

Thus, full-length and chimeric *Tetrahymena* actins affected differently in different developmental stages. Specific actin functions may be required in different stages.

Novel G protein-coupled receptor homologs in *Dictyostelium*.

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G protein-dependent signaling mediates a number of important functions in *Dictyostelium*. Thus far, only four highly homologous G protein-coupled chemoattractant receptors for cAMP (cARs) have been identified. However, there are a number of indications that additional, diverse types of G protein-coupled receptors (GPCRs) exist in *Dictyostelium*. First, the number of Gα subunits (8 which are well characterized and several putative ones in sequence databases) exceeds the number of known receptors. Second, G proteins have been implicated in responses to pterins and the cell density sensing factor CMF. In addition, *Dictyostelium* cells undergo chemotaxis to a variety of agents other than cAMP (e.g., folate, bacteria, LPA, and yeast extract). Presumably specific GPCRs exist which mediate these responses.

We identified two possible candidates, designated SSG217 and SSM331, in the sequence database of the *Dictyostelium* cDNA Project in Japan. Their complete sequences revealed the requisite seven putative transmembrane domains. Though substantially different from one another, both were found to be most similar to the cARs, conserving 23% residues in the region encompassing transmembrane domains III-VII including several that are widely conserved among GPCRs. Using RT-PCR, SSG217 was shown to be expressed in vegetative and early developmental stages while SSM331 is expressed only late in development following aggregation. These distinct expression patterns suggest that they have different functions. To assess these, the genes encoding the putative receptors were then disrupted by homologous recombination. Neither of the resulting knockout cell lines displayed obvious growth or developmental abnormalities, indicating that the putative GPCRs are dispensable or that redundant activities exist. Detailed analyses of the knockout cells are underway to evaluate subtle developmental defects and to assess their responses to known ligands such as folate and CMF.

2000-fold inducible gene expression by a tetracycline-controlled transactivator in *Dictyostelium*

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We established an inducible expression system that tightly controls expression of individual genes in *Dictyostelium discoideum (Dd).* The system consists of two components, one being an integrated plasmid encoding a eukaryotic tetracycline-controlled transactivator (tTA*) containing codons adapted to the preferred codon usage in *Dd*. The second component is an extrachromosomal plasmid containing the gene of interest. The ORF of this gene is preceded by a minimal promoter derived from the *Dd* actin15 promoter and a tTA*-binding region. In the presence of tetracycline, the transactivator is inactive and expression of the gene of interest is not or hardly detectable. In the absence of tetracycline, however, tTA* binds to its target and strongly activates transcription.

The system was tested with firefly luciferase as a reporter gene using several clonal cell lines expressing tTA* at different levels. As expected, in the presence of tetracycline luciferase activity was undetectable or very low. Removal of tetracycline from the medium strongly induced luciferase expression: within the cell lines expression levels varied from less than 10% to over 200% of the complete wild-type *Dd* actin15 promoter-driven luciferase expression level.

The kinetics and tetracycline concentration-dependency of induction were investigated in a clone with a very high inducible luciferase expression level. At a tetracycline concentration equal or below 0.01 ： g /ml, maximal luciferase expression was observed. Half-maximal expression was induced at 0.1 ： g tetracycline/ml, whereas only 0.05% of the maximal expression level was detected at tetracycline concentrations equal or above 1 ： g /ml. After removal of tetracycline from the medium, 50% of the maximal luciferase activity was induced within 16 hrs and full activity after 48 hrs.

Our results show that tight regulation of gene expression in *Dictyostelium* by nontoxic levels of tetracycline is feasible. This control system is a valuable tool for regulated expression of potentially toxic exogenous proteins and studying the effect of switchable expression of endogenous gene products in mutant and wild-type *Dd* axenic strains.

A twelve transmembrane guanylyl cyclase regulated by GTPγS and Ca^{2+}; a knock-out still aggregates

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As reported last year we have identified a guanylyl cyclase consisting of twelve putative transmembrane regions and two cyclase homology domains (DdGCA). This is the first guanylyl cyclase cloned with this topology, which is typical for adenylyl cyclases (e.g. DdACA). Overexpression of DdGCA in *aca*-null cells shows a 2.5 fold increase in guanylyl cyclase activity and no detectable adenylyl cyclase activity, demonstrating that the cloned gene encodes a guanylyl cyclase.

Recently we have been able to obtain a knock-out cellline. Up till now we have not been able to detect any phenotypical difference between these cells and wildtype cells (normal chemotaxis to cAMP and folic acid, osmotic shock, development to stalk and spores). In addition these cells show no significant reduction in guanylyl cyclase activity. Does this mean there are two (or more) guanylyl cyclases with compatible function or is the cloned guanylyl cyclase involved in pathways we have not identified yet?

To investigate the regulation of this guanylyl cyclase, without the background of the residual guanylyl cyclase(s), we mutated three amino acids, thereby creating an adenylyl cyclase ($DdGCA^{kqd}$) with which we measured activation in *aca*-nulls cells. The $DdGCA^{kqd}$ was able to rescue *aca*-null cells, creating small fruiting bodies. $DdGCA^{kqd}$/*aca*-nulls cells show a transient increase of cAMP levels, which peaks at 10 s, upon stimulation with 2'd-cAMP; folic acid or osmotic shock do not activate the enzyme. We also show that the adenylyl cyclase activity of $DdGCA^{kqd}$ is regulated by Ca^{2+} (strong inhibition). This inhibition is similar to the way membrane bound guanylyl cyclase in bovine eye is regulated via a guanylyl cyclase activating protein (GCAP). A strong activation is achieved by GTPγS (without GTPγS no activity could be measured). This activation suggest the regulation by a G-protein a typical feature of twelve transmembrane adenylyl cyclases.

So we here show the cloning of a new class of guanylyl cyclases that combines properties of mammalian membrane bound guanylyl cyclases (regulation by Ca^{2+} via GCAP) and adenylyl cyclases (regulation by GTPγS via G-proteins).

* The sequence has been placed into genbank™ with accession number #AJ238883

An F-Box/WD40 repeat-containing protein important for *Dictyostelium* cell type proportioning, slug behavior,and culmination.

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FbxA is a novel member of a family of proteins that contain an F-box and WD40 repeats and target specific proteins for degradation via the proteasomes. In fruiting bodies formed from cells where the *fbxA* gene is disrupted (*fbxA*$^-$ cells), the spore mass fails to fully ascend the stalk. Also, *fbxA*$^-$ slugs continue to migrate under environmental conditions where the parental strain immediately forms fruiting bodies. Consistent with this latter behavior, the development of *fbxA*$^-$ cells is hypersensitive to ammonia, the signalling molecule that regulates the transition from the slug stage to terminal differentiation. The slug is comprised of an anterior prestalk region and a posterior prespore region and the *fbxA* mRNA is highly enriched in the prestalk cells. The prestalk zone of the slug is further sub-divided into an anterior pstA region and a posterior pstO region. In *fbxA*$^-$ slugs the pstO region is much reduced in size and the prespore region is proportionately expanded. These results are therefore in accord with previous studies of an F-box/WD40 repeat-containing MEK kinase and of a putative ubiquitin conjugating enzyme, in suggesting that specific protein degradation forms part of the mechanism that regulates cell-type proportioning and culmination in *Dictyostelium*.

Involvement of a novel gene, *zyg1*, in zygote formation of *Dictyostelium mucoroides* and *D. discoideum* cells

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A novel gene, *zyg1*, has been isolated from *Dictyostelium mucoroides-7* (Dm7) cells using a cDNA library and differential plaque hybridization, as being preferentially expressed during the sexual development. The *zyg1* gene was expressed with a quite similar kinetics to a temporal change of zygote formation. Ethylene (inducer of zygote formation) known as a potent plant hormone actually enhansed the *zyg1* expression. Interestingly, *zyg1*-overexpression in *D. discoideum* Ax2 was found to induce giant cells and macrocyst-like structures by themselves without help of the opposite mating type, V12M2 cells, While *zyg1*- underexpression in Ax2 cells caused a failure of macrocyst formation in the presence of opposite mating type, V12M2 cells. These facts have strongly suggested that the *zyg1* gene may be involved in zygote formation in *Dictyostelium discoideum* cells. The involvement of *zyg1* gene in zygote formation of Dm7 cells is under investigation using *zyg1*-overexpressing and -underexpressing transformants derived from Dm7 cells.

A Protein from the Primary Wall of Macrocysts

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Few macrocyst-specific gene products have been identified in *Dictyostelium* and virtually none have been assigned a specific function. Using monoclonal antibodies, we have identified macrocyst-specific proteins in macrocysts produced from matings of *Dictyostelium discoideum* strains NC-4 and V-12 as well as in macrocysts produced by the homothallic strain *Dictyosteliu. mucoroides* Dm-7. The protein of 34 kilodaltons (M_r) from *D. mucoroides* was selected for further characterization. The protein was found to be uniquely present in the primary wall of macrocysts. It was localized to the primary wall by epifluorescent microscopy and confocal microscopy using a fluorescently labeled monoclonal antibody. The protein was not detected in a mutant lacking the primary wall. Western blot analysis on crude lysates probed with the monoclonal antibody showed the amount of this protein increased with time during macrocyst development. No specific protein, detectable by western blot analysis, was found in samples of vegetative amoebae or samples collected during sorocarp development. Enzymatic digestion of the protein with PNGase and EndoH provided no evidence for N-glycosylated moieties. Amino acid analysis of the amino terminal end produced the sequence XEIYNKDGNK... This partial sequence shows 100% identity with several porin proteins from the outer membrane of gram negative bacteria. A degenerate, gene-specific primer, whose design was based on this partial amino acid sequence, was used in 3' RACE to clone a one kilobase downstream region of the gene.

Folate Receptors Of *D. Discoideum* Vegetative Amoebae: Distribution And Trafficking

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Filopodia appear to serve a sensory role in amoebae from many phyla although other roles such as substrate-adhesion have not been ruled out. Vegetative amoebae of *D. discoideum* exhibit an array of branched long filopodia and this array is enlarged in the presence of any of the chemoattractant pteridines. We used an exogenously applied folate agonist and wild type (strain NC-4H) amoebae to investigate the distribution and possible fate of ligand-bound folate receptors in these amoebae.

A probe to study the distribution of folate receptors was constructed by binding the glutamate end of folic acid to lactalbumin-FITC conjugate. This probe (Folate∗) stimulates chemotaxis and enlargement of filopodial arrays. Thus, the physiological activity and the structure of Folate* validated its use as a ligand for folic acid receptors.

Late-log vegetative amoebae were harvested from suspension cultures into pH 7.3 phosphate-buffered saline. Fixed (using dilute formaldehyde/glutaraldehyde) or living (kept at < 5°C) amoebae were treated with Folate* and hexyl-rhodamine for examination by confocal microscopy. Hexyl-rhodamine is a membrane specific stain and was used a fluorescent counterstain to visualize filopodia.

Fixed amoebae exhibited a sparse punctate distribution of bound Folate* over their entire surface including filopodia. This distribution is consistent with the reported relatively-small number of folate binding sites (~10^5/cell). Live amoebae showed a significantly different appearance of Folate*. The ligand was not bound on any filopodia and there was relatively low level of Folate* bound onto the cell bodies. Most of the ligand appeared inside of many round ~1_m vesicle-like structures in the cytoplasm. It is important to note that the live cells were returned to room temperature when placed on the confocal microscope stage.

We interpret the presence of bound Folate* on filopodia of fixed amoebae as supporting a sensory role for their filopodia. However, our observations of live amoebae also supports a model that involves migration, aggregation, and endocytosis of ligand-bound folate receptors into what appear to be unusually large vesicles. This may be analogous to formation of caveolae in vertebrate cells. Our fixation protocol did not affect the general size or morphology of the cells but did block processes of receptor migration and endocytosis. This is consistent with a dependency of such processes on an array of proteins that are sensitive to cross linking fixation.

Endocytosis and removal of the bulk of the surface-bound Folate* is likely to be associated with a replacement with fresh folate receptors. Such a process is consistent with temporal sensing of chemoattractant gradients. Folate* may be a useful tool for addressing questions about the fate of receptor-bound pteridine chemoattractants and the form of folate that binds to receptors.

The extracellular factor inducing discoidin expression during development is different from CMF

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Development in *Dictyostelium* is regulated by the availability of nutrients and by extracellular factors which are secreted by the cells to monitor their denstity. Two extracellular factors were described, and both are glycosylated proteins: prestarvation factor, PSF, which is secreted during growth (Clarke et al., 1988), and conditioned medium factor, CMF, which is secreted during development (Gomer et al., 1991). Although the biological activity of PSF is readily detectable, its molecular nature is unknown. CMF was purified, its gene was cloned and disrupted, and CMF was shown to be essential for development (Jain et al., 1992).

We used the discoidins as molecular markers for the initiation of development. As described previously, discoidin is induced by medium conditioned by growing cells and by medium conditioned by developing cells (CM, which contains CMF). We present evidence that the factor responsible for discoidin induction in CM is different from CMF.

1. Recombinant CMF does not induce discoidin expression
2. CM's prepared from either wildtype cells or a CMF knockout strain (Brazill et al., 1998) both induce discoidin expression in wildtype cells
3. Both of these CM's also induce discoidin expression in the CMF knockout strain
4. Unlike CMF activity, the discoidin-inducing activity is higher in medium conditioned for 8h than in medium conditioned for 18h
5. the activity is heat labile (CMF is heat stable)

The factor detected in our assays could be either a new molecule or it could be PSF. So far we did not detect differences in biological activity between PSF and the discoidin-inducing factor:

1. Both medium conditioned by growing cells (containing PSF) and by starving cells (containing the discoidin-inducing factor) are able to induce discoidin expression during growth.
2. Both conditioned media are able to induce discoidin expression during development.

However, preliminary experiments suggest that PSF and the the discoidin-inducing factor in CM act on different elements of the discoidin promotor. This suggests that they are not identical.

Clarke et al., 1988, Differentiaiton 34, 79-87
Gomer et al., 1991, Development 112, 269-278
Jain et al., 1992, Genes and Development 6, 390-400
Brazill et al., 1998, Journal of Biological Chemistry 273, 8161-8168

A study of cell type proportion in relation to slug morphology in *Dictyostelium*

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Since the early studies on *Dictyostelium discoideum* it was noted that the proportion of its two main cells types is somehow regulated (Raper). This was observed in the following experimental results: (i) after removing a given cell type from the slug, a new slug is regenerated with roughly normal proportions (Bonner, 1955); (ii) the proportion of cell types is found to be more or less independent of the slug size (Bonner, 1948). However, a re-examination of these experiments reveals a wide scatter in the data; the regulation of cell type proportion seems to be far from precise. We have attempted to relate this scatter to the variations in the shape of the slug; first, by looking for correlations between slug shape and cell type proportion; second, by observing the changes in slug shape that occur after removing the posterior (mainly prespore) region. Preliminary results seem to support the shape dependency of the proportion.

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Heat shock cognate 90 (Hsc90) as a phosphoprotein that may be involved in transition of *Dictyostelium* cells from growth to differentiation.

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It has been demonstrated in *Dictyostelium discoideum* Ax-2 cells that 1) 90kDa and 101kDa proteins fail to be phosphorylated at a growth/differentiation check-point (PS-point) in the cell cycle under starvation conditions, 2) the highly phosphorylated state of proteins is generally unfavorable to cellular differentiation, 3) a part, at least, of 90kDa protein is located in the nucleus, and 4) the phosphorylation sites are limited to serine residues (Akiyama and Maeda, 1992). The present work was undertaken to examine more precisely the structure and function of 90kDa protein. The amino acid sequence of a part of purified 90kDa protein was found to be completely identical with that of heat shock cognate 90 protein (Hsc90) reported by Boves *et al*. (1995). Hsc90 is believed to be a member of heat shock protein 90 (Hsp90) family working as the molecular chaperone. To analyze the function of Hsc90, we attempted to obtain *hsc90*-null cells by means of homologous recombination. However, *hsc90*-disrupted transfomants were never isolated, thus strongly suggesting that the knock-out of *hsc90* gene might be lethal. This is consistent with the fact that geldanamycin, a specific inhibitor of Hsp90, greatly inhibits growth of Ax-2 cells. Interestingly, geldanamycin also seemed to impair transition of cells from growth to differentiation. We are now planning to inactivate *hsc90* at will, using the vector bearing the antisense *hsc90* under the control of actin6 (A6) promoter that is less active when cells were grown with bacteria. Preparation of *hsc90*-overexpressing cells and their analysis will be also promising to clarify the function of Hsc90, in association with the significance of its phosphorylation level.

Related G_ subunits function antagonistically during development.

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Like most eukaryotic organisms, *Dictyostelium* express multiple G protein G_ subunits that are important for the reception of different environmental signals. To examine potential relationships between the two most related G_ subunits and their signaling pathways, we conducted an extensive genetic analysis of Ga4 and Ga5 mutants. Contrasting phenotypes of *G_4* and *G_5* mutants in tip morphogenesis, responsiveness to folic acid, and cell localization suggested that these G_ subunits function antagonistically during development. Chimeric organisms composed of both G_ null mutants or both G_overexpression mutants displayed compensatory morphogenesis implying that these subunits promote complementary developmental processes. Strains lacking both subunits or overexpressing both subunits displayed phenotypes specific to each G_ subunit gene suggesting that these G_ subunits function independently of each other in many developmental processes. When expressed from the G_5 promoter, the G_4 subunit failed to fully rescue G_5 function or to produce phenotypes associated with elevated G_4 subunit expression, suggesting the interactions and distribution of the G_4 and G_5 subunits are different during development.

A Novel Factor(s) inducing the Morphogenesis of the Aggregation_minus Mutant lacking ERK2

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Many extracellular factors are known to play essential roles in the regulation of cell differentiation and morphogenesis in multicellular organisms. Most of efforts have been devoted to find such factors which enable Dictyostelium cells to differentiate under low cell density monolayer culture condition. DIF, CMF, CMF450, psi-factor and recent SDFs are successful examples of these efforts (Town et al., 1976; Gomer et al., 1991; Iijima et al., 1995; Oohata et al., 1997; Anjard et al., 1997). Here, we have considered a possibility that wild-type cells may secrete other factor(s) affecting morphogenesis.

Extracellular cAMP is a well-analyzed factor playing a central role in cell aggregation, at the initial stage of the morphogenesis. In order to see if there are any other factor(s) than cAMP, working for aggregation and morphogenesis, we used a set of mutants showing aggregation-minus phenotype due to the defects in cAR1 mediated signaling pathway for screening of the factor(s) in our assay system.

Previously, it was shown that cells lacking functional ERK2 can not aggregate because of lacking the activation of adenylyl cyclcase by extracellular cAMP stimulation. However, we found that the mutant cells have capability of morphogenesis to fruiting bodies under a condition with supplement of a certain factor(s) secreted by developing wild type cells. Under this condition, mutant cells formed extremely tiny tight aggregates without any aggregation streams. As this consequence, they developed to small Harley fruiting bodies and gave normal spore-to-stalk proportion in the fruiting bodies. When the spores of the fruiting bodies were plated on bacterial lawn, they germinated and multiply. The resulting cells gave their original phenotype, _aggregation-minus in the absence of the added factor(s). Furthermore, we observed that addition of cAMP did not give any aggregation forms in ERK2 minus cells. This shows that the factor(s) are not cAMP itself.

The suppression of the aggregation minus phenotype indicates that the factor(s) acts at the downstream of ERK2. As the characterized function of ERK2 is the activation of adenylyl cyclase, we have examined if the factor(s) were able to suppress the phenotype of the aggregation-minus mutants lacking the proteins which are necessary for the activation of adenylyl cyclase, such as CAR1, G-alpha2, G-beta, CRAC or ACA. But none of these mutants showed any sign of the morphogenesis in the presence of the factor(s). These results indicate that the production and detection of extracellular cAMP are essential for the function of the factor(s) and the factor(s) are important for the activation of adenylyl cyclase.

Thus, it was likely that the factor(s) act at the downstream of ERK2 only to elevate intracellular cAMP. However, from a observation that addition of 8Br-cAMP, a permeable analogue of cAMP, had no effect on the morphogenesis of the ERK2 mutant cells, besides of the activation of adenylyl cyclase, the factor(s) should commit a certain pathway which is indispensable for the aggregation and following morphogenesis.

Based on these findings above, we conclude that wild type cells secrete particular factor(s) which are essential for the initiation of morphogenesis and the activator of adenylyl cyclase in an ERK2 independent manner.

A Two-Domain Structure for Dynein-Microtubule Interactions: Using *Dictyostelium* to Map Functional Domains of a Large Polypeptide.

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Dynein is a high molecular weight motor protein important for microtubule-based motility in eukaryotic cells. It moves along a tubulin polymer through repetitive binding and release cycles that are tightly coordinated with force generation and nucleotide hydrolysis. ATP-sensitive contact of the motor with a microtubule has been mapped to a relatively small globular region (~125 aa) of the dynein heavy chain that lies at the distal end of an antiparallel coiled-coil stalk. This structure is unique among the three major families of cytoskeletal motor proteins and may also play a role in force production. We have made a series of 22 alanine substitutions within this contact region, and have expressed these in *Dictyostelium* within the context of the 380 kDa motor domain. While six substitutions had no obvious effect, the remainder showed distinct changes in microtubule binding activity. These not only enhanced (1) or reduced binding (11), but also affected nucleotide-stimulated release (4). The mapping work reveals the existence of two regions within the microtubule-binding domain that appear to act together to bind the motor to its substrate, and suggests a mechanism for coordinating binding with nucleotide hydrolysis. Moreover, it demonstrates the versatility of *Dictyostelium* as a protein expression factory.

An esterase from *Dictyostelium discoideum* involved in phagosome processing

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A cytoskeletally-associated glycoprotein of *Dictyostelium discoideum*, gp70, is an esterase and also called crystal protein because of its presence in crystalline structures called esterosomes. Although postulated to break down spore coats, the exact function of gp70 has not been defined. We report immunofluorescence microscopy and biochemical studies showing that gp70 was recruited to phagosomes and had a role in digesting bacteria. In comparison to wild type and cells overexpressing gp70, cells lacking gp70 grew more slowly on bacterial lawns or suspensions, and did not reach the same density in nutrient media. Immunoprecipitation studies showed that gp70 was secreted when cells were grown either in nutrient media or a bacterial suspension. Additional analyses revealed that disulfide bonds, predicted from the amino acid sequence, were present in the active protein isolated from detergent-insoluble cytoskeletons because the reductants dithiothreitol and b-mercaptoethanol retarded the mobility of purified gp70 on SDS gels and also inhibited its esterase activity in a dose-dependent manner. Esterase activity assays demonstrated the ability of gp70 to cleave the ester linkage between _- or _-naphthyl and 12-, 14- and 16-carbon acyl chains, which are the main fatty acids present in the lipid A moiety of bacterial lipopolysaccharides. The esterase activity of purified gp70 also was higher at pH 5 than at pH 7.5. Together, our results strongly indicate that despite its crystalline appearance, gp70 of vegetative cells is an active esterase involved in the degradation of phagosomal contents.

A novel gene trapping method used poly A signal sequences: An application to Dictyostelium genes

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PolyA trapping ,more recent addition to the gene trapping method, is a powerful method for mutating and isolating the flanking sequence of tagged clones. The trapping vector used contain the Bsr marker gene lacking in the polyA addition signal. Linearized vectors are introduced into cells by electroporation, allow to insert into the (close) vicinity to the 3' region of various genes, and then the fusion Bsr gene is now sufficient to activate its expression, because of supply of the poly A addition signal from trapped gene. (The transformants become selectable.) This type of fusion enables us to clone the 3' region of various developmental genes by 3'RACE, and the partial sequences obtained can be useful to get access to further information, comparing with ESTs of the Japan cDNA Project or sequences of the other data base.

We isolated 2000 transformants using the above selection strategy and screened 28 clones aberrant for developmental phenotypes. We determined the tagged sequences of 26 clones out of 28 clones: the range of tagged sequences are 40-600 bp and an average is about 150 bp. Three sequences within them have been found in ESTs of Japan cDNA Project; two sequences are very similar to genes found in gene banks of other organisms.

Characterisation of *Dictyostelium* mutants lacking extracellular matrix proteins.

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The products of the *ecmA* and *ecmB* genes (originally designated ST430 and ST310) are well characterised components of the *Dictyostelium* extracellular matrix, being found both in the slime sheath and in the stalk tube. It was previously shown that slug morphology was altered in null mutants lacking *ecmA*, whereas development in the *ecmB* null was apparently normal (1). More detailed analysis by SEM and TEM has revealed differences not previously detected. Over a certain size, the culminating *ecmA* null structures are prone to collapse, and do not form fruiting bodies of normal appearance. The *ecmB* nulls do consistently form fruiting bodies, but their stalks have an unusual slender and non-tapering shape. Changes in the appearance of the cells in the peripheral cell layer and within the papilla have also been observed. In both the *ecmA* and *ecmB* nulls the stalk tube constriction found below the funnel-shaped stalk tube entrance is absent. It is known that in this constricted region of the stalk tube, the cells are attached to one another by actin-containing junctions. As determined by ultrastructural analysis, we find that these junctions are significantly reduced in extent in the *ecmA* and *ecmB* nulls.

We have also examined a double null mutant lacking *ecmA* and *ecmB*. Slugs and fruiting bodies are formed, although a notable feature of migrating slugs is that many cells are lost into the trail. The double nulls exhibit a defect in the size regulation of aggregates. As cell densities are increased, the developing structures increase in size relative to wild-type structures. At high densities, there is a delay in subdivision of the aggregates, and multiple stalks are found within a single structure. At lower densities, a tendency for more than one stalk to be formed at the preculminant stage of development remains. This phenomenon is also observed to a lesser extent in structures lacking *ecmB* alone.

In order to investigate the possibility suggested by the *ecmA* and *ecmB* null phenotypes that components of the extracellular matrix may influence the properties of adjacent cells, the effect on disaggregated slug cells of placing them in contact with slime trails from wild-type and mutant slugs has been examined. The results of these experiments will be presented.

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Discovering the role of RasB in *Dictyostelium discoideum* growth.

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RasB, one of a large group of *Dictyostelium discoideum* Ras family proteins is expressed throughout growth and development, but present at higher levels during growth and early development. We have shown that overexpression of an activated version of RasB (RasB-G12T) in *Dictyostelium* Ax2 cells results in transformants that exhibit an aberrant actin cytoskeleton and a modest defect in cytokinesis. Relative to parental Ax2 cells, a large proportion (45%) of transformants exhibit a large flattened morphology when grown on a solid substratum and contain on average 2.5 nuclei per cell whereas wild type cells contain 1.1 nuclei per cell under the same conditions. In addition the transformants contain an increased number of actin crowns based on FITC-phalloidin staining. Based on these results we suggest that RasB might be involved in one or more signal transduction cascades regulating the actin cytoskeleton and/or cytokinesis.

The role of RasB in the process of cytokinesis is as yet unclear. *Dictyostelium* cells can undergo at least two forms of cell division: normal mitotically linked cytokinesis and an alternative form named" traction mediated cytokinesis". During traction mediated cytokinesis the cells pull themselves apart on a solid surface as a result of their poles moving in opposite directions rather than by cleavage ring and furrow formation. Using time-lapse microscopy we have shown that transformants containing activated RasB (G12T) protein are capable of undergoing both apparently normal mitotically linked cytokinesis and traction mediated cytokinesis but there is an increased failure rate of both forms of cell division compared to the parental strain.

Molecules that interact with *Dictyostelium* Ras proteins have not been identified. We are currently using affinity chromatography technology with various fusion proteins to identify effectors of RasB. We have identified three molecules (p60, p53 and p49) that interact specifically with pMAL-RasB and GST-RasB but not pMAL or GST alone. We are in the process of characterizing these putative RasB interacting molecules.

Dictyostelium cells with mutations in _lvsA_ have defects in cleavage furrow morphology

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In a genetic screen for essential cytokinesis genes, we isolated a novel gene that we named *large volume sphere A* (*lvsA*). Cells with mutations in this gene become large and multinucleate in suspension culture due to a defect in cytokinesis. To investigate the nature of this cytokinesis defect, we observed cells by video microscopy under both adherent and non-adherent conditions. Adherent *lvsA*$^-$ cells form successful cleavage furrows that constrict at a rate slightly slower rate than wildtype cells. Adherent *lvsA*$^-$ cells also localize GFP-myosin II to the cleavage furrow similar to wildtype cells. However, the phenotype of *lvsA*$^-$ cells in suspension was dramatically different. Instead of maintaining a single equatorial furrow, *lvsA*$^-$ cells attempting division in suspension developed a bloated swelling at the equator between two abnormally placed constrictions. Cells fixed in suspension showed that GFP-myosin II is localized to these abnormal constrictions. Interestingly, this morphology resembles the phenotype of Clathrin null cells as they fail to divide in suspension. This similarity to Clathrin null cells and sequence analysis of the LvsA protein suggests that LvsA may play a role in a membrane processing pathway that is essential for cytokinesis. To examine the role of the LvsA protein in cytokinesis, we used homologous recombination to introduce GFP in frame at the 5' end of *lvsA* in the genome. Cells expressing GFP-LvsA are able to grow in suspension, demonstrating that the protein is functional. Preliminary results indicate that LvsA may remain cytosolic throughout cytokinesis and other stages of the cell cycle.

Cre/*loxP*-mediated recombination in *Dictyostelium*

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The use of bacteriophage P1-derived Cre/*loxP*-mediated recombination systems greatly expands the repertoire of tools available for chromosome engineering in *Dictyostelium*. We have generated strains of *Dictyostelium* in which Cre recombinase mediates site-specific recombination at *loxP* sites. We disrupted the first intron of the cAMP phosphodiesterase gene, *regA*, with *pyr5-6* flanked by *loxP* sites in strain HL328. Strain HL328 is auxotrophic for uracil and resistant to 5-fluoroorotic acid (5-FOA). The resulting hypomorphic *regA* strain is independent of uracil for growth and sensitive to 5-FOA.

Transfection of that strain with a plasmid encoding the Cre-recombinase results in gene replacement by homologous recombination at the *loxP* sites. Recombinants that lost the *pyr5-6* gene grow in the presence of 5-FOA. Thus, the combination of the Cre/*loxP* system and the use of the *pyr5-6* gene, enables selection for both integration and excision. The use of Cre-recombinase driven by various promoters allows for transient, conditional, or constitutive control of gene function. Additional manipulations are possible on these strains, allowing for the identification of genetic modifiers.

Expression Analysis of Developmental Genes using Microarrayed DNA on Chips

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During the last few years we have discovered over 65 new genes by REMI mutagenesis that are required for proper morphogenesis. The functions of many of these genes have not yet been elucidated. Quantitative determination of their temporal and cell-type specific patterns of expression will help delineate their roles at various stages of development. We have prepared DNA microarrays (Chips) with PCR amplified regions of each of these newly discovered genes as well as probes from previously characterized developmental genes. Multiple copies of arrays with 629 different probes in an area of about 1mm2 were constructed using an automatic arrayer. Individual chips can be hybridized with fluorescently labelled copies of mRNA prepared at specific times in development. By comparing the red fluorescence of mRNA prepared from vegetative cells to the green fluorescence of mRNA prepared at 5, 10, 15, and 20 hours of development the increase and/or decrease of specific mRNA can be quantitatively estimated for each of the genes. We will also compare the complement of mRNA found in prespore and prestalk cells at the slug stage and during culmination. The chips carry probes from 269 fully sequenced genes as well as 359 cDNAs from the Japanese EST Project. For our pilot project we chose cDNAs that encode new proteins identified on the basis of sequence homology.
We plan on extending this analysis to strains carrying mutations in the 65 REMI genes as well as strains with previously defined mutations. The consequences of loss of specific genes to the pattern of gene expression will help delineate the dependent pathways that underlie development of Dictyostelium.

ORI-GENE; A tool for gene analysis based on evolutionary tree-Application to cDNA project of Dictyostelium discoideum.

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We have developed a computational tool, named ORI-GENE, to analyze results of homology searches from evolutional view. For the analysis, ORI-GENE provides the following two novel functions, first, it finds the organisms which have similar genes and visualizes their distribution pattern on the phylogenetic tree, second, it predicts the point, termed "origin", where each gene may have firstly appeared and classifies the genes by their origins. As the classification gives the set of genes acquired at the same point during evolution of organisms, it would be helpful to predict the gene function.

We also report the application of ORI-GENE to the *Dictyostelium* ESTs and discuss its capability to predict functions of unassigned genes via the classification and evolutional aspects of *Dictyostelium*.

Skp1 Isoforms are Differentially Modified and Compartmentalized in *Dictyostelium*

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Skp1 is an M_r 20,000 protein which belongs to the SCF-E3 ubiquitin ligase complex in many organisms. In *Dictyostelium*, Skp1 is encoded by two similar genes whose products differ by only a single amino acid, and is partially modified by a pentasaccharide, Gal_1-6Gal_1-Fuc_1-2Gal_1-3GlcNAc, *O*-linked to a hydroxyproline residue at position 143. Total Skp1 was localized by immunofluorescence in both the nucleus and patches of the cytoplasm throughout the life cycle, consistent with the presence of proteasomes in each of these compartments. Both isoforms were shown to be expressed at similar relative levels throughout the life cycle using RT-PCR. In strains expressing individual Skp1 isoforms tagged with the c-myc epitope, Skp1B was concentrated in the nucleus whereas Skp1A was concentrated in cytoplasmic patches. Furthermore, myc-tagged Skp1B migrated more slowly than Skp1A-myc by SDS-PAGE, suggesting differential glycosylation. To evaluate the role of the potential glycosylation difference in localization, the effect of prolyl hydroxylase inhibitors was investigated. _,_'-dipyridyl inhibited Skp1 prolyl hydroxylase *in vitro* and concentrations which inhibited incorporation of [^{3}H]fucose relative to [^{14}C]leucine *in vivo* reduced the apparent M_r of Skp1. Cells treated with _,_'-dipyridyl failed to concentrate Skp1B-myc in their nuclei. Thus the nuclear concentration of Skp1 appears to depend upon both an Ala residue at position 39 and the pentasaccharide moiety at position 143 of the protein.

The majority of expressed Skp1A-myc is hydroxylated at Pro143 but not glycosylated. As a first step to investigate this phenomenon, an assay was developed to measure the transfer of [^{3}H]GlcNAc from UDP-[^{3}H]GlcNAc to Skp1-HyPro143 or a synthetic Skp1 4-HyPro-peptide. The cytosolic fraction of the cell mediated transfer of radioactivity to Skp1 in a time-, concentration-, and HyPro-dependent fashion which was blocked by an anti-Skp1 antibody. Incorporated radioactivity was alkali-resistant and was recovered as $GlcNH_2$ after acid hydrolysis, consistent with linkage of GlcNAc to HyPro. The GlcNAc-transferase activity was purified 130,000-fold over DEAE-, phenyl-, reactive red 120-, Superdex-200-, dUMP-, UDP-GlcNAc- and Superdex-75-columns. The UDP-GlcNAc resin was synthesized by a novel method which linked UDP-GlcNAc via its 5-uridyl moiety, so as not to interfere with enzyme binding based on inhibitor studies. The activity behaved as a single component, had an apparent M_r of ~45,000 by gel filtration, a pH optimum of 7.5-8, and required DTT and a divalent cation. The purified enzyme consisted predominantly of a M_r 51,000 band after SDS-PAGE which was photoaffinity-labeled with 5-[^{125}I]ASA-UDP-GlcNAc in a UDP-GlcNAc-sensitive fashion. Its apparent K_m's for UDP-GlcNAc and Skp1 were submicromolar. The presence of the enzyme in the cytosolic fraction, its requirement for a reducing environment, and its high affinity for UDP-GlcNAc, strongly suggest that Skp1 is glycosylated by a HyPro GlcNAc-transferase which resides in the cytoplasm. The purified enzyme exhibited a very low specific activity suggesting that it may be down regulated in its purified form and that its activity may be regulated *in vivo*. *de novo* amino acid sequencing, using Q-TOF mass spectrometry in collaboration with Drs. H. Morris and A. Dell of the Imperial College, showed that the Skp1-HyPro GlcNAc-transferase is a novel protein whose sequence has not yet been encountered in the *Dictyostelium* sequence databases.

Microtubule Number and Organization in *Dictyostelium* in the Presence and Absence of a GFP-α-tubulin.

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Microtubules in *Dictyostelium* amoebae expressing a green fluorescent protein (GFP) tagged alpha (α)-tubulin are highly motile, exhibiting a wavelike movement reminiscent of the beating of cilia and flagella. Observation of the movement suggests that some of the GFP-labeled microtubules might in fact represent loosely arranged bundles composed of two or a few microtubules. Electron microscopy analysis of GFP-tubulin expressing and non-expressing (wild type /AX3) cell lines was undertaken to determine the organization of microtubules in the cytoplasm of vegetative stage amoebae.

Analysis of serially sectioned wild-type (AX3) cells shows numerous microtubules extending from the MTOC, and we frequently observe areas where two or more microtubules are closely associated over distances up to 1.9 μm (~1/5 of cell diameter). These observations suggest that some microtubule bundles do exist in amoebae and may contribute to the motility observed in live cells. However, as most microtubules are not associated in bundles, bundling per se cannot be a requirement for motility.

Analysis of microtubules in the GFP-tubulin expressing cells showed a significantly smaller number of microtubules emanating from the MTOC than was seen in wild-type cells. Also, only rarely were examples seen where two microtubules were closely associated. The number of microtubules observed by EM also appears to be less than observed in living cells suggesting that incorporation of GFP-tubulin into the microtubules may cause a decrease in the stability of the microtubules to the conditions used in preparing the cells for EM analysis. These results indicate that although the GFP-tubulin is a useful marker for microtubules in living cells, caution should be used in the interpretation of results obtained. This work was supported in part by a grant (#F98-USF-2) from the Florida Division of the American Cancer Society.

Identification Of The Gene For Polyphosphate Kinase In *Dictyostelium*

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The role of sterols in *Dictyostelium* growth and development is largely unknown. One approach to gaining an understanding of this role has been to study mutants resistant to polyene antibiotics, a class of drugs whose mode of action depends on the presence of sterol in the membrane. Not surprisingly, many of these mutants show alterations in the sterol biosynthetic pathway. There is, however, one class of mutants that become resistant despite no apparent change in the quality or quantity of sterol in the membrane. Some of these mutants show altered development, but, in general, they have proved refractory to further analysis. To try to understand these mutants, we have tried to clone the genes responsible using REMI combined with drug selection.
A pool of REMI mutants was plated on pimaricin containing plates and resistant plaques were seen at a frequency 1000x above the spontaneous mutation rate. On strain, HK5001, was purified and used for further study. It was highly resistant to pimaricin (eop $1.5x\ 10^{-1}$), and, in addition had slow growth and aberrant development phenotypes. The sterols of HK5001 were examined and were found to be normal. Both capture and recapitulation of the mutant phenotypes by homologous recombination were carried out successfully. A plasmid, pMS5, containing ~700 base pairs of flanking DNA 5' to the insertion, and ~2500 base pairs of flanking DNA 3' to the insertion, was obtained. Sequence analysis of pMS5 and the subsequently derived pMS7 showed no homology to any known sequence at the DNA level, but high homology at the amino acid level ($p=10^{-152}$) with the enzyme polyphosphate kinase. Polyphosphate kinase (PPK) is an enzyme responsible for the synthesis of long chain inorganic polyphosphate from ATP. Polyphosphate is ubiquitous and has been has been implicated in a wide range of cell functions. Polyphosphate kinase has heretofore only been described in bacteria. This identification represents the first PPK found in any eucaryotic organism. Further characterization of *Dictyostelium* PPK and polyphosphate will be described, and their possible role in *Dictyostelium* growth and development will be discussed.

SCAR interacts with RacC and Profilin and Regulates Endo-lysosomal and Phagosomal Processes.

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The protein Wasp has been shown to be important in Cdc42 and Rac GTPase-mediated cell signalling pathways that lead to rearrangements in the actin cytoskeleton of B and T lymphocytes. Most recently, a protein that is highly homologous to Wasp was discovered in *D. discoideum* during a genetic screen for second-site mutations that would suppress a developmental defect. Because this protein restores the normal developmental phenotype to cells that were defective in the cAMP receptor, it was termed Scar, for suppressor of cAMP receptor. Subsequently, a Scar homolog (Scar/Wave) was identified in mammalian systems. To understand the role of Scar in actin polymerization that may be important for endo-lysosomal and phagosomal function, we have biochemically analyzed a cell-line of the simple eukaryote *D. discoideum* that is genetically null for *scar*. Our studies indicate that fluid phase pinocytosis, macropinocytosis, and phagocytosis were reduced in the *scar*$^{-}$ cell-line. In addition, exocytosis of fluid phase material was also delayed in these cells, and there appeared to be a specific block in the movement of fluid phase from the lysosome to the post-lysosome organelle of the cell. Interestingly, disrupting the two genes encoding profilin (an actin monomer sequestering protein) in the *scar* null background further reduced the level of fluid phase pinocytosis, but restored phagocytosis to above normal levels. Finally, we have initiated biochemical and genetic studies to test if Scar can directly interact with Rho family GTPases. Yeast two-hybrid data indicates that Scar strongly interacts with RacC (a novel *D. discoideum* GTPase also implicated in regulating phagocytosis) when it is in the GTP-, but not GDP-bound form. Taken together, our data suggest a model in which Scar recruited by RacC may direct the polymerization of actin that is necessary for phagocytosis.

The phase of the cell cycle, cellular Ca^{2+} and mitochondrial activity are the early correlates of cell fate in *Dictyostelium discoideum.*

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In Dictyostelium, cell differentiation is regulated by the cell cycle phase at starvation. Cells that are in S or early G2 phase display a prestalk tendency while those in mid to late G2 exhibit a prespore tendency. Earlier work has shown that Ca^{2+} levels in growing cells reflect post-aggregation cell fate (Azhar et al., 1996). We show here that there is a correlation between cytosolic Ca^{2+}, mitochondrial activity and cell cycle phase. Cells that are in S or early G2 are either in a 'high' or 'low' calcium class and in turn display, respectively, 'high' or 'low' mitochondrial activity. On the other hand, cells in mid to late G2 invariably fall in a 'low calcium' and 'low mitochondrial activity' class. Hydroxyurea and nocodazole, both cell cycle inhibitors, influence the proportions of amoebae containing 'high' or 'low' Ca^{2+}, as well as cell fate, as expected on the basis of this correlation. In the mutant rtoA, which upon differentiation yields a disproportionate number of prestalk cells, an abnormally large fraction of amoebae fall in the 'high' calcium class. The initial cell-type choice in rtoA is independent of cell cycle position at starvation (Wood et al., 1996), implying that the calcium-cell cycle relationship is aberrant in rtoA. One reflection of this is an altered ability of the rtoA amoebae to transport Ca^{2+}. Our results suggest that there are cell cycle-dependent as well as independent initial cell-type choice mechanisms, both mediated by Ca^{2+}.

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A STUDY OF THE ROLES OF G_2 IN *Dictyostelium* DEVELOPMENTAL PROCESS BY TEMPERATURE-SENSITIVE G_2 MUTANTS.

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Dictyostelium discoideum grows as unicellular amoebae in nutrient rich environment and initiates a multicellular developmental program upon starvation. This developmental process is similar to some mammalian physiological responses in its molecular mechanism. Thus, *Dictyostelium* provides an excellent model for the study of these processes at the molecular level. G_2 is temporally expressed in the beginning stage of development. The previous studies with G_2 knockout mutants have provided some information about its role in the developmental process and indicated that it is critical for the initiation of chemotaxis and aggregation. However, its role in late- and post-aggregation processes is still unclear because knockout mutants can not go through the aggregation process. To address this problem, we generated two temperature-sensitive G_2 mutants by site-directed mutagenesis and expressed them in G_2 knockout cells. Both transformed cell lines had temperature-sensitive phenotypes. At the permissive temperature, the mutants function normally and development proceeds. When shifted to the restrictive temperature, development is blocked.

One advantage of the temperature-sensitive mutants as compared to knockout mutants is that we can let the cells go through any developmental stage at the permissive temperature, then shut off G_2 function by shifting to the restrictive temperature. By this means we study the role of G_2 at each stage of development. Also, the expression of some genes is dependent on the expression of G_2; these genes are not expressed in the G_2 knockout mutant which makes it difficult to differentiate the direct from the indirect functions of G_2. With the temperature-sensitive mutants, we can induce the expression of these genes by starving the cells at the permissive temperature then shift the cells to the restrictive temperature.

Our results indicate that G_2 is directly required for the initiation of aggregation. It may also play some role in the post-aggregative stages, as development was significantly delayed or stopped when cells at various stages were shifted to the restrictive temperature.

C-terminal, hexahistidine-tagged G_2: Comparison of GTPase activity between wild type and mutant _-subunits.

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In *Dictyostelium,* the transition from the unicellular state to a mature fruiting body requires coordinated signaling through a G-protein-linked, cAMP-responsive pathway. During aggregation, the cAMP receptor (cAR1) couples to the heterotrimeric G-protein, G2. Signal transduction activates multiple second messenger generating enzymes including adenylyl cyclase, guanylyl cyclase and phospholipase C. Gα2 is regulated by phosphorylation at serine-113 within the helical domain (Chen et al.). However, substitution of this residue results in a visibly normal phenotype, in both the non-phosphorylated mutants and one which mimics constitutive phosphorylation (S113A and S113D, respectively). In other systems, regulation of G-proteins has been shown to occur at the level of GTP hydrolysis, by stimulating the intrinsic GTPase activity of the protein. Accelerators of GTPase activity are well defined, including the GTPase activating proteins (GAP's) of small G-proteins such as Ras, to the more recent regulator of G-protein signaling (RGS) proteins, which serve as GAP's for heterotrimeric G-protein α-subunits.

Recent work (Skiba et al.) has identified the helical domain of transducin $G\alpha_t$ as a major determinant in the regulation by RGS proteins. In addition, mutation of critical amino acid residues within the yeast α-subunit Gpa1 has been shown to reduce the level of GTPase stimulation by the RGS protein Sst2 (DiBello et al. and Natochin and Artemyev). To determine if similar regulatory mechanisms exist in *Dictyostelium*, we have generated the cooresponding α-subunit mutations (G187S, S210D, S113A, S113D) in a Gα2 cDNA which encodes a C-terminal poly-histidine epitope to facilitate purification. When expressed in Gα2-minus cell lines, wild type and both phosphorylation mutants rescue the null phenotype. However, when plated on non-nutrient agar, both RGS mutants show abnormal development. G187S expressing cells will aggregate, but they form abnormal fruiting bodies which seemingly lack spore heads. In contrast, S210D cells fail to aggregate completely, although results show they may be defective in receptor-coupling. Imunoblot analysis indicates that all mutants express nearly equal amounts of protein. Purification of recombinant, His-tagged α-subunits is currently underway. GTP-binding and hydrolysis assays from each mutant protein should provide insight into possible regulatory mechanisms acting on G2.

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Analysis of cell motility using an under-agar folate chemotaxis assay: Contribution of Myosin II to cortical integrity.

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We have developed an under agar chemotaxis assay for vegetative amoebae undergoing folate chemotaxis. The assay is highly reproducible and particularly useful for visualizing cell behavior and localization of GFP labeled proteins during chemotactic movement. Using this assay, wild type cells move at speeds of up to 6.0 μm/min for a period of 9 hours. The movement is highly processive at the front of moving cells and more random in the cells lagging behind the front.

We have used this assay to analyze the movement of mutants with alterations in Myosin II function. Recent evidence has shown that Myosin II is involved in the cortical integrity necessary for the production of force[1]. Cells lacking myosin II (MHC-) cannot enter aggregation streams composed of wild-type cells. We have hypothesized that this defect is due to the inability of the myosin II mutant cells to generate force when pushing against a stiff barrier. In the under-agar chemotaxis assay, in order for cells to exit troughs and move under the agar in response to chemoattractant, they must push and flatten their leading edge (and subsequently the rest of the cell) under the agar. Time lapse imaging indicates that the trough edge is a barrier even to wild-type cells. Cells move along the agar edge, and periodically manage to squeeze under the agar. Therefore, the agar interface represents a mechanical barrier to protrusion, which must be deformed upward for the cell to move. If 2% or greater agar is used, mutants lacking myosin II are completely unable to penetrate this barrier, and remain in the trough. When the agar is reduced to 0.5%, the mutant cells are now able to exit the trough and move under the agar. These cells move chemotactically up the folate gradient, but their rate of movement is about 1/4 that of wild type cells. This result indicates that the lack of chemotactic movement of the mutant cells at the higher agar concentration is due to mechanical inhibition rather than their inability to recognize the gradient of folate.

We have recently shown that during development, the ability to penetrate aggregation streams is recovered in mutants lacking the essential light chain of myosin (ELC-) but not the regulatory light chain (RLC-). We hypothesize that the contractile function of myosin is not necessary for the cortical integrity allowing force to be produced. ELC- cells are still able to cross-link actin filaments and that is sufficient for normal force generation. Consistent with this hypothesis, ELC- mutants are able to force their way under the agar and chemotax in response to folate, while RLC- cells are not.

We are now using this assay in conjunction with GFP constructs and confocal microscopy, to determine the localization of specific cortical proteins during folate chemotaxis.

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Genes involved in the growth differentiation transition in Dictyostelium

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Using REMI and colony blots on discoidin expression, we have identified mutants with defects in the growth differentiation transition i.e. with misexpression of discoidin. In addition, previously constructed mutants including erk2$^-$, acr$^-$, hsc70$^-$, pkA$^-$, pianissimo$^-$ were checked for discoidin expression either alone or in combination with the gdt1 mutant.
gdt1 is a negative regulator of development and encodes a transmembrane protein with a putative kinase domain. It is expressed in growing cells and increasing with cell density, with the onset of development, gdt1 mRNA and protein is rapidly downregulated. Mixing experiments with wild type cells demonstrate that the gdt1 mutation is cell autonomous. In agreement with this observation, tests for PSF production indicate that gdt1- cells are not impaired in secretion of this factor. Though gdt1 mutants are accelerated in the very onset of development, they apparently do not sort out in mixing experiments with gfp marked wild type cells.
Combination with pkA mutants shows that gdt1$^-$ can partially rescue the pkA$^-$ phenotype: double mutants display strong discoidin expression even though they do not aggregate. Based on this observation we tested two putative PKA phosphorylation sites in the gdt1 protein and found that one was efficiently phosphorylated by recombinant PKA in vitro. Since gdt1$^-$/pkA$^-$ cells do not reach the levels of discoidin expression of gdt1$^-$ mutants, we conclude that the two genes are in parallel, interconnected signalling pathways.

Isolation and characterization of putative antisense RNAs

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In eukaryotes, antisense transcripts of structural genes have usually been detected fortuitously. So far, a systematic search for RNAs which are capable to form long continuous double stranded regions has not been performed.
Dictyostelium is one of the few systems where a regulatory function of an endogenous antisense RNA has been demonstrated. In an attempt to identify additional genes which are possibly regulated by antisense transcripts, we have employed specific monoclonal antibodies to isolate dsRNA from Dictyostelium cells.
A small amount of dsRNA (approx. 0,001% of total RNA) could be isolated by affinity to immobilized mAbs. Native gel electrophoresis with subsequent immuno-blotting and scanning force microscopy showed dsRNA molecules in the size range from 100 to 2000bp in the purified fraction. Using random primers, dsRNAs were amplified by RT PCR and cloned. Labelled run-on transcripts from isolated Dictyostelium nuclei were used to probe both orientations of in-vitro transcripts from the cloned fragments. The experiment demonstrated that at least some of the isolated sequences were indeed transcribed in both directions. Sequence analysis revealed long open reading frames demonstrating that fragments of structural genes had been isolated. Studies on the presumptive regulatory function of the complementary transcripts are under way.

Phosphatidylinositol 3-Kinases Regulate Homotypic Fusion of Phagosomes and Intraphagosomal pH Changes but not Internalization of Particles.

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The p110-related PI 3-kinases, DdPIK1 and DdPIK2, regulate macropinocytosis and fusion of acidic lysosomes, but not internalization of particles in *Dictyostelium*. Null mutants grow slowly using bacteria as a food source suggesting a possible defect in phagosomal maturation or membrane trafficking that leads to bacterial degradation. Microscopic techniques revealed that bacteria internalized into mutant cells or cells treated with the PI 3-kinase inhibitors LY294002 and wortmannin remained intact as single particles in phagosomes with closely associated membranes for up to 2-3 hours. In contrast, bacteria in control cells were partially degraded and found in spacious multiple particle phagosomes in control cells. To determine if PI 3-kinases regulated trafficking of lysosomal proteins to maturing phagosomes, latex bead containing phagosomes were purified from wild-type and LY294002 treated cells at various times after internalization. Thirty minutes and two hours after internalization of particles, phagosomes purified from drug and control cells contained comparable levels of lysosomal hydrolases, cysteine proteinases, and lysosomally associated proteins. Phagosomes from control and drug treated cells contained the proton pump complex and were acidic, but in drug treated cells the phagosomal pH did not increase over time at the rate observed for phagosomes in control cells. These results suggest that PI 3-kinases do not regulate trafficking of the known lysosomal proteins and that the kinases may directly or indirectly regulate homotypic fusion of single particle phagosomes to form multi-particle containing spacious phagosomes. This event may be coupled with pH changes that might be necessary to complete bacterial degradation.

Genetic Evidence that a Lysosomal Membrane Protein, DdLIMP and Profilin Interact to Regulate F-Actin and Macropinocytosis in *Dictyostelium.*

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Profilin is a G-actin and phosphoinositide-binding protein implicated in connecting signal transduction pathways with changes in the actin cytoskeleton that affect changes cell motility, membrane ruffling and endosomal membrane trafficking. Genetic disruption of *lmpA*, a *Dictyostelium* gene encoding a putative homolog of the LIMPII family of proteins, suppressed a *pro*$^-$ developmental phenotype, suggesting a functional connection between profilin and DdLIMP; a potential lysosome integral membrane protein. To further test this hypothesis, biochemical and microscopic assays were employed to examine rates of pinocytosis and phagocytosis, and transport of material along the endosomal pathway in wild-type, *pro*$^-$ (profilin null), and a double null mutant (*lmpA-/pro*$^-$) cell lines. *Pro*$^-$ cells, containing twice as much F-actin as control cells, were significantly defective in macropinocytosis, efflux of fluid phase and secretion of lysosomal hydrolases. Interestingly, *pro*$^-$ cells phagocytosed particles significantly faster than control cells. Reversal of the alterations in macropinocytosis, efflux and phagocytosis were observed in the *pro*$^-$*/lmpA*$^-$ mutant. Consistent with these observations, IF microscopy and subcellular fractionation indicated that DdLIMP associated with lysosomal, macropinosomal and early phagosomal membranes. Finally, the levels of F-actin in the *lmpA*$^-$ */pro*- mutant was intermediate between the levels observed in wild-type and the 2 fold higher levels observed in the *pro*$^-$ mutant. Our observations indicate possible opposing roles for profilin and a DdLIMP in linking changes in the actin cytoskeleton with regulation of endocytic processes. We propose a model that profilin along with PI 3-kinases and the Wasp-like protein Scar are necessary components driving the formation of macropinosomes; a process that is biochemically distinct from phagocytosis.

Characterisation of cAR1,cAR2 and cAR3 expression in cAR receptor mutants and their role in cell sorting.

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Cell movement in Dictyostelium is controlled by propagating cAMP waves. We are investigating the role of the cAR1, cAR2 and cAR3 receptors in the generation of cAMP waves as well as in chemotactic cell sorting. In these experiments we use a variety of mutant strains expressing only one or two of these receptors. Since it is to be expected that alterations in the expression level of one receptor will affect the expression of others it has become necessary to investigate the expression pattern of all other cAMP receptors in these strains. We use whole mount in situ hybridisation to characterise these mutants. We have optimised the procedure and can detect now expression at the single cell level.

Our data show that cAR1 is expressed in all cells throughout development, while expression of cAR3 is difficult to detect. cAR2 expression is first expressed at the mound stage in randomly scattered cells and in the slug is localised to the prestalk zone with little expression in anterior-like cells. Our data show that deletion of cAR3 does not affect the expression of the other cAMP receptors. Deletion of cAR4 results in reduced cAR2 and ecmA expression. Overexpression of cAR1 in a cAR3 background does not affect expression of the other receptors but overexpression of cAR3 in a cAR1 null background results in overexpression of the prestalk specific cAR2. Since cAR3 is thought to feed into GSK3 we are investigating the role of GSK3 in this upregulation.

We are also investigating hypothesis that cAR2 directs the sorting of pstA cells to the tip of the mound. This may occur if the high cAMP binding affinity of cAR2 allows pstA cells to respond to higher levels of cAMP, causing them to move better in response to high amplitude cAMP signals than cells that do not express cAR2. To this extent we have made a cAR2-GFP fusion protein. Synergy experiments with cells expressing this construct and cAR2 null cells and wildtype cells suggest that while cAR2 may be necessary for proper sorting of pstA cells, it is not itself *sufficient* to direct cells to the pre-stalkA zone.

Investigation of cAMP signalling using temperature sensitive ACA mutants

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The role of cAMP signalling in later development is still a matter of considerable debate. We have used a temperature sensitive mutant (tsACA2) of the Aggregation Specific Adenylyl Cyclase (ACA) to investigate the role of cAMP signalling in mounds and slugs. The mutant was selected (Kende Guo in Julian Gross' lab) from ACA null cells transformed with a library (C. Patrent & P. Devreotes) of expression vectors containing ACA gene amplified by error prone PCR under the control of an actin15 promoter then screening for development at 22°C and arrest at 28°C. We have shown that the cAMP relay response in aggregation competent tsACA2 cells was completely abolished by brief exposure (20 min) to 28°C whereas the cAMP response of wildtype cells was unaffected. Further characterisation showed that the basal catalytic activity was temperature sensitive at high temperature.

Initial characterisation of the mutant showed that it could be arrested at any stage of development by shifting to 28°C. Investigation of wave propagation showed that during aggregation and the mound stage, optical density waves could be reversibly inhibited by shifting from 22 to 28°C and back. At the same time the cells loose their co-ordination of movement. This clearly shows that the waves observed in the mound are due to propagating waves of cAMP. The role of ACA in cell sorting, wave propagation and co-ordination of cell movement in the slug stage are still under investigation.

The opportunistic pathogen *Pseudomonas aeruginosa* kills *Dictyostelium discoideum*: a new genetic model system for studying host/pathogen interactions?

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Microbial pathogens have evolved in relation to their mammalian hosts and have developed strategies for colonization, invasion, and pathogenesis. These infections are highly specific, involving specific host targets and signaling pathways. Some of these strategies involve the induction of host responses to facilitate the growth of the pathogen. In this case, the host becomes an active participant in the infection process.

One strategy to study the interactions between a pathogen and its host is the use of genetically tractable host models to study infections by identifying resistant host mutants. This approach may aid us in understanding the molecular mechanisms. With this in mind, we tested whether *Dictyostelium* could be used as a host model for the opportunistic pathogen *P. aeruginosa*. This gram-negative bacterium produces an array of secreted virulence factors during an infection. Although our immune system is equipped to fight such infection, individuals with a compromised immune system are at great risk of life-threatening pneumonia from *Pseudomonas* infections.

Incubation of *P. aeruginosa* with *Dictyostelium* cells in suspension or on agar plates (SM/5) leads to the lysis of *Dictyostelium* cells. In order to determine whether this interaction is based on a known virulence factor, we tested avirulent strains of *Pseudomonas* (courtesy of Fred Ausubel, Harvard University) for their virulence towards *Dictyostelium*. We identified one mutant that is unable to kill *Dictyostelium*. In fact, *Dictyostelium* feeds on this avirulent mutant, which carries an insertional mutation in the *lasR* gene. LasR is a transcription factor that is responsible for the expression of an array of virulence factors. *lasR* is induced at high population densities and this density or quorum sensing is an important regulator of pathogenesis.

We suggest that *Dictyostelium* can be used as a host to study the mechanisms of *Pseudomonas* infection. *Dictyostelium* must employ sophisticated strategies to utilize pathogenic bacteria as a source of nutrients, mechanisms that might be conserved in human cells.