INTERNATIONAL DICTYOSTELIUM CONFERENCE
Dicty 2000

30 July - 4 August, 2000

Dundee, Scotland

Organisers: Jeff Williams
Kees Weijer
Pauline Schaap

DICTYOSTELIUM 2000
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MEETING OVERVIEW

Time	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
09:00 - 10:20	arrivals	oral presentations	Oral presentations	oral presentations	oral presentations	oral presentations
10:20 - 11:00		coffee break	Coffee break	coffee break	coffee break	coffee break
11:00 - 12:20		oral presentations	Oral presentations	oral presentations	oral presentations	oral presentations
12:30 - 14:00		12.40 lunch	Lunch	12.40 lunch	13:00 start of tour and Glamis Castle visit	lunch
14:00 - 15:20	delegate registration	Oral presentations	Oral presentations	oral presentations		end of meeting
15:20 - 16:00		Coffee break	Coffee break	coffee break		
16:00 - 17:20		Oral presentations	Oral presentations	oral presentations		
18:30 - 20:00	welcome buffet	Dinner	Dinner	dinner		
20:00 - 22:00		poster session nos 1 - 16	poster session nos 17 - 32	poster session nos 33 - 49	banquet and ceilidh	

Please Note that all Posters will be up all week.

DICTYOSTELIUM 2000

Schedule of Oral Presentations:

Sunday 30 July 2000
Registration 14:00– 18:00
Buffet and Wine Reception from 18:00

Monday 31 July 2000
Unless otherwise indicated all talks are 15+5 min.

09:00 - 10:20 CHEMOTAXIS I Chair: Peter Van Haastert

Regulation of the activity and localization of PAKa via direct phosphorylation by Akt/PKB
Chang Chung, Gary Potikyan and Rick Firtel

Sensing and responding to chemoattractant gradients: role of P13K in controlling directional responses
Rick Firtel, John Moniakis, Chang Chung, Ruedi Meili, Tsuyoshi Araki, Masashi Fukuzawa, Tomaki Abe, and Jeff Williams

Scar, a WASp-related protein, is required for lateral pseudopod formation and expansion, chemotaxis and ligand mediated changes in F-actin levels in *Dictyostelium*
J Steiner, D Wessels, D R Soll, and C L Saxe

Filactin, a novel filamin- and actin-related protein
Michael Schleicher, Lars Israel, Daniela Rieger, Rolf Muller, Ludwig Eichinger, Francisco Rivero and Angelika Noegel

10:20 -11:00 Break

11.00 - 12.20 GENE REGULATION AND DIFFERENTIATION Chair: Alan Kimmel

The Activity of CRTF, a Transcription Factor for cAMP Receptor 1 Expression, is Altered by Cleavage and Regulated by cAMP Signaling
Xiuqian Mu, Seth Spanos and Alan Kimmel

The *cbf*A gene encodes a DNA-binding protein that supports growth of *Dictyostelium* amoebae and expression of retrotransposon TRE5-A transcripts
Thomas Winckler, Christina Tschepke, Ilse Zundorf and Theodor Dingermann

The MADS-box gene *srf*A plays multiple roles in *Dictyostelium* development
Ricardo Escalante, Juan Vicente and Leandro Sastre

Involvement of a novel gene, zygl, in zygote formation of *Dictyostelium* mucoroides and D *discoideum* cells
Aiko Amagai

Studies on possible functions of the *Dictyostelium* Hsp90 family in regulation of growth and differentiation
Tsuyoshi Morita, Kenji Saitoh and Yasuo Maeda

12:40 - 14:00 Lunch

14:00 - 15:20 CELL ADHESION AND CYTOKINESIS
Chair: Michael Schleicher

Adhesion Mechanism and Developmental Role of the Cell Adhesion Molecule gp150/LagC
Chi-Hung Siu, Jun Wang, Hau Shi, Liansheng Hou and Eric Huang
Dictyostelium DdCP224 is a microtubule-associated protein and a permanent centrosomal resident involved in centrosome duplication
Ralph Graf, Christine Daunderer, Andrea Hestermann and Manfred Schliwa
Protein sorting and organelle dynamics in cytokinesis
Guenther Gerisch 40'

15:20 – 16:00 Break

16:00 – 17:20 DIF SIGNALLING AND METABOLISM
Chair: Carl Saxe

A novel role of differentiation-inducing factor-1 (DIF-1) during early development of *Dictyostelium* assessed by the restoration of a developmental defect of the mutant lacking MAP-kinase, ERK2
Hidekazu Kuwayama, Masakazu Oyama, Yuzuru Kubohara and Mineko Maeda
Genetic Evidence that DIF-1 is the Inducer of pstO Differentiation
Christopher Thompson and Rob Kay
The DIF-inducible STAT, Dd-STATc, functions in both early and late development
Masashi Fukuzawa, Tsuyoshi Araki, Iris Adrian and Jeff Williams
Intermediates for stalk gene induction
Karin Weening and Pauline Schaap

Tuesday 1 August 2000
09:00 - 10:20 PROTEIN FACTORS Chair: Margaret Clarke

Purification of proteins with discoidin-inducing activity similar to PSF
A Kolbinger, J Kellermann, A Kisters, D Hatton, D Brock, R Gomer and B.W. Wetterauer
TagA, a putative serine protease/ABC transporter of *Dictyostelium* that is expressed at the onset of development and is required for the differentiation of a subpopulation of prespore cells
Adam Kuspa,, J Randall Good, Mathew Cabral and Jun Yang
A cell number-counting factor differentially modulates cAMP-induced cAMP and cGMP pulse sizes, and affects cell motility in addition to adhesion
Lei Tang, Robin Ammann, Hamann Alraaba, Richard Gomer
Elucidation of a cell-counting factor signal transduction pathway by isolation of second-site suppressors
Celine Roisin-Bouffay and Richard Gomer

10:20 -11:00 Break
11.00 - 12.20 cAMP SIGNALLING AND PATTERN FORMATION
Chair: Ikuo Takeuchi

The role of GskA and Aardvark in multicellular development

Adrian Harwood
Requirements for the Adenylyl Cyclases in development of *Dictyostelium*
Christophe Anjard, Fredrik Soderbom and William Loomis
Regulation of 5' Nucleotidase (5NT) in *Dictyostelium discoideum*
Charles Rutherford, Can Eristi, Muatasem Ubeidat, Chanpen Chanchao, Danielle Overall, Brad Joyce and Jeremy Goodin
Visualising Dd-STATa activation in the slug
Tomoaki Abe, Dirk Dorman, Kees Weijer and Jeff Williams

12:30 - 14:00 Lunch

14:00 - 15:20 CHEMOTAXIS II Chair: Angelika Noegel

Analysis of cell motility using an under agar folate chemotaxis assay
G Laevsky and D.A. Knecht
Phosphorylation-dependent internalization of cAMP receptor cAR1 and identification of novel *Dictyostelium* G protein-coupled receptors
R Shields, M Zhang, B Ibrahim and D Hereld
A Novel G*a* Protein that functions in the Adaptation/Deadaptation Pathway for cAMP Signaling
Joseph Brzostowski and Alan Kimmel
The internal phosphodiesterase RegA is essential for the suppression of lateral pseudopods during *Dictyostelium* chemotaxis
Deborah Wessels, Hui Zhang, Joshua Reynolds, Karla Daniels, Paul Heid, Sijie Lu, Adam Kuspa, Gad Shaulsky, William Loomis and David Soll

15:20 - 16:00 Break

16:00 - 17:20 METABOLISM AND ITS INTERFACE TO DEVELOPMENT Chair: Rob Kay

Delta 5 fatty acid desaturases from *Dictyostelium discoideum*
Tamao Saito, Mineko Maeda, Takahiro Morio and Hiroshi Ochiai
Superoxide generation during early *Dictyostelium* development
G Bloomfield and C Pears
Peroxisomal D-hydroxyacyl-CoA dehydrogenase deficiency causes anomalous lipid accumulation and developmental arrest in *Dictyostelium*
H. Kuwayama1, S. Matsuoka1, T. Saito2, H. Ochiai2 and M. Maeda1
Signaling pathways regulating resistance to the anticancer drug cisplatin are required for normal development
Stephen Alexander, Hannah Alexander, Guochun Li, Christopher Foote and Ma Xenia U Garcia, Gad Shaulsky and Adam Kuspa

Wednesday 2 August 2000

09:00 - 10:20 MEMBRANE AND PROTEIN TRAFFICKING Chair: Rex Chisholm

A myosin I is involved in membrane recycling from early endosomes
Eva Neuhaus and Thierry Soldati
Phagocytosis, Adhesion and the functions of Myosin VII

Richard Tuxworth, Deborah Wessels, David Soll and Margaret Titus
Functional characterization of a Dictyostelium Rho-GTPase dissociation inhibitor
Francisco Rivero and Heidrun Dislich
Evidence that the Spore Coat Protein SP85/PsB Mediates a Checkpoint for Cellulose Biosynthesis, Regulates Cellulose Packing, and Anchors Outer-Layer Proteins of the Spore Coat in *Dictyostelium*
Ping Zhang, Aiko C. McGlynn, Karen Kelley*, Gregory W. Erdos* and Christopher M.West

10:20 -11:00 Break

11.00 - 12.40 Ca^{2+} IN CHEMOTAXIS AND APOPTOSIS
Chair: Peter Newell

Prolyl oligopeptidase regulates cAMP-induced IP_3 signalling during development
Robin Williams and Adrian Harwood
Ca^{2+} signalling is not required for chemotaxis in *Dictyostelium*
David Traynor, Jacqueline Milne, Robert Insall, and Robert Kay
Evidence for the presence of the ALG-2/Alix pathway in *Dictyostelium*
Laurence Aubry, Beatrice Blot, Michel Satre, Remy Sadoul and Gerard Klein
Involvement of an Apoptosis Inducing Factor homolog in *Dictyostelium discoideum* cell death
Damien Arnoult, Jerome Estaquier, Franck Sureau, Jean Pierre Tissier, Alain Grodet, Marc Dellinger, Axel Kahn, Jean-Claude Ameisen and Patrice Xavier Petit
***Dictyostelium* cell death mutants**
Myriam Adam, Jean-Pierre Levraud and Pierre Golstein

12:40 - 14:00 Lunch

14:00 - 15:20 THE GENOME PROJECT Chair: Hideko Urushihara

The Dictyostelium Genome Project: progress and future prospects
Ludwig Eichinger 40’
The science and logistics of functional genomics
Steve Oliver, University of Manchester 50’

15:20 - 16:00 Break

16:00 – 17:15 FUNCTIONAL GENOMICS Chair: Jeff Williams

***Dictyostelium* cDNA project: Sequencing of growth phase cDNAs**
T Morio, H Urushihara, T Saito, M Katoh, H Kuwayama, J Williams, M Maeda, I Takeuchi, H Ochiai and Y Tanaka 20’
Making a gene catalogue of *Dictyostelium discoideum*
H Urushihara, E Koriki, T Morio, T Saito, H Ochiai, M Maeda and Y Tanaka 15’
Genome Scale Analysis of Gene Expression In *Dictyostelium*
Nancy Van Driessche, Richard Sucgang, Miroslava Ibarra, Jeffrey Tollett, John Halter, Adam Kuspa and Gad Shaulsky Mariko Katoh[6], Takahiro Morio[6], Hidekazu Kuwayama[6], Tamao Saito[7], Hideko Urushihara[6], Mineko Maeda[8], Ikuo Takeuchi[9], Hiroshi Ochiai[7], Yoshimasa Tanaka[6]. 15’
Analyses of Cell-Type Specific Genes Using DNA Microarrays
Negin Iranfar, Danny Fuller, Michael Laub and William Loomis 15’
Understanding developmentally regulated secretion through proteomics: a global analysis of the *Dictyostelium* prespore vesicle 10’

Supriya Srinivasan, Mathew Traini, Ben Herbert, Jenny Harry, Hannah Alexander, Keith L. Williams and Stephen Alexander

Break for 15' 17:30 – 19:00 OPEN SESSION ON COMMUNITY BUSINESS Chair: Richard Kessin. Please contact Rich directly with suggestions

Thursday 3 August 2000

09:00 - 10:20 EVOLUTION Chair: Bill Loomis

Evolutionary Origin of *Dictyostelium* and Its Relationship to Other Eukaryotes
Sandra L. Baldauf and Ingrid Wenk-Siefert[1,2] 25+5'

DNA double strand break repair enzymes and gene evolution
Natasha Zhukovskaya and Jeff Williams 5+5'

The Behaviour of Cheater Mutants in Chimeras with Wild-type
Dee N Dao, Turgay Tekinay, Stefan Pukatzki, Herbert Ennis and Richard Kessin

Chimeras and cheater clones in *Dictyostelium discoideum*
Joan Strassmann, Yong Zhu and David Queller

10:20 -11:00 Break

11.00 - 12.20 CHEMOTAXIS III Chair: Rick Firtel

Diffusion of water, proteins and second messengers in *Dictyostelium* cells
Eric Potma, Wim de Boeij, Douwe Wiersma and Peter van Haastert

Visualising cell-cell signalling during *Dictyostelium* development
Dirk Dormann, Carol Parent, Peter Devreotes and Cornelis Weijer

The aggregation process is mediated by RasC
Chinten Lim, George Spiegelman and Gerald Weeks

Ras Pathways – Multiple Routes to Cell Movement?
Robert Insall, Andrew Wilkins, Jonathan Chubb, Meenal Khosla, Derek Fraser, Paul Fisher and Gerald Weeks

13:00 – 19:00 Excursion to whisky distillery and Glamis Castle

20:00 - Banquet and Ceilidh (Celtic for party time)

Friday 4 August 2000

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One more choice for Dictyostelium discoideum cells: a 'plant-like' programmed cell death or a 'mammalian-like' apoptosis
I Tatischeff, A Grodet, J Tissier, I Duband-Goulet

***Dictyostelium* morphogenesis in the absence of two major extracellular matrix proteins**
A Early, M Grimson, T Kerr, M Melkus, A Morrison, J Williams and L R Blanton

Excitability and Synchronization in *Dictyostelium discoideum* and the Possible Relationship with the Equivalent Process in Neural Networks in Other Organisms
Seido Nagano

A Possible Mechanism of Slug Turning during Phototaxis
Kota Miura and Florian Siegert

10:20 -11:00 Break

11.00 - 12.00 SIGNALLING II Chair: Mineko Maeda

PKA from *Dictyostelium* as a sensor for cAMP concentration *in vitro* and *in vivo*
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Y Huang, C Parent, S Van Es, C Janetopoulos, H Patel, C Weijer and P Devreotes
Role of G-Protein carboxyl methylation in signal transduction
Y. Chen, J. Stock, and Edward C. Cox.

12:30 - 14:00 Lunch

POSTER SESSIONS

All posters can be displayed during the entire meeting. Please mount your posters on the poster boards according to the numbers indicated in the LIST OF POSTERS

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ORAL PRESENTATIONS

Monday 31 July 2000
09:00 - 10:20 CHEMOTAXIS I

Regulation of the activity and localization of PAKa via direct phosphorylation by Akt/PKB

Chang Y. Chung, Gary Potikyan, and Richard A. Firtel
Section of Cell and Developmental Biology, Center for Molecular Genetics, UCSD, 9500 Gilman Dr., La Jolla, CA 92093-0634

Chemotaxing *Dictyostelium* cells have a strongly biased axial polarity. The assembly of myosin II in the rear cell body is very important in defining axial polarity and in biasing the direction of movement by repressing extensions of lateral pseudopodia through cortical tension. PAKa, a structural homologue of mammalian PAK1 (P21-activated kinase 1), appears to be a key regulator for myosin II assembly in the posterior cell body. Regulation of the activity and localization of PAKa would be a key step to establish and/or maintain cellular polarity in migrating cells. We examined the regulation of the activity and localization of PAKa througha signaling pathway including PI3 kinase and Akt/PKB. *Dictyostelium* PAKa is mainly localized in the rear cell body of polarized cells and disruption of cell polarity by bathing cells with cAMP causes relocalization of PAKa. However, distribution of PAKa is rather uniform in both *akt/pkb* and *pi3k1/2* null cells and this localization is consistent with no localized myosin II assembly in *akt/pkb* null cells. We found a conserved Akt/PKB phosphorylation site (T579) and have demonstrated direct phosphorylation of this site by Akt/PKB *in vitro*. Mutations on T579 to Ala or Asp had significant effects on the activity and subcellular localization of PAKa. We suggest that direct phosphorylation of PAKa by Akt/PKB plays a very central role in regulating the localization and activity of PAKa and, in turn, controlling cellular polarity during chemotaxis.

Sensing and responding to chemoattractant gradients: role of PI3K in controlling directional responses

Rick Firtel[1], John Moniakis[1], Chang Chung[1], Ruedi Meili[1], Tsuyoshi Araki[2], Masashi Fukuzawa[2], Tomaki Abe[2], and Jeff Williams[2]
[1]*Section of Cell and Developmental Biology and the Center for Molecular Genetics, UCSD, La Jolla, CA 92093-0637;* [2]*Wellcome Trust laboratories, Department of Anatomy & Physiology, University of Dundee*

Chemotaxis in macrophage, neutrophils, and *Dictyostelium* cells is controlled by a set of coordinately regulated pathways that leads to cell polarization, polymerization of actin at the leading edge and protrusion of a new pseudopod, and contraction of myosin at the cell's posterior. We have been examining the role of PI3K, which appears to play a key role in chemotaxis in *Dictyostelium* and metazoans by directing responses to the leading through the localized production of PI(3,4,5)P_3 and PI(3,4)P_2, binding sites for the PH domain-containing proteins. In *Dictyostelium*, two of these are Akt/PKB and a novel protein DecA: localization of these proteins to the leading edge in a PI3K-dependent manner is required for their proper function in controlling chemotaxis. Akt null and DecA null strains have severe chemotaxis defects: Akt/PKB is required for proper cell polarization and cell movement, while DecA is required for proper level and kinetics of actin polymerization. The *Dictyostelium* p21-activated protein kinase PAKa, a structural homologue of metazoan PAK1, is required for the polymerization and contraction of myosin during cytokinesis and chemotaxis. During chemotaxis, Akt/PKB functions in part by regulating the subcellular localization of PAKa in the posterior of cells and its activation in response to chemoattractants, and thereby regulating myosin assembly.

In addition, we have identified an SH2-containing tyrosine kinase, SHK1, that also functions in this pathway, we think by acting as a negative regulator of PI3K. Evidence suggests that SHK1 is associated with the plasma membrane via binding of its SH2 domain to a pTyr residue. In SHK1 null cells, chemoattractant activation of PI3K is 5-10-fold elevated and the kinetics are highly extended, while SHK1 overexpressing cells exhibit severely reduce Akt response. SHK1 mutant strains, expected show highly aberrant chemotaxis. *Dictyostelium* has two Akt-family members, *bona fide* Akt/PKB, and PKBR-1, which is highly related in the kinase domain and C-terminal extension but has a N-terminal myristolyation site rather than a PH domain, and this thus constitutively membrane localized. Akt/PKB and PKBR-1 are both activated in response to chemoattractants via a G protein-coupled pathway. However, the activation of PKBR-1 is PI3K-independent. These data and the phenotypes suggest that SHK1 may be functioning at the level if PI3K or one of its regulators to control chemotaxis.

Scar, a WASp-related protein, is required for lateral pseudopod formation and expansion, chemotaxis and ligand mediated changes in F-actin levels in *Dictyostelium*

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Scar and related proteins of the WASp (Wiskott-Aldrich Syndrome protein) family have been shown to facilitate the polymerization of actin in vitro. The mechanism of action involves direct binding to actin and components of the Arp2/3 complex. In vivo Scar and WASp have been implicated in regulating the formation of lamellipodia and filopodia, respectively, and we previously showed that the absence of Scar in *Dictyostelium* cells leads to defects in F-actin organization. We now report on the ability of Scar null cells to move, form lateral pseudopods, and polymerize F-actin in response to chemoattractant. We found Scar$^-$ cells are unable to form lateral pseudopods and a localized increase of F-actin polymerization associated with new pseudopod formation was absent. Chemotaxis was severely impaired in these cells and cAMP stimulated F-actin polymerization was significantly reduced. Reintroduction of Scar, fused to GFP, reverted these phenotypes. Using this construct we also visualized Scar in living cells. Scar is highly enriched in the leading edge of cells and is present at the site of new pseudopod formation. We propose that Scar acts positively in a signaling cascade to regulate the site of new actin polymerization at the leading edge of cells and is essential for lateral pseudopod formation.

Filactin, a novel filamin- and actin-related protein

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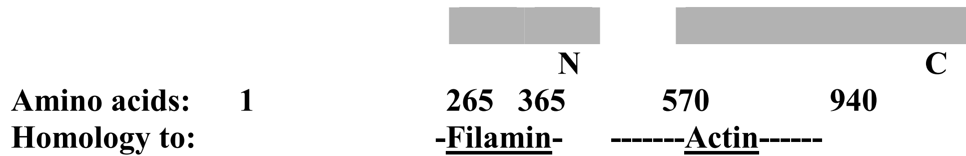
A search for genes that code for filamin-like proteins allowed the identification of several sequences from the German *Dictyostelium* Genome Project with mild homologies to this class of F-actin crosslinking proteins. Further studies revealed that *Dictyostelium* harbors an actin-related protein which is so far unique in evolution. The protein is composed of 940 amino acids and contains in its N-terminal half two regions with homology to filamin-like proteins; the C-terminus is an actin homologue ("Filactin", Fig 1A). Most likely, the filamin-related domains resemble in their structure IgG-like folds as they have been determined for the rod-region of the ABP120 gelation factor (Fucini et al., Nat. Struct. Biol. 4:223-230, 1997). Fitting the amino acid sequence of the actin part into the structure of muscle actin and extensive sequence comparisons show that filactin is by far closer related to muscle actin than to yet

identified actin-related proteins from the different ARP families (Fig. 1B). All amino acids that are required for ATP binding in the cleft between the actin subdomains 1/2 and 3/4 are present. We raised monoclonal antibodies against different domains of filactin and constructed a number of GFP fusion proteins which are currently used to characterize filactin at the biochemical and cell biological level.



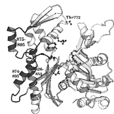


Fig. 1A: Domain structure of filactin

11.00 - 12.20 GENE REGULATION AND DIFFERENTIATION

The Activity of CRTF, a Transcription Factor for cAMP Receptor 1 Expression, Is Altered by Cleavage and Regulated by cAMP Signaling

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The promoter responsible for CAR1 expression during aggregation is activated by nM cAMP pulses and is repressed by a continuous exposure to cAMP, conditions which to lead to receptor adaptation. Deletion and mutation analyses of the CAR1 promoter defined an element essential for CAR1/G protein-regulated expression. A wild-type, but not mutant, 40bp fragment from this region directed transcriptional activation when concatamerized and linked to a minimal heterologous promoter. Mobility shift assay, UV-crosslinking, and DNase I footprinting was used to identify a protein, CRTF, that binds specifically to this DNA element in a Zinc-dependent manner. CRTF was purified by DNA-affinity chromatography, peptides were sequenced by Edman degradation, and full-length cDNAs were obtained. The deduced CRTF protein is ~100 kDa with a C-terminal, zinc finger-like motif that is required for DNA

binding. CRTF purified from growing cells represents a 40 kDa C-terminal fragment that retains DNA-binding activity.
CRTF-null strains, produced by homologous recombination, fail to develop under standard conditions or exhibit induced expression of CAR1 or other cAMP-regulated genes. Development is rescued by expression of 100 kDa CRTF protein, but not by expression of the 40 kDa truncation. Further, wild-type cells that express exogenous 100 kDa CRTF develop normally, but wild-type development is inhibited by expression of 40 kDa versions of CRTF. We suggest that full-length 100 kDa CRTF is an active form required for CAR1 gene induction; the 40 kDa DNA-binding trunctation functions as a repressor that lacks a transcriptional activation domain. This mechanism for cAMP-CRTF regulation may parallel that of Hedgehog-Ci/Gli signaling in other systems.

The *cbfA* gene encodes a DNA-binding protein that supports growth of *Dictyostelium* amoebae and expression of retrotransposon TRE5-A transcripts

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D. discoideum contains some 100 copies of the retrotransposable element TRE5-A (formerly named DRE). Both plus strand and minus strand RNAs are detected in growing *D. discoideum* cells. The A-module at the 5' end the TRE5-A has promoter activity and controls plus strand transcription of TRE5-A. The C-module at the 3' end of TRE5-A is thought to be critical for minus strand transcription. In experiments aimed to identify cellular proteins regulating TRE5-A expression and retrotransposition we analysed a nuclear protein which binds specifically to the C-module of TRE5-A. This protein, C-module-binding factor (CMBF), was purified from *D. discoideum* cells and the related gene, *cbfA*, was cloned. The *cbfA* gene predicts a protein of 139 kDa that does not contain extended similarities with proteins in the databases. It does contain, however, a cysteine-rich region that may form three distinct zinc finger-like structures. CMBF also contains a functional "AT-hook", a short peptide motif required for specific binding to AT-rich DNA sequences. This fits to observations that CMBF binds at two very AT-rich sites in the C-module, both of which have a central homopolymeric oligothymidine stretch of about 24 bp length.
We analysed cellular functions of CMBF by underexpressing the protein by translation stop codon suppression. One mutant cell line, JH.A, was analysed in detail. JH.A cells expressed only about 20% of wildtype CMBF, showed a pronounced growth defect and increased cell size in axenic cultures, and grew slowly on bacterial lawns. In JH.A cells the steady-state level of TRE5-A transcripts was drastically reduced, suggesting that CMBF is involved in the regulation of TRE5-A expression. The underlying mechanism is currently unknown but appears not to involve regulation of C-module or the A-module promoter strengths.

The MADS-box gene *srfA* plays multiple roles in *Dictyostelium* development

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srfA codes for a putative transcription factor of the MADS-box family. The study of *srfA* knock out strains revealed that this gene is required for several aspects of *Dictyostelium* development: terminal spore differentiation, proper slug migration and culmination. The deffect in spore differentiation can not be overcome by the activation of PKA, suggesting that DdSRF may function downstream of this kinase in the signal transduction pathway that control final spore formation. Under experimental conditions that favor direct culmination (buffered filters) only a transient arrest at the finger stage could be observed in the mutant,

before an otherwise normal morphogenesis that gives rise to a well-proportioned fruiting body. However, under experimental conditions that encourage slug migration (non- buffered conditions), the mutant strains display a much more severe phenotype that compromises both slug behavior and culmination.
The promoter region of *srfA* has been cloned, sequenced and its functional activity studied by in situ *LacZ* staining. *srfA* displays a complex temporal and cell-type-specific pattern of expression that involves virtually all *Dictyostelium* cell types. This complexity is achieved by the use of three alternative promoters. The activity of the proximal promoter region (P1) is restricted to a subset of prestalk cells. Little or no expression was observed in the lower cup and basal disc during culmination. The middle promoter (P2) stains preferentially prestalk cells under normal conditions of filter development. Interestingly, the activity of this promoter in the posterior prespore cells is strongly induced by experimental conditions that encourage the morphogenetic pathway of slug migration. The distal promoter (P3) displays a dual pattern of expression. Before culmination the promoter drives *lacZ* expression to few cells scattered along the structure in a typical anterior-like cell (ALC) pattern. Later on a strong staining was extended to the spores by the end of culmination.
Work is currently in progress in our laboratory to determine the regulatory pathways that control *sfrA* expression from these promoters to further understand the roles played by this transcription factor in *Dictyostelium* development.

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

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A gene, zyg1, was isolated by differential screening from Dictyostelium mucoroides 7 (Dm7) cells, as one specifically expressed during their sexual development. The zyg1 gene encodes for a novel protein (ZIG1; deduced MW 29.4 kDa) consisting of 268 amino acid. Although the gene has several pkC phospholylation sites, it has neither transmembrane domains nor specified signal sequences. The expression of zyg1 mRNA was initiated after 2 h of starvation and reached to the maximum level at 8 h under submerged conditions. The expression pattern is a quite similar to a temporal change of zygote formation during the sexual development (macrocyst formation). Actually, zyg1-overexpressing cells derived from Dm7 was found to form zygotic giant cells even at the vegetative stage coupling with the precocious overexpression of zyg1 mRNA. In addition , they eventually formed macrocysts even under conditions favouring to asexual fruiting body formation. Interestingly, when the zyg1 mRNA was overexpressed in AX2 (heterothallic strain) cells which need the opposite mating type strain (V12M2) for macrocyst formation, they constructed giant cells and finally macrocyst-like structures without any help of the opposite mating type strain. As expected, antisense mediated zyg1-iactivation in AX2 cells greatly inhibited macrocyst formation in the presence of opposite mating type strain, V12M2. From these results, it is evident that the zyg1 gene has an essential role in zygote formation beyond the difference of homothallic and heterothallic strains. The function of ZYG1 as a possible signal transducer phospholylated by pkC is discussed.

Studies on possible functions of the *Dictyostelium* Hsp90 family in regulation of growth and differentiation

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Heat shock protein 90 (Hsp90), which is ubiquitous and a highly conserved 90 kDa molecular chaperone, plays important roles in the cell-cycle regulation, cell differentiation, and apoptosis. Hsp90 generally forms complexes with many protein kinases (eg., Src, Raf, CK2, and CDK4) and transcriptional regulators such as steroid hormone receptors to keep their conformations stable until they are stabilized by conformational changes associated with signal transduction. In *Dictyostelium discoideum* cells, we recognized three members of Hsp90 family, Hsc90 (heat shock cognate protein 90), Dd-GRP94 (*Dictyostelium discoideum* glucose-regulated protein 94), and Dd-mtHsp90 (*Dictyostelium discoideum* mitochondrial heat shock protein 90). The phosphorylation level of Hsc90 was specifically reduced in response to the initiation of differentiation, and the knock-out of *hsc90* seemed to be lethal. On the other hand, the expression of *dd-grp94* was found to be greatly reduced within 60 min of starvation, possibly coupling with Ca^{2+}-depletion from intracellular Ca^{2+} stores such as the endoplasmic reticulum (ER). *dd-grp94*-overexpressing cells (*grp94*^{OE}-cells) collected without forming distinct aggregation-streams and never formed normal fruiting bodies, being remained as spherical cell masses ever after 72 hr of starvation. Antisense mediated gene inactivation of *dd-grp94* seemed to be lethal. With respect to Dd-mtHsp90, its expression was found to be greatly reduced in response to starvation, as the case for *dd-grp94*. Interestingly, although Dd-mtHsp90 localizes in the plasma membranes of vegetatively growing cells, it translocates to mitochondria in response to the growth/differentiation transition (GDT) of starving cells. The function of Dd-mtHSP90 is under investigation.

14:00 - 15:20 CELL ADHESION AND CYTOKINESIS

Adhesion Mechanism and Developmental Role of the Cell Adhesion Molecule gp150/LagC

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Previous work in our laboratory has demonstrated that gp150 is a Ca^{2+}/Mg^{2+}-independent cell adhesion molecule encoded by the lagC gene. To investigate the mechanism by which gp150 mediates cell-cell adhesion, a GST fusion protein which contains the complete extracellular domain of gp150 was expressed in bacteria and purified. The cell-to-substratum binding assay was used to determine whether gp150 mediated cell binding via homophilic or heterophilic interactions. Both KAx-3 cells and AK127 (lagC-null) cells were developed for 16 h. Cells of both strains attached equally well on gp150 fusion protein-coated Petri dishes, suggesting the presence of heterophilic receptors on these cells. To determine whether gp150 is capable of homophilic interaction, both KAx-3 cells and AK127 cells were transfected with an expression vector containing the gp150 cDNA driven by the actin 15 promoter. Although transformants expressed functional gp150 on the cell surface during vegetative growth, cells at this stage failed to form EDTA-resistant aggregates and showed only background levels of attachment to substrate-immobilized gp150 fusion protein. These results thus indicate that gp150 is incapable of homophilic binding and mediates cell-cell adhesion only via heterophilic interaction. To map the heterophilic binding site in gp150, fusion proteins containing different segments of gp150 were subjected to the cell-to-substratum binding assay. Fusion proteins were also used to inhibit cell-cell reassociation. Results have led to the localization of the binding site to a region between Ser300 and Val360, which is characterized by a-helical structure in the C-terminal half..

Immunofluorescence labeling of gp150 has revealed a unique temporal and spatial expression pattern during development. To further investigate the regulation of lagC expression and the potential role of gp150 in cell-type differentiation, we have cloned the lagC gene, including ~2 kb of the 5'-flanking DNA. To define the promoter region of the lagC gene, the green

fluorescence protein (GFP) cDNA was fused to different fragments of lagC 5'-DNA and then cloned into an expression vector. Analysis of the transformants indicated that a 550 bp-segment in the 5'-DNA is sufficient to confer proper transcriptional regulation of GFP. Time-lapse video microscopy is being used to examine the temporal and spatial location of GFP-expressing cells. Results are consistent with the immunofluorescence labeling data, showing a low level of GFP expression during the aggregation stage and a rapid induction of GFP expression in cells mostly in the periphery of the loose aggregate. The mound structure is surrounded by this layer of highly fluorescent cells. Some of these cells eventually move to the tip, while a large number remain at the posterior end of a slug which eventually give rise to a brightly fluorescent disc structure at the fruiting body stage.

Dictyostelium DdCP224 is a microtubule-associated protein and a permanent centrosomal resident involved in centrosome duplication

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Monoclonal antibodies raised against isolated *Dictyostelium discoideum* centrosomes were used for immunoscreening of a size-fractionated cDNA library. Finally, a cDNA encoding a 224-kD Dictyostelium discoideum centrosomal protein (DdCP224) was isolated. DdCP224 was detected at the centrosome throughout the entire cell cycle. In interphase it localizes to the corona and microtubules, during mitosis it is found at spindle poles, spindle microtubules and kinetochores. Centrosomal localization, unlike kinetochore localization, does not require microtubules, suggesting that DdCP224 is a genuine centrosomal component. DdCP224 exhibits sequence identity to a weakly conserved class of microtubule-associated proteins including human TOGp, Xenopus XMAP215 and yeast Stu2p. Stu2p is a permanent SPB resident and has a size of only ~90 kDa corresponding to the N-terminal half of the Dictyostelium and human proteins. The functions of the N- and C-terminal halves of DdCP224 were investigated in the corresponding GFP-fusion mutants. Surprisingly, the N-terminal construct showed only cytosolic localization, whereas the C-terminal construct localized exclusively to the centrosome. This is unexpected because Stu2p is localized at the spindle pole body. Full-length DdCP224-GFP was found at centrosomes and along microtubules. In addtion, it bound to pig brain microtubules in vitro, unlike the two truncated mutants. Thus, centrosome binding is determined by the C-terminal half and microtubule binding may require the interaction of the N- and C-terminal halves. Interestingly, more than ~50% of all cells expressing full-length DdCP224-GFP contained supernumerary centrosomes not associated with the nucleus. In addition, these mutant cells had a cytokinesis defect, since ~50% were multinuclear. However, the two defects do not seem to be directly linked since supernumerary centrosomes did not occur at higher frequency in multinuclear than in mononuclear cells. These results, together with the frequent occurrence of abnormally shaped centrosomes, indicate that DdCP224 is involved in centrosome duplication. Such a function has not been discussed for any other member of this protein family so far.
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Protein sorting and organelle dynamics in cytokinesis

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In mitotic cells of *Dictyostelium*, actin-binding proteins distribute either to the polar regions or to the cleavage furrow. In anaphase most of the filamentous actin assembles in ruffles at the two poles of a dividing cell, and a number of regulatory proteins including coronin, cofilin, its activator Aip1, and Arp3 colocalize with the polar actin assemblies. In contrast, cortexillins are translocated to the midzone, where they prime a cleavage furrow. Basal activities in translocation of cortexillins and in the rescue of cytokinesis are localized to a 84 amino-acid

segment of the C-terminal domain. Myosin II, which also accumulates in the midzone, stabilizes the furrow in its position. This function is essential in cells that divide in suspension, but is dispensable when cells are attached to a substrate. Cytokinesis is characterized by an actin cycle comprising polymerization at the cell poles, flow to the midzone, and depolymerization in the cleavage furrow. This cycle can be cut-off by preventing actin disassembly. For entrapping actin in the midzone, we have used a GFP-tagged, slowly dissociating actin-binding fragment of talin. Cortexillins I and II are essential constituents of the machinery that is set up in the midzone of a dividing cell for the disassembly of actin. To study organelle dynamics in cytokinesis and other cell functions, GFP fusion proteins have been constructed for the specific labeling of the endoplasmic reticulum, the Golgi apparatus, or the contractile vacuole system in live cells. Whereas the ER stays intact, the other organelles undergo distinct cycles of reorganization during mitosis.

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2. Two-step positioning of a cleavage furrow by cortexillin and myosin II. Weber, I., Neujahr, R., Du, A., Koehler, J., Faix, J. and Gerisch, G. Current Biology **10**, 501-506 (2000).
3. Cytokinesis without myosin II. Gerisch, G. and Weber, I. Review. Curr. Opin. Cell Biol. **12**, 126-132 (2000).
4. The contractile vacuole network of *Dictyostelium* as a distinct organelle: its dynamics visualized by a GFP marker protein. Gabriel, D., Hacker, U., Koehler, J., Mueller-Taubenberger, A., Schwartz, J.-M., Westphal, M. and Gerisch, G. Journal of Cell Science **112**, 3995-4005 (1999).
Microtubule-dependent Golgi disassembly and reconstitution during mitosis in *Dictyostelium discoideum*. Schneider, N., Gerisch, G. and Schwartz, J.-M. In: GFP in Motion, compiled by B. Ludin and A. Matus. Trends in Cell Biology **9**, supplement on CD-ROM (1999).

16:00-17.20 DIF SIGNALLING AND METABOLISM

A novel role of differentiation-inducing factor-1 (DIF-1) during early development of *Dictyostelium* assessed by the restoration of a developmental defect of the mutant lacking MAP-kinase, ERK2

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In the last *Dictyostelium* meeting, we have reported that the developing wild-type cells of *Dictyostelium* secrete a diffusible factor(s) which is able to rescue the developmental block in the mutant lacking MAP-kinase ERK2 encoded by the gene erkB (1). In this meeting, we demonstrate that DIF-1 (differentiation-inducing factor of stalk cell) can mimic the role of the factor(s), and we discuss the mechanism of the action of DIF-1 in the erkB-null mutant. The mutant cannot form multicellular aggregates because of its defect in cAMP signaling. In the presence of 100 nM DIF-1, however, the mutant cells formed tiny slugs, which eventually developed into small fruiting bodies. By contrast, DIF-1 never rescued the developmental arrest of other *Dictyostelium* mutants defective in cAMP response lacking adenylyl cyclase A (ACA), cAMP receptors (cAR1 and cAR3), heterotrimeric G protein, the cytosolic regulator of ACA (CRAC), and the catalytic subunit of cAMP-dependent protein kinase (PKA-C). Most importantly, it was found that DIF-1 did not affect cellular cAMP level but rather elevated the transcriptional level of PKA during the development of erkB-null. These results suggest that DIF-1 may rescue the developmental defect in erkB-null via the increase in PKA activity in some way. Furthermore, since DIF-1 has been believed to act only in the later developmental stages, when prestalk- and stalk-specific genes are expressed, the present

finding is the first conclusive evidence to show that DIF-1 plays a crucial role in the early development other than inducing prestalk- and stalk-cell differentiation.

(1) A diffusible factor involved in MAP-kinase ERK2-regulated development of *Dictyostelium.* Maeda, M. and Kuwayama, H. (2000) *Dev. Growth Differ*. In press.

Genetic Evidence that DIF-1 is the Inducer of pstO Differentiation

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Although it has been proposed that DIF-1 plays a central role in the control of stalk and spore cell differentiation during *Dictyostelium* development, most evidence comes from cell culture rather than genetics. Therefore, we have taken a reverse genetic approach to generate a mutant that does not produce DIF-1.

Firstly, we identified the gene encoding the enzyme which catalyses the final step in DIF biosynthesis, the des-methyl-DIF-1 methyltrasferase (*dmtA*). The product of this gene appears to be indistinguishable from the endogenous enzyme and its developmental regulation closely mirrors that of DIF-1. Furthermore, *dmtA* disruptants produce no detectable des-methyl-DIF-1 methyltrasferase activity. Cell associated DIF-1 is undetectable in the *dmtA* knockout strain as assayed by chlorine labelling. The sensitivity of this assay means that the mutant produces less than 10% of wild type DIF-1.

The *dmtA* mutant has a clear developmental phenotype: it forms long, thin slugs which tend to break and produces aberrant culminants with the stalk tube lying mostly on the substratum. The sorus is barely raised or often fallen. The phenotype is rescued by development on agar containing DIF-1. Standard prestalk and prespore markers are expressed at levels comparable to wild type, but markers of a specific subset of prestalk cells (pstO) are <u>not</u> expressed and the prespore zone shows a complimentary expansion. However, other specific prestalk markers (e.g. pstA) are unaffected which raises the possibilty that their expression is driven by a DIF-1 independent mechanism.

To establish how some prestalk cell types and stalk cell differentiation may be induced in the *dmtA* mutant, we have characterised the stalk inducing activities of the mutant by HPLC. This reveals: 1. The major stalk inducing bioactivity (DIF-X) is novel and absent (or present at much lower levels) in the wild type. 2. The dmtA mutant produces a small amount of activity (less than 0.5% compared to wild type) that runs as DIF-1. We are investigating whether DIF-X or the residual DIF-1 may drive pstA gene expression.

We have thus produced a mutant which reveals the minimal “DIFless” phenotype and it provides strong evidence that DIF-1 is the inducer of pstO cell differentiation. Furthermore, the mutant reveals a novel stalk inducing activity (DIF-X), the characterisation of which is ongoing.

The DIF-inducible STAT, Dd-STATc, functions in both early and late development

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University of Dundee, UK

At the slug stage Dd-STATc is selectively localised in the nuclei of pstO cells. When DIF is added to cells at four hours of development in shaken suspension Dd-STATc becomes tyrosine phosphorylated and rapidly translocates to the nucleus. Nuclear import requires a centrally located, c. 50aa sequence that is the binding site of a protein with homology to the oxysterol binding proteins (OSBPs). We have recently isolated a null mutant of OSBP but Dd-STATc shows normal nuclear translocation. There are, however, a number of additional Dictyostelium OSBPs and there may, therefore, be redundancy in the Dd-STATc nuclear translocation pathway. We have also isolated a null mutant of Dd-STATc and it is a “slugger” mutant (i.e. it remains as a migratory slug under environmental conditions that cause the

parent strain to culminate). We recently showed in a collaboration with Rick Firtel's group that Dd-STATa, the best characterised Dictyostelium STAT is the repressor that prevents premature expression of ecmB and it is also needed for optimal chemotaxis to cAMP. Interestingly, the Dd-STATc null strain over-expresses and ectopically expresses a prestalk marker: the late promoter of the PDE gene. Also, the Dd-STATc null is a rapidly developing mutant, that initiates wave formation and csA expression approximately two hours prematurely. Thus, as with Dd-STATa, the Dd-STATc protein appears to act as a repressor of gene expression at the slug stage but it has additional, early functions. The availability of the null mutant has allowed us to test the biological function of tyrosine phosphorylation and consequent dimerisation. Nuclear translocation can be uncoupled from tyrosine phosphorylation because a point mutant of Dd-STATc, where the site of tyrosine phosphorylation is altered to phenylalanine (the "Y-F" mutant), retains the ability to translocate to the nucleus in response to DIF. However, all aspects of the phenotype of the Dd-STATc null are "rescued" by expression of the intact Dd-STATc gene but not by the Y-F mutant. Thus dimerisation is not required for nuclear translocation but it is required for biological function, presumably because the DNA binding sites of STAT proteins are dimeric.

Intermediates for stalk gene induction

Karin E. Weening and Pauline Schaap

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Earlier data showed that a slow sustained increase in cytosolic Ca^{2+} levels mediates induction of the stalk gene *ecmB* by DIF. We have applied several strategies to find other components of this signaling pathway. Since stalk cell differentiation can be considered as a form of programmed cell death (PCD) we have studied effects of overexpression of metazoan apoptosis genes in *Dictyostelium* and have tried to retrieve homologs of such genes from the *Dictyostelium* genome databases. So far homologs have only been found for the Alzheimer′s disease gene ALG-2, a Ca^{2+} binding protein, and its interacting protein AIP1. Mouse ALG-2 also appears to be lethal when expressed in *Dictyostelium*. As a second strategy we selected a number of REMI mutants that were specifically defective in DIF-induced *ecmB* gene expression. The rescued gene of one of these mutants harbours a calponin domain with five LIM domains as predominant structural features. The mutant forms mounds, but is incapable of forming tips or of participating in tip formation when mixed with wild-type cells. Chimeras of the mutant with at least 50% wild-type cells can form fruiting structures. The mutant cells in such structures express *ecmB* in the upper and lower cup, but never in the stalk cells.

Tuesday 1 August 2000

09:00 - 10:20 PROTEIN FACTORS

Purification of proteins with discoidin-inducing activity similar to PSF

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The initiation of development of *Dictyostelium* is triggered by starvation. However, cells do not passively experience the lack of nutrients, but actively anticipate it and prepare for development in the "prestarvation response". Growing cells secrete a protein, PSF, which accumulates with increasing cell density and induces early developmental genes. PSF is also secreted during the first 2 hours of development. The amount of PSF necessary for gene induction is affected by the concentration of bacteria, which counteract its effects. This

competition allows the population to measure cell density relative to the amount of food bacteria. Discoidin I is one of the targets of PSF, and discoidin induction is one of the earliest events at the initiation of development (Clarke and Gomer, 1995).
We developed a fast bioassay for discoidin I induction and used it to purify an activity from conditioned starvation media. The activity copurifies with a protein complex of ca. 450 kDa consisting of three proteins: 50-55 kDa, 80 kDa and 120 kDa. N-terminal and internal sequencing revealed that the 50-55 kDa and 120 kDa proteins are identical to cAMP phosphodiesterase (PDE), while the 80 kDa fraction yielded peptide sequences of a novel protein. The functional significance of the association of PDE with the 80 kDa protein is unclear so far.
Partial purification of PSF from conditioned growth medium in Margaret Clarke's lab indicated that PSF has a molecular weight of 70 kDa (Burdine and Clarke, 1995). Several experiments suggest that the activity in media conditioned by growing cells and the activity in media conditioned by starving cells (6 hours) have common components in spite of the size difference.

1. Both CM's are active during growth as well as during development.
2. Accumulation of the activity declined after a few hours of development (similar to PSF).
3. The activity in both CM's is heat labile (similar to PSF).
4. From both CM's, active fractions can be purified, and these contain 50-55 kDa and 80 kDa polypeptides.
5. Both CM's activate discoidin I expression via the dNCE promoter fragment (Vauti et al., 1990).

In conclusion, we have partially purified a secreted factor with the properties of PSF. Like counting factor, it appears to be a complex of polypeptides.

Clarke M and Gomer RH (1995) Experientia *51*, 1124-1134.
Vauti F, Morandini P, Blusch J, Sachse A and Nellen W (1990) Mol. Cell. Biol. *10*, 4080-4088.
Burdine, V., and Clarke, M. (1995) Mol. Biol. Cell *6*, 311-325.

TagA, a putative serine protease/ABC transporter of *Dictyostelium* that is expressed at the onset of development and is required for the differentiation of a subpopulation of prespore cells.

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The *tag* genes in *Dictyostelium* are predicted to encode multi-domain proteins consisting of serine protease and ATP-binding cassette (ABC) transporter domains. We have identified a novel member of the tag gene family, *tagA*, which is involved in cell type differentiation. The *tagA* gene is expressed at its highest level after 2 hours of development and at lower levels later, whereas TagA protein accumulates through the first 6-10 hours of development and decreases thereafter. In wild-type cells, TagA protein and *tagA* promoter activity can be detected in ~10% of the cells at 4 hours of development and in ~80% of the mature spores. This indicates that TagA as a prespore-specific protein. However, TagA mutants harboring an *ecmA::GFP* prestalk reporter construct form developing structures with 2-3 times more GFP-positive cells than that of the parental strain. In migrating slugs, most of these additional prestalk cells appear to be in the posterior prespore region. TagA mutant cells that activate the *tagA* promoter to high levels, as measured by a *tagA::lacZ* expression construct, also co-localize with prespore cells during development. Upon culmination, these cells are found in the basal disc and lower cup region of terminal fruiting bodies, but are absent from the spore population. Within developing *tagA*$^-$/wild-type mixtures, *tagA* mutant cells form most of the prestalk cells of the chimera, but do not appear to induce inappropriate differentiation of the

wild-type cells, indicating that the *tagA* phenotype is cell autonomous. These results suggest that the initial population of *tagA* expressing cells, in the absence of TagA function, adopt a prestalk cell fate similar to that of PstB cells and this may account for the additional prestalk cells observed in the mutant. However, the *tagA* mutant cells do produce prespore cells and, ultimately, spores. This suggests that there may be distinct mechanisms for prespore cell differentiation and that TagA is absolutely required only for the differentiation of an initial population of prespore cells. Expression of the *tagA* gene at two hours of development provides evidence that commitment to the prespore cell fate, at least in some cells, occurs earlier than previously believed.

A cell number-counting factor differentially modulates cAMP-induced cAMP and cGMP pulse sizes, and affects cell motility in addition to adhesion

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Little is known about how the size of a tissue or group of cells is regulated. *Dictyostelium* amoebae aggregate in dendritic streams which break up into groups of ~2 x 10^4 cells. A secreted 450 kDa protein complex, counting factor (CF), regulates the size of these groups. High levels of CF cause the streams to break into small groups, while cells lacking CF form huge groups, with the resulting fruiting bodies toppling over. The aggregation is mediated by relayed pulses of cAMP. Cells also respond to cAMP with a short cGMP pulse, and other workers have found that mutants with abnormally large cGMP pulses form large groups, while mutants with small cGMP pulses form small groups. We find that CF slowly down-regulates the cAMP-induced cGMP pulse by inhibiting guanylyl cyclase activity. In contrast, a five-minute exposure of cells to CF increases the cAMP-induced cAMP pulse. The effect of CF on group size can be reproduced by mimicking CF's effect on the cAMP pulse. Pulsing streaming wild-type cells with a high concentration of cAMP results in the formation of small groups, while reducing cAMP pulse size with exogenous phosphodiesterase during stream formation causes cells to form large groups.

We previously found that CF decreases cell-cell adhesion. Computer simulations predicted that in addition to decreasing adhesion, increasing the random component of cell motility would cause a stream of cells to break up. *Dictyostelium* motility is being characterized by several groups. In streaming cells, during the first 80 seconds after the arrival of a pulse of cAMP, the protein composition of the cytoskeleton undergoes several changes, in part due to the cGMP-induced phosphorylation of myosin II. We are using wild-type cells expressing a GFP-myosin heavy chain construct (a gift from Dr. Jim Spudich, Stanford University) to monitor the motility of cells. We have made chimeras by mixing these cells with a 10 fold excess of *smlA*, wild-type, or *countin* cells. We monitor the aggregating cells and the distribution of myosin by time-lapse observations with a confocal microscope. Preliminary experiments indicate that in the *countin* chimeras, where the CF concentration is low and the mixtures form large fruiting bodies, the GFP myosin cells move slowly, and cAMP pulses cause the GFP myosin to change from a roughly uniform distribution to a concentration at the cell cortex. In the *smlA* chimeras, where the CF concentration is high and the mixtures form small fruiting bodies, the cells are much more motile, and the GFP myosin tends to remain concentrated at the rear of the cell. In addition to changes in myosin, a cAMP pulse causes a ~30 second transient increase in the amount of F actin. We found that streaming *countin* cells have a low basal level of F actin, while smlA cells have a high basal level. 2D protein gels show that *smlA* cells have relatively high levels of a protein at 120 kD which by amino acid

sequencing appears to be the 120 kD actin binding protein (ABP); this is present at a lower level in wild-type cells and is barely detectable in *countin* cells.
The combined observations indicate that although regulating only the cGMP pulse size or the cAMP pulse size is sufficient to control group size, CF affects both pulses simultaneously. Possibly through this pathway, CF regulates both cell-cell adhesion and cell motility. The computer simulations predict that by regulating adhesion and motility, CF can then regulate group size.

Elucidation of a cell-counting factor signal transduction pathway by isolation of second-site suppressors

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Starved *Dictyostelium* cells form aggregation streams which break up into evenly sized groups of ~2 x 10^4 cells. We previously found that this group size is regulated by a secreted 450 kD protein complex called counting factor (CF). Transformants oversecreting CF (*smlA*), or wild-type cells developing in a high concentration of exogenous CF, have streams which break up into many small groups, while transformants lacking CF have streams which do not break up and thus form large groups.
In an attempt to identify other genes that are involved in size determination, we have executed a genetic screen to find second site suppressors of *smlA* using random insertional mutagenesis (REMI) with a blasticidin-resistance plasmid. We isolated 26 mutants that allow *smlA* cells to form normal size fruiting bodies. Adhesion assays and mixing experiments performed on these mutants show that they fall into two classes. One group (19) appears to be insensitive to the signal secreted by *smlA* cells, suggesting that they are defective in sensing the CF signal. The other group (7) is still sensitive to the CF secreted by *smlA* cells, suggesting that the disruption was not in the CF signal transduction pathway. We have cloned and sequenced flanking DNA from 2 of the suppressors that appear to have CF signal sensing defects. One of the suppressors is a ubiquitin and raises an interesting possibility. We have seen that high levels of CF decrease the levels of gp24 and gp80 adhesion molecules, and this ubiquitin could be involved in a CF induced degradation of the adhesion molecules. Disruption of this ubiquitin gene could permit high levels of the adhesion molecules to accrue, and consequently large groups to form. Another of the suppressors, Ddkel1, has two kelch domains, and has sequence similarity to the intracellular protein muskelin. In mice, muskelin modulates cell-adhesive behaviour by mediating signal transduction from thrombospondin-1, a regulated macromolecular component of extracellular matrix. We are currently testing the hypothesis that Ddkel1 may have a similar function in the signal transduction pathway between CF and cell-cell adhesion.

11.00 - 12.20 cAMP SIGNALLING AND PATTERN FORMATION

The role of GskA and Aardvark in multicellular development

Adrian J. Harwood
MRC-LMCB, University College London, UK

The Wg/Wnt-1 signalling is a key component of pattern formation in metazoa and the proteins GSK-3 and ∃-catenin are central to this pathway. Protein homologues of these

proteins exist in *Dictyostelium* and we are investigating their function. The homologue of GSK-3, gskA, also regulates pattern formation in *Dictyostelium* and we have previously shown that cAMP, via the receptor cAR3, regulates GskA activity to control the prespore:pstB ratio. This pathway requires the tyrosine kinase, ZAK. *Aardvark* (*aar*) encodes a ∃-catenin homologue. As in metazoa, Aar is required for both the formation of adherens junctions and cell signalling. I will discuss our recent results.

Requirements for the adenylyl cyclases in development of *Dictyostelium*

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Two distinct genes, *acaA* and *acrA*, encode adenylyl cyclases that function during development prior to fruiting body formation in Dictyostelium. ACA accumulates during the aggregation stage and is necessary for secretion of cAMP for chemotaxis as well as activation of the cAMP dependent protein kinase PKA. In the absence of ACA, overexpression of PKA permits cells to continue through development to form fruiting bodies. However, they do not secrete cAMP to attract neighboring cells and form aggregates only by accretion when plated at high cell densities. No measurable cAMP accumulates in these *acaA*(PKA-C) cells prior to culmination.

The product of *acrA*, ACR (which has also been referred to as ACB), is very low during the early stages of aggregation but accumulates dramatically thereafter such that it accounts for the majority of the measured adenylyl cyclase activity by culmination. Strains carrying null mutations in *acrA* aggregate normally and proceed to the culmination stage but form few viable spores. Addition of 10 mM 8-Bromo-cAMP to *acrA*$^-$ strains does not result in a significant increase in the number of viable spores in although it induces the spore specific gene *spiA* and increases the number and stability of non-viable propidium iodide permeable spore-like structures. However, *acrA*$^-$ cells that ectopically express a G-protein independent form of ACA are able to make detergent resistant viable spores. It appears that ACR is responsible for cAMP production late in development but that this role can be carried out by ACA if it is overexpressed.

To determine whether ACR activity is required for development of *acaA*(PKA-C) cells lacking ACA but overexpressing PKA, we constructed a strain, TL130, in which both *acaA* and *acrA* were inactivated while PKA was overexpressed. No adenylyl cyclase activity was found at any point during 24 hours of development of these *acrA*$^-$ *acaA*$^-$ (PKA-C) cells using assays optimized for either ACA or ACR. TL130 cells showed the same phenotype during early development as the parental *acaA*(PKA-C) cells in that they aggregated only at high cell densities and then formed tipped mounds that developed to the finger stage. The cell type specific genes, *cotB* and *ecmA*, were expressed at the same levels as in *acaA*(PKA-C) cells. These results suggest that, as long as the catalytic subunit of PKA is overexpressed, development can proceed to the finger stage and cell type divergence can occur in the absence of cAMP synthesis. However, the finger-like structures did not form fruiting bodies although a few scattered stalks and spores-like strucutures were observed.

When these *acrA*$^-$ *acaA*$^-$ (PKA-C) cells were mixed with 10% wild type cells, the chimeras developed well and formed fruiting bodies indicating that the wild type cells provided signals leading to culmination. Fluorescently labelled mutant cells could be seen both in the anterior (prestalk) and posterior (prespore) of the slugs and throughout culminants. However, all of the detergent resistant viable spores were derived from the small number of wild type cells. It appears that there is a cell autonomous requirement for ACR to form viable spores.This is somewhat surprising since the levels of PKA were found to be high at 22 hours of development in the *acrA*$^-$ *acaA*$^-$ (PKA-C).

The phenotypes of these mutants demonstrate that cell type divergence and sorting can proceed in the absence of cAMP as long as PKA is overexpressed. However, terminal differentiation of viable spores depends on intracellular cAMP supplied by either ACA or ACR. This cell autonomous requirement for cAMP apears to be acting through a PKA-independent pathway that has yet to be characterized.

Regulation of 5’ Nucleotidase (5NT) in *Dictyostelium discoideum*.

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5’ Nucleotidase (5NT) in *Dictyostelium discoideum* is an enzyme that shows high substrate specificity to 5’AMP. The enzyme has received considerable attention in the past because of the critical role played by cyclic AMP in cell differentiation in this organism. Degradation of cAMP by cAMP phosphodiesterase (PDE) produces 5’AMP, the substrate of 5NT. During the time course of development, the enzyme activity of 5NT increases and becomes restricted to a narrow band of cells that form the interface between the prestalk/prespore zones. We have purified a polypeptide associated with 5NT-enzyme activity. Protein sequence of this peptide was obtained by Mass Spectrometry and Edman Degradation. PCR amplification of genomic DNA using degenerate oligonucleotides and a search of sequences of a cDNA project yielded DNA fragments with sequence corresponding to the peptide sequence of 5NT. In addition, a clone was found that corresponded to the classical “alkaline phosphatase” (AP) as described in several organisms. The sequences of the 5NT and AP cDNAs were not similar, indicating they are the products of separate genes and that both genes exist in *Dictyostelium*. Analysis of the expression of *5NT* during *Dictyostelium* development by Northern blotting determined that the gene is developmentally regulated. Southern blot analysis showed a single form of the *5NT* gene. Targeted gene disruption and knockout mutagenesis using the *5NT* sequences was attempted. Analysis of Blasticidin resistant strains by PCR amplification of genomic DNA, Southern and Northern blotting revealed five strains in which the *5NT* gene had been disrupted. All of these strains formed multi-tipped mounds at the beginning of the culmination stage and were delayed at this stage before finally completing development. Knockout mutagenesis of the *AP* gene is in progress. Both cDNA and genomic clones of the *5NT* gene have been cloned. In addition, a 1200 bp upstream fragment has been cloned and sequenced. Deletions of these fragments have been prepared in order to identify regulatory sequences and protein-binding sites.

Visualising Dd-STATa activation in the slug

Tomoaki Abe, Dirk Dorman, Kees Weijer and Jeff Williams

University of Dundee

DdSTATa is a *Dictyostelium* homologue of the metazoan STAT (Signal transducer and activator of transcription) proteins. DdSTATa translocates to the nucleus in response to extracellular cAMP and it is localised in the nuclei of most cells at the tight aggregate stage. By the slug stage nuclear enrichment becomes confined to a subset of the pstA cells located at the extreme slug tip. Because cAMP directs nuclear translocation of Dd-STATa during early development, and because of the evidence suggesting that the slug tip is a source of cAMP signalling, we determined whether cells in the rear of the slug can be induced to accumulate Dd-STATa by exposure to cAMP. Two Dd-STATa:GFP fusion constructs were made, one using the Actin 15 (semi-constitutive) promoter the other using the pspA (prespore-specific) promoter. Transformants with both constructs gave the same result. When a micro-injection

needle was used to introduce pulses of cAMP into the rear of slugs there was a rapid movement of Dd-STATa to the nuclei of cells surrounding the needle. This result suggests that cAMP signalling from the tip is responsible for the tip-localized Dd-STATa nuclear accumulation observed in uninjected slugs, it shows that prespore cells are competent to respond to cAMP by Dd-STATa activation and it suggests that cAMP signalling is in some way limiting in the rear of the slug. Co-injection of cAMP with the cAR1 specific inhibitor IPA (2',3'-O-Isopropylidene-Adenosine) strongly inhibited nuclear translocation, suggesting that the effect of cAMP is mediated by the cAR1 receptor. Dd-STATa also translocates into nuclei surrounding the lesion point when a slug is cut in two. We found that various mechanical disturbances, in addition to bissection, e.g. poking with a glass needle tip or injecting a bubble of nitrogen gas, causes the rapid nuclear localisation of DdSTATa in the area immediately surrounding the site of mechanical disruption. Injection of IPA alone did not abolish the nuclear localisation around the tip of an injection needle, suggesting that it is not cAMP that mediates the stress induced DdSTATa translocation. There appear therefore to be a least two distinct ways in which Dd-STATa can be activated at the slug stage: by binding of cAMP to cAR1 and through a pathway that seems to be activated by tissue stress and/or mechanical damage.

14:00 - 15:20 CHEMOTAXIS II

Analysis of cell motility using an under agar folate chemotaxis assay:

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We have developed an under agar chemotaxis assay for vegetative amoebae undergoing folate chemotaxis. We have used this assay to analyze the movement of mutants with alterations in cortical function. Recent evidence has shown that Myosin II is involved in the cortical integrity necessary for the production of force. Cells lacking myosin II (mhcA-) cannot enter aggregation streams composed of wild-type cells and become distorted during their interactions with wild-type cells. We have hypothesized that this behavior is due to a lack of cortical stiffness, leading to the inability of mutant cells to apply force or resist applied forces. In the under agar chemotaxis assay, cells move out of troughs and under the agar, and continue moving up the folate gradient at speeds of up to 6.0Φm/min for a period of 9 hours. Folate has been shown to have both chemotactic and chemokinetic effects on Dictyostelium cell motility.

In order to exit the troughs, the cells must locally deform the agar upward and at the same time flatten themselves. Cell height is reduced from about 12Φm to 4 Φm when under 2% agar and the cross sectional surface area increases accordingly. The greater the agar concentration, the flatter the cells until at a density of 3% agar the wild-type cells are no longer able to exit the trough. Presumably this is because they are either unable to deform the agar or flatten themselves sufficiently to squeeze underneath, or both. Therefore, the agar appears to represent a mechanical barrier to protrusion at the edge of the trough and beyond.

Mutants lacking myosin II are able to move under 0.5% agar. If the density of the agar is increased to 2%, these mutants are completely unable to penetrate this barrier, while the wild type can still efficiently move. The movement of the mutants in 0.5% is abberant in that the uropods of cells are greatly stretched and cells become fragmented over time. Cells lacking the essential light chain of myosin have no contractile activity, yet they are able to penetrate and move in aggregation streams normally and they move to the same extent as wild-type cells under the agarose. We hypothesize that the actin filament cross-linking function of myosin provides sufficient cortical integrity to allow cells to apply force to external objects. These results indicate that myosin is a major contributor to cortical stiffness and that the contractile function of myosin is not necessary for this aspect of myosin function.

This system is ideally suited to determining the localization of GFP-fusion proteins during motility and chemotaxis. The dynamics of localization of a variety of cytoskeletal proteins are currently being analyzed. The implications of this data for mechanisms of cell motility will be discussed.

Phosphorylation-dependent internalization of cAMP receptor cAR1 and identification of novel *Dictyostelium* G protein-coupled receptors

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Like other G protein-coupled receptors (GPCRs), the aggregation-stage cAMP receptor of *Dictyostelium*, cAR1, undergoes reversible ligand-induced phosphorylation of C-terminal domain serine residues. Over-expression of phosphorylation-deficient alleles results in a dominant mound-stage arrest, consistent with a role for phosphorylation in the inactivation of cAR1 at this stage. Following prolonged cAMP stimulation as occurs in mounds, we have found that phosphorylation-deficient mutant receptors remain cell-surface-accessible whereas wild-type receptor binding sites are reduced by more than 70 percent. Under these conditions, GFP-tagged wild-type cAR1 was found to be internalized. Together these findings indicate that cAR1 phosphorylation is a prerequisite for internalization and suggest that cAR1 must be cleared from mound-stage cells by this and possibly other mechanisms for development to proceed.

Towards identifying the cAR1 kinase, we have found that cAR1 phosphorylation in intact cells can be inhibited by H89, an ATP analog, at doses well above that required to inhibit PKA and sufficient to inhibit a variety of other kinases. Surprisingly, H89 was also found to inhibit cAR1 *dephosphorylation*, suggesting that a kinase dynamically regulates the phosphatase's activity. Other inhibitors are being evaluated to better define cAR1 kinase's identity.

We are also making efforts to identify novel *Dictyostelium* GPCRs which could provide new opportunities to exploit this system for the study of GPCR regulation. Our searches of the databases of the *Dictyostelium* cDNA Project in Japan and the international genome sequencing consortium have revealed more than a dozen GPCR candidates including a close-knit family which is strikingly similar to mammalian G protein-coupled neurotransmitter receptors. Further analysis of these is expected to uncover new signaling mechanisms and their biological significance.

A Novel G∀ Protein That Functions in the Adaptation/Deadaptation Pathway for cAMP Signaling

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The extracellular, pulsatile release of cAMP during early development of *Dictyostelium* directs the chemotactic migration of cells and initiates developmental gene expression. The cAMP signal is transduced by 7-transmembrane receptor (7-TMR)/G-protein pathways to transiently activate adenylyl cyclase (AC). While G∃(is implicated in AC activation, adaptation mechanisms are unknown. Phosphorylation of mammalian 7-TMRs is linked to adaptation. Although cAMP receptor 1 (cAR1) phosphorylation is contemporaneous with adaptation in *Dictyostelium*, studies show that it is not required for adaptation. We identified a novel G∀ (G∀ 9) and studied adaptive cAMP signaling in *g∀9*-null strains and strains expressing constitutively activated forms of G∀ 9 (*g∀9CA*). *g∀9*-nulls aggregate faster and form significantly smaller aggregation territories relative to WT cells, suggesting these cells

are hyper-responsive to developmental signals. This possibility is supported by the observation that *g∀9*-nulls chemotax faster than WT cells to a cAMP point source. Time-lapse video microscopy of developing cells show that *g∀9*-nulls produce optical density waves more frequently than WT cells, suggesting that they initiate cAMP wave propagation more frequently. Biochemical experiments confirmed that *g∀9*-nulls have an accelerated formation of cAMP pulses. When G∀9 is expressed in *g∀9*-nulls, cAMP pulse frequency and the developmental phenotype revert to wild-type. These results suggest the intriguing possibility that G∀9 functions in the adaptation/deadaptation pathway for cAMP signaling. *g∀9CA* strains are defective in AC activation, are developmentally delayed and form extremely large territories, suggesting that G∀9 functions to inhibit AC, and, perhaps, to adapt the system. However, AC activation and deactivation appears normal in *g∀9*-nulls. Therefore, removal of G∀9 from the pathway is not sufficient maintain AC activation, suggesting redundant adaptation mechanisms may exist. Presently, we are testing the hypothesis that cAR phosphorylation and G∀9 work synergistically in adaptation.

We also tested the hypothesis that *g∀9*-nulls are hypersensitive to secreted factors other than cAMP during early development. We identified a new, secreted aggregation promotion factor (APF) complex that potentiates chemotaxis. *g∀9*- nulls are hypersensitive to APF, responding faster and at lower cell densities, consistent with the loss of an inhibitory response. Conversely, *g∀9CA* strains are less sensitive to this factor. We purified APF to homogeneity and sequenced the proteins by mass spectrometry. Interestingly, two proteins are secreted proteases. In other organisms, signaling networks initiated by secreted proteases, like the *Drosophila* Toll receptor pathway, are crucial for development. The contribution of proteases to APF activity will be tested. Two other proteins are novel and their genes are being cloned. Our observations suggest that G∀9 is part of a pathway that controls cell movement and senses cell density. This network may provide a model in which to understand chemotaxis of neutrophils and assembly of mammalian tissues.

The internal phosphodiesterase RegA is essential for the suppression of lateral pseudopods during *Dictyostelium* chemotaxis

Deborah Wessels[a], Hui Zhang[a], Joshua Reynolds[a], Karla Daniels[a], Paul Heid[a], Sijie Lu[b], Adam Kuspa[b,c], Gad Shaulsky[c], William F. Loomis[d] and David R. Soll[a]

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During aggregation, *Dictyostelium* amoebae respond to outwardly moving, non-dissipating waves of cAMP. In the front of each wave, cells experience an increasing temporal and a positive spatial gradient while in the back of each wave the gradients are reversed so that cells experience a decreasing temporal and a negative spatial gradient. Since the waves are roughly symmetric, the duration of the two phases is equal. Behavior of *Dictyostelium* in each portion of the natural wave (front, peak, back) is distinctly different. One of the most crucial aspects of behavior in natural waves is suppression of lateral pseudopod formation in the front of the wave, the only portion of the wave during which cells move in a directed fashion towards the source of cAMP, the aggregation center.

During the natural wave, the activity of the cytoplasmic phosphodiesterase RegA is cyclically inhibited by the MAP kinase ERK2, leading to cyclic accumulation of internal cAMP and cyclic activation of the cAMP-dependent protein kinase PKA. This pathway has been implicated in the regulation of developmental genes. We now demonstrate that RegA also plays a fundamental role in the motile response of cells to natural waves of cAMP through the suppression of lateral pseudopods in the front of a natural wave.

Using a computer assisted motion analysis system, we found that *regA*$^{-}$ mutant cells had little sense of direction during aggregation. Furthermore, when labeled wild type cells were followed in a field of aggregating *regA*$^{-}$ cells, they also failed to move in an orderly direction, indicating that signaling was impaired in mutant cell cultures. However, when labeled *regA*$^{-}$ cells were followed in a field of aggregating wild type cells, they again failed to move in an orderly fashion, primarily in the deduced fronts of waves, indicating that the chemotactic response was also impaired. Since wild type cells must assess both the increasing spatial gradient and the increasing temporal gradient of cAMP in the front of a natural wave, the behavior of *regA*$^{-}$ cells was motion-analyzed first in spatial gradients in the absence of temporal waves and then in simulated temporal waves in the absence of spatial gradients. Our results demonstrate that RegA is not involved in assessing the direction of a spatial gradient of cAMP nor in distinguishing between increasing and decreasing temporal gradients of cAMP. However, RegA is essential for specifically suppressing lateral pseudopod formation during the response to an increasing temporal gradient of cAMP, a necessary component of natural chemotaxis. We discuss the possibility that RegA functions in a network that regulates myosin phosphorylation by controlling internal cAMP and PKA levels, and in support of that hypothesis demonstrate that myosin II does not localize in a normal fashion to the cortex of *regA*$^{-}$ cells in an increasing temporal gradient of cAMP.

16:00 - 17:20 METABOLISM AND ITS INTERFACE TO DEVELOPMENT

Delta 5 fatty acid desaturases from *Dictyostelium discoideum*

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Dictyostelium membrane contains unusually high concentrations of dienoic fatty acids. To understand the function of polyunsaturated fatty acids in the membrane, we analyzed delta 5 fatty acid desaturases that regulate the production of dienoic fatty acids.

We found cDNA fragments putatively encoding amino acid sequences characteristic of fatty acid desaturase in EST database of the *Dictyostelium* cDNA project. Using these sequences, we identified two functional delta 5 desaturases that were 66% identical to each other and 39-42% identical to the first found delta 5 desaturase of *Mortierella alpina*. Both of the *Dictyostelium* enzymes contained an N-terminal cytochrome b5 domain that shared 43% identity with cytochrome b5 of *Oryza satiba*. We could confirm their function as delta 5 desaturase by the gain of function mutation in the yeast *Saccharomyces ceravisiae*. Despite these similarities, those two desaturases showed different substrate specificities and expression patterns.

Using knockout and overexpression mutantions, we investigated the roles of delta 5 desaturases in regulation of the levels of unsaturated membrane lipids during acclimation to change in environmental temperature.

Superoxide generation during early *Dictyostelium* development

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Recent studies have demonstrated a role for reactive oxygen species, such as peroxide and superoxide, during various cellular processes in mammalian and plant cells, in which they specifically affect a number of signalling systems. Using the tetrazolium dye XTT, we have established that aggregating *Dictyostelium* amoebae generate significant amounts of superoxide anion. We have attempted to test the hypothesis that this superoxide has a functional role in the developmental process. The superoxide-scavenging tetrazolium dyes MTT, NBT, and XTT are all able to prevent aggregation, as is the chemically unrelated scavenger tiron. Cells overexpressing cytosolic superoxide dismutase also show an aggregation defect: when grown on plates of *Klebsiella aerogenes* they totally fail to aggregate, while on non-nutrient agar they undergo normal aggregation only when plated at high density. This is consistent with a role for intracellular superoxide in signalling events during aggregation.

Peroxysomal D-hydroxyacyl-CoA dehydrogenase deficiency causes anomalous lipid accumulation and developmental arrest in *Dictyostelium*

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Mutation of 17?-hydroxysteroid dehydrogenase 4-homolog (HSD4) that is involved in peroxisomal lipid ?-oxidation causes Zellweger (*Cerebro-Hepato-Renal*) syndrome in human. HSD4 is composed of three distinct functional domains, D-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and sterol carrier protein from the N-terminus. The *Dictyostelium* counterpart (DdHSD) of this enzyme is highly homologous to the mammalian ones, but the central domain, enoyl-CoA hydratase, is missing. Disruption of the gene (Ddhsd4) which encodes this enzyme by homologous recombination causes severe developmental defects in bacterially grown cells. Bacterially grown mutant cells aggregate by random collision to form loose mounds on non-nutrient agar, but never show further development. Such defect is complemented by the introduction of only N-terminal D-hydroxyacyl-CoA dehydrogenase domain having the peroxisome-targeting signal at its C-terminus. Chemotactic response toward cAMP is greatly impaired. Expression levels of the genes encoding cAMP receptor (cAR1) and G-protein ∀2 are suppressed in the mutant. Biochemical analyses demonstrate that the bacterially grown mutant cells accumulate several lipids that are not usually detected in bacterially grown wild type cells. These results suggest that peroxisomal lipid metabolism plays a crucial role in *Dictyostelium* development.

Signaling pathways regulating resistance to the anticancer drug cisplatin are required for normal development

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Cisplatin is a chemotherapeutic agent that is widely used to treat a variety of human malignancies. However, its efficacy is limited by the fact that some types of cancers are resistant to initial treatment and other tumors acquire resistance during the course of therapy. Despite the widespread occurrence of this problem, little is known about the underlying molecular genetic causes of this resistance, in part because the responsible genes have been difficult to identify in drug resistant tumor cell lines.

To address this gap in our knowledge, we took an unbiased insertional mutagenesis approach to isolate mutants that show a heritable increased resistance to the drug. To date we have

isolated 7 mutants and identified 6 of the genes. The results indicate that multiple signaling pathways are involved in cisplatin resistance. Moreover, the mutants are not resistant to other DNA damaging agents indicating that the resistance is specific. Further study of two of the pathways is revealing the details of the mechanisms that control resistance to the drug.
Sphingosine-1-P lyase is a key enzyme regulating the level of S-1-P in the cell, which in turn has been implicated in regulating cell proliferation and cell death pathways in other systems. We hypothesized that the increased resistance to cisplatin in S-1-P lyase null cells is the direct result of the increase in S-1-P. This idea is being tested by manipulating the level of S-1-P using both genetic and pharmacological approaches and determining the effect on cisplatin resistance. For example, we have isolated the sphingosine kinase gene and are in the process of generating null and overexpressor strains to regulate the S-1-P levels. Significantly, the S-1-P lyase mutant also has a dramatic developmental phenotype and the loss of this enzyme is manifest at all stages of development.
The *reg*A phosphodiesterase is involved in regulating cAMP levels during development and therefore affects the activity of the cAMP dependent protein kinase A. *reg*A and PKA are expressed at low levels in growing cells. We predicted that PKA mutants would also show differences in cisplatin resistance compared to the wild-type. Indeed, PKA-R null and PKA-C overexpressor strains both show an even greater level of cisplatin resistance than the *reg*A null strain.
Overall, we have uncovered signaling pathways that were previously unsuspected of modulating resistance to an important anticancer drug and demonstrated that this strategy can be used to identify new targets for chemotherapeutic agents.

09:00 - 10:20 MEMBRANE AND PROTEIN TRAFFICKING

A myosin I is involved in membrane recycling from early endosomes

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We investigate the function of unconventional myosins in Dictyostelium discoideum, an organism especially attractive for the investigation ofcytoskeleton-related processes in the endocytic pathway. The molecularmechanisms of membrane trafficking in this simple eukaryote show extensivesimilarities to mammalian cells. Nevertheless, the apparent linearity of theendosomal fluid-phase flux was thought to illustrate a major discrepancybetween these organisms. In addition, geometry-based mechanisms have beenproposed to account for the sorting of membranes and fluid-phase in theendocytic pathway, yet little is known about the involvement of theactin-myosin cytoskeleton.Our findings obtained by a combination of molecular genetics, cell surfacelabeling, biochemical fractionation and a variety of microscopy techniques,now demonstrate that (1) a major trafficking route recycles plasma membranecomponents from an endosomal compartment back to the cell surface and that(2) a myosin I is directly involved in this pathway. D. discoideum myosin IBfunctions in the recycling of plasma membrane components from endosomes backto the cell surface. Cells lacking MyoB (myoA-/B-, and myoB- cells) andwild-type cells treated with the myosin inhibitor BDM accumulated a plasmamembrane marker on intracellular endocytic vacuoles. An assay based onreversible biotinylation of plasma membrane proteins demonstrated thatrecycling of membrane components is severely impaired in myoA/B null cells.In addition, MyoB was specifically found on magnetically purified earlypinosomes. Total internal reflection fluorescence microscopy revealed thatmaturation of pinosomes in myoA-/B- cells was clearly affected, as they wereless efficient at concentrating marker molecules than wild-type cells.Moreover, the number of vacuolin-positive post-lysosomes was significantlyreduced. Using a rapid-freezing cryo-EM method, we observed an

increasednumber of small vesicles tethered to big endocytic vacuoles in myoA-/B-cells. This accumulation of vesicles suggests that the defects in membranerecycling result from a disordered morphology of the sorting compartment.In summary, we show that D. discoideum cells, like mammalian cells,efficiently recycle plasma membrane components from an early endocyticcompartment, emphasising that major transport steps are conserved betweenthese organisms. We further show that MyoB is involved in this membranerecycling step. Our findings indicate that MyoB may affect the surface tovolume characteristics of tubules and vacuoles. The present work highlightsthat actin and myosin may be implicated in the sorting of membrane andcontents, process further supplemented by the extensively studied signal-and lipid-based sorting mechanisms. We propose that the actin-myosincytoskeleton may build the mechanical support to shape the morphology ofintracellular compartments, thereby providing the foundation forgeometry-driven sorting processes.

Phagocytosis, Adhesion and the Functions of Myosin VII

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Myosin VII is a member of the superfamily of unconventional myosins that function as motor proteins on actin filaments. Myosin VII a particularly interesting member of this family because natural mutations occur in human and murine populations and lead to sensory disorders. In humans, mutations in the myo7A gene result in Ushers syndrome (the most common form of combined blindness and deafness) whilst in mice, mutations cause deafness and loss of balance.
The Dictyostelium myosin VII is encoded by the myoi locus. Disruption of the gene demonstrates a specific requirement for myosin VII during phagocytosis. The organisation of the actin cytoskeleton and other actin-based processes are normal, including pinocytosis. Here we describe the basis of the phagocytic defect in myosin VII null cells.
A detailed kinetic analysis of phagocytosis in control and myosin VII null cells suggests that the defect lies in the adhesion of particles to the cell surface prior to engulfment. Direct measurement of particle adhesion confirms this defect. However, the kinetics of internalisation appear to be relatively normal once the process is initiated.
We demonstrate also that the motility of myosin VII cells is reduced by 50%. 3D reconstruction of moving cells indicates that reduced motility is due to an inability to adhere correctly to the substratum.
These data demonstrate that myosin VII functions in the control of adhesion, both of particles during phagocytosis and of the cell itself to the substratum. Several previously characterised phagocytosis mutants also show defects in cell adhesion and motility, supporting a scenario in which cell adhesion in the absence of focal contacts is essentially an attempt at phagocytosis of an infinitely large particle.

Functional characterization of a *Dictyostelium* Rho-GTPase dissociation inhibitor

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To better understand the role of signal transduction cascades involving Rac GTPases in cytoskeleton-dependent processes, we have started the functional characterization of a *Dictyostelium* Rho-GTPase dissociation inhibitor (RhoGDI). Using protein sequences of human RhoGDIs as query, we identified several EST clones encoding a *Dictyostelium*

homologue. DdRhoGDI shares 33-37% identity and 51-59% similarity to several known RhoGDIs from animal species.
Analysis of the data released by the *Dictyostelium* genome project allowed us to establish the structure of the *rdiA* gene. Since the *rdiA* gene is interrupted by a single intron located upstream of the translation start codon, we used PCR on genomic DNA to amplify a product encompassing the full-length coding sequence of DdRhoGDI. Southern blot analyses using hybridization under low stringency conditions showed that *rdiA* exists as a single copy in the *Dictyostelium* genome. The *rdiA* gene is expressed at highest levels at early stages of development, but the protein product is present at constant levels throughout the complete developmental cycle.
To gain insight into the function of DdRhoGDI, we have generated a knock-out strain using homologous recombination with a vector carrying a blasticidin resistance cassette. Monoclonal antibodies generated against bacterially expressed DdRhoGDI recognized a single 25 kDa protein in AX2 cells and no bands in the *rdiA* minus mutants. In immunofluorescence studies a pattern of diffuse punctate staining was observed in AX2 cells that was absent in *rdiA* minus mutant. Cells deficient in DdRhoGDI are multinucleate, grow slowly in axenic medium, display pinocytosis and phagocytosis defects and minor alterations in the developmental cycle.

Evidence that the Spore Coat Protein SP85/PsB Mediates a Checkpoint for Cellulose Biosynthesis, Regulates Cellulose Packing, and Anchors Outer-Layer Proteins of the Spore Coat in *Dictyostelium*

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The *Dictyostelium* spore coat resembles a protein-cellulose-protein sandwich at the cell surface. SP85 (PsB) has been identified as an important structural component of the coat that forms a heteromeric multimer in prespore cells, and binds cellulose and SP65 and other coat proteins after secretion. SP85-null spores produce coats with reduced bouyant densities, increased porosity and rates of germination, and reduced SP65. Expression of the N- and C-terminal domains of SP85 exert dominant negative effects that mimic some phenotypes of SP85-null spores. The dominant negative activity of SP85 domains has been exploited further by expressing hybrids of the N-terminal domain with the Cys-rich C_1-domain and the terminal C_2-domain. The NC_1-fusion was found to block the differentiation of 85% of spores at a stage after coat protein secretion (PSV exocytosis), but prior to cellulose synthesis based on Calcofluor labeling and quantitation of glucose after trifluoroacetolysis. When NC_1 was expressed in dcsA-null (cellulose-null) cells, prespore cells remained amoeboid and osmotically-stable, showing that NC_1 blocked differentiation prior to cell dehydration and functioned independently of cellulose. The blockade was exerted transcellularly in interstrain chimeras. The results suggest that SP85 is involved in a cell surface checkpoint for cell dehydration and cellulose synthesis that is executed after coat protein secretion. The checkpoint was executed in SP85-null spores, suggesting that NC_1 functions by blocking normal down-regulation of inhibition by SP85.

In the absence of SP85, all cells produced cellulose based on Calcofluor labeling, but it was organized differently based on partial sensitivity to acid hydrolysis and the round shape of the spores. Expression of the NC_1- or NC_2-fusions in SP85-null cells resulted in multi-fold hyperproduction of acid-sensitive at the expense of normal cellulose. These results suggest that SP85 is required for the crystalline packing of cellulose, and for capping the level of glucose that is polymerized.

Electron microscopy revealed that SP85-null and NC_2-spores had emaciated, discontinuous outer protein layers and germinated rapidly. In contrast, the few spores that appeared when NC_1 was expressed, and spores expressing a C_1C_2-fusion, formed hypertrophic outer layer-like material that did not require endogenous SP85. Correlated with evidence that NC_1 bound cellulose and SP65, and that NC_2 bound other outer layer coat proteins, these data implicate a cross-bridging activity for SP85 in anchoring of outer layer proteins to cellulose.

Thus SP85 appears to have a central role in outside-in signaling to initiate cellulose synthesis, in the crystalline packing of cellulose into a physically useful form, and in the attachment of outer layer proteins to the cellulose core. The contribution of a single protein to these multiple functions suggests that SP85 might have an important integrative role to ensure sequential coordination of these events.

11.00 - 12.20 Ca^{2+} IN CHEMOTAXIS AND APOPTOSIS

Prolyl oligopeptidase regulates cAMP-induced IP_3 signalling during development

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In *Dictyostelium*, the loss of prolyl oligopeptidase (PO) activity has been show to produce resistance to the effect of lithium on early aggregation (Williams et al 1999). This is due to increased levels of IP_3 in the cell, allowing cells to bypass the block of inostiol monophosphatase caused by lithium. This has clinical significance in the treatment of unipolar and bipolar depression, since these patients can be treated with lithium. Interestingly, unipolar and bipolar depression have been found to be accompanied by altered levels of plamsa PO (Maes et al, 1995).

Depression is associated with lowered PO activity and may correspond to our dpoA knockout strain (LisA). In contrast mania is associated with elevated PO activity. To investigate the consequence of this elevation we have overexpressed the *Dictyostelium* PO gene (dpoA). An over-expression construct, comprising the entire open reading frame and the endogenous promoter of dpoA, gives up to a seven-fold increase in enzyme activity in growing cells. Like LisA, the over-expressing strain shows wild-type fruiting body formation, however this strain shows lithium hypersensitivity. I will describe the analysis of this over-expressing strain, and provide a current model for the effect of increased PO levels on IP_3 signalling. The implications of this work in the treatment of manic depression will also be discussed.

Williams, R.S. et al. (1999) Loss of a prolyl oligopeptidase confers resistance to lithium by elevation of inositol (1,4,5) trisphosphate. *EMBO J.* **18**, 2734–2745.

Maes, M. et al. (1995) Alterations in plasma prolyl endopeptidase activity in depression, mania, and schizophrenia: effects of antidepressants, mood stabilizers, and antipsychotic drugs. *Psychiatry Res*. **58**, 217–225.

Ca^{2+} signalling is not required for chemotaxis in *Dictyostelium*

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Dictyostelium cells can rapidly move towards a source of cyclic-AMP. This chemoattractant is detected by G-protein-linked receptors, such as cAR1, which trigger a signalling cascade including a rapid influx of Ca^{2+}. It has been proposed that chemotaxis requires Ca^{2+} signalling: for instance, introduction of Ca^{2+} chelators into cells virtually paralyses them. However, chelator experiments may deplete cells of Ca^{2+} and lower basal cytosolic Ca^{2+} to

unphysiological levels. Therefore additional tests for the role of Ca^{2+} signalling in chemotaxis are required.
We have disrupted an $InsP_3$ (inositol 1,4,5-trisphosphate) receptor-like gene, *iplA*, to produce null cells in which Ca^{2+} entry in response to chemoattractants is abolished, as is the normal increase in cytosolic Ca^{2+} that follows chemotactic stimulation. However, imaging experiments show that the resting cytosolic Ca^{2+} concentration in the mutant cells is similar to wild-type cells. We find that the production of cyclic-GMP, cyclic-AMP and the activation of the MAP kinase, DdERK2, triggered from the cyclic-AMP receptor, are little perturbed in the mutant. Mobilization of actin into the cytoskeleton, in response to cyclic-AMP, also follows similar kinetics to wild-type. Mutant cells chemotax efficiently towards cyclic-AMP or folic acid and their sensitivity to cyclic-AMP is similar to wild-type. Finally, they move at similar speeds to wild-type cells in the presence or absence of chemoattractant. We therefore conclude that Ca^{2+} signalling is not necessary for chemotaxis to cyclic-AMP.

Evidence for the presence of the ALG-2/Alix pathway in *Dictyostelium*

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ALG-2 is a 22kDa Ca^{2+}-binding protein, which has been demonstrated to be linked to apoptosis in mammalian cells. Lately, this protein has been shown to interact in a Ca^{2+}-dependent manner with Alix, an SH3-binding domain containing protein. In *Dictyostelium*, we identified two isoforms of ALG-2, Dd-ALG-2a and Dd-ALG-2b and a homologue of Alix, Dd-Alix, which share a high degree of similarity with their mammalian homologues. These three proteins are expressed in vegetative cells as well as during development. Analysis of knock-out strains and overexpressors is underway. In *Dictyostelium*, alteration of intracellular Ca^{2+} has been shown to affect prestalk cell differentiation. However, only a few Ca^{2+}-dependent proteins have been associated to the differentiation signaling machinery. It is tempting to envision a role for ALG-2 isoforms as Ca^{2+} targets that might regulate *Dictyostelium* development. The presence in the *Dictyostelium* of the ALG-2/Alix couple suggests that *Dictyostelium* shares a novel common signaling cascade with mammalian cells and analysis of its role in a simpler organism should help to better understand its function in more complex systems.

Involvement of an Apoptosis Inducing Factor homolog in *Dictyostelium discoideum* cell death

Damien Arnoult[1], Jérome Estaquier[1], Franck Sureau[2,3], Jean Pierre Tissier[4], Alain Grodet[1], Marc Dellinger[5], Axel Kahn[3], Jean-Claude Ameisen[1] and Patrice Xavier Petit3*
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Mitochondria play a pivotal role in apoptosis in multicellular organisms by releasing apoptogenic factors such as cytochrome *c* and Apoptosis Inducing Factor (AIF). The cell death of the unicellular organism *Dictyostelium discoideum* (Dd) induced by protoporphyrin IX in presence of light or actinomycin D has several apoptosis-like features. These features include the disruption of the mitochondrial transmembrane potential ()Pm), exposure of the

phosphatidyl residues at the external surface of the plasma membrane, intense vacuolization, and loss of DNA content, but no oligonucleosomal DNA fragmentation.

These events are accompanied by pronounced autophagy and release of pseudo-apoptotic corpses that are engulfed by neighboring cells.

We have cloned a putative cell death effector that is a Dd mitochondrial flavoprotein homolog of mammalian AIF. Here we show that DdAIF is translocated from the mitochondria to the cytoplasm after the onset of cell death. Cytoplasmic extracts from dying Dd cells triggered the breakdown of isolated *Dictyostelium* and mammalian nuclei in a cell free extracts system, and this process was inhibited by a polyclonal antibody specific for DdAIF. We believe that DdAIF is an evolutionary cell death inducer because its overexpression causes apoptosis in mammalian 293T cells. Our findings indicate that the cell death machinery in Dd involves mitochondria and an AIF homologue, suggesting the evolutionary conservation of a least part of the cell death machinery in the cells of both unicellular and multicellular organisms.

Dictyostelium cell death mutants

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No abstract provided.

14:00 - 15:20 THE GENOME PROJECT

The *Dictyostelium* genome project: progress and future prospects

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The first complete genome of a bacterial organism, *Haemophilus influenzae*, was published in 1995. Now, five years later, complete sequences of 30 prokaryotic genomes, 3 eukaryotic genomes and 7 eukaryotic chromosomes including chromosomes 21 and 22 from man 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 mammalian is in progress and powerful techniques for functional analysis are being further developed.

The entire *Dictyostelium* genome is being sequenced by an international consortium including the Baylor College of Medicine, the Sanger Centre, the Pasteur Institute, and the GSC Jena in collaboration with the Institute of Biochemistry in Cologne. The genome has a size of 34 Mb split into six chromosomes ranging from four to seven Mb. The chromosomes have been purified by PFGE and shotgun cloned into pUC18. This way individual chromosomes were enriched to about 50 %. Currently sequencing and assembly of chromosomes 2 and 6, the largest and smallest chromosome, are in progress.

In the German part of the project we have already produced over 160,000 sequence reads (4 fold coverage) for chromosome 2. Due to the abundance of complex repetitive elements (10 % of the genome) and the contamination of the PFGE purified chromosomes with the other chromosomes a special assembly strategy has been adopted. We have used blast homology searches and read pair information to assemble larger contigs. The now existing contigs cover all chromosome 2 specific genes. For contigs larger than 3 kb more than 1,700 non-overlapping gene models were predicted using GlimmerM, a gene prediction program originally developed for *Plasmodium falciparum*.

The sequence information and clone ressources so far available are already extremely useful for the *Dictyostelium* community. They provide the basis to investigate gene families, to search for orthologous genes, and to initiate systematic functional studies including chip

profiling and knockouts. The *Dictyostelium* genome project is funded by the DFG, the NIH, the EC and there is an application under consideration by the MRC.

Genome Project websites:
http://www.uni-koeln.de/dictyostelium/
http://genome.imb-jena.de/dictyostelium/
http://dictygenome.bcm.tmc.edu/
http://www.sanger.ac.uk/Projects/D_discoideum/

The science and logistics of functional genomics

Professor Steve Oliver,
University of Manchester , UK

Dictyostelium cDNA project: Sequencing of growth phase cDNAs

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In an effort to understand genetic networks involved in multicellular development, we are carrying out a cDNA sequencing project with the aim of identifying and characterizing genes expressed during development by analysis of expressed sequence tags (ESTs). As reported before, we have identified about 5000 non-redundant EST groups through the sequencing analysis of 19000 cDNA clones derived from slug stage.To obtain further sequence information from *Dictyostelium* genes, we have started the second phase of the cDNA sequencing project. We picked up 10000 cDNA clones prepared from growing cells. After subtracting clones of highly overlapping with the slug cDNAs, the remaining 8100 clones are subjected to sequencing. So far we found a number of novel genes expected to play an important role in regulation of cellular activities such as cell proliferation and differentiation. The current status of the sequencing project will be reported.

In addition to expanding the sequence information, expression analyses of the cloned cDNAs are ongoing using microarray technology, in collaboration with Baylor College of Medicine, and *in situ* hybridization. The expression profile data enables us to classify the clones into co-expressing gene groups. We are planning to investigate promoter elements responsible for certain expression pattern by comparing promoter sequences of co-expressing genes and mutant promoter analyses. The investigation, in conjunction with functional analyses of putative transcription factors obtained in the cDNA project and the genome project, will provide helpful information for elucidation of the network of transcriptional regulation during multicellular development.

Making a gene catalogue of *Dictyostelium discoideum*

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One apparent purpose of cDNA projects is to identify genes in an organism. To finish up the sequencing project of slug-stage libraries, SS and SL, we attempted to make a gene catalogue in *D. discoideum* (Dd), which contains identity and functional category of each gene. The overall process of catalogue making includes sequence clustering, gene identification, functional classification, and library analysis.To determine gene identity, the descriptions in

the highest blast search results against protein databases (DAD, PIR and SWISSPROT) were obtained followed by processing such as addition of “strong similarity to”, “weak similarity to” and so on.Functional classification basically followed that of *Saccharomyces cerevisiae* (Sc) available from MIPS database. Since Sc classification was too detailed to find overall views, and since novel categories of genes did exist in Dd, as those for movement and multicellular organization, we modified the functional categories to fit Dd genes and made Sc-Dd conversion table for functional categories. To avoid literal discrepancy between the two organisms, we tried to assign the orthologous genes in Dd to the same categories of Sc. This was performed by introducing “keyword” list for functional identity. The keywords were extracted from blast descriptions as essential words to indicate gene functions, and then were correlated to functional categories by searching MIPS database, followed by Sc-Dd category conversion. The keyword-function table thus obtained was used as a reference to assign each gene to functional categories in Dd.The identity and function list of non-redundant sequences in SS and SL libraries makes a gene catalogue in Dd slug. Addition of gamete specific ESTs by Urushihara’s group and Dd genes deposited to EEBJ/GenBank/EMBL database but absent in Dicty_cDB completes the current gene catalogue in Dd, which will be convenient for searches of homologous genes. Summary of gene numbers in each category roughly reflects gene expression profiles of given libraries.

Genome Scale Analysis of Gene Expression in *Dictyostelium*

Nancy Van Driessche[1,2], Richard Sucgang[3], Miroslava Ibarra[1], Jeffrey Tollett[1,4], John Halter[5], Adam Kuspa[1,2,3] and Gad Shaulsky[1]. Mariko Katoh[6], Takahiro Morio[6], Hidekazu Kuwayama[6], Tamao Saito[7], Hideko Urushihara[6], Mineko Maeda[8], Ikuo Takeuchi[9], Hiroshi Ochiai[7], Yoshimasa Tanaka[6].

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Detection of gene expression at the genome-scale is becoming a versatile tool in modern biological exploration. By providing data on the expression of most genes in the genome, DNA microarrays have been used for discovery of gene function by associating the expression patterns of novel genes with that of well-characterized genes. They have also been used as a means of phenotyping and as a tool in pharmacological explorations since mutations and drug treatment result in characteristic and informative patterns of gene expression that can be used for profile matching.

In an effort to bring the power of DNA microarray technology to bear on the investigation of gene function in *Dictyostelium*, we have collected a set of nearly 8,000 hybridization targets that represent 75% of all the genes in the genome. Most of the hybridization targets for the microarray are cDNA clones that represent genes expressed during growth, development and the sexual cycle. Targets from genes discovered by genome sequencing and by REMI mutagenesis were also included. RNA samples collected from *Dictyostelium* cultures under various conditions of growth and development were analyzed by hybridization to the array. Several protocols were developed that allow improved probe labeling, internal controls for consistent quantification and which adapt the method to the unique properties of *Dictyostelium* genes.

Refining the collection of hybridization targets, completing the collection of all the genes in the *Dictyostelium* genome, and annotating the array are ongoing projects. Most of our efforts are now directed towards the description of gene expression during the cell cycle,

developmental cycle and sexual cycle of *Dictyostelium*. These studies will serve as the basis for genome-scale discovery of gene function.

Analyses of Cell-Type Specific Genes Using DNA Microarrays.

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The way in which Dictyostelium develops makes it exceptionally favorable for global expression analyses using DNA microarrayed on glass slides (chips). Synchronous development of billions of cells together with the ease of separating the major cell types allows high resolution studies of temporal changes in gene expression as well as comparison of mRNA accumulation patterns in prespore and prestalk cells. We have prepared and interrogated chips carrying 700 sequenced Dictyostelium genes in a pilot project aimed at optimizing the technique for studies with Dictyostelium. This is a large enough gene set to generate massive amounts of data and yet small enough that each gene can be scrutinized individually. A test set of 45 developmental genes that have been previously characterized using Northern blot techniques was included to provide comparison to published studies. We have carried out multiple independent analyses of RNA collected every two hours throughout development of the wild type strains, AX4 and NC4, as well as RNA collected from isolated prespore and prestalk cells. Microarrays were prepared on either polylysine coated slides arrayed with the robot designed in the Brown lab at Stanford or on silanized slides arrayed with a Molecular Dynamics robot. RNA was copied into Cy5 labelled DNA and compared to Cy3 labelled DNA prepared from time-averaged RNA made by pooling aliquots of samples collected throughout development. Increases and decreases in relative signal were normalized to the relative signal found with vegetative RNA (T=0). Independent determinations for >90% of the individual genes varied less than 10% at a given time point. Expression of at least 175 genes was found to change by more than a factor of 2 at one stage or another.

The cell type specificity of previously established prespore and prestalk genes was confirmed by probing the microarrays with RNA of density-separated cell types collected from Percoll gradients. Moreover, we uncovered a half dozen new prespore genes and at least that many new prestalk enriched genes. Many of the prestalk enriched genes encode cytoskeletal components. The cell type specificity of several of these newly recognized genes was confirmed on Northern blots. The apparent degree of cell type enrichment seen on Northerns was compared to the values from microarray analyses to establish a conversion factor.

The cell type specific genes were clustered on the basis of their temporal patterns of expression during development. Together with other developmentally regulated genes, these clusters will be used to analyze the consequences of mutations in critical genes controlling progress through the developmental stages.

We are in the process of determining the contribution of *de novo* synthesis to accumulation of specific mRNAs by measuring their turnover following inhibition of RNA polymerase at various stages of development. Further uses of these techniques will be discussed.

Understanding developmentally regulated secretion through proteomics: a global analysis of the Dictyostelium prespore vesicle

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In multicellular organisms the spatial and temporal control of proteins secretion regulates important developmental events such as tissue specification and the interactions of cells with their immediate extracellular environment. However, the biochemical mechanisms that underlie protein secretion within the context of developing embryos are not well understood, primarily because the complexity of vertebrate embryos has precluded a detailed characterization at the molecular level.

In contrast, 80% of the cells within each developing *Dictyostelium* aggregate are involved in the coordinate fusion of the prespore vesicles (PSVs) with the plasma membrane at the terminal stages of spore differentiation and fruiting body construction. The ability to purify PSVs from large numbers (10^{10}) of synchronously developing *D. discoideum* cells has allowed their biochemical analysis, a prerequisite for understanding the mechanisms that underlie developmentally regulated protein secretion.

The PSVs are post-Golgi vesicles that appear *de novo* in prespore cells soon after aggregation and cell fate specification. During the slug stage, the PSVs accumulate the contents of the future spore coat, the spore coat proteins, and await the signal for fusion. During culmination, an unknown developmental signal triggers the PSVs to fuse synchronously with the plasma membrane and secrete their contents into the extracellular space. The spore coat proteins then assemble into a highly ordered trilamellar spore coat, comprised of inner and outer layers of cross linked spore coat proteins that flank a middle cellulose layer

To understand the regulation of protein secretion from the PSVs we need to answer the following questions: 1) What is the origin of the PSVs during development? 2) What is the biochemistry underlying the developmental signal that allows the PSVs to fuse with the plasma membrane? 3) What are the molecular motors that regulate the physical transport of the PSVs from the cell interior to the plasma membrane? 4) What enzymes contained in the PSVs are involved in spore coat assembly? To answer these questions it is imperative to identify the proteins that constitute the PSVs. The purification of the PSVs from *Dictyostelium* prespore cells[1] has allowed us to identify their regulatory as well as structural protein components using proteomic analysis[2]. To our knowledge, this is the first comprehensive identification of the proteins of a transport vesicle.

PSV proteins were separated by two-dimensional polyacrylamide gel electrophoresis, and the proteins were subjected to peptide mass fingerprinting and protein identification using protein and EST databases. This global protein discovery approach has given us a snapshot of the proteins in the PSVs, and allowed us to make educated inferences about the roles of these proteins within the context of regulated secretion. The 80-100 proteins within the PSVs fall into several functional categories which begin to reveal a global picture of the regulation of PSV biogenesis, developmental maturation, fusion and spore coat assembly. This information should be applicable to a more general understanding of developmentally regulated protein secretion.

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09:00 - 10:20 EVOLUTION

Evolutionary Origin of *Dictyostelium* and Its Relationship to Other Eukaryotes

Sandra L. Baldauf and Ingrid Wenk-Siefert[1,2]
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Four groups of organisms have been variously classified as "slime molds": the **dictyostelids** (cellular slime molds, e.g. *Dictyostelium discoideum*), **myxogastrids** (acellular or plasmodial slime molds, e.g. *Physarum polycephalum*), **acrasids** (e.g. *Acrasis rosea)* and **protostelids** (1). Morphology-based classification traditionally divides these taxa into two distinct groups - the Acrasidae (**dictyostelids plus acrasids**), and the Myxomycetes (**protostelids plus myxogastrids**). However, these classifications have been long considered problematic, particularly the Acrasidae (1).

The acrasids resemble the dictyostelids in having uninucleate amoebae, which aggregate to form a multicellular fruiting body. However, unlike dictyostelids, acrasid amoebae have lobose pseudopodia, do not respond to cAMP signaling, do not aggregate by streaming, and form fruiting bodies lacking both a cellulosic stalk and terminally differentiated cells. Meanwhile, some protostelids strongly resemble myxogastrids in being amoeboflagellates and forming small plasmodia, but others more strongly resemble dictyostelids in having a purely amoeboid trophic stage. The dictyostelids, myxogastrids and protostelids also share several strong characteristics with each other, such as amoebae with filose pseudopodia and fruiting bodies with sterile, cellulosic stalks (1).

Very little sequence data exists for any of these taxa, other than *Dictyostelium*. Therefore molecular phylogenetic data have consisted largely of small ribosomal subunit RNA trees. These generally show the myxogastrids and dictyostelids arising separately and relatively early in eukaryote evolution (2). However these data are now known to be seriously flawed (3). We have sequenced elongation factor-1∀ and tubulins from myxogastrid, protostelid and acrasid slime molds. Analyses of these data strongly suggest a number of reclassifications as follows.

1) The **acrasids** are unrelated to the other slime molds, but are instead close relatives of the vahlkampid amoebae (4), which in turn are most closely related to the Euglenozoa (5). 2) The rest of the slime molds, i.e. the protostelids, dictyostelids and myxogastrids, form **a single exclusive grouping, the Mycetozoa**. Thus these taxa are more closely related to each other than they are to any other eukaryotes (6). 3) The **protostelids are probably not monophyletic**, rather some are closer relatives of dictyostelids than myxogastrids, while others may represent the earliest branches in the Mycetozoa. 4) This holophyletic Mycetozoa is the **closest living relative of the animal-fungal clade** that has been characterized to date (5).

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DNA double strand break repair enzymes and gene evolution

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During the purification of a Dictyostelium transcription factor we fortuitously isolated two proteins that bind non-specifically to free DNA ends. These two proteins were partially sequenced by mass spectrommetry and were shown to be the Dictyostelium orthologs of Ku70 and Ku80. These two proteins form a dimer that binds to free DNA ends in a non-sequence

specific manner. In vertebrates double strand breaks are repaired by the combined action of the Non-Homologous End-Joining (NHEJ) proteins: XRCC4, DNA ligase IV, Ku70, Ku80 and DNA-PK. DNA-PK, a protein kinase most closely related to the (PI) 3-kinase family, phosphorylates protein substrates when its regulator, the Ku70-Ku80 dimer, binds to DNA. The C terminus of Ku80 in vertebrates contains a short (12aa), conserved region that forms the binding site for DNA-PK. A conserved terminus for Ku80 and a DNA-PK gene have thus far only been observed in vertebrates; neither Drosophila nor the nematode Ku80 has a conserved C terminal segment and our analysis of their genome sequences failed to reveal DNA-PK. NHEJ proteins are also important for V(D)J recombination in the immune system and this has been used to rationalise their apparent absence from non-vertebrates. When we sequenced the Dictyostelium Ku80 we found that it contains a perfect match, over the last 12 aa, to the C terminus of human Ku80. This predicted the presence of DNA-PK and, by searching the combined genomic and cDNA databases, we found a DNA-PK ortholog. Remarkably, therefore, Dictyostelium possesses a gene that is present in man but absent in flies and worms. This raises the obvious question of its function in Dictyostelium and suggests that Dictyostelium can be used to investigate this important process.

The Behavior of Cheater Mutants in Chimeras with Wild-type

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We have isolated a mutant called cheater A (*chtA*), which in chimeras with wild-type, preferentially forms spores. Reconstruction experiments confirm that *chtA* cells increase in the population. Without a wild-type partner, the mutant is defective in culmination and has an altered ratio of prespore and prestalk cells. The cheater mutants aggregate at the same rate as the wild-type, and in tipped aggregates some can be found in the nascent tips. Later the cells sort to the prespore zone. In chimeras with wild-type that express ∃-galactosidase from a prespore promoter, the wild-type cells initially induce the prespore gene in the rear of slugs, but then these cells migrate to the prestalk zone and finally to the upper cup of the spore mass. Cheater cells prefer the rear of the chimeric slug, but the greater the number, the more they are found in the anterior regions of the prespore zone of the slug. The presence of wild-type reduces the number of mutant cells that express a prestalk marker and thus when wild-type is present, mutant differentiation into prespore and spore cells occurs more frequently.

As reported last year by our laboratory and also by Margaret Nelson, Jeff Williams and Rick Firtel, the gene codes for an F-box protein that brings phosphorylated target proteins into contact with the ubiquitination machinery. It thus promotes highly specific degradation of a target or targets. We postulate that this destruction is essential for culmination. We are now attempting to determine the nature of these ubiquitination targets. In one effort, we have isolated suppressors. In this case the *chtA* mutant, which normally cannot form spores, is mutated again so that rudimentary fruiting bodies and spores are recovered. One of the suppressing genes is *dhkA*, a receptor histidine kinase, and another apparently codes for a protein that regulates autophagy.

The *chtA* mutant would probably not survive in the wild because of its failure to form spores when developed by itself. However it is important to realize that in cases in which the cells are not all genetically uniform, there is a severe competition for access to the spore population. We suggest that subverting endogenous cell signaling is one mechanism by which cells can become disproportionately represented in the spore population. Recently, the ability of one wild-type strain to contribute disproportionately to spore rather than stalk populations has been observed in wild populations by Strassmann, Queller and their colleagues, as described at this conference.

Chimeras and cheater clones in *Dictyostelium discoideum*

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Although *Dictyostelium discoideum* is widely used as a model organism for multicellular development, its multicellular stage forms by aggregation rather than from descendants of a single cell. In this sense it is more like a social group than a single organism, and it seems potentially vulnerable to exploitative strains that preferentially become reproductive spores instead of non-reproductive, altruistic stalk cells. We used microsatellite genotyping to show that this vulnerability is not just theoretical. Genetically diverse clones collected from the field readily form chimeric multicellular fruiting structures. These chimeric mixtures are often not fair, in the sense that one clone contributes less than its proportional share to the cells destined to become the dead stalk. Clones that do not contribute their share to the stalk can be considered to be cheaters. If such chimeras are frequent in nature, molecular inter-cellular signalling mechanisms may serve the selfish interests of particular clones and may therefore be quite different from signals in cooperative development of metazoans that develop from a single cell.

11.00 - 12.20 CHEMOTAXIS III

Diffusion of water, proteins and second messengers in *Dictyostelium* cells

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The main constituent of cells is water, which is the medium for the diffusion of proteins and small molecules such as cAMP, cGMP and Ca^{2+}. Despite the importance of water in living cells, not much is known about its physical properties. We report on nonlinear coherent anti-Stokes Raman scattering (CARS) to specifically probe the OH vibration in H_2O. The microscope system is equipped with a high repetition rate femtosecond laser to excite and record OH stretch vibration. By changing the medium from buffered H_2O to D_2O, this microscope enables real-time mapping of H_2O concentration gradients in single living cells at high spatial resolution. The results indicate that the *Dictyostelium* plasma membrane is relatively resistant to water permeability suggesting the absence of water channels. Indeed, water permeability of the plasma membrane is increased several-fold in transformants expressing an *Arabidopsis* water channel (cells provided by W.F. Loomis). Water diffusion within the cell is relatively high except for a one-micron layer immediately below the plasma membrane, which shows very slow water diffusion.

Protein diffusion was measured in living cells using transformants expressing soluble GFP. The GFP was photobleaching in a small volume element of about 0.5-micrometer in diameter. Using recovery of the fluorescent signal in that volume element, diffusion of GFP was determined. The results suggest that intracellular GFP diffusion is about 2-fold slower than in buffer, and is not very different in the cytosol or the nucleus.

The role of diffusion of second messengers for chemotaxis was evaluated in models calculating gradients of soluble and membrane-bound second messengers. For details see abstract and poster: Modelling of signal localisation in *Dictyostelium* cells: membrane verses cytosol by Marten Postma and Peter J.M. van Haastert.

Visualising cell-cell signalling during *Dictyostelium* development

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Starving *Dictyostelium* cells communicate by propagating waves of cAMP. CRAC contains a PH (Pleckstrin homology)-domain and is essential for the activation of the adenylate cyclase ACA. Upon cAMP receptor stimulation CRAC is transiently recruited to the plasma membrane. We used cells expressing a CRAC-GFP fusion protein to investigate the cAMP relay during different stages of the life cycle. As cAMP waves propagate along aggregation streams CRAC-GFP translocates to the leading edge of the cells that have received the cAMP signal. We observe a close correlation between CRAC-GFP translocation and dark field wave propagation in the aggregation and mound stages. We can also visualise the range and dynamics of cAMP signal propagation from cell to cell in these stages.

We also studied cells expressing the GFP-tagged PH-domain of the mammalian GRP1 protein, which specifically recognises PtdIns(3,4,5)P_3 (PIP3). During early aggregation the membrane localisation of the PH domain changes in response to cAMP waves, while it more permanently associated with the plasma membrane in cells in streams and mounds. There is still a weak modulation of GRP1 binding in response to cAMP waves and most cells show a clear graded distribution of membrane association with maximal binding at the leading edge of the cell. We will also describe the signalling in slugs using strains expressing the CRAC and GRP1PH domains under the control of celltype specific promoters.

The aggregation process is mediated by RasC.

Chinten James Lim[1], George B. Spiegelman[1,2] and Gerald Weeks[1,2]
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The RasC protein is one of seven known members of the *Dictyostelium* Ras subfamily. It is expressed throughout growth and development with peak expression occurring during aggregation and early slug formation. Whole mount *in situ* staining for ∃-galactosidase activity of transformants expressing LacZ from the *rasC* promoter revealed enrichment of *rasC* expression in the prestalk cell population with lower but detectable levels of expression in the prespore cell population from late aggregation to early culmination. During the later stages of culmination, *rasC* expression was enriched in the prespore cell population as evidenced from intense staining observed in the ascending sorogen. We generated a *rasC* null strain by homologous recombination and showed that the null strain was deficient in aggregation. The aggregation defect was circumvented by supplementing starved null cells with periodic exogenous pulses of cAMP; the resulting aggregates proceeded to form fruiting body structures that resembled those of the wildtype parental strain and produced detergent resistant spores, suggesting a defect in cAMP relay. This was confirmed by the finding that the synthesis of cAMP in response to 2'-deoxy-cAMP stimulation of null cells was greatly reduced relative to wildtype levels. In addition, the null cells exhibited reduced overall random motility and reduced motility in folate and cAMP chemoattractant gradients. In response to cAMP stimulation, null cells exhibited a slightly reduced level of Erk2 phosphorylation when compared to wildtype cells. This suggests the action of RasC is not directly upstream of Erk2 as in the classical Ras-MAPK activation cascade, but may be peripherally involved in the regulation of Erk2 activation.A number of potential components of the Ras signaling pathway had been previously identified as being involved in aggregation: the Ras-GEFs AleA and GefB, the ras interacting protein Rip3 and the MAP kinase Erk2. The identification of the Ras protein involved has been elusive; null disruptions of the Ras homologs, RasG, RasD and RasS did not produce aggregation defects. The results presented

here suggest that RasC is the Ras protein involved in mediating the signaling processes required for aggregation.

Ras Pathways - Multiple Routes to Cell Movement?

Robert Insall, Andrew Wilkins, Jonathan Chubb, Meenal Khosla*, Derek Fraser†, Paul Fisher† & Gerald Weeks*

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Dictyostelium has an unexpectedly large number of genes connected with Ras signalling. There are at least ten Ras-family genes, compared with five in *Drosophila*, which is more complex and has a larger genome. Similarly, there is a large family of RasGEFs (the proteins which activate Ras) comprising at least ten members.We are trying to discover why so many Ras pathways appear to be needed. Knockouts of individual *ras* genes have thus far always yielded phenotypes, which suggests that they each have distinct functions. RasGEF knockouts, however, mostly do not have clear phenotypes. We would also like to know whether Ras pathways are organized linearly, with each Ras being controlled by a single GEF, or in promiscuous networks. Recent data suggests that RasGEF and Ras disruptants have different phenotypes, which argues for networks.Nearly all observed phenotypes of Ras pathway mutants affect the cytoskeleton and cell motility. It remains to be seen whether Ras pathways are particularly important in movement processes, or if these phenotypes are just particularly visible in *Dictyostelium*.

9.00-10:20 SIGNALLING I

One more choice for *Dictyostelium discoideum* cells: a "plant-like" programmed cell death or a "mammalian-like" apoptosis

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The eukaryotic microorganism *Dictyostelium discoideum (Dd)* is at the crossroads of a primitive "animal-like" unicellularity during growth and a "plant-like" multicellularity at the end of the starvation-induced development. The multicellular-specific programmed cell death of *Dictyostelium* was studied by using the HMX44 mutant, characterized by its unicellular amoebae unable to complete normal development but with a differentiation into 100% dead stalk cells inducible *in vitro* [1].

We have shown that, in the absence of aggregation and differentiation, the *Dd* (Ax-2) axenic strain can undergo another form of cell death that closely resembles apoptosis of most mammalian cells. This death has been studied in two different experimental conditions that are inhibiting *D. discoideum* multicellular development. The first condition was obtained by keeping *Dd* stationary cells about one week in their HL5 growth medium, thus leading to death of *D. discoideum* cells after axenic growth in suspension. In the second experimental condition, *Dd* unicellular death was induced by starving the cells in a “conditioned” cell-free medium inhibiting normal aggregation of the cells in suspension. This "death-inducing"

medium was prepared by previous starvation of a first ($4x10^7$ cells/ml) *Dd* cell population that was agitated in suspension during 22 hours in 17mM potassium phosphate buffer (pH 6.8) and then made cell-free by centrifugation 5min at 700g. In both cases of these *Dd* unicellular-specific cell deaths, light microscopy and scanning- and transmission electron microscopy revealed morphological features known as hallmarks of apoptosis for higher eukaryotic cells. Although "ladder" DNA fragmentation was lacking for *Dd* unicellular death, apoptosis was further corroborated by flow cytometry measurements [2].

Dictyostelium morphogenesis in the absence of two major extracellular matrix proteins

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We have generated null mutants that lack two major and related protein components of the slime sheath and stalk tube, encoded by the ecmA and ecmB genes. Previous analysis of mutants singly lacking EcmA or EcmB proteins revealed distinct morphological abnormalities. While the ecmB nulls do consistently form upright fruiting bodies, albeit with stalks of an unusual slender and non-tapering shape, the culminating ecmAnull structures are more severely perturbed and are prone to collapse. Some of the ultrastructural changes seen are very similar, however. For both mutants, there are changes in the appearance of cells within the papilla, and the constriction of the stalk tube just below the funnel-shaped stalk tube entrance is missing. Consistent with this, the ring of actin-containing cell-cell junctions, normally located in the constricted region, is significantly reduced in extent in both mutants.

We have now performed a similar analysis of mutants lacking both EcmA and EcmB. This has shown that in addition to the probable loss of the actin ring, there is an apparent misplacement of many of upper cup cells to below the spore mass. The patterning of several cell-type specific lacZ markers has been examined. The most obvious change is seen for ST(stalk tube)-lacZ, which is now expressed ectopically in scattered cells within the papilla, and there is a tendency for more than one stalk to be formed within a single structure. Surprisingly, the upper cup staining seen using ecmB-lacZ seems unaffected.

Another intriguing feature of the ecmA/B double null is the loss of size regulation. As cell densities are increased, the developing structures increase in size relative to wild-type structures. At very high densities, morphogenesis is severely affected. Time-lapse video microscopy has been used to study the formation of the ecmA/B double null structures. Lastly, in order to investigate the possibility that components of the extracellular matrix may influence the properties of adjacent cells, suggested by ultrastructural changes in the ecmA and ecmB null mutants, the interaction between cells and slime trails derived from wild-type and mutant slugs has been examined in vitro.

Excitability and Synchronization in *Dictyostelium discoideum* and the Possible Relationship with the Equivalent Process in Neural Networks in Other Organisms

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During aggregation of *Dictyostelium* amoebae, they produce periodic bursts of cAMP, but this oscillatory production ceases when aggregation has been completed. I have shown that the simple characteristics of receptor kinetics could lead to the synchronization of cAMP production, and that this synchronization plays an essential role in the observed changes in signaling patterns. The synchronization mechanism that has been found in *Dictyostelium* has now also been theoretically generalized as a receptor-product coupling scheme for the global synchronization of limit cycle oscillators (Nagano, 2000).

Although this has yet to be confirmed experimentally, the aggregation of a certain minimum number of amoebae appears to be a prerequisite for bursts of production of cAMP to commence, and to sustain when the leakage rate of cAMP from the mass of amoebae into the extracellular medium is low. If this is the case, a cluster can only be a center for aggregation for the whole system when the cluster contains more amoebae than a minimum value.

When many *Dictyostelium* amoebae are put in a given plane by restricting the motion of the cells, the propagation of production of bursts of cAMP can clearly be seen along the arrays of amoebae, and the phenomenon looks very similar to the propagation of signals in neural networks. I plan to show videos of simulations of the processes described above, and to discuss relationship between signal propagation in neural networks and a possible controlling mechanism for the formation of a slug.

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A Possible Mechanism of Slug Turning during Phototaxis.

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Dictyostelium slugs show highly sensitive phototaxis. How light interferes with the movement of up to 10^5 of individual cells and turns the slug toward a light source is currently unknown. To address this question, we analyzed in detail slug and individual cell behavior during phototactic turning. Phototaxis is initiated in the anterior tip region. During directed migration towards light the anterior tip swung laterally back and forth between the light axis (serpentine movement) as if slugs were repetitively correcting their orientation. Thus, directed migration during phototaxis involves small repetitive turnings. In addition, the analysis of slug turning behavior showed that the initial turning of the tip resembles a lever arm action. A simultaneous analysis of cell movement during turning showed that some cells on the irradiated side moved across the anterior zone toward the distal side resulting in an asymmetric cell accumulation. This cell behavior was also observed during serpentine movement. To further investigate how such cell movement occurs, we irradiated a spot of light to slugs flattened under a thin agar sheet. In this agar-overlay condition, slugs became disc-shaped and cell movement was rotational. Light was irradiated to a quarter of the whole slug while rest was kept in the dark. After spot irradiation some cells showed a directed movement toward the irradiated spot. Since light irradiation induces a release of cAMP from slug cells (Miura & Siegert, 2000), we conclude that spot irradiation induced a localized release of cAMP which then triggered the directed movement of cells toward the irradiated spot. From these results, we propose a mechanism of slug turning: laterally irradiated light is focused at the distal side of the slug by a lens effect and locally induces cyclic AMP release. Some prestalk cells react to this cAMP burst and accumulate at the side away from the light source, thus increasing the volume on that side and decreasing the volume on the other side. The asymmetric depletion and accumulation of cells causes the observed lever arm movement of the tip and the subsequent bending towards the light source.

K. Miura and F. Siegert. (2000) Light affects cAMP signaling and cell movement activity in *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci.* 97:2111-16.

11.00-12.00 SIGNALLING II

PKA from *Dictyostelium* as a sensor for cAMP concentration *in vitro* and *in vivo*

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In *Dictyostelium*, PKA is a heterodimer comprised of one regulatory (R) and one catalytic (C) subunit. The R-subunit resembles closely the mammalian RII type and can interact with mammalian C-subunits. However, unlike its mammalian counterpart, the *Dictyostelium* R-subunit does not associate as a dimer. This facilitates its use in both cAMP binding and R-C interaction studies.

We fused the *Dictyostelium discoideum* R-subunit to a gene encoding a green fluorescent protein (GFP) and expressed it in *E. coli*. Random insertion of either S65T or w7 mutant GFP within the *Dictyostelium* R-subunit resulted in the isolation of over 120 clones, a number of which encoded in frame fusion proteins, whereas others displayed frameshifts following the GFP encoding region (Biondi et al., 1998).

In order to obtain fluorescence resonance energy transfer (FRET), we chose one clone expressing a truncated R-subunit, containing a single cAMP binding site (site A), fused to a potential donor GFP. Incubation in the presence of fluorescently labelled cGMP allowed fluorescence energy transfer *in vitro*. FRET change, in the presence of unlabelled cAMP, was used to determine the true K_D for cAMP which is about 20 nM. Such competition experiments are now being used for determination of cAMP concentration in biological fluids or cell extracts.

In an attempt to measure cAMP concentration *in vivo*, two GFPs with donor and acceptor properties were inserted within a single R-subunit. Sites were selected in which both donor and acceptor GFPs were in close proximity and on the same face of the R subunit. FRET occured as determined by limited protease digestion of the double R-GFP fusion. Additionally, FRET changed when the double R-GFP fusion bound to the C-subunit. These results indicate that the R-subunit changes its conformation upon binding to the C subunit.

An alternative methodology to distinguish the R-C complex *in vivo* involved labelling both R- and C-subunits with GFPs. GFP addition at either the C- or the N-terminus of the C-subunit resulted in fully regulated catalytic activity. Insertion within the protein, however, abolished activity. Preliminary results suggest that one R- and C-GFP pair displays FRET and that this FRET is abrogated in the presence of cAMP. Additionally, C-GFP constructs are being employed to determine the localisation of the catalytic subunit within *Dictyostelium*.

A FRET based cAMP reporter system will allow monitoring of intracellular cAMP levels not only in *Dictyostelium*, but also in any eucaryotic cell.

Biondi, R. M., Baehler, P. J., Reymond, C. D., and Véron, M. (1998). Random insertion of GFP into the cAMP dependent protein kinase regulatory subunit from *Dictyostelium discoideum*. Nucl. Acids Res. *26*, 4946-4952.

G Protein and Lipid Signaling in Chemotaxis

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Dictyostelium is becoming recognized as the premier genetic system to study the mechanism of chemotaxis. Previous studies have shown that cAR1, the chemoattractant receptor, is uniformly localized on the cell membrane, while G∃ is slightly polarized. The distribution of these proteins is unaltered during cAMP stimulation. We now report that G∀ is also uniformly distributed before and after cAMP stimulation. Preliminary evidence suggests that FRET can be used to monitor G∀2/G∃ interactions in living cells. In contrast, PH(CRAC) translocates to the leading edges of differentiated cells in response to cAMP stimulation, and similar behavior has been observed for PH(PKB) and for PH(Atk) in neutrophils. Here we examine the binding preferences of a number of PH domains, including those of CRAC, Btk (Bruton tyrosine kinase), Grp1(general receptor for phosphoinositol 1), and additional PH domains found in the database. In vegetative cells, PH(CRAC) is largely localized in the cytosol and strongly labels macropinosomes. In contrast, although PH (Btk) and PH(Grp1) localize in the cytosol, they do not label macropinosomes strongly. In differentiated cells, PH(CRAC) shows robust translocation from cytosol to the cell membrane in response to cAMP. However, neither PH(Btk) nor PH(Grp1) shows a strong response to cAMP. In dot blot assays, PH(CRAC) shows a stronger preference for PI(3,4)P_2 than for PI(3,4,5)P_3; whereas the binding specificity of lipids of PH(Btk) and PH(Grp1) is stronger for PI(3,4,5)P_3 than for PI(3,4)P_2. We conclude that chemotaxis is accomplished through a cascade of signal events and that the lipid production may mediate this important process. Also the lipids that are at the leading edges of differentiated cells and the lipids that are on macropinosomes are mainly PI(3,4)P_2 rather than PI(3,4,5)P_3.

Role of protein carboxyl methylation in signal transduction

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The reversible carboxyl methylation of signal transduction proteins at glutamate side chains plays an important role in the modulation of chemotaxis responses in virtually all motile prokaryotes. Eukaryotic signal transduction proteins such as the heterotrimeric G-proteins and Ras-related GTPases are subject to an analogous carboxyl methylation modification at the ∀-carboxyl group of a C-terminal prenylcysteine residue. Here we use *Dictyostelium discoideum* as a model system to study the role of the prenylcysteine carboxyl methyltransferase (PCMT) in signaling processes.

A preliminary characterization of protein carboxyl methylation in *Dictyostelium* indicated a predominant methylated species of approximately 25 kD that most likely corresponds to a Ras related GTPase. Methylation of this protein increased dramatically in cell-free extracts in response to addition of GTP?S. Transient increases were observed in intact cells in response to a pulse of 1.0 ΦM cAMP. There appears to be only one PCMT homologue in the *Dictyostelium* genome. Northern analysis indicated a single message expressed at the highest level during early development. The phenotype of a deletion mutant indicates a cell-density dependent defect in aggregation and differentiation. Our results suggest that prenylcysteine carboxyl methylation of Ras-related GTPases may play an important role in signal transduction in *Dictyostelium*.

POSTER ABSTRACTS

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Progress toward DNA sequence assembly of chromosome 6.

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In collaboration with the Dictyostelium Genome Sequencing Consortium.

We have accumulated sufficient DNA sequence data to begin the assembly of chromosome 6. In collaboration with the Sanger Center, we have generated shotgun reads from genomic libraries enriched for chromosome 6 clones (chromosomal material provided by E. Cox, Princeton) and from sublibraries produced from yeast artificial chromosomes (YACs) containing 150-kb to 300-kb segments of chromosome 6. We are basing our assembly strategy on the known positions of the YAC clones and on the chromosome 6 HAPPY map recently completed by Paul Dear's group (MRC). Although we and others in the consortium have attempted assemblies of the entire data set, we have found that it is most useful to first sort the individual sequence reads into YAC-specific bins and perform smaller assemblies on the culled data. This results in high-quality assemblies (contigs) of 2-12 kb in length that can be extended by repeating the sorting process with sequence data from the end of each contig. The high-density of HAPPY map markers allow us to place most contigs with a degree of accuracy sufficient to direct the specialized finishing experiments that we will have to perform to fill gaps.

To mine our data prior to completion, we have initiated a project in search of Hypothetical Genes (HG): predicted protein coding regions found in the contigs that we are constructing. We will annotate these predicted genes and make them available on the Baylor website. Current progress concerning the assembly and preliminary annotation of chromosome 6 will be described.

A genetic approach to studying the biology of the opportunistic pathogen *Pseudomonas aeruginosa*.

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Microbial pathogens have developed sophisticated strategies for infecting mammalian hosts. Some of these strategies involve the induction of cellular host responses to facilitate the growth of the pathogen such that the host becomes an active participant in the infection process. We have chosen a genetic approach to study the interactions between a pathogen and its host. We are employing *Dictyostelium discoideum* as a host for infections with the opportunistic pathogen *Pseudomonas aeruginosa*. This gram-negative bacterium produces an array of secreted virulence factors during an infection, and causes life-threatening infections for individuals with
compromised immune systems or chronic lung disease.

We have developed conditions under which *P. aeruginosa* infects *Dictyostelium* cells, which leads to rapid lysis of the host cells. We have identified two independent virulence mechanisms that are important for the infection of *Dictyostelium* cells. One mechanism involves the injection of the cytotoxin ExoU into the host cell through a type III secretion mechanism. *Pseudomonas* mutants that are incapable of injecting ExoU into host cells are also

unable to kill *Dictyostelium* amoebae. ExoU is a novel virulence factor that causes rapid lysis of epithelial cells. The mechanism by which ExoU mediates lysis is not understood.
The other mechanism that is responsible for killing *Dictyostelium* involves the expression of virulence genes in a cell-density dependent fashion. One avirulent *P. aeruginosa* mutant 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. This density or quorum-sensing mechanism is an important regulator of pathogenesis, because it guarantees that certain virulence factors are only made when *P. aeruginosa* is present at high numbers.
We plan to carry out large-scale mutagenesis screens to isolate mutant alleles that confer resistance to *P. aeruginosa.* We hope to identify host components that are targeted by virulence factors, like ExoU, or those that are regulated by the quorum-sensing mechanism. This knowledge could be used to develop strategies to deal with *P. aeruginosa,* a human pathogen that is otherwise resistant to a large number of antibiotics.

Analysis of *cbfA* gene function by *amber* translation stop codon suppression

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CMBF is a nuclear DNA-binding protein specific for the C-module DNA sequence of the retrotransposon TRE5-A. The CMBF-encoding gene, *cbfA*, could not be disrupted by homologous recombination. In this study we analysed CMBF function by stably underexpressing full-length CMBF by translation stop codon suppression. An *amber* (UAG) translation stop codon was introduced into the chromosomal copy of the *cbfA* gene, and a suppressor tRNA gene was expressed in the same cell. *Amber* stop codon suppression apparently worked at relatively low frequency. In a resulting mutant cell line, JH.A, functional CMBF protein was reduced by about 80%. The JH.A cells showed a pronounced growth defect both in axenic culture and on bacterial lawns, suggesting that the *cbfA* gene supports growth of *D. discoideum* amoebae. We propose to use translation stop codon suppression to analyse the functions of genes that cannot be destroyed by homologous recombination.

An improved gene-trap method using polyA trap vectors and 3'RACE

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REMI mutagenesis has been utilized as a powerful tool for identifying genes based on their functions. However, low yield of mutants as a result of frequent insertion of the vector plasmid into intergenic regions caused difficulty in large-scale screening of mutants which show wide spectrum of phenotypes, such as mutants defective in multicellular development. To overcome the problem, we have established the poly A trap method, an improved method for insertional mutagenesis using newly designed vectors. The poly A addition signal of the Bsr cassette was removed from these vectors so that the vector forced to use authentic polyA signals for proper expression of the Bsr gene. The vectors contain the bsr casette which lack signal sequences for poly A addition and termination of transcription. Therefore, the insertional events preferentially inserted into 3'-end of a certain gene survive using a poly A signal to properly express the Bsr gene. We examined whether the vector was actually inserted in the vicinity of poly A signal and found that 28 out of 43 events inserted in known Dicty genes have been inserted using authentic poly A signals of the genes. So far we have

screened 30,000 transformants and isolated 220 mutants which showed aberrant development. As the Bsr gene is expressed as a fusion transcript with the 3'-end sequence of the tagged gene, it is easy to amplify the sequence by 3'-RACE. The amplified sequences can be used as a signature for identification of the tagged genes by comparing to the Dictyostelium cDNA and genomic database. We have already obtained signature sequences of the 220 developmental mutants with an average length of 125 bp. In virtue of the advantages mentioned above, the poly A trap method will be a helpful tool for elucidation of genetic network in multicellular development.

Dictyostelium **- Model for imaging individual molecules on the cell surface**

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We present the first detection and analysis of single fluorescence-labeled lipids and proteins in the slime mold *Dictyostelium*. For precise characterization of the initial signaling events in the well-described *Dictyostelium* experiments at the level of individual molecules are superior. This is because signaling events start out from an initial singular molecular interaction leading to a final signaling cascade. The concepts, as applied here, have been previously applied *in vivo* at the single molecule level on fluorescence labeled receptors, lipids, and ion channels in human cells.Our experimental setup consists of an inverted microscope, with excitation between 450 and 1050 nm, and image detection on an ultrasensitive CCD-camera or avalanche photodiodes. This setup allows images of individual fluorophores and fluorescent proteins within a time-frame of a few milliseconds at a signal-to-background ratio of 30.
Individual Cy3 and Cy5 labeled lipids were detected on the cell membrane of *Dictyostelium*. Depending on the lipid type, we observe confinement of lipids due to inhomogeneities on the plasma membrane, which are interpreted with the lipid-raft model. Further, we have applied the single-molecule microscopy to the detection of 14-3-3-eYfp and CRAC-eGFP fusion proteins, which were attracted to the cell membrane of *Dictyostelium*.

Transcriptional regulation of *spiA* in the development of *Dictyostelium discoideum*

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In order to better understand the molecular mechanisms of cellular differentiation in *Dictyostelium discoideum*, we have examined the regulatory sequences of the spore-specific gene *spiA,* which begins to be expressed only at mid-culmination[1]. This region includes at least two essential *cis*-acting elements: a novel AT-rich element and a G-box half site. We have carried out experiments to look for a possible sporulation-specific *trans*-acting factor that would account for restriction of *spiA* transcription to the culmination stage. We employed electrophoretic mobility shift assays (EMSA) with a labeled 95bp fragment of the *spiA* promoter, thought to contain key *cis*-acting elements, together with nuclear extracts from various developmental stages. Two retarded complexes were observed: Complex I one was detected only with culmination stage extracts, while Complex II was formed with nuclear extracts from both the finger and culmination stages of development. The protein (or proteins) involved in Complex I formation is evidently a good candidate for the factor that we are seeking.

1.Richardson, D.L., C. B. Hong & W. F. Loomis (1991) A prespore gene, Dd31, expressed during culmination of *Dictyostelium discoideum*. *Dev. Biol.* **144**:269-280.

5'-Nucleotidase in *Dictyostelium*: Cloning, Expression in *E. coli* and Immunological Studies.

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During the time course of development, the enzyme activity of 5N-Nucleotidase (5NT) increases and becomes restricted to a narrow band of cells that form the interface between the prestalk/ prespore zones. Substrate specificity studies using purified enzyme showed a preference for 5N-AMP, and, therefore this activity was termed 5NT. The enzyme has received considerable attention in the past because of the critical role played by cAMP in cell differentiation in this organism. In our lab 5NT was purified and sequenced. By searching a cDNA databank, a cDNA fragment was found with nucleotide sequence corresponding to the peptide sequence of 5NT. Another clone was found that corresponded to the classical alkaline phosphatase (AP) in other organisms. The sequence of 5NT and AP cDNAs were not similar, indicating that they are products of separate genes and that both genes exist in *Dictyostelium*. Northern blot analysis showed that the 5NT gene is developmentally regulated. Southern blot analysis showed a single form of the 5NT gene. To test if AP and 5NT are products of two different genes western blot analysis was performed. The cDNAs for 5NT and AP were inserted downstream of the T7 promoter in expression vectors that have a stretch of six histidine residues at the C-terminal. Upon induction with isopropyl-$-D-thiogalactopyranoside (IPTG), the recombinant proteins were synthesized and accumulated in the form of inclusion bodies. The recombinant proteins were then purified on a Talon-Metal Affinity column. Characterization by sodium dodecyl sulfate - polyacrylamide gel electrophoresis showed that the proteins accumulated at level of approximately 10-15% of the total protein in *E. coli*. The initial results from these western blots indicate that 5NT and AP are two different, developmentally regulated proteins.

Dictyostelium IQGAP-like protein GAPA is localized to the cleavage furrow during cytokinesis

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Dictyostelium IQGAP-like protein GAPA was identified by a REMI mutation that causes cytokinesis defects. GAPA-null cells are highly multinucleate in both dish and suspension cultures. Observation by video microscopy revealed that GAPA-null cells are defective in the final cleavage of the cytoplasmic bridge during cytokinesis. In addition, the results from the mutant cells lacking both GAPA and DdRasGAP1/DGAP1, another IQGAP-like protein, suggest that IQGAP-like proteins also play some role in the furrow progression. In this study, subcellular localization of GAPA was determined using GFP-fusion protein. GFP-GAPA mainly associated with the plasma membrane evenly in interphase. During cytokinesis GFP-GAPA was accumulated at the cleavage furrow. This accumulation started at an early stage of cytokinesis. Even in the absence of myosin II, GFP-GAPA was accumulated at the cleavage furrow. Conversely, GAPA-null cells are able to localize myosin II at the cleavage furrow, indicating that GAPA and myosin II are localized to the furrow independently. These results suggest that IQGAP-like proteins are involved in myosin II-independent cytokinesis observed in *Dictyostelium* cells grown on a surface.

Generation and Characterisation of a Comitin-deficient Mutant in *Dictyostelium discoideum*

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The actin cytoskeleton of eukaryotic cells is important for cell architecture, endo- and exocytosis, phagocytosis and vesicle transport. This broad range of functions is supported by many actin-binding proteins. One of them is comitin, a 24 kDa actin-binding protein, which was first identified in *D. discoideum* but appears to be present in mammalian cells as well. Its 185-amino-acids polypeptide chain can be separated into a N-terminal core domain which shows homologies to domains of plant lectins and a C-terminal domain including six repeats of the GYP(P)Q-motive. The N-terminal core domain is capable of binding to F-actin and to mannose residues including glycoproteins and glycolipids on the cytoplasmic surface of membrane vesicles. Immunofluorescence studies indicate that comitin is localised on the cytosolic site of the Golgi apparatus and on vesicles distributed throughout the cell.

To investigate comitin's *in-vivo*-functions we have generated a comitin knock-out mutant by gene replacement. We observed an altered response to hyperosmotic stress. When AX2 and mutant cells were treated with 0.4 M sorbitol, a marked reduction in survival rate of the comitin-minus mutant in comparison to AX2 was observed. In addition, in immunofluorescence analysis with the anti-comitin antibodies mAb 190-23-5 and mAb 190-340-8 comitin distribution was found to be altered in response to hyperosmotic stress. Deletion of the comitin gene also impaired phagocytosis of TRITC labelled yeast cells. GFP-actin-fluorescence experiments in mutant and AX2 cells phagocytosing yeasts displayed that mutant cells were not impaired in reshaping the actin into phagocytic cups and in forming the phagosome. Impaired phagocytosis of yeast cells in the comitin knock-out mutant therefore appears to be reside mainly in initial uptake rates and not in the downstream processing of phagosomes. The mutant is unaltered in growth rates, cell size, development, pinocytosis, exocytosis of lysosomal enzymes, chemotaxis and motility as compared to wildtype AX2 cells.

DLIM1 and DLIM2: two cytoskeleton-associated novel LIM domain proteins

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**These authors contributed equally to this work*

A LIM domain is defined by a unique double Zinc-finger sequence motif found in a diverse class of proteins including transcription factors, proto-oncogene products and cytoskeletal components. Several LIM domain proteins have been observed to mediate specific protein-protein interactions via their LIM domains and, in this capacity, LIM domains are believed to influence subcellular protein localization and regulate protein function. We have identified two LIM domain containing proteins from *Dictyostelium*, DLIM1 and DLIM2. DLIM1 and DLIM2 are comprised of double- and single-copy of LIM domains, respectively. The mRNA transcript of DLIM1 is most abundant during growth and early development, whereas, transcription of DLIM2 is developmentally regulated. To understand the cellular dynamics of both proteins, we have used a green fluorescent protein (GFP)-tagged version of DLIM1 and DLIM2. Both GFP-DLIM1 and GFP-DLIM2 expressing cells exhibit a similar localization pattern for each protein with preferential localization at the cell periphery and at regions of intercellular contacts. The pattern of GFP fluorescence in both the cells also coincided with that of the actin staining. In our study of living cells, we observed that both GFP-DLIM1 and GFP-DLIM2 are strongly enriched in phagocytic cups and macropinosomes and are gradually

released within less than 1 min after formation of the phagosome or the macropinosome, respectively. In aggregation-competent cells which acquire a typical cylindrical shape, both proteins accumulated to high levels at the leading edge. Moreover, GFP-DLIM1 was also observed to play a role in exocytosis since GFP-DLIM1 was present on the membrane of the exocytic-vacuole immediately before exocytosis. Location and dynamic behaviour of both DLIM1 and DLIM2 indicate that they might play a regulatory role in the organization of the cytoskeleton. We have now generated DLIM1$^-$ and DLIM2$^-$ single mutants as well as a DLIM1$^-$/DLIM2$^-$ double mutant by homologous recombination. Consequences of the absence of these proteins at single cell level and at the multicellular stage are being investigated.

Dictyostelium Rap function on the cell growth and cytoskeleton

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Rap is highly conserved, multifunctional GTPase protein found in all eukaryotic cells. In mammalian cells Rap is activated by a variety of extracellular stimuli, suggesting that the protein regulates diverse cellular processes, including cell proliferation, morphogenesis, endocytosis/exocytosis and cell motility. *Dictyostelium discoideum* has a single *rap* gene and the encoded protein shares 76% amino acid identity with the human Rap1A protein, making *Dictyostelium* an excellent organism in which to investigate the Rap function.

Repeated failure to obtain gene disruptants, suggested that *rap* might be an essential gene in *Dictyostelium*. We therefore attempted to reduce the level of Rap by conditionally expressing *rap* antisense RNA from the folate repressible, discoidin promoter. Transformed cells were selected in the presence of folate and maintained at low cell density. Reduced Rap expression level was induced by allowing the transformants to grow in the medium without folate. Under these conditions, cell growth gradually decreased and eventually cells died. Prior to cell death, the Rap protein level had been reduced to about 10% of its original value. The *rap* antisense transformant expressing −10% of the normal level of Rap protein exhibited reduced rates of phagocytosis and fluid phase endocytosis. They were also more sensitive than wild type cells and exhibited reduced cGMP accumulation and tyrosine phosphorylation of actin in response to osmotic stress. Finally they are, on average, smaller than wild type cells, exhibit abnormal actin distribution and reduced random motility. These results indicate that *rap* is an essential gene for growth and is important for a number of cellular processes that require cytoskeletal function.

Genomic structure of the *Dictyostelium discoideum* family of Rho-related proteins

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Small GTPases of the Rho family of Ras-related proteins are molecular switches involved in the reorganization of the actin cytoskeleton in a variety of organisms. As a first step towards a functional characterization of the *Dictyostelium* Rho-related proteins, we have started a search for additional members of this family taking advantage of the ongoing genome sequencing project. We have sequenced and/or assembled more than 70 kb genomic DNA encoding 15 *rac* genes and 1 pseudogene. Comparison with EST sequences revealed that every *rac* gene is interrupted by at least 1 and up to 4 introns. We have derived a consensus sequence for the splicing sites. For a particular gene, *racC*, extensive alternative splicing was identified. Comparing genomic and EST sequences, the requirements for mRNA processing at the 3’ end have been analyzed.

We have studied the pattern of expression of novel rac genes through the developmental cycle. Whereas *racG*, *racH* and *racI* mRNAs are present at all stages, *racJ* and *racL* are strongly developmentally regulated, being expressed exclusively at late stages.
The amino acid sequence of the Rac proteins has been analyzed in the context of Rho-related proteins of other organisms. Of special interest are RacD and RacA. RacD posseses a 50 residues long C-terminal extension and lacks the prenylation motif characteristic of Rho proteins. RacA also lacks a prenylation motif and possesses a 400 residues long C-terminal extension that contains a proline-rich region, two BTB domains (supposed to be involved in homo- and heterotypic interactions) and an unknown C-terminal domain. We have also identified homologues for RacA in *Drosophila* and mammals.

Dictyostelium Cells use Two Nonclassical Pathways to Target Soluble Proteins for Secretion.

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The endogenous lectin discoidin-I and the Ca2+-dependent cell adhesion molecule DdCAD-1 are soluble cytoplasmic proteins which are synthesized in great abundance during the early phase of Dictyostelium development. Interestingly, both proteins have been found to associate with the ecto-surface of the plasma membrane. Previous work from our lab has implicated the involvement of contractile vacuoles in transporting DdCAD-1 to the cell surface. Similar to DdCAD-1, discoidin-I is targeted for the cell surface and secretion via the contractile vacuole. Discoidin-I has been found to co-localize with contractile vacuoles by immunofluorescence labeling and subcellular fractionation studies have shown that contractile vacuoles contain a substantial amount of cellular discoidin-I. Biotinylated discoidin-I can be imported into isolated contractile vacuoles in the absence of cytosolic factors and become resistant to protease digestion in the absence of detergent, suggesting that the protein is protected within the lumen of these vacuoles. Interestingly, in addition to contractile vacuoles, discoidin-I has been found to concentrate in certain membrane blebs of 0.5 to 1 mm in diameter, suggesting that it might be shed into the medium via evagination of these membrane blebs. These vesicles have been recovered from the medium and shown to contain discoidin-I but not DdCAD-1. Therefore, at least two nonclassical pathways are being utilized to target discoidin-I for secretion in Dictyostelium.

Functional analysis of the gp64 homologue of *Dictyostelium*

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The gp64 is membrane protein of Polysphondylium pallidum and seems to inhibit branching formation on the primary stalk. The gp64 gene translates to 320 amino acids (including 36 cysteine residues) and then, these protein products are digested at COOH-terminal region. The mature gp64 consists of 279 amino acids and attaches GPI anchor(1). We used Dictyostelium for further analysis of the function of this protein. We searched Dictyostelium cDNA database for gp64 homologue, and selected one clone (clone name: SLC529). We identified a full-length SLC529 cDNA . This clone has 377 amino acid residues including 40 cysteines. From the sequence, the protein product of this clone seems to be a GPI anchoring membrane protein.

And SLC529 resembles gp64 in hydrophobicity: this clone has hydrophobic regions both NH3- and COOH- terminal regions respectively. We carried out genomic Southern blot analysis and found that this SLC529 is single copy clone in genome. Then, we produced anti-sense RNA and over expression transformants of the gene, and observed the phenotypes of these mutants. Both mutants showed phenotypes similar to wild type, but anti-sense RNA mutants tended to develop rapidly. Now we are trying to make the knockout construct.

Molecular Cloning and COOH-terminal Processing of Gp64, a Putative Cell-Cell Adhesion Protein of the Cellular Slime mold Polysphondylium pallidum. R.Manabe, T,Saito, T.Kumazaki, T.Sakaitani, N.Nakata, and H.Ochiai. J. Biol. Chem. (1994) 269:528-53

Analysis of the transcriptional regulation of the membrane protein gp64 of *Polysphondylium pallidum*

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The cysteine-rich membrane protein gp64 of the cellular slime mold *Polysphondylium pallidum* inhibits branching formation on the primary stalk. The mRNA of gp64 is expressed extensively under the growth, then quickly begins to decrease during the aggregation, and reexpressed weekly at the late stage(1). As an approach to reveal its transcriptional regulation, we analyzed the gp64 promoter.

Using the inverse PCR technique, we isolated about 5 kb genomic region surrounding of the gp64 gene. A primer extension analysis identified a major transcription start site 65 bp upstream of the translation start codon. The promoter region of the gp64 gene contains sequences homologous to a TATA box at position - 47 to - 37 and to an initiator (Inr, PyPyCAPyPyPyPy) at position - 3 to + 5 from the transcription start site. The gp64 promoter-*lacZ* fusion reporter assay detected strong reporter expression during growth, and the axenic medium (A-medium) repressed its promoter expression. Partial deletion analysis of the promoter revealed two positive vegetative regulatory elements extending between - 187 to - 177 and - 110 to - 62 bp from the transcription start site(2).

DNA binding protein assay of the gp64 promoter by a DNA-electrophoretic mobility shift assay revealed the presence of the protein(s) binding to two positive vegetative regulatory elements, and both binding sites included the GATTTTTTA consensus sequence. The competition analysis suggest that the upstream element and the surrounding contain two or three protein binding sites.

(1) Molecular Cloning and the COOH-terminal Processing of Gp64, a Putative Cell-Cell Adhesion Protein of the Cellular Slime Mold *Polysphondylium pallidum.* Manabe, R., Saito, T., Sakaitani, T., Nakata, N. and Ochiai, H., J. Biol. Chem. (1994) 269: 528-535

(2) Promoter analysis of the membrane protein gp64 gene of the cellular slime mold *Polysphondylium pallidum.* Takaoka, N., Fukuzawa, M., Saito, T., Sakaitani, T. and Ochiai, H., Biochim. Biophys. Acta (1999) 1447: 226-230

Functional analysis of a newly identified cysteine-rich protein in *Dictyostelium discoideum*

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gp64 of the cellular slime mold *Polysphondylium pallidum* is a Cys-rich and GPI anchored membrane protein. A gp64 overexpressing mutant arrested at the finger-like stage of the development. This means the gp64 seems to inhibit branching formation of *P. pallidum*.

To analyze how this protein regulates the development in *P. pallidum*, we tried to search a gp64 homolog using *Dictyostelium* cDNA database, because *Dictyostelium* is superior to *Polyshondylium* as a model organism. As the result, we gained 4 clones (SSA813, SSK554, SLA769 and SLC529) and analyzed one of the clones.

SLA769 is 952 bp in length and its genome sequence does not contain any introns. This clone does not have a GPI anchor cleavage and attachment site and its C-terminal region is not so hydrophobicity. It therefore seems that SLA769 is not membrane protein unlike gp64. PSORT prediction also indicates that SLA769 is not membrane protein, but nuclear one.

It is interesting how this Cys-rich protein interacts with in nucleus and regulates development. In order to analyze the function of this clone, I made antisense and overexpression constructs, introduced into the cells by electroporation and developed them in the light or the dark conditions. An antisense mutant in dark tends to remain slug after 24 hr in comparison with that of Ax2 strain. But an overexpression mutant developed normally. Now, I am trying to isolate KO mutants.

A gene encoding prespore-cell-inducing factor in *Dictyostelium discoideum*: its cloning and characterisation by targeted gene disruption.

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Factors exist in conditioned medium of *D. discoideum* are able to induce differentiation of amoebae into prespore cells at a very low cell density (~10^2 cells/cm^2). We have purified a heat labile and non-dialysable factor with apparent molecular mass of 106 kD on SDS-PAGE, which we named P factor (psi, prespore-inducing factor). Based on partial amino acid sequences of the purified P factor, we isolated the corresponding cDNA clones. The cDNA encodes a novel protein containing both proline and zinc-rich regions. As N-terminal sequencing proved the first 19 amino acids are excised, predicted molecular mass of the matured secreted protein is 60kD. Candidates of Ax2 backboned knockout mutant strains of the P factor gene were created by targeted integration. Although these mutant strains developed normally, conditioned medium (CM) derived from these mutants showed apparently reduced prespore-cell-inducing activity in submerged culture. Rescuing the mutant strains by transforming actin15-P expression vector caused overproduction of P factor protein and the CM showed 20-fold higher prespore-cell-inducing activity than CM derived from wild-type (Ax2) when V12M2 was used as a tester strain. These results support the idea that R factor is one of important factors for prespore-cell-differentiation in *D. discoideum*.

1. Oohata. A.A., Nakagawa, M., Tasaka, M. and Fujii, S. (1997) Development, 124, 2781-2783.
2. Nakagawa, M., Oohata, A.A., Tojo, H. and Fujii, S. (1999) Biochem. J., 343, 265-271.

Positioning of prestalk cell sub-types and prespore cells in slugs of *D. discoideum* - Analysis and simulations based on balance of force -

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The slug of *Dictyostelium discoideum* consists of several cell types; prestalk and prespore are the two major cell types, and at least three sub-types, pstAB, pstA, and pstO, can be distinguished in the prestalk cell population. These cell types show a fairly constant spatial distribution during slug migration. It has been suggested from cell motility experiments using

cells from the first finger stage that the difference in the ability of chemotactic movement may be the cause of the sorting pattern of cell types (1). However, it is not clear what properties of the cells are directly involved in the positioning of cell types in slugs and whether the same differences are responsible for the maintenance of the spatial pattern during migration.
In our previous study (2), we proposed a continuous mathematical model to explain the morphogenetic movement of cell aggregates and sorting of two cell types. The model was based on the assumption that the motive force of each cell is in dynamical equilibrium with intrinsic resistance, which acts against cell movement, and with the resistance from the surrounding cells. We have now extended this model to cell aggregates containing more than two types of cells. These cell types are assumed to have different magnitudes of motive force and intrinsic resistance coefficient. Analytical studies using this model show that (i) cells are sorted out according to their motive force, (ii) at steady state, cell types differing in motive force are distributed to form intersecting ellipsoids, in which neighbouring cell types are separated from each other by spherical boundaries, and (iii) if the resistance coefficient also differs between cell types the boundary becomes elongated along the direction of slug migration. This effect of the resistance on the boundary shape indicates that, besides the motive force, the resistance against cell movement may be important in determining the spatial pattern of cell type distribution. We also carried out computer simulations using the model. Although cell movements were restricted in two-dimensional space, simulations approximately reproduced the sorting behaviour of cell types and overall movement of the cell aggregate.
1. Evidence for positional differentiation of prestalk cells and for a morphogenetic gradient in *Dictyostelium*. Early, A., Abe, T. and Williams, J. Cell 83 (1995) 91-99.
2. Theoretical model for morphogenesis and cell sorting in *Dictyostelium discoideum*. Umeda, T. and Inouye, K. Physica D 126 (1999) 189-200.

Developmental changes in the spatial expression of genes involved in myosin function in *Dictyostelium*

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We analyzed the spatial expression patterns of the genes involved in myosin function by *in situ* hybridization at the tipped aggregate and early culmination stages of *Dictyostelium.* Myosin heavy chain II (MHC-II) mRNA was enriched in the anterior prestalk region of the tipped aggregates, whereas it disappeared from there and began to appear in both upper and lower cups of the early culminants. Similarly, mRNAs for essential light chain, regulatory light chain, myosin light chain kinase A and myosin heavy chain kinase C were enriched in the prestalk region of the tipped aggregates. Surprisingly, however, expression of these genes was distinctively regulated in the early culminants. These findings suggest the existence of mechanisms responsible for the expression of particular genes.

Altered cell type proportioning in Dictyostelium lacking AMP deaminase

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One of the strains recovered in our near-saturation REMI screen for morphological genes in Dicytostelium, DG1114, was picked because it forms thick stalks and glassy sori with less than 5% the normal number of spores. Homologous recombination with the disrupted gene generated strains with the same gross phenotype. Slugs of these strains were found to have more than twice the normal proportion of prestalk cells and a compensatory reduction in prespore cells as judged by the number of stained cells in derivatives expressing the prestalk marker *ecmA::lacZ* or the prespore marker *cotB::lacZ*. Moreover, the level of *ecmA* mRNA was at least 3 fold higher in the mutants than in wild type at all stages following aggregation and the level of *cotB* mRNA was reduced. When whole slugs of strains carrying the mutation as well as a *ecmA::lacZ* construct were stained for ?-gal activity the strongly stained region extended more than half way back from the tip. However, when whole slugs carrying the mutation as well as a *ecmO::lacZ* construct to mark PST-O cells were stained for ∃-gal activity there was no increase in the number of stained cells and these were found at the characteristic collar just behind the tip. It appears that the mutation results in an increase in PST-A prestalk cells with a concomitant decrease in the number of prespore cells.

The sequence of the gene disrupted in these cells is predicted to encode a 89 kDa protein with greater than 50% identity to AMP deaminases in humans, *Arabidopsis* and yeast. This enzyme catalyzes the conversion of AMP to IMP and has been implicated in metabolic myopathy in human patients. We found that there is considerable AMP deaminase activity throughout development of Dictyostelium and that it is completely missing at all stages in the mutant cells. Therefore, we have designated the gene *amdA*.

The concentration of cell-associated AMP, IMP, cAMP, adenosine and inosine, metabolites that might be affected by loss of AMP deaminase, were determined in wild type and *amdA*$^-$ strains. Only inosine was found to be significantly affected. It accumulated during aggregation of *amdA*$^-$ cells to reach levels greater than 3 fold higher than in wild type cells and continued to increase somewhat thereafter. We also measured the amount of adenosine, inosine and AMP secreted during early development. *amdA*$^-$ mutant cells released the same amount of adenosine as wild type cells and only slightly more inosine. However, they secreted 8 times more AMP than wild type cells during the first 5 hours of development as well as during the subsequent two hours. Secretion of AMP may account for the fact that it doesn't accumulate to high levels within the mutant cells.

Since cell type proportioning is size invariant and able to regulate when disturbed, it is thought to be mediated by secreted signal(s). Therefore, we determined whether loss of AMP deaminase affects release of such signals or the response to them. We mixed wild type and *amdA*$^-$ mutant cells that carried the *ecmA::lacZ* construct with unmarked cells and let them develop to the slug stage before dissociating and counting the number of stained cells. Wild type cells developing in the presence of an equal number of *amdA*$^-$ mutant cells did not form more prestalk cells than usual nor did the presence of wild type cells affect the abnormally high proportion of prestalk cells formed by the mutant cells. Likewise, development in chimeras with wild type cells did not increase the number of spores of the mutant strain. The cell autonomous nature of the phenotype indicates that loss of AMP deaminase affects how the cells respond to cell type proportioning signals rather than signaling itself.

We have found that addition of 10 mM AMP, IMP, adenosine or inosine has no significant effect on cell type proportioning as judged by the number of stained cells in a wild type strain carrying a *ecmA::lacZ* construct. Nor did addition of adenosine deaminase affect cell type proportioning of wild type cells carrying a *cotB::lacZ* construct. These results indicate that cell type proportioning is unaffected by the intercellular concentration of AMP, IMP, adenosine or inosine. However, AMP deaminase activity within cells affects the proportion that differentiate into PST-A cells. It will be interesting to see if overexpression of *amdA* results in a decrease in PST-A cells.

Patterns of cellulose deposition in the developing stalk of *Dictyostelium discoideum*

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The synthesis of cellulose by *Dictyostelium discoideum* is a developmentally regulated process that does not occur in the vegetative amoebae. During culmination, cellulose is a primary component of the stalk, a structure that is synthesized by the population of prestalk cells in a highly regulated manner and that serves to lift the spores above the surface substrate. Stalk formation has been described as a two-step process: (1) synthesis of the stalk tube by the prestalk cells as they move up the outside of the stalk, trailing cellulose behind them as they move; and (2) differentiation of the stalk cell walls after the prestalk cells enter the apex of the stalk tube, when they stop moving, vacuolate, enlarge, and synthesize a cell wall around themselves. The forces generated by the stalk cell enlargement were hypothesized to provide both the force required to lengthen the stalk and the mechanical strength to support the entire structure. This study, using cryotechniques to preserve and process developing culminants, shows that the stalk has a more complex cellulosic architecture than was once believed, and the deposition patterns of cellulose to the components of the stalk are under strict spatial and temporal control. Careful analysis of these mid to late culminants reveals that a second layer of cellulose is deposited on the inside of the stalk tube, perpendicular to the initial layer of vertically oriented microfibrils. This inner layer, therefore, runs radially around the inside of the stalk, and begins to be generated some distance below the entrance to the stalk tube, which suggests that stalk cells inside the stalk remain motile and move in a circumferential manner around the inside of the stalk, depositing the trailing, stalk tube mode of cellulose behind them. It is only after this radial stalk tube layer is deposited that stalk cell wall formation begins. The preliminary evidence in this study suggests that the elegant architecture of the stalk appears to be the product of not only prestalk cell movements, but stalk cell movements as well, and the result is a reinforced stalk tube that is capable of directing upward the pressures produced by the enlarging stalk cells.

Implication of elongation factor-2 (EF-2) for transition of *Dictyostelium* cells from growth to differentiation

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In the cell cycle of *Dictyostelium discoideum* Ax-2 cells, a growth/differentiation check-point, PS-point (putative shift point), has been specified in the mid-late G2 phase (Maeda et al., 1989). As previously reported, 90kDa and 101kDa proteins specifically fail to be phosphorylated during differentiation from the PS-point (Akiyama and Maeda, 1992). The 90kDa protein has been identified as a HSP90 family, as presented in this meeting by Morita et al. On the other hand, the partial amino acid sequence of 101kDa protein purified by 2D-SDS-PAGE showed complete identity with that of elongation factor-2 (EF-2) reported by Toda et al. (1989). EF-2 is an essential factor required for translocation of ribosome along mRNA during translation. The activity is generally regulated by phosphorylation, and the dephosphorylated state is an active form. This indicates that protein synthesis may be augmented particularly at the initiation of differentiation from the PS-point. Although

transformants (*ef-2*) overexpressing *ef-2* grew at the same rate with parental Ax-2 cells at the vegetative growth phase, starved *ef-2*-cells exhibited more rapid differentiation than Ax-2 cells under submerged conditions in BSS (Bonner's salt solution; Bonner, 1947). On agar, *ef-2*-cells also formed aggregation-streams earlier than Ax-2 cells. We attempted antisense-mediated inactivation of *ef-2* using pDNeo2, but we never obtained G418 resistant clones, thus suggesting that EF-2 is essential for growth as well as for differentiation.

Exploring the Role of Functional Redundancy Among the cAMP Receptors in *Dictyostelium*

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The developmental cycle of *Dictyostelium discoideum* is dependent on a family of four differentially regulated cAMP receptors (cAR's). Of the four members, cAR2 has been shown to be critical for completion of development and changes in gene expression after aggregates have formed (Saxe et al., Genes & Dev. 7:262-272. 1993). While cAR1 and cAR3 have been shown to have overlapping functions (Insall et al., Mol. Biol. Cell, 5:703-11. 1994), these receptors when overexpressed cannot rescue strains lacking *carB*, the gene for cAR2. These observations may be explained by the vast differences in affinity between cAR1 and cAR3 (high affinity for cAMP) and cAR2 (low affinity for cAMP), or because cAR2 uniquely couples to signaling components in cells in which it is expressed, or both. We have used two molecular genetic approaches to understand not only the potentially unique role cAR2 plays in development, but also to determine what role affinity plays in the regulation and signal transduction properties of the entire family of cAMP receptors in *Dictyostelium*. Using site-directed mutagenesis and random chimeragenesis, a panel of receptor alleles spanning a wide range of affinities was created to test whether or not the cAMP receptors in *Dictyostelium* are functionally redundant. Our data reveal that although each allele can bind cAMP and activate the enzyme adenylyl cyclase in response to ligand binding, not every receptor can rescue various receptor null strains. We believe that the ability of certain alleles to rescue receptor null strains is affinity dependent. Alleles with relatively high affinities ($K_i < 1$ μM) can rescue cAR1/cAR3 null strains, while those of relatively low affinites ($K_i > 5$ μM) can rescue cAR2 null strains. Our data suggest that the cAMP receptors in *Dictyostelium* are functionally redundant with respect to all stages of the developmental program, and that all cAR's can functionally interact with the appropriate G proteins and other intracellular proteins responsible for completion of the developmental program.

The effects of over- and mis-expression of cAR2 and cAR3 on development.

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Dictyostelium cells sense cAMP through cAMP receptors (cAR's), of which there are four sub-types (cAR's 1-4). Various data suggest that each sub-type plays a different role in the control of development. We have set out to examine the roles cAR's 2 and 3 using strains which over-express tagged forms of these receptors from ubiquitous and cell-type specific promoters. We have found that over-expression of cAR3 gives cells a rearguard (possibly pre-stalk B) differentiation preference, resulting in altered cell type proportions. This could be caused by an inhibition of GSK3 activation. We have also shown that over-expression of cAR2 is not sufficient to direct sorting to the tip of aggregates, and does not appear to have an effect on cell type proportions.

Random mutagenesis of the G∀2 protein of *Dictyostelium*: identification of important functional amino acids.

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Dictyostelium discoideum is being used to study signal transduction mediated by the heterotrimeric G proteins which consist of three subunits,∀,∃ and (. These proteins are part of a large family conserved throughout the eukaryotes that relay signals from ligand-bound receptors to effector enzymes. The effectors produce second messengers that can induce changes in a wide variety of cellular responses such as gene expression, cytoskeletal organization, and metabolism. This project is designed to identify amino acid residues that are important to ∀ subunit function through the use of *Dictyostelium* G∀2 and the approach of Polymerase Chain Reaction (PCR)-generated random mutagenesis. This PCR mutagenesis is an efficient method of introducing random mutations throughout a target DNA sequence. We have mutagenize the 1175-nucleotide G∀2 cDNA and achieved an error range of 0-4 amino acid changes per 357 amino acids of G∀2. The total mutant frequency obtained is close to the optimum for most applications of random mutagenesis and will greatly facilitated identification of important amino acid residues in G∀2. The primary screen for functional mutations is simple. Since G∀2 is essential to cell aggregation in *Dictyostelium* and since only wild type or functional protein expressed by the vector can rescue the aggregation phenotype, a lack of phenotypic rescue of the g∀2 null cells allows us to quickly identify important functional mutations of the G∀2 protein. Roughly 30 of the 85 G∀2 aggregation-minus, protein-expressing mutants have been sequenced using G∀2 specific primers. The results of 26 clones are presented. The overall frequency of mutagenesis is similar to that estimated by sequencing G∀2 bacterial clones. Using sequence homology between the G∀2 and G∀t (transducin), each of the mutations has been located in the 3-dimensional crystal structure of Gt∀. Out of the 14 single amino acid changes found, seven occur within the "helical domain" of ∀-subunit and seven are in the "GTPase domain". Two of these lie in previously identified regions within the GTPase domain, one in the receptor binding domain and one in the $(binding domain. Interestingly, two of the point mutations D154V and S155P occur at sequential amino acids in the loop between the ∀D and ∀E helices of the helical domain. The multiply mutated clones represent at least an additional twelve amino acid residues of functional significance to G∀2.

Cell-Density Sensing Mediated by a Protein Kinase C and a Regulator of G-protein Signaling (RGS protein)

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. When *Dictyostelium discoideum* starves, it senses the local density of other starving cells by simultaneously sensing and secreting a glycoprotein called conditioned medium factor (CMF). When the density of starving cells is high, the corresponding high density of CMF allows signal transduction through the chemoattractant cAMP receptor cAR1 to occur. Binding of cAMP to cAR1 activates a heterotrimeric G protein whose alpha subunit is G∀2. We have shown previously that CMF regulates cAMP signal transduction in part by

decreasing the cAMP stimulated GTPase activity of G∀2. CMF accomplishes this regulation through the activation of a second G protein, G∀1, and phospholipase C.

To discover other proteins involved in CMF regulation of G∀2, we have examined proteins that might act downstream of phospholipase C and upstream of G∀2. Phospholipase C catalyzes the conversion of PIP_2 to IP_3 and diacylglycerol (DAG). We find that addition of a membrane permeable DAG analog mimics the effect of CMF in that it decreases the GTPase activity of G∀2, suggesting the CMF acts through the creation of DAG. DAG is a known activator of protein kinase C (PKC). Addition of CMF to cells causes an increase in PKC activity. In addition, the PKC inhibitor bisindolylmaleimide inhibits the ability of CMF to stimulate aggregation at low cell density. This argues that CMF works through the activation of PKC. Using degenerate PCR, we have identified a novel genomic DNA sequence with high homology to PKC. Finally, we have identified an activity in cell lysates that can stimulate the GTPase activity of G∀2.

A temperature-sensitive mutant defective in adenylyl cyclase activation due to single point mutation in the *pia* gene

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Dictyostelium mutant HSB1, obtained by chemical mutagenesis of AX2 cells, is defective in adenylyl cyclase (ACA) activation, fails therefore to aggregate, but is completely rescued following complementation with wild-type cells (Bozzaro et al., Dev. Biol. 1987, 123, 540-548).

Recently we found serendipitously that the mutant is temperature sensitive: HSB1 cells aggregate and form fruiting bodies at temperatures below 17oC, with an optimum at 13oC; they remain, instead, as single cells at and 18oC. The developmental stage sensitive to temperature is aggregation: if cells are allowed to aggregate at the permissive temperature, they complete development upon shifting at 23oC, whereas a temperature shift at any time before or at early aggregation inhibits further development.

Consistent with the previous finding that ACA activation is defective in the mutant, GTP(S fails to stimulate the enzyme activity in HSB1 cell lysates, but stimulation is restored upon addition of AX2 cell cytosol.

To investigate whether HSB1 is defective in cytosolic regulators of ACA, such as PIA or CRAC, the mutant was transfected with expression vectors containing the genes encoding PIA or CRAC under the control of the actin promoter. Wild type *pia,* but not *crac,* completely rescued the mutant, suggesting that HSB1 cells contain a mutated form of the *pia* gene. The HSB1 *pia* gene was thus cloned and sequenced; a single point mutation was found, resulting in the aminoacid exchange $D>G^{917}$. The mutated PIA accumulates in HSB1 cells in amounts comparable to wild type PIA in AX2 cells, suggesting that the mutation does not interfere with synthesis and stability of the protein.

The findings that HSB1 cell aggregates develop normally when shifted from 13oC to 23oC, and that a temperature shift of cells at earlier stages inhibits further development, indicate that PIA is necessary for ACA activation only up to aggregation.

Expression and role of adenylyl cyclases during late development in *Dictyostelium*

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Cyclic AMP signalling controls cell movement and developmental gene expression in *Dictyostelium discoideum*. Three adenylyl cyclases, ACA, ACG and ACB have been identified[1,2,3]. Null mutants show defects in aggregation, spore germination[4] and terminal differentiation respectively. To further elucidate their role in pattern formation, we performed two sets of experiments, using *in situ* hybridization and LacZ-promotor constructs.

First, we studied the expression pattern of the adenylyl cyclases themselves in wild-type cells. *ACA* expression is present in the tip of the slug and in the anterior like cells, whereas *ACG* is strongly expressed in spores and very weakly in prespore cells. Interestingly, in contrast to the *spiA* gene, *ACG* is also expressed in *acb*$^-$, suggesting that the regulation of its expression is different from that of spore genes.

Second, we looked at the expression pattern of prestalk and prespore genes in null mutants for *acb* and *acg*. Our results show that the patterns are similar to wild-type in these mutants.

Prespore gene expression requires micromolar concentration of cAMP. *ACA* is predominantly expressed in the tip and development of *aca*$^-$ can be rescued by overexpression of the catalytic subunit of PKA[5]. Moreover, both *acb* and *acg* null mutants are not defective in prespore gene expression. This prompted us to search for other adenylyl cyclase genes in the *Dictyostelium* gene bank. We found a putative adenylyl cyclase gene homologous to an adenylyl cyclase from rat testis (sAC)[6]. In contrast to all other mammalian adenylyl cyclases, sAC is a soluble protein and the two catalytic domains are closely related to cyanobacterial adenylyl cyclases. Knock-out and expression analysis of the *Dictyostelium* homologue are in progress and first data will be shown.

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Modelling of signal localisation in *Dictyostelium* cells: Membrane Verses Cytosol

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Chemotactic cells like Dictyostelium and Neutrophils are able to sense external concentration differences of chemoattractants and turn this signal into a localised intracellular response. Cytosolic proteins and actin networks translocate, leading to directional cell movement and cell polarisation. Chemotactic cells are extremely sensitive, directional movement is observed with concentration differences of 2.5 % over the length of the cell.

When a chemoattractant molecule binds to a receptor located at the plasma membrane a transduction cascade is triggered, leading to production of second messengers in the cell. An external concentration gradient leads therefore to a difference in receptor occupation, giving rise to a gradient of second messengers in the cell.

In order to understand the basic properties and constraints of such a signal transduction pathway, we studied a simple but general quantitative model, including production, degradation and diffusion of second messenger molecules. We come to the conclusion that cytosolic second messengers, in contrast to membrane-bound second messengers, are not able to transduce an external gradient into a strong internal gradient. We also show that in order to

understand the extreme sensitivity for concentration differences of only a few percent, a non-linear amplification system is required. A model will be presented that includes the translocation to the activated receptor of a solluble enzyme that participates in the production of the second messenger, thereby localising the production of the second messenger only at one side of the cell. The amplification model does not require adaptation mechanisms to explain several features observed in CRAC-GFP jumping experiments.

Identification and characterization of DdPDE3, a cGMP-specific phosphodiesterase

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In *Dictyostelium* cAMP and cGMP play important functions as first and second messengers in chemotaxis and developments. Two cyclic nucleotide phosphodiesterases (DdPDE 1 and 2) have been identified previously, an extracellular dual-specificity enzyme and an intracellular cAMP-specific enzyme (encoded by the psdA and regA gene, respectively). Biochemical data suggest the presence of at least one cGMP-specific PDE that is activated by cGMP. Using bioinformatics we identified a partial sequence in the *Dictyostelium* EST database that shows a high degree of amino acid sequence identity with mammalian PDE catalytic domains (DdPDE3). The deduced amino acid sequence of a full-length DdPDE3 cDNA isolated in this study predicts a 60 kDa protein with a 300 amino acid C-terminal PDE catalytic domain, which is preceded by about 200 amino acids that are rich in asparagine and glutamine residues. Expression of the DdPDE3 catalytic domain in *E. coli* shows that the enzyme has Michaelis-Menten kinetics and is far more active towards cGMP (K_m = 0.25 ΦM) than toward cAMP (K_m = 145 ΦM); cGMP does not stimulate enzyme activity. The enzyme requires divalent cations for activity; Mn^{2+} is preferred to Mg^{2+} , whereas Ca^{2+} yields no activity. DdPDE3 is inhibited by 3-isobutyl-1-methyl-xanthine (IBMX) with an IC_{50} of about 50 ΦM. Overexpression of DdPDE3 in *Dictyostelium* confirms these kinetic properties without indications for its activation by cGMP. The properties of DdPDE3 resemble those of mammalian PDE9 which also shows the highest sequence similarity within the catalytic domains. Mutant *stm*F lacks a cGMP-stimulated cGMP-PDE. Experiments show unaltered Northern and Southern blots of DdPDE3 in this mutant. In addition, *stm*F possesses a small cGMP-hydrolyzing activity that has all the cgaracteristics of DdPDE3, suggesting that we have not cloned the cGMP-stimulated cGMP-specific PDE that id defective in *stm*F mutants. DdPDE3 is the first cGMP-specific PDE identified in lower eukaryotes.

Proteins that are Potentially Involved in the Initial Signal Transduction Leading to Cell Migration

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Concluding from available data there is an extensive crosstalk between proteins below the plasma membrane regulating the directed cell motility of *Dictyostelium discoideum* amoebae. Distinct binding domains like pleckstrin-homology (PH), poly-proline, actin-binding, or WD domains are directly involved in targeting soluble effector proteins. We have identified several potential candidates for such proteins in *Dictyostelium discoideum*, including homologues of gephyrin found in vertebrate tissues, and diaphanous from *Drosophila*. Furthermore, we have characterized two clones encoding *Dictyostelium* proteins with PH domains.

Gephyrin first isolated from mammalian brain copurifies with the glycin receptor of the post synaptic membrane and plays an important role as a bridge protein between the glycin receptor

on the one hand, and microtubules and actin filaments on the other. Its strong homology to proteins involved in the synthesis of the molybdenum cofactor found in *Drosophila*, *E.coli* and *Arabidopsis* suggests an involvement in several cell functions. Our clone coding for a gephyrin homologue of *Dictyostelium discoideum* is about 1 kB in size. Its derived protein sequence comprises 330 amino acids including the C-terminus of the putative protein. Aligning this *Dictyostelium* protein to the 736-amino acid chicken gephyrin shows 40% sequence identity and 55% similiarity between the C-terminal half of chicken gephyrin and the partial *Dictyostelium* protein. The 1-kB clone is currently used for screening various cDNA libraries of *Dictyostelium discoideum* in order to retrieve the full-length clone.

Nuclear Accumulation of Skp1 Depends on its Glycosylation in *Dictyostelium*

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Skp1 is a subunit of SCF-E3 ubiquitin ligases and other protein complexes. In *Dictyostelium*, Skp1 is modified by an unusual pentasaccharide *O*-linked to a hydroxyproline at position 143. The pentasacchriade is the product of a novel 6-enzyme glycosylation pathway that resides in the cytoplasm. *Dictyostelium* Skp1 is the product of two loci encoding isoforms that differ by a single amino acid. Skp1A and Skp1B mRNAs were found at similar relative levels throughout the life cycle based on RT-PCR. When myc-tagged Skp1A and Skp1B were expressed behind a promoter active during growth and early development, they were similarly glycosylated based on Western blotting and mass spectrometry. In contrast, Skp1B was not glycosylated when expressed behind a promoter active in prespore cells, but was subsequently glycosylated upon return of the cells to growth medium. Based on immunofluorescence localization, Skp1 accumulated in both the nucleus and actin-rich and -poor regions of the cytoplasm. Whereas expressed Skp1A-myc and Skp1B-myc are both accumulated in the nucleus, Skp1A-myc whose glycosylation was blocked by mutation of its attachment site did not. Two other mutant alleles of Skp1A-myc that were not glycosylated, as well as Skp1B-myc expressed in prespore cells, also failed to accumulate in the nucleus.∀,∀'-dipyridyl, which was shown to be an inhibitor of the prolyl hydroxylase required for Skp1 glycosylation, also inhibited nuclear accumulation. Finally, nuclear accumulation occurred in a mutant that attached only the core disaccharide to Skp1. Thus Skp1 glycosylation was developmentally regulated, not strictly co-translational, and was required as competence factor for Skp1's accumulation in the nucleus of *Dictyostelium*.

Convergent Evolution of a non-Golgi alpha1,2-Fucosyl-transferase that Modifies Skp1 in the Cytoplasm of *Dictyostelium*

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Skp1 is a subunit of the SCF-E3 ubiquitin ligase complex that targets cell cycle and other regulatory factors for degradation. In *Dictyostelium*, Skp1 is modified by an unusual pentasaccharide containing the type 1 blood group H structure at its core. In order to address how the third sugar, an alpha1,2-linked fucose, is applied, a GDP-Fuc:Skp1-alpha1,2-fucosyltransferase (FTase) was purified over 3 million-fold to near homogeneity identifying a polypeptide FT85 as a candidate for the enzyme. Submicrogram quantities of FT85 were

subjected to *de novo* sequencing in a Q-TOF mass spectrometer. Degenerate primers based on the peptide sequences were used to amplify putative FT85 DNA. The complete genomic sequence was obtained by use of a novel linker-PCR method and alignment with sequences from the *Dictyostelium* genome project. An ORF of 768 amino acids was found which corresponded to the estimated M_r of 85,000 for FT85. FT85 was found to be required for Skp1 fucosylation as an FT85-null strain produced Skp1 with a slightly reduced Mr. In addition, extracts from FT85-null cells exhibited negligible Skp1-FTase activity. FT85 contained no sequences expected to target the FTase to the secretory pathway, the location of all other known eukaryotic fucosyltransferases. Sequence homology studies revealed that the Skp1-FTase is most closely related to members of the NRD2-family (also known as family 2 or family E) class of glycosyltransferases that reside in the cytoplasm of prokaryotes. These enzymes glycosylate lipid-linked polysaccharide precursors before they are flipped across the plasma membrane to assemble into the capsule and other extracellular structures. We hypothesize that *Dictyostelium* recruited its non-Golgi Skp1-FTase from this original pool of cytoplasmic glycosyltransferases, rather than re-target a Golgi fucosyltransferase to carry out this function. This is a striking example of convergent evolution of H-type alpha1,2-fucosyltransferases.

Molecular analysis of DdNek2, the first non-vertebrate homologue of the human centrosomal NIMA-related kinase Nek2

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NIMA-related kinases (**n**ever **i**n **m**itosis, **A**spergillus nidulans) are a ubiquitous family of serin/threonine kinases promoting cell cycle-dependent events such as G2/M progression, chromosome condensation and centrosome splitting. Human Nek2 is unique among this kinase family since it is an integral component of the human centrosome (1). Recently, a cDNA sequence of a Dictyostelium member of this protein family was identified in the cDNA-project of the University of Tsukuba/Japan. Clone SLD805 contains a complete open reading frame encoding DdNek2 which has 43% amino-acid identity to human Nek2. Within the catalytical domain (~65% of the entire sequence) both sequences are even 54% identical. Moreover, both proteins have a similar domain structure and share appoximately the same length of ~50 kDa. Thus, DdNek2 is the first non-vertebrate homologue of human Nek2. DdNek2 was expressed in *E. coli* as a fusion with maltose-binding protein (MBP). By contrast to human Nek2, bacterially expressed DdNek2 is catalytically active, i.e. it exhibits autophosphorylation and uses "/$-casein as an artifical substrate. MBP-DdNek2 was expressed in *Dictyostelium* as well. One-step isolation of MBP-DdNek2 from cytosolic *Dictyostelium* extracts by affinity chromatography on amylose resin resulted in a highly pure catalytically active enzyme preparation. Thus, MBP is a well suited fusion-tag for expression and high purification of native proteins not only in *E. coli* but also in *Dictyostelium*.

Immunofluorescence analyses with antibodies raised against bacterially expressed MBP-DdNek2 and expression of a GFP-DdNek2 fusion protein in *Dictyostelium* showed that DdNek2 is localized at the centrosome during the entire cell cycle. The GFP-DdNek2 mutants frequently exhibit supernumerary centrosomes indicating a role of DdNek2 in centrosome dupliation. Recently, a centrosomal 280-kDa substrate protein of human Nek2, called C-Nap1, could be identified (2). Interestingly, antibodies against human C-Nap1 specifically recognized a 280-kDa band in immunoblots of Dictyostelium centrosome preparations. Furthermore, immunofluorescence analysis revealed that anti-C-Nap1 antibodies bound to the Dictyostelium centrosome, as in human cells, only during interphase. Dissociation of C-Nap1 at the onset of mitosis, presumably triggered by its phosphorylation, is thought to facilitate centrosome splitting (2). The immunological evidence for a putative C-Nap1 homologue in *Dictyostelium* and the presence of DdNek2 at the centrosome suggests that the important

functional role of Nek2 in centrosome duplication is conserved from *Dictyostelium* to humans in a very similar fashion despite of considerable differences in morphology and duplication of human and *Dictyostelium* centrosomes.
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A gene that might be involved in the link between calcium and the cell cycle in *Dictyostelium discoideum*.

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The cell cycle phase - at starvation - and cellular calcium levels - both at the time of starvation and during subsequent development - influence cell fate in *D. discoideum*.
Our aim is to understand the genetic basis of the link between calcium and the cell cycle. Towards this end we have used a *D. discoideum* vegetative stage cDNA library to try and complement temperature-sensitive cell cycle mutations in the yeast *S. cerevisiae* that also have an associated 'calcium phenotype'. As part of this exercise, a screen for the yeast *cdc24-4* mutation was carried out. At the restrictive temperature (37^0C) the mutant is arrested in G1; a second aspect of the mutant phenotype is an increased calcium influx into the cell. A cDNA capable of restoring the growth defect in the *cdc24-4* mutant turned out to encode the *D. discoideum* ribosomal protein S4. The cDNA suppresses the phenotype of *cdc24-4* but not of *cdc7* or *cdc31*, both of which are genes that affect the same stage in the cell cycle as *cdc24-4*. Further, we have screened, under conditions that allow for S4 overexpression, eight different yeast mutations that fall in the missense, nonsense and frameshift categories. This showed that the rescue of *cdc24-4* was not due to translational errors but was a specific effect. The calcium status of yeast cells as analysed using chlortetracycline (a probe for sequestered calcium) revealed that at the restrictive temperature the *cdc24-4* mutant had a 7.7-fold increase in sequestered calcium as compared to the wild type. Upon rescue by the *D. discoideum* cDNA, the same *cdc24-4* mutant had a 2.5-fold higher calcium level than the wild type. Thus, rescuing the growth phenotype concomitantly results in a 3.1-fold reduction in sequestered calcium. However, the *cdc24-4* mutant phenotype cannot be rescued by a lowering of the calcium level *per se*. The possible involvement of the cDNA in the calcium- cell cycle link in *D. discoideum* is being studied.
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Transcriptional coactivator SnwA - phosphorylation, oligomerisation, and stability

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Coactivator and corepressor complexes were recently found to play a central role in the regulation of transcription. Our interest in signal transduction in *Dictyostelium* led us to the discovery of a novel type of coregulator – the SnwA protein. It was identified in every eukaryotic organism examined, which indicates ancient and important regulatory function. The interactions of SNW proteins from higher eukaryotes (SKIP, NCoA-62) with steroid/vitamin D receptors, ski/sno/SMAD transcription factors, and CBF1/NotchIC complex implicate their role in a surprisingly large number of pathways. Importantly, SNW proteins

were also found in association with spliceosomes (Neubauer, G. et al., 1998, *Nat. Genet.* 20(1): 46-50).

The in vitro binding properties and the distribution in vivo of SnwA as well as its N-terminal and C-terminal domains were analysed using myc- and His- tagged proteins. The cells overexpressing the protein or its truncated versions accumulate high levels of degradation products in both cytosolic and nuclear fractions, the fractions differ in the products pattern, however. We also mapped the region required for nuclear localisation of the protein.

SnwA is highly resistant to salt extraction of the nuclei. Extracted fraction (1M KCl) migrates as a 180 kDa complex on native PAGE, which is suggestive of dimer formation and in contrast to the mobility of cytosolic SnwA (80 kDa). We employed yeast two hybrid assay to provide additional evidence for SnwA-SnwA dimer formation and to localise the binding regions. In parallel, we used sonication in low salt buffers and sucrose gradient fractionation to obtain high molecular weight complexes that contain SnwA. The size of the complex is dependent upon micrococcal nuclease treatment and suggests nucleosomal and/or RNA association.

Phosphorylation plays an important role in spliceosome assembly. Remarkably, SnwA exists in three isoforms, which can be interconverted by alkaline phosphatase treatment. Nuclear-specific isoforms N1 and N2 show differential sensitivity to cycloheximide in vivo.

The dimerisation/ oligomerisation and the existence of phospho-isoforms of SnwA may be important for the understanding of the function of this coregulator, which, we hypothesise, may coactivate transcription by spliceosome targeting.

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Homologous expression and analysis of proteins encoded by retrotransposon TRE5-A

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Gene therapy is an experimental strategy in molecular medicine aimed to introduce expression vectors into cells in order to produce a protein of interest in these cells. The expressed proteins may either compensate for loss-of-function mutations of genes or to vaccinate against infectious particles. The stable integration of expression vectors into the host cell genome for gene therapy purposes suffers from two major drawbacks: low transfection rates and more or less random insertion of the expression plasmids into the host cell genome. The first problem can be partially overcome by using retrovirus-based expression vectors, taking advantage of the very efficient, evolutionary selected capability of retroviruses to infect cells. However, retroviruses tend to insert randomly in their host's genomes, such that a risk of affecting functional genes such as tumor suppressor genes may cause secondary side effects, e.g. cancer. Hence it is desirable to combine the high insertion efficiency of retrovirus-based vectors with the DNA sequence-specific or position-specific integration of some nonviral transposable elements.

Mobile elements are "selfish" genetic entities which replicate and migrate within host cell genomes. A "life cycle" of retrotransposons includes transcription of the elements into RNA intermediates. These transcripts are reverse transcribed by means of encoded reverse transcriptase enzymes. The copy DNAs are inserted into the host genomes by element-encoded endonucleases (integrases). The *Dictyostelium* repetitive element, TRE5-A (fomerly known as DRE), is present with about 100 copies in the genome of NC4-derived strains of *Dictyostelium discoideum*. TRE5-A elements show a remarkable position-specificity of integration, inserting 50$\pm$4 base pairs upstream of tRNA genes. Since tRNA gene-flanking regions do not contain open reading frames, mobilized TRE5-A elements never cause insertion

mutations upon retrotransposition. The TRE5-A element encodes two open reading frames (ORFs). In ORF2 is an endonuclease (EN) domain located, which is thought to be essential for position-specific integration, and a reverse transcriptase that catalyses cDNA synthesis of TRE5-A RNAs. In order to get more information about the TRE's integration mechanism, we expressed the TRE5-A-derived EN protein (ENp) and the protein encoded by ORF1 (ORF1p) in *D. discoideum*. Recombinant proteins were analysed for subcellular localization both by biochemical fractionation and confocal laser scanning microscopy of GFP-tagged derivatives. Both ORF1p and ENp showed strong tendency to form aggregates which were deposited in the cytoplasm of *D. discoideum* cells. Preliminary results to characterize the endonuclease activity of ENp are discussed

Cloning and characterization of a Dictyostelium gene homologous to Yeast Ssn6

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By means of REMI, we have identified a *Dictyostelium* gene (*trfA*) highly homologous to a *Saccharomyces cerevisiae* gene encoding a TPR (tetratricopeptide repeat) protein, Ssn6 (Cyc8), which functions as a global transcriptional repressor for diverse genes. The deduced amino acid sequence of the *Dictyostelium* gene product, TRFA, contains ten consecutive TPR units as well as Gln repeats, Asn repeats, and a region rich in Glu, Lys, Ser and Thr. The sequences of some of the ten TPR units in TRFA are more than 70% identical to the corresponding units in Ssn6.

The *trfA*$^-$ cells produced smooth plaques on a bacterial lawn, and also failed to aggregate normally when starved on a plain agar plate. Individual cells showed weaker chemotactic responces to both cAMP and folate. although adenylyl cyclases of the *trfA*$^-$ cells were activated by cAMP as in the wild-type cells.

In a rich medium, they grew more slowly and stopped growing at a lower density than the wild-type cells. Furthermore, they divided into cells of various sizes and tended to be much smaller than the wild-type cells. These pleiotropic defects of the *trfA*$^-$ cells and localization of TRFA in the nucleus suggest the possibility that *Dictyostelium* TRFA may regulate the transcription of diverse genes required for normal growth and early development.

Mutational analysis of the promoter Box A of the ribonucleotide reductase small subunuit gene

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Ribonucleotide reductase catalyzes the reduction of ribonucleotides to deoxyribunucleotides, the rate-limiting step in *de novo* generation of dNTPs. We have previously shown that the gene that encodes the small subunit of ribonucleotide reductase, *rnrB*, is expressed in prespore cells during multicellular development (Tsang *et al.* 1996 BBA 1309: 100-8). This correlates well with the DNA synthesis activity that has been observed in these cells, both spatially and temporally (Zimmerman and Weijer 1993 Dev. Biol. 160: 178-85, Durston and Vork 1978 Exp. Cell Res. 115: 454-457, Zada-Hames and Ashwoth 1978 Dev. Biol. 307-20). To understand the mechanism leading to cell type-specific expression of this gene, we have performed deletion analysis using a *rnrB-lacZ* fusion gene. We have found

that two GC-rich boxes, called box A and box B, can independently confer cell type-specific expression of *rnrB*. We have examined box A in more detail. The box A sequence is GGAACCAAAATTGCGCTAA. We have found that a deletion construct containing only the 3' half of box A sequence (only the GCGC element) is expressed in both prespore and prestalk cells (Bonfils, Gaudet and Tsang 1999 JBC 274: 20384-90). This observation suggests that the 5' half of box A may act as a repressor element in prestalk cells.

To further investigate the mechanism controlling cell type-specific expression of *rnrB*, we have constructed point mutants in box A and fused them to the *lacZ* gene. Box A-M1 has mutations in the 5' half of box A, GGAATTAAAATTGCGCTAA, while box A-M2 has mutations in the 3' half, GGAACCAAAATTGCTATAA (underline represent mutated bases). Cells harboring the box A-M1 contruct show weak expression, while cells haboring the box A-M2 control have no detectable expression. This suggests that the GCGC element near the 3' end of box A is a transcriptional activator binding site. We have also looked at the effect of these mutations on the full length *rnrB* promoter. Our results indicate that M1 slugs show significant expression in the prestalk zone. This is consistent with the idea that the 5' half of box A consists of a prestalk repressor element.

To understand how expression of *rnrB* in prespore cells is controlled we have done electrophoretic mobility shift assays. We have detected the presence of a developmentally-regulated box A-binding factor in nuclear extracts of post-aggregative cells (Bonfils, Gaudet and Tsang 1999 JBC 274: 20384-90). We have performed competition assays to determine whether the box A-binding factor binds to the 5' or the 3' part of the box A element. Our results show that an oligonucleotide corresponding to M1 competes efficiently for binding to this factor, while M2 is a poor competitor. Therefore, the 3' half of box A is important for binding to the box A-binding factor. Moreover, this indicates that this factor does not bind the putative repressor element in the 5' half of box A. The box A-binding factor detected in these extract could possibly be a transcriptional activator.

We propose that box A could function as follows. Box A consists of two functional elements: the 5' and the 3' halves. The 3' half would be an activator of gene expression in postaggregative cells, while the 5'-box A may act to repress expression in prestalk cells.

A developmentally regulated ammonium transporter AmtA affects spore viability in *Dictyostelium discoideum*

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In *Dictyostelium* development, ammonia acts as a key morphogen as well as cAMP and DIF. To study the regulatory mechanism by ammonia during development, we have isolated a putative ammonium transporter gene *amtA* from the slug cDNA library of the *Dictyostelium* cDNA project. The 1572 bp sequence of *amtA* contained two introns and encoded a 49.1 kDa protein consisting of 463 amino acids. The predicted amino acid sequence shared 38, 35 and 30% identity with the sequences of ammonium transporters of *Bacillus subtilis* (NRGA), *Saccharomyces cerevisiae* (Mep2p) and *Arabidopsis thaliana* (AtAMT2), respectively. Northern analysis showed that expression of the 1.7 kb *amtA* mRNA is developmentally regulated; it first appeared at 4 hours after starvation, and then gradually increased during the late development.

The *amtA*-null mutant did not show any detectable difference from the parental strain Ax2 in synchronous development on nitrocellulose filter, but viability of spores in sori significantly decreased in 2 weeks compared with that of Ax2 spores. Recently, it has been reported that high concentration (more than 100 mM) of ammonium phosphate accumulated in sori promotes spore dormancy through stimulation of osmotically and ammonia-stimulated adenylyl cyclase, ACG (1). Taken together, it is possible that AmtA plays an important role in

maintaining spore dormancy through the regulation of ammonium level. To examine this possibility, we are comparing the amount of ammonium ion in sori of *amtA* disruptant to that of the wild-type strain.

Cotter DA, Dunbar AJ, Buconjic SD, and Wheldrake JF (1999). Ammonium phosphate in sori of *Dictyostelium discoideum* promotes spore dormancy through stimulation of the osmosensor ACG. *Microbiology* 145:1891-1901.

Overexpression of PKACAT in *splA*⁻ Spores Rescues the Phenotype

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The maintenance of spore dormancy is a complex process mediated by an interaction of signal transduction cascades through different protein kinases. Recently, a dual specificity kinase, SplA, has been shown to be important in spore differentiation and maintaining spore dormancy in *Dictyostelium* [1]. Strains that have this kinase disrupted develop normally through fruiting body formation, however they do not maintain spore dormancy; spores spontaneously lyse and die within the fruiting body. Spore staining with propidium iodide, a cell viability marker, demonstrates most spores have compromised cell membranes. Also, calcofluor staining, which stains cellulose, shows an incomplete spore wall surrounding *splA*⁻ spores [1]. Previous studies have established actin tyrosine phosphorylation as a marker of spore dormancy and maturation. Maximal phosphorylation occurs after one to two days of spore maturation. *splA*⁻ spores show a considerably lower level of phosphorylated actin compared to controls. PKA activity has been shown to promote spore dormancy through activation of ACG by osmotic pressure elicited by ammonium phosphate present in the matrix within the fruiting body. We have overexpressed the catalytic subunit of PKA to investigate the relationship between cAMP signaling and SplA. *splA*⁻/PKACAT⁺ cells demonstrate rescued propidium iodide staining indicating intact cell membranes. Rescued cells also synthesize complete spore coats as demonstrated by complete calcofluor staining. Overexpression of PKA rescues actin tyrosine phosphorylation in spores to near wild type levels.

In vegetative cells, the story regarding tyrosine phosphorylation is different. Cells phosphorylate actin in response to acute stress such as osmotic pressure or temperature shift. Interestingly, vegetative *splA*⁻ cells phosphorylate actin in response to osmotic stress to a similar level as in wild type cells. This suggests that the pathways leading to actin tyrosine phosphorylation are different between spores and cells. Also, PKA may act in a synergistic manner with SplA in a signal transduction pathway possibly leading to spore dormancy and maturation.

1. Nuckolls, G.H., *et al.*, The Dictyostelium dual-specificity kinase splA is essential for spore differentiation. Development, 1996. **122**: p. 3295-3305.

A Network Model for the Regulation of Dormancy in Spores of *Dictyostelium discoideum*

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The asexual fruiting body of *Dictyostelium discoideum* is normally formed 24 hours after vegetative amoebae are plated on agar surfaces or developed in 96 well microtitre plates.

Complete spore dormancy with heat resistance is not obtained until an additional 12 to 24 hours have passed during which 50% of the spore actin is tyrosine phosphorylated. The extracellular signals initiating spore encapsulation and maintenance of dormancy are received by multiple histidine kinases believed to control the accumulation of cAMP produced by one or more spore adenylyl cyclases. The cAMP controls the activity of PKA resulting in protein phosphorylation cascades ending with the phosphorylation of actin and immobilization of the spore cytoskeleton. The histidine kinases and adenylyl cyclases are multidomain proteins with 'PAS' sequences allowing simultaneous reception of both extracellular and intracellular signals. Thus, a mutation in any single domain is unlikely to disrupt the network leading to spore lethality, except under extreme conditions.
The activation of spores by heat shock, hypoosmotic shock, bacterial products, and autoactivation triggers a PLC pathway to overcome the cAMP inhibitory network reactivating the cytoskeleton through calmodulin function. The understanding of the complexity of the dormancy regulating network may require inactivation of each signaling component one domain at a time.

R and C subunits of the cAMP-dependent protein kinase (PKA) as FRET partners.

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The green fluorescent (GFP) protein is currently used in various *in vivo* cell biology studies, for example to monitor the localization of proteins fused to GFP or gene expression. Since the GFP gene was cloned, many mutants with different spectral properties have been produced. In this respect, FRET (fluorescence resonance energy transfer) can be used to detect physiological changes that depend on protein-protein interactions. Such interactions are monitored by measuring FRET between proteins of interest fused to GFP mutants with overlapping excitation and emission spectra. The appearance or desappearance of FRET reflects the distance between the GFPs, since FRET efficiency decreases as a function of d^6.

We decided to use this technique to develop a sensor for cAMP. PKA represents a sensitive indicator of intracellular cAMP levels. In *Dictyostelium,* PKA is composed of regulatory and catalytic subunits that associate in an inactive holoenzyme. Upon binding of cAMP to the R subunit, the holoenzyme dissociates, releasing an active C subunit. The approach we chose consists in producing R and C subunits tagged with two different GFPs (ECFP and EYFP), either at the N-terminus or at the C-terminus. Thus, various combinations of different R and C fusion subunits were tested in *Dictyostelium*, in order to determine the best FRET partners and to be able to measure *in vivo* variations of intracellular cAMP concentrations. Analysing the rate of dissociation of the holoenzyme in various physiological conditions would be of obvious interest as well.

The best result (only 3% change in FRET) was obtained with the co-transformant involving C-terminal fusion subunits, after adding cAMP (100uM) to cell lysates and 8-bromo-cAMP (1 mM) to living cells. This suggests that the binding efficiency between the FRET partners may not be optimal. The GFPs may also not be close enough or not correctly oriented for FRET to occur. On the other hand, the endogenous PKA may interfere with its GFP-tagged couterpart. In an attempt to lower this background, we used a *Dictyostelium* PKAC minus strain to express our constructs. The analysis of these transformants are currently under way.

Another approach consists in measuring intramolecular FRET by using the regulatory subunit tagged to its N- and C-terminus with two different GFPs. We added a cyan GFP (ECFP) to the N-terminal end of a fusion R subunit (described in Biondi et al., 1998) that contains a S65T-GFP at the C-terminus. This new construct display a small change in FRET (3-4%) in

Dictyostelium cells, the best result being obtained with cell lysates after adding cAMP (200 uM).

A role for YakA, AcaA and PKA in the oxidative stress response of Dictyostelium discoideum.

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YakA is a protein kinase required for the transition from growth to development of Dictyostelium cells. It is required for the decrease in specific mRNAs and the induction of PkaC that occurs when cells are starved. YakA also controls the cell cycle by regulating the interval between cell divisions. Here, we report that

YakA is required for the oxidative stress response of *Dictyostelium* cells. The mean doubling time of cells from *yakA* null, *pkaC* null, *acaA* null and *pkaC* over-expressing strains that were submitted to several different stress conditions indicate a role for YakA, PKA and cAMP in the induction of growth arrest that follows oxidative stress. *yakA*-null cells are extremely sensitive to oxidative stress generated by nitric oxide and hydrogen peroxide and a second-site mutation in *pkaC* suppresses this sensitivity. Our findings indicate that the adenylyl cyclase AcaA and PkaC are necessary for the growth inhibition observed and reveal a novel role for PKA and cAMP in the regulation of stress responses in *Dictyostelium*.

A Flavohemoglobin, FhpA, from Macrocyst-Forming *Dictyostelium mucoroides*

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A 1.3 kbp cDNA, prepared from *Dictyostelium mucoroides* amoebae 8 hours after initiating macrocyst formation, hybridized to a transcript abundant in amoebae forming macrocysts, but rare or absent in amoebae forming sorocarps. The differential expression of this transcript, confirmed by both northern blot analysis and primer extension, suggested this gene product was developmentally regulated. Moreover, development under reduced O_2 (5%) did not alter the abundance of this transcript. BLAST analysis of the open reading frame (ORF) revealed significant amino acid identity with flavohemoglobin genes in *Dictyostelium discoideum* (288 out of 397 residues, 74% identity), yeast (179 out of 399 residues, 45% identity), and bacteria (185 out of 399 residues, 46% identity). The deduced protein, hereafter referred to as FhpA, for this ORF in *D. mucoroides* had a molecular mass of 44 kD. Kyte-Doolittle analysis and the apparent lack of a signal peptide in FhpA supported a cytosolic location for this protein. Nucleotide analysis of *fhpA* cDNA and the corresponding genomic DNA revealed a single 65 bp intron which contained the consensus sequence (GT-AG) found in most introns and was located 249 bp downstream of the proposed start codon. Consistent with other *Dictyostelium* genes, the A-T content of *fhpA* was 71%. The nucleotide sequence 5' to the *fhpA* structural gene, presumably containing the promoter region and associated cis-acting elements, was obtained by genomic walking and subsequently sequenced. Not typical of the genus *Dictyostelium*, this analysis showed that 319 bp 5' to the ORF encoding FhpA was not A-T rich (48%). Further attempts to identify the regulatory elements involved in the expression of *fhpA* and to assign function to FhpA have been initiated.

Delirium A, not such a crazy mutant...

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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 is involved in development for 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 different from apoptosis (1). In order to identify genes involved in Dictyostelium cell death, a program of REMI electroporation and PCD resistant mutant screen was set up using HMX44 as recipient strain. Transfected cells were tested for resistance to death upon differentiation. Surviving cells (cell death mutants) were recovered by their ability to regrow in rich medium. Twelve cell death resistant mutants have been isolated so far using this approach. Sequences flanking plasmid insertion have been recovered for six of them. Flanking sequences from mutant Delirium A have been used to perform homologous recombination in AX2; development in DlrA- cells did not proceed further than a loose aggregate. Expression analysis of early developmental markers and cAMP response in DlrA- cells compared to AX2 will be described.

Involvement of the glucose-regulated protein 94 (Dd-GRP94) in starvation response of *Dictyostelium discoideum* cells

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Upon deprivation of nutrients, *Dictyostelium discoideum* Ax-2 cells arrest proliferation and initiate the developmental program including induction of altered genes expression which are necessary for differentiation. Recently, we found the member of Hsp90 family present in the endoplasmic reticulum (ER), Dd-GRP94 (*Dictyostelium discoideum* glucose-regulated protein 94), which contains a typical ER signal sequence at its N-terminus and a so-called ER retention sequence (HDEL) at its extreme C-terminus. In general, GRP94 is induced by glucose starvation and depletion of Ca^{2+} in intracellular Ca^{2+} stores, which lead to accumulation of malfold protein in ER and induce ER stress. Unexpectedly, however, the expression of *dd-grp94* was found to be greatly reduced within 60 min of starvation, possibly coupling with Ca^{2+}-depletion from intracellular Ca^{2+} stores such as ER. *dd-grp94*-overexpressing cells (*grp94*^{OE}-cells) collected without forming distinct aggregation-streams, and never formed normal fruiting bodies, being remained as spherical cell masses ever after 72 hr of starvation. Also, prespore differentiation as well as maturation into spore and stalk cells were markedly impaired in the *grp94*^{OE}-cells. Incidentally, antisense-mediated gene inactivation of *dd-grp94* seemed to be lethal. These data strongly suggest importance of the Dd-GRP94 in the differentiation of starving cells.

Identification of 4th STAT protein in *Dictyostelium discoideum*

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Based on information obtained from the *Dictyostelium* Genome Database, we recently isolated the fourth DdSTAT, DdSTATd. DdSTATd consists of 815 amino acids and has typical DdSTAT homology with three other known DdSTATs: a, b and c.

We produced a fusion construct of DdSTATd-rgfp under the control of Actin15 promoter and visualised the nuclear-localisation pattern in slugs. The result showed that DdSTATd-rgfp localises in the nuclei of both prestalk and prespore cells. Knock-out mutant of DdSTATd did

not show strong morphological abnormality. Finer anaylysis of the mutant is under way and the data will be presented.

Connectivity clustering of gene expression patterns in Dictyostelium development

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A novel algorithm based on the mutual connectivities of patterns was developed. The program reveals natural tendencies of data to cluster in analogy to the physical phenomenon of percolation. Moreover, the program generates background statistics for each element in a cluster. Gene expression data derived from probing microarrayed Dictyostelium genes was analyzed by connectivity to reveal clusters based on the abundance of their products at different stages of development. Three natural temporal clusters were found: genes that increased soon after the initiation of development; genes that increased following aggregation; and genes that decreased or changed little during development. Natural subdivisions within each of the major clusters were also observed. The program has several advantages over other commonly used methods for clustering such as self-organizing maps and K-means which force gene expression data into a fixed number of predetermined clustering structures. Connectivity clustering is a stochastic version of linked graph methods and is probabilistic in nature. As such, it accomadates the possibility that one gene participates in multiple clusters.

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