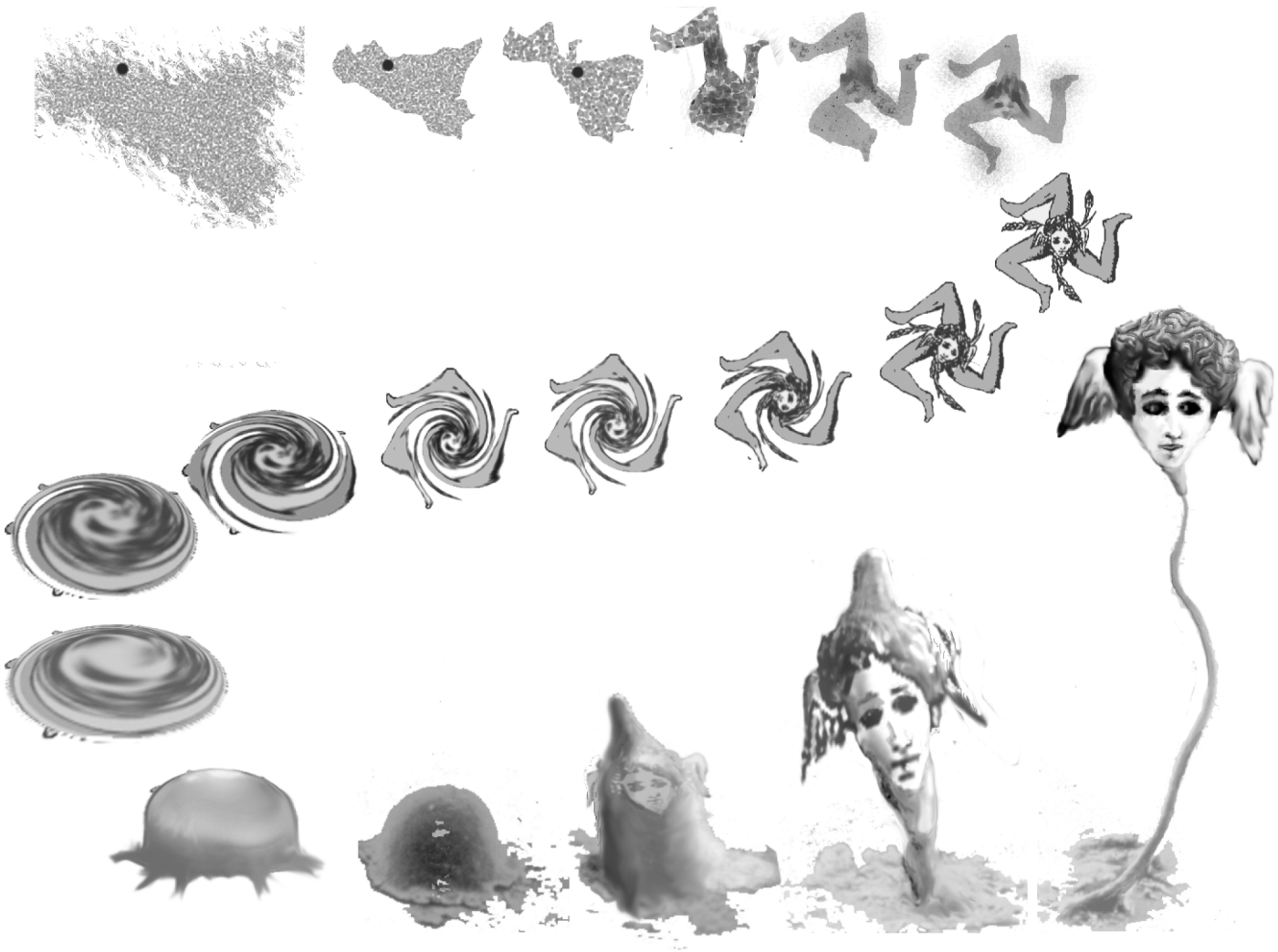
DICTY 2002



Congress Center Torre Normanna
Altavilla Milicia (PA)
22-27 Settembre 2002

INTERNATIONAL DICTYOSTELIUM CONFERENCE 2002

Altavilla – Hotel Torre Normanna
September 22-27, 2002

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time	Sunday, September 22	Monday	Tuesday	Wednesday	Thursday	Friday, September 27
9:00-10:20		*Genomics* G. Shaulsky M.A. Rajandream A. Noegel G. Glöckner J. A. Pachebat H. Urushihara	*Cytoskeleton II* R. Chisholm R. Gräf J. Faix R. Insall M. Schleicher I. S. Näthke	*Signals & development* J. Williams R.H. Gomer D. Brazill W.F. Loomis G. Schaulsky N. Van Driessche	*Pattern formation I* R. Kay C.J. Weijer A.R. Kimmel J. Ryves P. Thomason K. Miura	*Adhesion/Autophagy* Y. Maeda 8:30 P. Fey 8:50 M. Benghezal 9:10 G.P. Otto *Pattern formation II* E. Cox 9:30 D. Blumberg 9:50 C. Thompson
10:40-11:10		*Coffee break*	*Coffee break*	*Coffee break*	*Coffee break*	*10:10 Coffee break*
11:10-12:10		*Actin & motors* M. Schleicher G. Gerisch E. D. Korn D. J. Manstein M. Sameshima	*Endo-/Phagocytosis* R. Kessin A.Müller-Taubenberger M. Clarke S. Bozzaro M. Maniak	*Cell cycle & cell fate* H. MacWillimas A. Kuspa C. Pears S. Hirose C. West	*Gene regulation* T. Winckler P. Golstein P. Folk P.L. Beech D. Ratner	10:30 R. Escalante 10:50 M. Maeda 11:10 C. Anjard 11:30 D. Dorman
12:45-14:00		*Lunch*	*Lunch*	*Lunch*	*Lunch*	*12:00 Lunch*
14:00-15:20		*Chemotaxis I* P. Schaap R. Firtel C. Parent H. R. Luo M. Iijima	*CGMP/chemotaxis II* R. Firtel P.J.M. Van Haastert M.E. Meima D.R. Soll D. Wessels	*Chemotaxis III* P. Fisher D. Hereld J.A. Brzostowski B.E. Snaar-Jagalska W. Jang	Excursion to Monreale and Palermo	End of the meeting
15:20-15:40		*Coffee break*	*Coffee break*	*Coffe break*		
15:40-17:00	Registration	*Genetics* W. Loomis T. Winckler T. Muramoto J. King R. Mutzel	*Ca and IP3* P. Newell B. Coukell D. Malchow A. J. Harwood C. Schlatterer	*Stress responses* G. Weeks J. G. Williams A. De Lozanne G. Mendez Souza I. Tatischeff		
17:00-19:00		Leisure, workshops, etc.	Leisure, workshops, etc.	Leisure, workshops, etc.		
19:00-20:00	Welcome party	*Dinner*	*Dinner*	*Dinner*		
20:00-22:00		Poster session 1	Poster session 2	Poster session 3	20:00 Banquet	

Note: Posters will be up all week

POSTER SESSION 1	POSTER SESSION 2	POSTER SESSION 3
70 Abe T.	86 Dondero F.	103 Adam M
71 Beck P.	87 Kriebel WP.	104 Kibler K
72 Hamlin E.	88 Letourneur F.	105 Cotter D
73 Barth C.	89 Manstein D.	106 Fukusawa M
74 Kuwayama H.	90 Mahadeo D.	107 Zhukovskaja N
75 Lay S.	91 Rivero F.	108 Oohata A
76 Moreno N.	92 Sasaki K.	109 Maeda M
77 Szafranski K.	93 Steenbergen J.	110 Alvarez-Curto E
78 Thomason P.	94 Ueda M.	111 Saran S
79 De Lozanne A.	95 Voss E.	112 Shaw C
80 Daniels K.	96 Weissenmeyer B.	113 Weening K
81 Baik M.	97 Wessels D.	114 Williams RSB
82Gräf R.	98 Arigoni M.	115 Yagura S
83 Strassman J.	99 Zhang H.	116 Katoh M
84 Heuser J.	100 Balest A.	117 Blumberg D
85 Mai A.	101 Brock D.	118 Ikeno D
	102 Dalton E.	119 Sevcikova

Monday, 23-09-2002

Session I: Genomics – 9:00-10:40

Chair: Gad Shaulsky

1 - The *Dictyostelium* genome project: an update
Marie-Adèle Rajandream

2 - Sequence and analysis of chromosomes 1 and 2
G. Glöckner, L. Eichinger, K. Szafranski, J. Pachebat, A. Bankier, P. Dear, R. Lehmann, C. Baumgart, G. Parra, J. Abril, R. Guig, K. Kumpf, B. Tunggal, the Dictyostelium Genome Sequencing Consortium, E. Cox, M. A. Quail, M. Platzer, A. Rosenthal and A. Noegel

3 - Gene prediction and annotation in *D. discoideum*
K. Szafranski, R. Lehmann, G. Parra, R. Guigo, L. Eichinger, M. Platzer, A. Noegel, and G. Glöckner

4 - HAPPY mapping the *Dictyostelium discoideum* genome
J.A. Pachebat, A.T. Bankier, B.A. Konfortov, G. Glöckner, K. Szafranski, R. Sucgang, M.-A. Rajandream, R Davies, G. Bloomfield, the Dictyostelium Genome Sequencing Consortium, P. H. Dear.

5 - Large-scale analysis of full-length cDNAs and their start points in *Dictyostelium discoideum*
Hideko Urushihara, Shintaro Katayama, Hidekazu Kuwayama, Takahiro Morio, Shinji Obara, Tetsuo Ida, and Yoshimasa Tanaka

Coffee Break 10:40-11:10

Session II: Actin and motors – 11:10-12:30

Chair: Michael Schleicher

6 - Dynamic organization of the actin cytoskeleton
O. Medalia, I. Weber, G. Gerisch, and W. Baumeister

7 - Biochemical and biological properties of tail chimeras of *Dictyostelium* myosin II
E. D. Korn, S. Shu and X. Liu

8 - Functional characterization of members of the myosin I family from *Dictyostelium discoideum*
Ulrike Dürrwang, Setsuko Fujita-Becker, Muriel Erent, and Dietmar J. Manstein

9 - Structure and role of actin rods in *Dictyostelium discoideum* spores
M. Sameshima, Y. Kishi, D. Mahadeo, Masako Osumi, T. Sugo, and D. Cotter

Session III: Chemotaxis I – 14:00-15:20

Chair: Pauline Schaap

10 - Regulation of chemotaxis in *Dictyostelium*.
Alex Sobko, Ruedi Meilli, Susan Lee, Sylvain Merlot, Binggang Sun, Kyung Chan Park, Young-Hoon Han, Kosuke Takeda, Heidi Szemenyie, and Rick Firtel

11 - The adenylyl cyclase ACA is localized at the uropod of chemotaxing cells
P.W. Kriebel, and C.A. Parent

12 - Inositol pyrophosphates regulate cAMP-triggered chemotaxis by modulating CRAC- PI(3,4,5)P3 interaction
Hongbo R. Luo, Y. E. Huang, C. Chen, A. Saiardi, M. Shimizu, E. Nagata, P. Devreotes and S. H. Snyder

13 - PI3-phosphatase, PTEN Regulates Sensing of Chemoattractant Gradients
Miho Iijima and Peter Devreotes

Coffee Break 15:20-15:40

Session IV: Genetics – 15:40-17

Chair: William Loomis

14 - Transfer RNA gene-targeted retrotransposons continue to shape the *Dictyostelium* genome
Peter Beck, Monika Baik, Oliver Siol, Theodor Dingermann and Thomas Winckler

15 - Construction of a gamete-specific gene pool and RNAi-mediated functional analysis in *Dictyostelium discoideum*
Tetsuya Muramoto, Katsuya Suzuki, Shinji Obara, Yoshimasa Tanaka, and Hideko Urushihara

16 - Axenic parasexual genetics in *Dictyostelium discoideum*
J. King and R. Insall

17 - Molecular phylogenetic analysis of evolutionary strategies in social amoebae
Thomas Winckler, Annette Bäuerle, Theodor Dingermann, Rolf Marschalek, Rupert Mutzel, and Sandra L. Baldauf

Poster session I – 20:00-22:00

Tuesday, 24-09-2002

Session V: Cytoskeleton II - 9:00-10:40

Chair: Rex Chisholm

18 - DdCP224 and DdEB1: two *Dictyostelium* MAPs with a role at the centrosome and in microtubule plus end dynamics
R. Gräf, A. Hestermann, and M. Rehberg

19 - Signaling molecules as regulators of cytokinesis and cell motility
Jan Faix, Igor Weber and Jibi Jacob.

20 - The Arp2/3 complex and the control of actin polymerization in *Dictyostelium*
Simone Blagg, Jason King, Karl Saxe & Robert Insall

21 - *Dictyostelium* Ste20-like kinases in signalling pathways to the cytoskeleton
Rajesh Arasada, Hyun-Ju Son, Ludwig Eichinger, Michael Schleicher

22 - Cytoskeletal regulation by the Adenomatous Polyposis Coli protein in *Dictyostelium*
Ian P. Newton, Pauline Schaap, Inke S. Näthke

Coffee Break 10:40-11:10

Session VI: Endo-/phagocytosis - 11:10-12:30

Chair: Richard Kessin

23 - Visualizing individual steps during phagocytosis
A. Müller-Taubenberger, I. Weber, and G. Gerisch

24 - Dynamics and fusion in the early endocytic pathway of *Dictyostelium*.
M. Clarke, J. Kohler, J. Heuser, and G. Gerisch

25 - The *Dictyostelium* homologue of human Nramp1 is required for efficient phagocytosis and resistance to pathogenic bacteria
Barbara Peracino, Carina Skriwan, Alessandra Balest, Alessandra Balbo, Barbara Pergolizzi, Angelika A. Noegel, Michael Steinert and Salvatore Bozzaro

26 - The *Dictyostelium* LC-FACS protein contributes to fatty acid uptake and endocytosis
K. von Löhneysen, N. Pawolleck, H. Rühling, and M. Maniak

Session VII: cGMP/chemotaxis II - 14:00-15:20

Chair: Richard Firtel

27 - A novel cGMP-signaling pathway mediating myosin phosphorylation and chemotaxis in *Dictyostelium*
Leonard Bosgraaf, Henk Russcher, Janet L. Smith, Deborah Wessels, David R. Soll and Peter J.M. Van Haastert

28 - Identification of two novel phosphodiesterases in *Dictyostelium*
Marcel E. Meima, Karin E. Weening, Daniel E. Rozen, Ricardo M. Biondi and Pauline Schaap.

29 - The regulation of cell behavior in the natural wave
David R. Soll

30 - rasC null mutants exhibit defects in cellular translocation and pseudopod formation during basic motile behavior and chemotaxis
Deborah Wessels , David R. Soll , Tien Pham, Spencer Kuhl , Ryan Keller , Rebecca Brincks , C. James Lim , Karl Zawadzki and Gerald Weeks

Coffee Break 15:20-15:40

Session VIII: Calcium and IP3 - 15:40-17

Chair: Peter Newell

31 - Ca^{2+}/calcineurin-mediated up-regulation of a novel gene family in *Dictyostelium*
Barrie Coukell, Yi Li, and Anne Cameron

32 - W 7, a calmodulin antagonist, enhanced cAMP oscillations and caused release of stored calcium.
D. Malchow, D.F. Lusche and C. Schlatterer

33 - Prolyl oligopeptidase and inositol phosphate metabolism during early development
R.S.B. Williams, E. C Dalton, B. Serreck, G.W. Mayr and A.J. Harwood

34 - Are there two types of calcium channels in the plasma membrane?
Christina Schlatterer, Ralph Schaloske, Karen Bezares-Roder, Dieter Malchow

Poster session II – 20:00-22:00

Wednesday, 25-09-2002

Session IX: Signals and development - 9:00-10:40

Chair: Jeffrey Williams

35 - A single cell-density sensing factor stimulates distinct signal transduction pathways through two different receptors
William J. Deery, Tong Gao, Robin Ammann, and Richard H. Gomer

36 - A Phospholipase D regulates quorum sensing in *Dictyostelium discoideum*
Yi Chen, Vanessa Rodrick, Yi Yan, and Derrick Brazill

37 - Genetic modules expressed during early development of *Dictyostelium*
Negin Iranfar, Danny Fuller, and William F. Loomis with the cooperation of the Japanese EST Project

38 - Beyond development: transcriptional profiling of the *Dictyostelium* cell cycle, spore germination and de-differentiation
Chad Shaw, Nancy Van Driessche,, Miroslava Ibarra, Sujata Sharma, Ezgi Okyay,, Takahiro Morio,, Mariko Katoh, Hideko Urushihara, Yoshimasa Tanaka, Junji Chida, Aiko Amagai, Yasuo Maeda, Dana Mahadeo, David Cotter, Adam Kuspa,, and Gad Shaulsky

39 - Microarray phenotyping of the PKA and YakA pathway
Nancy Van Driessche, Chad Shaw,, Sujata Sharma, Miroslava Ibarra, Trushar Surang, Adam Kuspa,, and Gad Shaulsky,

Coffee Break 10:40-11:10

Session X: Cell cycle and cell fate - 11:10-12:30

Chair: Harry MacWilliams

40 - Prespore cell-cycle arrest during *Dictyostelium* development
Guokai Chen, Gad Shaulsky and Adam Kuspa,

41 - A homologue of Cdk8 required for optimum growth, aggregation and spore cell differentiation.
Hsiu-Hsu Lin, Hao-Jen Huang, Christine Michaelis, Gerry Weeks and Catherine Pears

42 - A transcriptional switch at growth/differentiation transition (GDT) of *Dictyostelium* cells: cis- and trans- elements of the dia1 and fkbp2 genes regulated during the GDT
Shigenori Hirose, Aiko Amagai and Yasuo Maeda

43 - Evolutionary and functional implications of the complex glycosylation of Skp1, a cytoplasmic/nuclear glycoprotein associated with polyubiquitination
Christopher M. West, Hanke van der Wel, Suzanne Z. Fisher, Howard Morris, Maria Panico, Thanai Paxton, Anne Dell, Lee Kaplan, and Eric A. Gaucher

Session XI: Chemotaxis III - 14:00-15:20

Chair: Paul Fisher

44 - G protein-coupled cAMP receptor activation is regulated by hydrophobic residues near the cytoplasmic end of the receptor's third transmembrane domain
M. Zhang and D. Hereld

45 - Gα9-mediated inhibition of developmental signal-response
Joseph A. Brzostowski, Carole A. Parent and Alan R. Kimmel.

46 - Internalisation of G-protein coupled cAMP receptors upon stimulation followed at the single-molecule level in *Dictyostelium discoideum*.
A. Sergé, S. de Keijzer,, M. Zhang, D. Hereld, H.P. Spaink, T. Schmidt and B.E. Snaar-Jagalska

47 - A secreted cell-number counting factor regulates metabolic pathways to control group size in *Dictyostelium*
Wonhee Jang, and Richard H. Gomer,

Coffee Break 15:20-15:40

Session XII: Stress responses - 15:40-17

Chair: Gerry Weeks

48 - A STAT regulated stress-induced signalling pathway in *Dictyostelium*
Tsuyoshi Araki, Masatsune Tsujioka, Tomoaki Abe, Pauline Schaap, Takahiro Morio, Hideko Urushihara, Mariko Katoh, Mineko Maeda, Yoshimasi Tanaka, Ikuo Takeuchi and Jeffrey G. Williams

49 - LvsA, a *Dictyostelium* BEACH protein with essential roles in cytokinesis and osmoregulation.
A. De Lozanne, W. Wu, J. Yajnik, & M. Siano

50 - Regulation of *Dictyostelium* stress responses by YakA, PKA and KeaA.
Raquel Bagattini, Luciana Mantzouranis and Glaucia Mendes Souza

51 - More about the two programmed cell deaths of *Dictyostelium discoideum*
I.Tatischeff

Poster session III – 20:00-22:00

Thursday, 26-09-2002

Session XIII: Pattern formation I - 9:00-10:40

Chair: Robert Kay

52 - The role of cAR2 and cAR3 in differentiation and the control of movement.
Nicholas Oswald, Gerti Weijer and Cornelis J Weijer

53 - Receptor-dependent and tyrosine phosphatase-mediated inhibition of GSK3 regulates cell fate choice
Leung Kim and Alan R. Kimmel

54 - Regulation of GskA and Aardvark during *Dictyostelium* development
J. Ryves, J Reynolds, and A.J. Harwood

55 - Control of morphogenesis by histidine kinase signaling
Peter Thomason and Ted Cox

56 - Correlating the slug behavior with its internal three-dimensional cell movement
Kota Miura

Coffee Break 10:40-11:10

Session XIV: Gene regulation - 11:10-12:30

Chair: Thomas Winckler

57 - Differential localization and roles in mitochondrial division of two FtsZs in *Dictyostelium*
P.R. Gilson, X.-C. Yu, D. Hereld, C. Barth, A. Savage, S. Lay, P. R. Fisher, W. Margolin, and P.L. Beech

58 - Developmental interactions of the *Dictyostelium* F-box protein ChtA/FbxA with a histidine kinase signaling pathway
David I. Ratner, Turgay Tekinay, Herbert L. Ennis, Mary Wu, Margaret K. Nelson, Jakob Franke, and Richard H. Kessin

59 - Coding-region control of PKA and its inhibition by LRR-bearing DeliriumA
Myriam Adam, Jean-Pierre Levraud, Marie-Françoise Luciani, Fabienne Savoy, Karen Brennan, Christophe Reymond, Matthias Hentze, and Pierre Golstein

60 - Transcriptional coactivator SnwA/SKIP– integrating diverse functions
P. Folk, R. Blatny, O. Tolde, I. Fukova, K. Martinkova, M. Skruzny, L. Hamplova, A. Blahuskova, and F.Puta

Friday, 27-09-2002

Session XV: Adhesion/Autophagy – 8:30-9:30

Chair: Yasuo Maeda

61 - SadA, A novel adhesion receptor In *Dictyostelium*
Petra Fey, Stephen Stephens, Margaret Titus, and Rex L. Chisholm

62 - Synergistic roles of members of the TM9 superfamily of proteins
Mohammed Benghezal, Sophie Cornillon, Leigh Gebbie, Laeticia Alibaud, Franz Brückert, François Letourneur, Pierre Cosson

63 - Autophagy is essential for *Dictyostelium* development
Grant P. Otto, Mary Y. Wu, Nevzat Kazgan, and Richard H. Kessin.

Session XVI: Pattern formation II – 9:30:11:50

Chair: Edward Cox

64 - The novel anti-adhesive protein AMPA effects cell type specific differentiation and pattern formation.
Daphne D. Blumberg, Hoa Ho, and Timothy R. Varney.

65 - Microarray and mutational analysis of DIF Signaling
Chris Thompson, Chad Shaw, Mimi Ibarra and Gad Shaulsky

Coffee Break 10:10-10:30

66 - Regulated expression of the MADS-box transcription factor SrfA mediates activation of gene expression by PKA during *Dictyostelium* sporulation
Ricardo Escalante and Leandro Sastre

67 - Microarray-assisted in situ hybridization of *Dictyostelium* prestalk genes.
Mineko Maeda, Keiko Nishio, Haruyo Sakamoto, Takahiro Morio, Hideko Urushihara, Yoshimasa Tanaka, Negin Iranfar, Danny Fuller and William F. Loomis

68 - The *Dictyostelium* homolog of the DBI neuropeptide is the precursor of the spore differentiation factor SDF-2
Christophe Anjard and William F. Loomis

69 - Analysis of slug cell polarisation
Dirk Dormann, Gerti Weijer and Cornelis J. Weijer

Poster session 1 - Monday, 23-09-2002
20:00-22:00

70 – A rapid, generally applicable method to produce gene-disruption constructs based on in vitro transposition
Tomoaki Abe and Jeff Williams

71 – How to observe "jumping" tRNA-gene targeted retrotransposons in the *Dictyostelium* genome
Peter Beck, Theodor Dingermann and Thomas Winckler

72 – Sequencing and finishing strategies for *D. discoideum* at the Sanger Institute
Nancy Hamlin, Paul Davis, David Saunders

73 – Transcription and transcript processing in *Dictyostelium* mitochondria
C. Barth, B. Jayawardena, U. Greferath, P. Le and P. R. Fisher

74 – Generation of *D. discoideum* mutants disrupted in gene for transcription factors by the PCR-generated gene disruption construct
Hidekazu Kuwayama, Shinji Obara, Takahiro Morio, Mariko Katoh, Hideko Urushihara, Satoru Kuhara, and Yoshimasa Tanaka

75 – Functional studies on two nuclear encoded mitochondrial proteins, SdhA and Cpn60, in *Dictyostelium discoideum*.
S. T. Lay, M. Kotsifas, C. Barth, P. R. Gilson and P. R. Fisher

76 – Isolation of genes whose expression is dependent on the MADS-box transcription factor SRFA.
N. Moreno, J.J. Vicente, L. Sastre and R. Escalante

77 – Distribution of transposable elements in the genome of *D. discoideum*
K. Szafranski, A.A. Noegel, G. Glöckner

78 – A series of Gateway expression vectors for use in *Dictyostelium*
Peter Thomason

79 – The *Dictyostelium* family of BEACH-domain containing proteins.
A. De Lozanne, N. Wang and W. Wu

80 – The development of monoclonal antibodies against phosphorylation state-specific proteins in *Dictyostelium* in the developmental studies hybridoma bank
Karla J. Daniels and David R. Soll

81 – Expression of the transcription regulator CbfA (CMBF) is required for growth-development transition
Monika Baik, Peter Beck, Theodor Dingermann and Thomas Winckler

Poster session 2 - Tuesday, 24-09-2002

20:00-22:00

86 – Copper resistance and copper pumps in the slime mold *Dictyostelium discoideum*
B. Burlando, F. Dondero, J. Camakaris and A.Viarengo

87 – The adenylyl cyclase ACA is localized at the uropod of chemotaxing cells
P.W. Kriebel, and C.A. Parent

88 – The AP-1clathrin adaptor complex is required for efficient pinocytosis and phagocytosis.
B. de Chassey, A. Dubois Y. Lefkir, and F. Letourneur

89 – Mutations in the relay loop region result in dominant-negative inhibition of myosin function
Georgios Tsiavaliaris, Setsuko Fujita-Becker, Renu Batra, Dmitrii I. Levitsky, F. Jon Kull, Michael A. Geeves & Dietmar J. Manstein

90 – Investigating the role of CRAC in chemotaxis.
D.C. Mahadeo, F.I. Comer, and C.A.Parent

91 – Role of *Dictyostelium discoideum* Rho-related proteins RacG and RacH in cytoskeleton-dependent processes
Baggavalli P. Somesh, Heidrun Dislich and Francisco Rivero

92 – A study on a pinocytosis-deficient REMI mutant isolated from *Dictyostelium discoideum*
Kazunori Sasaki, Hitomi Yamaguchi, Aiko Amagai and Yasuo Maeda

93 – Cryptococcus neoformans virulence is enhanced by intracellular growth in *Dictyostelium discoideum*
J. N. Steenbergen, J. D. Nosanchuk, and A. Casadevall

94 – Single-molecule imaging of signaling molecules in living *Dictyostelium* cells.
Masahiro Ueda,, Yasushi. Sako, Toshiki Tanaka, Peter.N. Devreotes and ToshioYanagida

95 – 2D, 3D DIAS and DIAS 4.0: Existing and emerging software packages for analyzing the motility and shape of *Dictyostelium* amoebae and other cell types
E.R. Voss and D.R. Soll

96 – The calcineurin inhibitor Gossypol impairs growth, cell signaling and development in *Dictyostelium discoideum*
Barbara Weissenmayer, Katrina Böckeler, Rupert Mutzel

Poster session 3 - Wednesday, 25-09-2002

20:00-22:00

103 – Coding- region control of PKA-C : defining the target sequence
Myriam Adam, Karen Brennan, Matthias Hentze, Pierre Golstein

104 – Intercellular communication genes in *Dictyostelium* development
K. Kibler, T. Nguyen, J. Svetz, N. VanDriessche, M. Ibarra, C. Shaw, and G. Shaulsky

105 – Actin tyrosine phosphorylation patterns during spore aging in wild type and mutants of *Dictyostelium discoideum*
Dana Mahadeo, Tracy Marr, Keith Gale, Yoshiro Kishi, Kashif Kirmani, Masazumi Sameshima and David Cotter.

106 – The *Dictyostelium* stalk cell inducer DIF regulates nuclear accumulation of a STATc protein by controlling its export from the nucleus
Masashi Fukuzawa, Tomoaki Abe and Jeffrey G. Williams

107 – The evolution of SH2 signalling: a biologically functional *Dictyostelium* STAT protein with an aberrant SH2 domain
N. V. Zhukovskaya, M. Fukuzawa, K. A. Jermyn, M. Tsujioka, T. Abe T. Kawataand J. G. Williams

108 – A novel prespore-inducing factor with low molecular weight in *Dictyostelium*: its partial purification and characterization
A.A. Oohata, and Y.Takaya

109 – Low molecular weight factors secreted by wild-type and DIF-less mutant of *Dictyostelium*, that are involved in the restoration of a mutant erkB-
Masatsune Tsujioka, Tatsuyoshi Yamamoto, Christopher R. Thompson, Robert R. Kay and Mineko Maeda

110 – Regulation and function of adenosine kinase in *Dictyostelium discoideum*
Elisa Alvarez-Curto and Pauline Schaap.

111 – Structure and function of ACG
Shweta Saran and Pauline Schaap,

112 – Clusters of gene expression in dicty time course data
Chad Shaw, Nancy Van Driessche, Mariko Katoh, Mimi Ibarra, Gad Shaulsky

113 – Opposite activity patterns of the aggregative and late pdsa promoters in *Dictyostelium* slugs. Requirement for novel cell-cell interactions.
Karin E. Weening, Irene Verkerke-Van Wijk, Christopher R. Thompson Richard H. Kessin, Gregory J. Podgorski and Pauline Schaap

114 – The Genetics of valproic acid resistance

The *Dictyostelium* genome project: an update

Marie-Adèle Rajandream

Pathogen Sequencing Unit, The Wellcome Trust Sanger Institute, Genome Campus, Hinxton, Cambridge, CB10 1SA, UK

The *Dictyostelium* genome is one of the most of difficult to sequence because of its biased (high A+T) base composition and because of the presence of low complexity intergenic regions. Large highly similar complex repeat structures make up approximately 10% of the genome and this complicates the process of assembling shotgun reads into large contigs. Nevertheless, the chr 2 sequence has been published, with about 180 gaps and certain other areas have also been finished to a high standard. For instance the 1/3 of chr 6 for which the Sanger Institute is responsible, constitutes 1.46 Mb and currently exists as 75 contigs, the largest of which is 197 Kb. The shotgun phase for the other chromosomes is essentially complete, to an approximate 10-fold coverage, with the exception of chr 3. Combined, this has required 762,528 reads at the three genome centres (Baylor, Sanger and Jena).

The genome centres are now cooperating closely, with the objective of producing a unified assembly from these reads. This will be annotated to common standards and should produce a comprehensive gene catalogue next year.

To this end, an automatic assembly procedure is under development at the Sanger Institute. Currently this whole genome assembly produces 10,000 contigs on average from the shotgun reads of the various chromosome-enriched, short-insert libraries. This large number is mainly due to missed overlaps. The problem can be addressed by making use of read pair information providing there are sufficient paired reads to work with. An assembly programme which exploits read pair information is in place at the Sanger Institute. The aim is now to improve the read data so that the read pair based assembly can become an effective way of obtaining an automatically produced contig set with a minimum level of redundant or low quality sequence.

In addition to a high percentage of paired shotgun reads, read pairs from large insert clones (spanning approximately 10-20 kb) are needed, particularly for resolving the larger repeats. This together with HAPPY and REMI-RFLP mapping information would help resolve the bigger gaps. Sequence skims from YACs verified versus the HAPPY map are also indispensable in this process as they can provide coverage for regions not well represented in any of the shotgun or large insert libraries. This combined approach has previously been successful with the *P. falciparum* genome, which presents similar difficulties to the *Dictyostelium* one.

At present more sequence is being generated by the genome centres to increase the proportion of paired reads. Efforts are also underway to construct large insert libraries and to probe the YAC and cYAC libraries to find clones which could span the physical gaps. This is all underpinned by the HAPPY map from Paul Dear's team (at the MRC Laboratory of Molecular Biology, Cambridge) which is complete for chromosomes 2 and 6 and in progress for the remaining chromosomes.

Sequence and analysis of chromosomes 1 and 2

[*]G. Glöckner, [†]L. Eichinger, [*]K. Szafranski, [‡]J. Pachebat, [‡]A. Bankier, [‡]P. Dear, [*]R. Lehmann, [*]C. Baumgart, [§]G. Parra, [§]J. Abril, [§]R. Guig_, [*]K. Kumpf, [†]B. Tunggal, the *Dictyostelium* Genome Sequencing Consortium, _E. Cox, ¶M. A. Quail, [*]M. Platzer, [#]A. Rosenthal and [†]A. Noegel

*IMB Jena, Dept of Genome Analysis, Beutenbergstr. 11, 07745 Jena, Germany; †Center for Biochemistry, Medical Faculty, University of Cologne, Joseph-Stelzmann-Str. 52, 50931 Köln, Germany, ‡PNAC Biotech, Laboratory of Molecular Biology, MRC Centre, Hills Road, Cambridge CB2 2QH, UK; §Grup de Recerca en Informatica Biomedica, Institut Municipal d'Investigació Mèdica, Universitat Pompeu Fabra, Centre de Regulació Genòmica, 08003 Barcelona, Spain; _Princeton University, Princeton, NJ 08544, USA; ¶The Sanger Centre, Wellcome Trust Genome Campus, Hinxton, Cambridgeshire CB10 1SA, UK; #Friedrich Schiller Universität Jena, 07743 Jena, Germany

We report on the sequence of two of the six *Dictyostelium discoideum* chromosomes. Chromosome 1 (C1) is close to completion, sequence and analysis of chromosome 2 (C2), the largest of the six chromosomes, has recently been published (Glöckner *et al.*, Nature 418, 79-85, 2002).

C1 and C2 comprise approximately 5 and 8 megabases (Mb) of sequence representing about 40 % of the genome. Shotgun sequences were assembled by a seed- and BLAST-based cyclic assembly strategy which, after finishing, resulted in 49 C1 and 152 C2 contigs. Read pair information, a HAPPY map, previously mapped genes and a circular YAC library were used to validate the assembly and to order the contigs along the chromosomes.

Despite an A+T content of nearly 80% C1 encodes 1,760 and C2 2,799 predicted protein coding genes. This gene density, about 1 gene per 2.7 kb, is surpassed only by *Saccharomyces cerevisiae* (one per 2 kb) and is similar to that of *Schizosaccharomyces pombe* (one per 2.5 kb). If we assume that the other chromosomes have a similar gene density, we expect around 11,000 genes in the *D. discoideum* genome.

Analysis of the C2 encoded genes showed that a significant number of the genes have higher similarities to genes of vertebrates than to those of other fully sequenced eukaryotes. This analysis strengthened the view that the evolutionary position of *D. discoideum* is located prior to the branching of metazoa and fungi but after the divergence of the plant kingdom, placing it close to the base of metazoan evolution.

Gene prediction and annotation in *D. discoideum*

[1]K. Szafranski, [1]R. Lehmann, [2]G. Parra, [2]R. Guigo, [3]L. Eichinger, [1]M. Platzer, [3]A. Noegel, and [1]G. Glöckner

1IMB Jena, Dept of Genome Analysis, Beutenbergstr. 11, 07745 Jena, Germany; 2Grup de Recerca en Informatica Biomedica, Institut Municipal d'Investigació Mèdica, Universitat Pompeu Fabra, Centre de Regulació Genòmica, 08003 Barcelona, Spain; 3Center for Biochemistry, Medical Faculty, University of Cologne, Joseph-Stelzmann-Str. 52, 50931 Köln, Germany

Gene prediction and annotation is the most important step in large scale genomic sequencing projects. The information which constitutes the basis for a specific organism is hidden in the sequence. The extraction of genes and their surrounding regions, the most meaningful part of a genome, is a challenge since the signals determining genes are rather weak.

For *ab initio* gene finding these prediction programs use features which are also used by the cellular machinery to identify and correctly transcribe genes. These features include promoters, splice signals, polyA signals etc.. There are only few signals which are in common for all eukaryotes. Many features are singular to each species and most likely co-evolved with the parts of the transcription machinery to fit in its special requirements. Thus, for every species a given gene prediction program has to be adapted to the genome in question.

For the analysis of chromosomes 1 and 2 we used a version of GeneID, which was trained on 160 previously known *Dictyostelium* genes. We will present examples of predicted gene models and discuss the reliability of these predictions in the light of confirmed gene structures.

Classification of predicted genes is the next step in the annotation process. We decided to use the Gene Ontology system (http://www.geneontology.org/) for a computer-assisted classification. The obtained results are only preliminary, since in an initial phase the classification process was done automatically. A comparison of the automated classification with a manual classification will be presented to show the strengths and weaknesses of our currently available *Dictyostelium* annotation database (http://genome.imb-jena.de/dicdi/Dictyosteliuminfo.html)

HAPPY mapping the *Dictyostelium discoideum* genome

[‡]J.A. Pachebat, [‡]A.T. Bankier, [‡]B.A. Konfortov, [*]G. Glöckner, [*]K. Szafranski, [$]R. Sucgang, ¶M.-A. Rajandream, ¶R Davies, ¶[‡]G. Bloomfield, the *Dictyostelium* Genome Sequencing Consortium, [‡]P. H. Dear.

‡PNAC Biotech, Laboratory of Molecular Biology, MRC Centre, Hills Road, Cambridge CB2 2QH, UK; *IMB Jena, Dept of Genome Analysis, Beutenbergstr. 11, 07745 Jena, Germany; $Baylor College of Medicine, 1 Baylor Place, Houston, TX 77030, USA; ¶The Sanger Centre, Wellcome Trust Genome Campus, Hinxton, Cambridgeshire CB10 1SA, UK

As part of the *Dictyostelium* sequencing project an accurate high-resolution HAPPY map of the *D. discoideum* AX4 genome is being made. The HAPPY map is an important aid to the sequencing centres as it defines the order of sequence contigs along each chromosome and aids gap closure (Glöckner *et al.*, Nature 418, 79-85, 2002).

HAPPY mapping is an *in vitro* PCR based technique that examines the co-segregation of sequence-tagged site (STS) markers in a panel of randomly broken genomic DNA. This technique is accurate and avoids many of the artefacts associated with physical maps.

The HAPPY map of the *Dictyostelium* genome is being assembled on a chromosome-by-chromosome basis, using a combination of STS markers supplied from the chromosome specific sequencing projects and previously mapped genes. On completion the HAPPY map is expected to consist of over 5000 markers, with an average spacing of 6.8 kb along the 34 Mb genome.

Here we report on the production of high-resolution HAPPY maps for each of the *Dictyostelium* chromosomes.

Large-scale analysis of full-length cDNAs and their start points in *Dictyostelium discoideum*

Hideko Urushihara[1], Shintaro Katayama[2], Hidekazu Kuwayama[1], Takahiro Morio[1], Shinji Obara[1], Tetsuo Ida[2], and Yoshimasa Tanaka[1]

[1]Institute of Biological Sciences, and [2]Institute of Information Sciences and Electronics, University of Tsukuba, Tsukuba-shi Japan

Isolation of complete cDNAs is an essential requirement for precise determination of gene structures and locations in the genome. To complement the gene identification by large-scale EST analysis of the *Dictyostelium* cDNA Project, we have constructed full-length cDNA libraries by an oligo-capping method from the vegetative, aggregating, migrating and culminating cells. In this paper, we report about the 28,979 full-length cDNA clones so far analyzed.

Nucleotide sequences were determined from both ends of each clone (in collaboration with Yuji Kohara at National Institute of Genetics), yielding altogether 48,001 reads. Comparison of gene expression profiles among the four libraries demonstrated that the most remarkable change occurs at the aggregation stage, which is consistent with the results of expression analysis using DNA microarray (Development 129:1543-1552, 2002). The sequence reads were assembled by the PHRAP program and clustered into 2,926 independent genes. These numbers indicate high clone redundancies in the full-length libraries due to the intrinsic bias that only cDNAs reverse-transcribed up to the 5' cap site of mRNAs were cloned. Apparently, values of full-length cDNAs are in determination of the exact gene structure rather than in the gene finding itself.

The forward sequences of full-length libraries without any ambiguous base callings at 5' ends, consisting 1,731 cDNA contigs, were used to analyze the transcription initiation sites. The relevant sequences and contigs will be called TIS-contigs and TIS-sequences, respectively. When TIS-contigs containing 2 or more TIS-sequences were examined, it was found that the transcription start sites were not 'points' but 'zones'. Although there is a possibility that the 5' end of some TIS-sequences had been artificially altered during preparation of the libraries, TISs do seem to be variable. Detailed examination showed that about one tenth of the TIS-contigs have "major" TISs where more than half of the TIS-sequences of the relevant contigs start. It should be noted, however, that despite the considerably large variation in TIS, surprisingly few TIS-contigs showed alteration in the start point of possible ORF. In addition, stage-dependent variation of TIS was observed in some TIS-contigs. If above findings are supported by further experimental analysis, we should regard broader genome regions as regulatory upstream sequences.

Dynamic organization of the actin cytoskeleton

O. Medalia, I. Weber, G. Gerisch, and W. Baumeister

Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, D-82152 Martinsried, Germany

The actin network in the cortex of *Dictyostelium* cells undergoes rapid reorganization in chemotaxis, phagocytosis, and cytokinesis. For instance, in dividing cells the actin is polymerizing at the cell poles, filaments are translocated to the midzone and are disassembled in the cleavage furrow. GFP-fusions of the Arp2/3 complex or of actin itself reveal the dynamics of reorganization on the scale of seconds.

Any procedure to preserve the ultrastructure of the membrane-anchored actin network in its transient functional state should instantly arrest the structure of the cells in order to visualize macromolecular complexes and cell organelles in their native 3-dimensional organization. We have used electron tomography of vitrified cells to reconstruct different types of actin networks together with their connections to the plasma membrane. It is planned to extend cryo-electron tomography to *Dictyostelium* mutants altered in specific regulatory proteins.

Biochemical and biological properties of tail chimeras of *Dictyostelium* myosin II

E. D. Korn, S. Shu and X. Liu

National Institutes of Health, Bethesda, MD USA

The actin-activated ATPase activity of myosin II of *Dictyostelium discoideum* is activated by phosphorylation of its regulatory light chain whereas myosin II from *Acanthamoeba castellanii* is inhibited by phosphorylation of three serines at the tip of the non-helical tailpiece at the end of the coiled-coil tail of its heavy chain. In previous studies (Liu *et al.* (2000) PNAS 97, 12553-12558), we constructed a chimera of the head of *Dictyostelium* myosin II (Dd-Wt) and the tail of *Acanthamoeba* myosin II (Ch-Ac) and, as a control, a chimera of the head of Dd-Wt and the tail of smooth muscle myosin II (Ch-Sm). Smooth muscle myosin II, like Dd-Wt, is activated by regulatory light chain phosphorylation. Unexpectedly, we found that both chimeras were about 20-fold more active than Dd-Wt and their actin-activated ATPase activities were essentially unregulated by either heavy or light chain phosphorylation. *In vitro*, Ch-Ac formed short, bipolar filaments similar to Dd-Wt (and Ac-Wt) and Ch-Sm formed very large side-polar filaments similar to Sm-Wt. In contrast to Dd-Wt, the polymerization of which is regulated by heavy chain phosphorylation, polymerization of both chimeras was unregulated.

The single myosin II of *Dictyostelium discoideum* is required for cytokinesis and growth in suspension culture, for differentiation and development beyond the mound stage, and for capping of Con A receptors. Myosin II contributes to, but is not essential for, motility and chemotaxis. In collaboration with T. Q. P. Uyeda, AIST, Japan, and C. Parent, NCI, USA, we have investigated the ability of Ch-Ac and Ch-Sm to rescue these functions of myosin II when expressed in myosin II-null cells, using expression of Dd-Wt as a control. Ch-Ac localizes to the periphery of vegetative cells, like Dd-Wt, but Ch-Sm concentrates mostly in a single cortical patch. Both chimeras localize properly to the cleavage furrow and support cytokinesis and growth in suspension culture. Both chimeras support Con A capping and co-localize with the Con A cap, like wild-type myosin, but Ch-Ac redisperses more slowly than Dd-Wt and Ch-Sm not at all. Both chimeras partially rescue motility and chemotaxis. Neither chimera, however, supports full development to fruiting bodies. We conclude: (1) neither regulated ATPase activity, nor regulated polymerization nor bipolar filaments (if Ch-Sm forms side polar filaments *in vivo* as it does *in vitro*) are required for many functions of myosin II; (2) no specific sequence may be required for localization of filamentous myosin II to the cleavage furrow; and (3) the chimeras are unable to replace wild-type myosin at either of the two developmental steps that require myosin II (Springer *et al.* (1994) Development 120:2651-2660).

Functional characterization of members of the myosin I family from *Dictyostelium discoideum*

Ulrike Dürrwang, Setsuko Fujita-Becker, Muriel Erent, and Dietmar J. Manstein

Max-Planck Institute for Medical Research, Dept. of Biophysics, Jahnstr. 29, 69120 Heidelberg, Germany

Motor domain constructs of *D. discoideum* class I myosins were analyzed using steady-state, stopped-flow, and direct functional assays. Motor domains fused to two α-actinin repeats, acting as artificial lever arm, produced fast actin-sliding velocities of up to 2 µm/s for MyoB, MyoD and MyoE at 30°C. The regulation of these class I myosins by heavy-chain phosphorylation was examined using an *Acantamoeba* myosin I heavy chain kinase (gift of Dr. E.D. Korn, NIH) to generate the activated form, λ-protein phosphatase to generate the inactivated form, and genetically activated or inactivated forms of the myosin I constructs. Phosphorylation or replacement of the TEDS site serin by a Glu-residue leads to a more than 5-fold increase of motile activity for each of the constructs examined. In comparison to *Dictyostelium* myosin II (KD = 14µM), transient kinetic measurements showed the ADP affinity of MyoB, MyoD, and MyoE to be increased. KD values are 1 µM for MyoB, 2 µM for MyoD, and 7 µM for MyoE. In the case of MyoB and MyoD actin binding strongly affects the affinity for ADP, while coupling between the actin- and nucleotide-binding sites appears to be much weaker for MyoE. KAD values of 62 µM for MyoB, 104 µM for MyoD, and 23 µM for MyoE were obtained. Additionally, MyoD displayed marked differences in its interactions with ATP and Mg^{2+} compared to class II myosins.

Structure and role of actin rods in *Dictyostelium discoideum* spores

M. Sameshima[1], Y. Kishi[1], D. Mahadeo[2], Masako Osumi[3], T. Sugo[4], and D. Cotter[2]

1 Electron Microscopy Center, The Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, Tokyo 113-8613, Japan.; 2 Department of Biological Sciences, University of Windsor, Windsor, Ontario, Canada N9B 3P4.; 3 Department of Chemical and Biological Sciences, Faculty of Science, Japan Women's University, Tokyo 112-8681, Japan.; 4 Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical School, Tochigi 329-0498, Japan

In a recent study, we found in dormant spores of *D. discoideum* that tyrosine residues on approximately 50% of actin molecules are phosphorylated (1,2). The high levels of actin phosphorylation may be required for maintenance and viability of dormant spores. The rod like structures visible by electron microscopy that can be stained with fluorescent phalloidin and an anti-actin antibody, appears in dormant spores. Since the rods are densely composed of fibers that are approximately twice as thick as classical actin filaments, this is a new type of actin rods and may contain novel factors such as actin-modulating proteins. Here, details of new rod structures were examined by employing quick freeze and high pressure freeze methods (3). We also found that one of the important enzymes for cellular methylation, S-adenosyl-L-homocysteine hydrolase (SAHH), may accumulate into the actin rods (4).

The rods first appeared in premature spores as bundles composed of straight and parallel fibers cross-linked at 10 nm intervals on average. On cross-sectional images, the fibers were found to be tubules approximately 13.0 nm in diameter arranged hexagonally. Since three electron-dense structures similar to actin filaments form the tubule wall, we term them actin tubules. Formation of the actin rods begins during late culmination stage and proceeds until two days after completion of fruiting bodies. The physical events occur in the following order; association of several modules of bundles, closely packing and decrease in diameter of actin tubules, elongation of rods across the nucleus or the cytoplasm. Shortly after exposure to conditions for germination, rods were shortened and then disappeared. In shortened rods, the actin tubules were again unpacked and arranged in a parallel and hexagonal fashion.

In spores expressing GFP-actin (linker sequence is DPGGG), only dot or short rod-like fluorescence was observed. Correspondingly the spore shape was round instead of capsule-like. Moreover, viability of the spores was reduced 5 days after the end of sporulation. In spores expressing GFP-cofilin, rod-like fluorescence was observed only in the cytoplasm. The rods were fragmented by pressure from the cover glass indicating that the actin rods may be effective in absorbing physical pressure. Thus the rods seem to be required for spores to complete the dormant state and to maintain viability.

When supernatants of spore homogenates were incubated under an actin-polymerizing condition, tubular structures approximately 13 nm in diameter, which is similar to that of the actin tubules of intact spore actin rods were reconstructed. After two additional repeat of the incubation under actin de-polymerizing and polymerizing conditions, *Dictyostelium* SAHH was concentrated in the pellets. Rod-like fluorescence of GFP-SAHH was observed both in the nucleus and the cytoplasm of the mature spores, while it diffusely distributed in the cytoplasm and the nucleus of amoeboid cells. In dormant spores SAHH my be recruited to actin rods, as a result SAHH activity could be restricted and cellular methylation is maintained to a significantly lower level than amoeboid cells.

Regulation of chemotaxis in *Dictyostelium*.

Alex Sobko, Ruedi Meilli, Susan Lee, Sylvain Merlot, Binggang Sun, Kyung Chan Park, Young-Hoon Han, Kosuke Takeda, Heidi Szemenyie, and Rick Firtel

Section of Cell and Developmental Biology, Center for Molecular Genetics, UCSD, La Jolla, CA 92093-0634

Previously we identified several pathways that regulate the formation of the leading edge and cell polarity. These include the PI3K pathway, where we have demonstrated that PI3K localizes to the leading edge, where it is actived downstream from Ras and controls the spatially-restricted localization of PH domain-containing proteins, including Akt/PKB and PhdA, work from our lab, and CRAC, work of Carole Parent and Peter Devreotes. These proteins then regulate different aspects of cell polarity and leading and trailing edge functions. We have also demonstrated that the tumor suppressor PTEN, a 3' inositol-PI(3,4,5)P3/PI(3,4)P2-specific phosphatase negatively regulates this pathway and is required for restricting the formation of PI(3,4,5)P3/PI(3,4)P2-containing lipid domains, and thus PH domain localization, to the leading edge. We have further demonstrated that MEK1 and ERK1 localize to the leading edge and are required for proper chemotaxis. We also showed that the control of MEK1's subcellular localization is regulated, in part, via chemotractant-mediated MEK1 SUMOylation. We have continued to examine the regulation of events controlling regulation at the leading edge in chemotaxing cells. I will highlight some of our recent findings and the relevance to the control of chemotaxis and other signaling pathways in vertebrate cells.

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The adenylyl cyclase ACA is localized at the uropod of chemotaxing cells

P.W. Kriebel, and C.A. Parent

Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda MD 20892-4255

In *Dictyostelium discoideum* cAMP is important in signal relay and development. Upon starvation, *D. discoideum* amoebae secrete cAMP, which is detected by G protein-coupled receptors (cARs) that specifically bind cAMP. In this system, cAMP acts as a chemoattractant and the binding of cAMP to cARs activates a multitude of signaling pathways giving rise to chemotaxis, the synthesis and secretion of additional cAMP via the activation of ACA (signal relay), and changes in gene expression. In an attempt to gain insight into the role of cAMP in chemotaxis, we sought to visualize the cellular distribution of ACA in live chemotaxing cells. We expressed the ACA-YFP fusion protein in aca- cells (ACA-YFP/aca-) and found that it rescued the developmental defect of the aca- cells and retained wild type biochemical properties. Surprisingly, microscopic imaging of ACA-YFP/*aca*- cells revealed plasma membrane labeling that was highly enriched at the uropod of chemotaxing cells. Moreover, this polarized cellular distribution was dependent on the actin cytoskeleton since the addition of inhibitors of actin polymerization led to a uniform distribution of ACA-YFP. Closer examination of ACA-YFP/*aca*- cells using confocal imaging, revealed that ACA-YFP also labeled intracellular vesicles, which appeared to traffic during chemotaxis. To further explore ACA's role in chemotaxis, the capacity of *aca*- cells to respond to chemoattractant gradients was studied. We found that *aca*- cells responded well to the gradient but failed to produce streams, which are generated when cells align in a head to tail fashion. The observations that ACA is essential for streaming and that ACA-YFP is enriched at the uropod, suggest a mechanism for streaming. In this model the release of cAMP from the posterior of the cell attracts and orients other cells so that the cells align head to tail. To further explore this hypothesis, cell mixing experiments were performed where cells expressing ACA-YFP at the posterior were mixed with *aca*- cells. We found that although both ACA-YFP/*aca*- and *aca*- cells consistently streamed to the uropods of cells expressing ACA-YFP, under no circumstances did ACA-YFP/*aca*- cells align to the back of *aca*- cells. These results support the theory that cAMP is released from the uropod during chemotaxis and streaming. Our findings show that the polarized distribution of adenylyl cyclases has impact on the biology of *D. discoideum* development. We propose that a similar distribution of adenylyl cyclases could be present in other cell types since compartmentalization of cAMP metabolism has been suggested in a variety of mammalian cells.

Inositol pyrophosphates regulate cAMP-triggered chemotaxis by modulating CRAC-PI(3,4,5)P3 interaction

Hongbo R. Luo[1], Y. E. Huang[2], C. Chen[1], A. Saiardi[1], M. Shimizu[2], E. Nagata[1], P. Devreotes[2] and S. H. Snyder[1]

1Dept. of Neuroscience, 2Dept of Cell Biology, Johns Hopkins Univ. Sch. of Med., 725 N. Wolfe St., Baltimore, MD 21205

Inositol phosphates possess multiple roles in biology. Best characterized is inositol 1,4,5-trisphosphate ($InsP_3$), a major second messenger for multiple intracellular signaling pathways. A variety of higher inositol polyphosphates exist including the recently discovered diphosphoinositol-pentakisphosphate ($InsP_7$) and bis-diphosphoinositol tetrakisphosphate ($InsP_8$) which contain energetic pyrophosphate bonds. Inositol polyphosphates have been implicated in regulation of mRNA transport from the nucleus, vesicular trafficking, apoptosis, DNA homologous recombination, and nonhomologous DNA end joining (NHEJ). However, their exact cellular and molecular functions have not been fully clarified. In this study, we used *Dictyostelium discoideum* to elucidate the molecular mechanisms of inositol polyphosphates. The concentration of inositol polyphosphates is extremely high in *Dictyostelium*, which makes it easy to label and analyze them. In fact, many inositol polyphosphates, including $InsP_6$, $InsP_7$ and $InsP_8$, were first identified in this system. The intracellular level of inositol polyphosphates can also be regulated in *Dictyostelium*. During development, a 25-fold accumulation of $InsP_7$ and $InsP_8$ was observed, indicating potential developmental functions for these inositol pyrophosphates.

After searching the *Dictyostelium* genomic sequence, We identified 3 genes containing the unique inositol-binding motif, which is shared by many inositol polyphosphate kinases. We cloned and identified one of them as $InsP_6K$. The endogenous $InsP_6K$ gene was disrupted by homologous recombination. The $InsP_6K$ null cells could no longer make $InsP_7$ and $InsP_8$. Upon starvation, *Dictyostelium* cells stop dividing and a portion of the cells secrete cAMP. Cells then form aggregate using pulses of cAMP as a chemoattractant. Cyclic AMP binds the cAMP receptor (CAR1) on the cell membrane and triggers downstream signaling pathways leading to chemotaxis. The $InsP_6K$ knockout cells displayed a higher sensitivity to cAMP stimulation, suggesting $InsP_7$ might be a regulator in the pathway triggered by the cAMP. One of essential steps in this pathway is the binding of CRAC (cytosolic regulator of adenylyl cyclase) to PI(3,4,5)P3. It was shown that CRAC binds PI(3,4,5)P3 via its PH domain. We demonstrated that $InsP_7$ can also bind the same PH domain and compete for its binding with PI(3,4,5)P3 both *in vitro* and *in vivo*. Deletion of $InsP_6K$ enhanced the binding between PI(3,4,5)P3 and CRAC, thus increasing the downstream activity after cAMP stimulation.

PI3- phosphatase, PTEN Regulates Sensing of Chemoattractant Gradients

Miho Iijima and Peter Devreotes

Department of Cell Biology, Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205, USA.

Mechanisms of gradient sensing and chemotaxis are conserved in the mammalian leukocytes and *Dictyostelium* amoebae. Both cells use G protein linked signaling pathways. Although, chemoattractant receptors and G proteins are evenly distributed along the plasma membrane, PH domains specific for PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 bind to the membrane at the leading edge of the chemotaxing cell. This suggests that the local production of these phosphoinositides through regulation of PI3Ks and PTEN phosphatases is a key mechanism for directional sensing. Translocation of specific PH domain containing proteins may control actin polymerization and pseudopod formation at the leading edge. However, the connection of the signaling events to directional movement is still unknown.

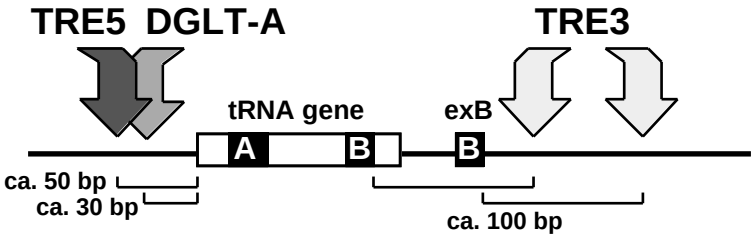
To analyze the importance of phosphoinositide, in the signaling events involved in directional movement, we disrupted a *Dictyostelium* PTEN homologue and studied its role in chemotaxis. We found that disruption of PTEN dramatically prolonged the PH domain relocation response in cells exposed to a uniform increase in chemoattractant. Parallel increases in intracellular cAMP are augmented, while increases in cGMP are unaffected. Chemoattractant-elicited actin polymerization responses are greatly increased and prolonged. In exogenous chemotactic gradients, PH domains cover a broad region at the front of the cell and F-actin filled pseudopodia are extended from a correspondingly wider region, causing the cells lacking PTEN to follow a circuitous route towards the attractant. Expressed PTEN-GFP reversed the phenotypes of the *pten*- cells and localized to the rear of chemotaxing cells. These results suggest that the regulation of PTEN is essential for efficient directional movement because specific phosphoinositides direct physiological responses such as actin polymerization to the leading edge of the cell.

Transfer RNA gene-targeted retrotransposons continue to shape the *Dictyostelium* genome

Peter Beck, Monika Baik, Oliver Siol, Theodor Dingermann and Thomas Winckler

Johann Wolfgang Goethe-Universität (Biozentrum), Institut für Pharmazeutische Biologie, Marie-Curie Strasse 9, D-60439 Frankfurt am Main, Germany

The *Dictyostelium* genome is highly packed with genes and does not leave much space for molecular invaders to expand and freely move from one location to another. For mobile elements such as retrotransposons a useful evolutionary strategy to integrate away from coding sequences is to selected the vicinity of tRNA genes as integration sites. tRNA genes are superior landmarks for integration of mobile elements since they are numerous, scattered on all chromosomes, and usually devoid of genes in their close neighborhood. The *D. discoideum* cells host seven non-long terminal repeat (LTR) retrotransposons (TREs) that make up about 4% of the entire genome. TREs integrate very precisely at genomic loci ca. 50 bp upstream (TRE5) or about 100 bp downstream (TRE3) of tRNA genes. In addition to the TREs the LTR retrotransposon DGLT-A is frequently found ca. 30 bp upstream of tRNA genes (see figure). The recently finished chromosome 2 sequence has allowed a close look at the distribution of TREs in the *D. discoideum* genome. The TREs have managed to spread all over chromosome 2 and occupy 75% of the tRNA loci present on this chromosome. Most tRNA genes are associated with more than one TRE. Although the structures of the TREs and their genomic distributions have been analysed in detail, the question remained whether the TREs continue to shape the modern *D. discoideum* genome by retrotransposing to new genomic locations. A genetic selection protocol recently developed in our lab allowed us to observe "jumping" TREs *in vivo. D. discoideum* cells lacking a functional wildtype UMP synthase gene were equipped with a cloned UMP synthase gene variant that was tagged with a "bait" tRNA gene. The artificial UMP synthase gene was frequently disrupted by insertions of TRE5-A and produced mutants resistent to 5-fluorouracil selection. TRE5-A appears to be the only member of the TRE family that displays significant tRNA gene-targeted retrotransposition activity in the modern *D. discoideum* genome. The relatively high retrotransposition frequency of TRE5-A (about 100 *de novo* insertions were isolated from 10^7 cells) suggests that TRE5-A retrotransposition significantly contributes to the evolution of the *D. discoideum* genome.



Construction of a gamete-specific gene pool and RNAi-mediated functional analysis in *Dictyostelium discoideum*

Tetsuya Muramoto, Katsuya Suzuki, Shinji Obara, Yoshimasa Tanaka, and Hideko Urushihara

Institute of Biological Sciences, University of Tsukuba, Tsukuba-shi 305-8572, Japan

We have been studying the sexual process of *Dictyostelium discoideum* as a model system for fertilization. To comprehend the genetic system controlling the sexual maturation and cell fusion, we are performing the extensive analysis of cDNAs from the fusion-competent cells or gametes of KAX3. However, the initial analysis of cDNA libraries revealed that a large fraction of their clones were housekeeping genes such as those for ribosomal proteins and elongation factors. In the present study, therefore, we constructed a gamete-specific subtraction library (FC-IC) to enrich the sexuality-related genes, and analyzed the cDNA clones therein.

Nucleotide sequences of all 903 clones in the FC-IC library were determined from both ends (in collaboration with Yuji Kohara at National Institute of Genetics). The obtained 1,481 sequences were clustered into 341 non-redundant cDNA fragments derived from 272 independent genes. By the determination of expression specificities by real-time RT-PCR, we finally collected 68 specific genes to construct a gamete-specific gene pool. Among them, *rasG* (1,947-fold increase) and a new Ras family gene (427-fold increase) are the top two in the entire pool. Genes with possible relevance to the sexual process such as cell adhesion proteins and mating-type related genes were also found. Although half of the specific genes were functionally annotated and demonstrated that genes categorized in ‘signal transduction’ and ‘multicellular organization’ are prevalent in the pool, the rest were completely novel sequences without any hits by homology search.

One of them, FC-IC0003, was analyzed in detail. It has a region similar to the gamete-specific hydroproline-rich glycoproteins a2 in *Chlamydomonas reinhardtii*, and is nearly 30 times more abundantly expressed in the gametes. Since this gene was found to be mating-type specific as well, we named it as *gmsA* (gamete and mating type specific gene A). In addition to the common proline-rich repeats, gmsA possesses a SCP family of extracellular domain at 5’, and a papain family cysteine protease domain at 3’ regions, and possibly locates on cell surface to mediate cell interactions. When the RNAi construct for *gmsA* was introduced into KAX3 cells, the resulted transformants showed reduction both in expression level of *gmsA* and in competency of sexual cell fusion. These results strongly suggest the involvement of *gmsA* in the sexual process of *D. discoideum.*

Axenic parasexual genetics in *Dictyostelium discoideum*

J. King and R. Insall

School of Biosciences, University of Birmingham, Edgbaston, Birmingham, UK B16 9LJ

We have developed a system whereby diploid strains of *Dictyostelium* can be readily isolated, maintained in axenic culture using recessive selectable markers, transformed and segregated to give recombinant, haploid progeny. In addition this system also allows the manipulation and replacement of genes which would prove lethal in a haploid cell.

Dictyostelium cells are normally haploid, containing 7 chromosomes in a single nucleus throughout the G1 phase of the cell cycle. In addition to this normal life cycle, *Dictyostelium* also contain elements of a parasexual life cycle in which at low frequency, two parental haploid cells fuse together to form a single diploid cell with 14 chromosomes contained within a single nucleus. These diploid cells can then either go through the normal cell cycle, producing diploid progeny or can revert to the haploid state to produce recombinant progeny.

Using this system we have been able to perform repeated rounds of recombination leading to the isolation of haploid cells with multiple gene disruptions by combining existing mutants.

In addition, the isolation of diploid strains which can be maintained in axenic culture has enabled us to disrupt one copy of the *arp2* gene (a lethal mutation in a haploid cell) and isolate a haploid strain wherein the only expressed copy is a tagged, extrachromosomal version.

Molecular phylogenetic analysis of evolutionary strategies in social amoebae

Thomas Winckler[1], Annette Bäuerle[2], Theodor Dingermann[1], Rolf Marschalek[1], Rupert Mutzel[3], and Sandra L. Baldauf[4]

1 Institut für Pharmazeutische Biologie, Universität Frankfurt/M. (Biozentrum), Marie-Curie-Strasse 9, D-60439 Frankfurt am Main, Germany; 2 Fachbereich Biologie, Universität Konstanz, D-78457 Konstanz, Germany; 3 Fachbereich Biologie, Chemie, Pharmazie, Institut für Biologie - Mikrobiologie, Freie Universität Berlin, Königin-Luise-Strasse 12-16, D-14195 Berlin, Germany; 4 Department of Biology, University of York, Heslington, York YO105DD, UK

Starving populations of Dictyostelid amoebae aggregate chemotactically into multicellular mounds that undergo morphogenesis to erect a fruiting body which consists of stalk structures supporting spheres of spores. Amoebae of species belonging to individual genera construct a variety of morphologically distinct fruiting bodies. These may or may not sacrifice part of the initial population in the formation of dead stalk cells, and coordinate chemotactic aggregation by the generation, reception, and relay of chemically widely diverse signal molecules. In order to gain insight into the evolutionary constraints behind these adaptive solutions, we have constructed phylogenetic trees based on small subunit ribosomal rRNA and protein (EF-1α) sequences. Our results suggest that early Dictyostelids communicated via a peptide-based signalling system, while cAMP and folate systems may have evolved later. Formation of cellular stalks consisting of terminally differentiated, non-viable cells, appears to be a primitive trait since *Acytostelium* species are most closely related to *Polysphondylium pallidum*. The last common ancestor to modern social amoebae therefore was a multicellular organism coordinating aggregation via a peptide acrasin, and sacrifying part of the population for the construction of a cellular stalk.

DdCP224 and DdEB1: two *Dictyostelium* MAPs with a role at the centrosome and in microtubule plus end dynamics

R. Gräf, A. Hestermann, and M. Rehberg

Adolf-Butenandt-Institut / Zellbiologie, Universität München, Schillerstr. 42, D-80336 München, Germany,

Microtubules interact with huge protein complexes not only with their minus ends but also with their peripheral plus ends. The centrosome at their minus ends nucleates and organizes the microtubule cytoskeleton whereas the microtubule plus end complex is thought to mediate interactions of microtubule tips with cortical actin and membrane proteins. We have characterized two *Dictyostelium* microtubule-associated proteins, DdCP224 and DdEB1, with a dual function since they are both centrosomal components and concentrated at microtubule tips (Gräf *et al.* (2000) JCS 113, 1747; Rehberg & Gräf (2002) MBC, in press). DdCP224 is an essential protein that belongs to the XMAP215-family. It is involved in centrosome duplication because overexpression provoked formation of supernumerary centrosomes. In order to achieve underexpression of DdCP224, the endogeous promoter was replaced by the actin-6 promoter and cells were grown at low densities in Klebsiella suspensions. Underexpression strongly reduced cell growth and resulted in a striking disappearance of supernumerary centrosomes. Thus, DdCP224 is essential and the appearance of supernumerary centrosome is dependent of the protein dosage. Moreover, underexpression, caused nocodazole hypersensitivity and delayed microtubule regrowth after drug removal. Thus, DdCP224 regulates microtubule plus end dynamics and is required for microtubule elongation. A further DdCP224 function became evident in a mutant overexpressing a GFP-fusion protein where the N-terminal half and the C-terminal fifth of DdCP224 was deleted. These mutants often contained very long, bundled microtubule that curved along the plasma membrane suggesting that DdCP224 plays a role in the interaction of microtubule tips with the cell cortex. DdCP224 and DdEB1, the *Dictyostelium* EB1homologue, colocalized at the centrosome and MT tips, and were part of the same cytosolic protein complex suggesting that they act together in their functions. DdEB1 is not only the largest known EB1-homologue but also the first that is also a genuine centrosomal component. Microscopic and biochemical analyses of tagged DdEB1 deletion mutants revealed that microtubule binding requires homo-oligomerization mediated by a coiled-coil domain. A DdEB1 null mutant was viable but retarded in prometaphase progression due to a defect in spindle formation. As spindle elongation was normal, DdEB1 seems to be required for the initiation of the outgrowth of spindle microtubules.

Signaling molecules as regulators of cytokinesis and cell motility

Jan Faix, Igor Weber* and Jibi Jacob.

A. Butenandt-Institut für Zellbiologie, LMU-Muenchen, Schillerstr. 42, 80336 Muenchen, Germany, and * Max-Planck-Institut für Biochemie, 82152 Martinsried, Germany

Motile processes in eukaryotic organisms during chemotaxis or cytokinesis are controlled by small GTP-binding proteins, PI-kinases and PH-domain containing proteins, however the underlying molecular mechanisms are still poorly understood. These proteins are components of complex signal transduction pathways that integrate and, after activation, relay extracellular and cell-cycle dependent signals to downstream targets in order to regulate actin cytoskeleton dynamics. A typical example is the actin-bundling protein cortexillin, a downstream target of activated Rac1A (Faix *et al.*, EMBO J. 20, 3705-3715, 2001). Cortexillin belongs to the α-actinin/spectrin superfamily of actin-binding proteins and its activity is crucial for cytokinesis in *Dictyostelium* cells. The IQGAP-related and Rac1-binding protein DGAP1 specifically interacts with the C-terminal actin-bundling domain of cortexillin I. This C-terminal domain of cortexillin I was previously shown to be sufficient for targeting to the cleavage furrow and rescue of cytokinesis of cortexillin I-mutants. Like cortexillin I, DGAP1 is enriched in the cortex of interphase cells and translocates to the cleavage furrow during cytokinesis. The activated form of the small GTPase Rac1A recruits DGAP1 into a quaternary complex with cortexillin I and II. In DGAP1-null mutants, a complex can still be formed with a second IQGAP-related protein, GAPA. The simultaneous elimination of DGAP1 and GAPA, however, prevents complex formation and localization of the cortexillins to the cleavage furrow. This leads to a severe defect in cytokinesis, which is similar to that found in cortexillin I/II double-null mutants. The observations defined a novel signaling pathway in which activated Rac1A and its IQGAP-related effector proteins form a quaternary complex with its down-stream target cortexillin.

The wide range of functional roles of small GTPases that are linked to the dynamic reorganization of the actin cytoskeleton in *Dictyostelium* cells, however, indicates a complex hierarchy of molecules regulating cytokinesis. In a search for the upstream components of the Rac1A/DGAP1/cortexillin pathway we have recently identified a gene that encodes a novel RacGEF (guanine nucleotide exchange factor). RacGEF-null mutants show severe defects in cytokinesis and also in cell motility. Complementation of the knock-out mutant with the complete RacGEF tagged with GFP rescues the phenotype, demonstrating the specificity of this RacGEF in these processes.

The arp2/3 complex and the control of actin polymerization in *Dictyostelium*

Simone Blagg, Jason King, Karl Saxe & Robert Insall

School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK

The seven-membered Arp2/3 complex is thought to control a large proportion of new actin polymerization during cell movement. In particular, it is thought to connect signalling to actin dynamics through the adapter proteins Scar1 and WASP. It is still not clear which actin-based processes do and do not require the Arp2/3 complex. It is also unknown how control of the Arp2/3 complex is divided between Scar1, WASP and other proteins such as CARMIL.

We have been concentrating on two aspects of the Arp2/3 complex. Firstly, we have been using multiple methods to generate loss-of-fuction mutants in members of the Arp2/3 complex. This work is complicated by the clear lethality of simple gene disruptions. Secondly, we have studied the control of the Arp2/3 complex by Scar1 and signalling. The precise roles of Scar1, and the way its function is regulated by signalling, are the subject of much current debate. One clear conclusion is that additional regulatory proteins must be bound to Scar1 during the resting state. We will describe our progress in identifying these proteins and understanding how they are regulated.

Dictyostelium Ste20-like kinases in signalling pathways to the cytoskeleton

Rajesh Arasada[1], Hyun-Ju Son[1], Ludwig Eichinger[2], Michael Schleicher[1]

1Adolf-Butenandt-Institute/Cell Biology, Ludwig-Maximilians-Universität, München, FRG; 2Institute of Biochemistry I, Medical Faculty; Universität zu Köln, Köln, FRG

We cloned and characterised two members of the *Dictyostelium discoideum* STE20-like kinase family and named them DST-1 and DST-2 (***D****ictyostelium* **ST**E-20-like kinase). Based on homology and domain structure both belong to the GCK subfamily of STE20–like kinases. DST1 displays highest similarity to SOK-1 (**S**TE20/**o**xidant stress response **k**inase-**1**) that is activated by oxidative stress. Both proteins are 75% identical in their catalytic domains and 31% identical in their regulatory domains. DST-1 phosphorylates severin, a gelsolin-like F-actin fragmenting protein (Eichinger *et al.*,1998, J.Biol.Chem.273:12952-12959).

DST-2 is most homologous to human MST-1 (**M**ammalian **ST**E20-like kinase **1**) with 71% amino acid identity in the kinase domain. DST-2 was expressed during all stages of *D. discoideum* development. *In vitro* phosphorylation assays with recombinant DST-2 and various potential substrates showed that DST-2 phosphorylates itself, myelin basic protein, as well as severin. A series of C-terminal deletions of DST-2 indicated that the regulatory domain of DST-2 contains elements that inhibit its catalytic activity. Phosphorylation experiments in the presence or absence of the catalytic subunit of cAMP-dependent protein kinase (PKA) from bovine heart showed that DST-2 was phosphorylated and activated by PKA *in vitro. In vivo*, DST-2 might therefore be a downstream target of PKA and could be involved in the regulation of the remodelling of the actin cytoskeleton via the actin-fragmenting protein severin.

Cytoskeletal regulation by the Adenomatous Polyposis Coli protein in *Dictyostelium*

Ian P. Newton, Pauline Schaap, Inke S. Näthke

Division of Cell & Developmental Biology, University of Dundee, Scotland, UK

Mutations in the Adenomatous Polyposis Coli (APC) gene are responsible for familial and sporadic colon cancer. A well-characterised function of the APC protein is its ability to bind to a large multi-protein complex that regulates the degradation of beta-catenin. Many of the truncated forms of APC found in colonic tumours of patients, are unable to target beta-catenin for degradation and the resulting increase in the activity of TCF/Lef has been implicated in the transformation that results from such APC mutations. However, the truncated forms of APC found in tumours also lack other critical domains and the importance of additional functions of APC is gaining significant recognition. In particular the ability of APC to regulate the cytoskeleton may be crucial for its role as a tumour suppressor. APC binds directly and indirectly to microtubules and increases their stability. In interphase, APC accumulates at dynamic microtubule ends to support the formation of parallel microtubule arrays. In mitosis, APC localises to microtubule ends that are embedded in kinetochores where mitotic spindles are attached to chromosomes. In addition to regulating microtubules, APC may also affect the actin cytoskeleton. A number of proteins that bind to APC also interact with actin and APC has been described in f-actin and microtubule-associated pools. The distribution of APC between these two pools appears to be finely tuned, but little is known about the physiological significance of the interactions between APC and f-actin. An intriguing possibility is that APC co-ordinates microtubule and f-actin networks. One important question that arises from these observations is how the increasingly long list of diverse functions and protein interactions assigned to APC are co-ordinated.

A number of known binding partners for APC, including GSK3 and beta-catenin (= aardvark) are expressed in *Dictyostelium* and their importance in differentiation has been well established. In addition, both the *Dictyostelium* microtubule and f-actin cytoskeleton and their associated proteins are closely related to those in other organisms and appear to be regulated similarly. These findings suggest that *Dictyostelium* is likely to contain protein(s) with similar functions to APC. However, only proteins distantly related to specific domains of APC have been identified in *Dictyostelium* so far, raising the possibility that a number of smaller proteins that are related to specific domains of APC perform the individual tasks of these domains to cumulatively provide the functions that the large APC protein carries out in other organisms.

To determine whether *Dictyostelium* can serve as an experimental system to study the functions of APC, in particular its ability to regulate cytoskeletal organisation, we expressed a number of large APC fragments or the entire APC protein in *Dictyostelium* and determined the effects on cytoskeletal organisation, cell migration and development. We found that different domains of APC slowed or abrogated normal development to varying degrees and preliminary results suggest that changes in cytoskeletal organisation may be responsible for these effects. We will present our data to confirm that *Dictyostelium* provides a useful experimental system to investigate the function of APC, particularly its ability to regulate cytoskeletal proteins. Furthermore, the high degree of similarity between the APC-binding site of beta-catenin and the corresponding regions in *Dictyostelium* aardvark predicts that aardvark can bind to mammalian APC. Thus, *Dictyostelium* may enable us to determine how the cytoskeletal functions of APC are related to its role in the regulation of beta-catenin/aardvark.

Visualizing individual steps during phagocytosis

A. Müller-Taubenberger, I. Weber, and G. Gerisch

Cell Dynamics Group, Max-Planck-Institute for Biochemistry, Am Klopferspitz 18a, 82152 Martinsried, Germany

Phagocytosis is the uptake of large particles (> 0.5 μm) into cells by a mechanism that is based on the local rearrangement of the actin cytoskeleton. In lower unicellular organisms phagocytosis is associated with food uptake, while in higher eukaryotic cells like neutrophils and macrophages it contributes to the immune response and defence against invading pathogens. During phagocytosis at least four stages can be distinguished: (1) particle adhesion via interaction with a receptor, (2) activation of a signaling cascade that finally leads to spatial remodeling of the actin cytoskeleton, (3) engulfment of the particle, and (4) removal of the actin-coat and fusion with lysosomes.

In order to gain insight into actin-based processes related to phagocytosis we tagged proteins known to regulate actin-filament dynamics with GFP. The Arp2/3 complex visualized by GFP-fusions to either Arp3 or p41-Arc, is recruited within seconds to sites where actin polymerization is induced.

DAip1 (actin interacting protein 1) enhances the activity of the actin-depolymerizing protein cofilin and localizes to phagocytic cups. DAip1-null cells are significantly impaired in phagocytosis, supporting the view that the proper turnover of actin-filaments is necessary for efficient phagocytosis.

Furthermore, we showed that double-null mutants of the ER-resident Ca^{2+}-binding chaperones calreticulin and calnexin are massively disturbed in phagocytosis, suggesting that the Ca^{2+}-storage capacity of calreticulin and calnexin might directly modulate the actin system during particle uptake. GFP-fusions of calnexin and calreticulin revealed a close transient apposition of the endoplasmic reticulum to the nascent phagocytic cup. This finding is in line with recent data acquired by mouse macrophage proteome analysis, establishing calreticulin and calnexin as constituents of phagosomes.

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Dynamics and fusion in the early endocytic pathway of *Dictyostelium*.

M. Clarke[1], J. Kohler[2], J. Heuser[3], and G. Gerisch[2]

1Program in Molecular and Cell Biology, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA; 2Max-Planck-Institut für Biochemie, D82152 Martinsried, Germany; 3Washington University School of Medicine, St. Louis, MO, USA.

We have examined two aspects of endocytosis in *Dictyostelium*. First, we have monitored trafficking of vacuolar proton pumps in the endocytic pathway. We created a fusion between VatM, the 100-kDa transmembrane subunit of the V-ATPase, and GFP. When expressed in *Dictyostelium* cells, VatM-GFP was correctly targeted to contractile vacuole and endo-lysosomal membranes, and was competent to direct assembly of the V-ATPase enzyme complex. In cells whose endosomes were pre-labeled with TRITC-dextran and then fed yeast particles, VatM-GFP was brought to newly formed yeast phagosomes as a component of the membrane of acidic endosomal vesicles, which clustered about the phagosome. VatM-GFP accumulation in the phagosome membrane and TRITC-dextran entry into the phagosome occurred with the same time course, suggesting transfer via a direct fusion of endosomes with phagosomes. Several minutes were required before the intensity of the VatM-GFP labeling of new phagosomes reached the level observed in older phagosomes, suggesting that this fusion process was progressive and continuous. VatM-GFP was retrieved from the phagosome membrane prior to exocytosis of the indigestible remnants of the yeast particle.

We have also used fluorescent fluid phase markers and GFP-labeled microtubules to visualize the uptake, dynamics, and fusion of early endosomes in *Dictyostelium.* Consecutive labeling with two fluorescent fluid phase markers demonstrated that within the first few minutes after uptake, new macropinosomes underwent fusion with pre-existing endosomes. The fusing endosomes, which represent the mixing compartment, displayed extreme shape changes and rapid transport about the cell in association with microtubules. The great plasticity of endosomes at this stage of maturation was also evident by electron microscopy. The constant undulatory motion of microtubules was implemental in establishing contact with endosomes. Treatment of cells with agents that selectively disrupted either actin filaments or microtubules confirmed that endosome dynamics are microtubule-based. Further maturation of endosomes led to loss of pleiomorphy in favor of a spherical shape, inability to fuse with new macropinosomes, and diminished motility.

In summary, pre-existing endosomes deliver both their content and membrane proteins to new phagosomes and macropinosomes. This mechanism allows efficient recycling of the V-ATPase. These interactions in the early endocytic pathway appear to involve complete fusion rather than a "kiss-and-run" mechanism.

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The *Dictyostelium* homologue of human Nramp1 is required for efficient phagocytosis and resistance to pathogenic bacteria

Barbara Peracino, Carina Skrivan[*], Alessandra Balest, Alessandra Balbo, Barbara Pergolizzi, Angelika A. Noegel[§], Michael Steinert[*] and Salvatore Bozzaro[#]

Dipartimento di Scienze Cliniche e Biologiche, Ospedale S. Luigi, 10043 Orbassano (Italy); *Institut für Molekulare Infektionsbiologie, Universität Würzburg, Röntgenring 11, 97070 Würzburg (Germany) and §Institut für Biochemie I, Universität zu Köln, Joseph-Stelzmann-Str. 52, 50931-Cologne (Germany)

The *Nramp1* gene confers on macrophages resistance to infection by endosymbiontic bacteria of the genus *Legionella, Salmonella, Mycobacteria,* and of the protist *Leishmania*. The *Nramp1* gene product is a lysosomal membrane protein with 12 putative transmembrane domains and a divalent metal transport motif. The protein appears to act as iron and manganese transporter, but its mechanism of action in pathogen infection is still unclear.

We have cloned the *Dictyostelium* homologue of human Nramp1. *Dictyostelium Nramp1* encodes a protein of 53kDa with 11 putative transmembrane domains. The overall homology with the human gene is 55%, with 90% in the putative consensus transport signature. The *Nramp1* gene is transcribed during the growth phase and downregulated upon starvation. To gain insights into its function, GFP fusion proteins with Nramp1 or the vatB subunit of the V-H^+ ATPase were expressed in cells. GFP-vatB was inserted in the membranes of all acidic compartments, in addition to the contractile vacuole system. In contrast, GFP-Nramp1 decorated membranes of only a subset of acidic vesicles. Late, but not early, endosomes and phagosomes were decorated with GFP-Nramp1, whereas GFP-vatB labeled both early and late endo- and phagosomal vesicles. Cells expressing an *Nramp1* antisense gene construct displayed reduced *E. coli, L. pneumophila* and *M. avium* phagocytosis, but were better hosts than control cells for intracellular growth of *L. pneumophyla* and *M. avium.*

The *Dictyostelium* LC-FACS protein contributes to fatty acid uptake and endocytosis

K. von Löhneysen, N. Pawolleck, H. Rühling, and M. Maniak

Abteilung Zellbiologie, Universität Kassel, Heinrich-Plett-Str. 40, 34132 Kassel, Germany

We have produced monoclonal antibodies directed against protein constituents of magnetically purified phagosomes[(1)]. One antigen that was enriched on phagosomes as compared to total cell homogenate had over 40% identity to mammalian Long-Chain Fatty-Acyl-CoenzymeA Synthetases (LC-FACS). These proteins contribute to cellular metabolism in various ways. For one, they are present in mitochondria or peroxisomes, where they mediate the beta-oxidation of fatty acids. Secondly, in the endoplasmic reticulum LC-FACS-proteins contribute to the synthesis of membrane lipids. Third, LC-FACS is involved in the uptake of fatty acids and therefore assumed to localise to the plasma membrane. Using GFP-fusions targeted to these compartments e.g.[(2)] we could not detect an overlap with the distribution of the *Dictyostelium* enzyme. Instead, the *Dictyostelium* LC-FACS associated with endocytic vesicles containing the vacuolar H^+-ATPase[(3)] minutes after their formation. Subsequently, its amount was decreased on the surface of late neutral endosomes.

Cells lacking the LC-FACS protein due to gene disruption by homologuous recombination showed a reduced accumulation of exogeneously supplied fatty acids in intracellular organelles. When assayed for their kinetic properties in endocytosis, the uptake-rates of particles and fluid were found to be similar in mutants and wild-type cells. LC-FACS mutants may require a slightly prolonged time span for transit of endocytosed marker, i.e. the time between internalisation and exocytosis.

Surprisingly phagocytosis was sensitive to TriacsinC, a potent inhibitor of LC-FACS. Therefore we considered the possibility that a genetic deficiency in LC-FACS could produce synthetic phenotypes if combined with known endocytosis mutations. Indeed, a double knockout strain lacking both LC-FACS and vacuolin, a protein from late neutral endosomes[(4,5)], showed endocytosis defects that were not observed in the corresponding single mutants. We therefore conclude that LC-FACS contributes to endocytosis in addition to its role in fatty acid uptake.

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A novel cGMP-signaling pathway mediating myosin phosphorylation and chemotaxis in *Dictyostelium*

Leonard Bosgraaf[#], Henk Russcher[#], Janet L. Smith[^], Deborah Wessels[*], David R. Soll[*] and Peter J.M. Van Haastert[#]

Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, the Netherlands; ^ Boston Biomedical Research Institute, 64 Grove Street, Watertown, Massachusetts 02472-2829, U.S.A.; * W.M. Keck Dynamic Image Analysis Facility, Department of Biological Sciences, University of Iowa, Iowa City, IA 52242, U.S.A.

Chemotactic stimulation of *Dictyostelium* cells results in a transient increase in cGMP levels, and transient phosphorylation of myosin II heavy and regulatory light chains. In *Dictyostelium*, two guanylyl cyclases and four candidate cGMP-binding proteins (GbpA-D) are implicated in cGMP signaling. GbpA and GbpB are homologous proteins with a Zn^{2+}-hydrolase domain. A double *gbpA/gbpB* gene disruption leads to a reduction of cGMP-phosphodiesterase activity and a ten-fold increase of basal and stimulated cGMP levels. Chemotaxis in *gbpA*$^{-}$*B*$^{-}$ cells is associated with increased myosin II phosphorylation compared to wild type cells; formation of lateral pseudopodia is suppressed resulting in enhanced chemotaxis. GbpC is homologous to GbpD, and contains Ras, MAPKKK and Ras-GEF domains. Inactivation of the *gbp* genes indicates that only GbpC harbours high affinity cGMP-binding activity. Myosin phosphorylation, assembly of myosin in the cytoskeleton as well as chemotaxis are severely impaired in mutants lacking GbpC and D, or mutants lacking both guanylyl cyclases. Thus a novel cGMP-signaling cascade is critical for chemotaxis in *Dictyostelium*, and plays a major role in myosin II regulation during this process.

Identification of two novel phosphodiesterases in *Dictyostelium*

Marcel E. Meima, Karin E. Weening, Daniel E. Rozen, Ricardo M. Biondi and Pauline Schaap.

School of Life Sciences, University of Dundee, MSI/WTB complex, Dow Street, Dundee DD1 5EH, UK.

We identified two homologous novel genes *PdeD* and *PdeE* that each harbour a binuclear Zn^{2+} binding domain and two cyclic nucleotide binding domains. Knockout and overexpression studies showed that *PdeE* encodes a low affinity cAMP phosphodiesterase, while *PdeD* encodes a cGMP stimulated cGMP phosphodiesterase. pdeD null mutants displayed extensively streaming aggregates, prolonged elevation of cGMP levels after chemotactic stimulation and reduced cGMP-PDE activity. This phenotype is very similar to that of the streamer F mutants, which are defective in a cGMP stimulated cGMP phosphodiesterase activity. *PdeD* transcripts were lacking in *stmF* mutant NP377, indicating that this mutant carries a *PdeD* lesion. Expression of a YFP-PdeD fusion protein in pdeD null cells restored the normal cGMP response. When purified by immunoprecipitation, the YFP-PdeD fusion protein displayed cGMP-PDE activity, which was retained in a truncated construct that contained only the binuclear Zn^{2+} binding domain. The cyclic nucleotide binding domains of PdeD most likely mediate allosteric activation of phosphodiesterase activity by cGMP.

The regulation of cell behavior in the natural wave

David R. Soll

Department of Biological Sciences, University of Iowa, Iowa City, Iowa 52242 USA.

Using a newly developed contextual framework for interpreting the behavioral defects o of mutants in basic motile behavior and chemotaxis, a set of protocols testing these behaviors, and computer-assisted 2D and 3D dynamic image reconstruction and analysis systems, a number of regulatory and cytoskeletal mutants have recently been analyzed. The results of these analyses suggest parallel dependent pathways emanating from the different phases of the wave, and distinctions between molecular components playing a role in basic motile behavior and molecular components playing roles exclusively in upstream regulatory pathways emanating from the different phases of the wave. A number of characterized mutants will be considered including *regA*$^{-}$, *REGA* point mutations, *pkaR*$^{-}$, *scar*$^{-}$, *rasC*$^{-}$, *Ddmek1*$^{-}$, *aar*$^{-}$, *torA*$^{-}$, myo I *mutants (myoA*$^{-}$, *myoB*$^{-}$, *myoC*$^{-}$, *myoA*$^{-}$*/B*$^{-}$, *myoB/C*$^{-}$, *myoF*$^{-}$, *myoA*$^{-}$*/F*$^{-}$), myosin RLC phosphorylation mutants, myosin light chain kinase A deletion mutant, myosin heavy chain phosphorylation mutants, *mhc*$^{-}$, cGMP binding proteins, etc. All of these analyses have been done in collaboration (W.F. Loomis, A. Kuspar, C. Saxe, G. Weeks, R. Firtel, A. Harwood, P. Devreotes, M. Titus, R. Chisolm, E. Smith, P. van Haastert). The results of these studies suggest unexpected relationships between particular regulatory pathways and target processes involved in the basic motile behavior of a cell.

rasC null mutants exhibit defects in cellular translocation and pseudopod formation during basic motile behavior and chemotaxis

Deborah Wessels [1*], David R. Soll [1], Tien Pham[1], Spencer Kuhl [1], Ryan Keller [1], Rebecca Brincks [1], C. James Lim [2], Karl Zawadzki [2] and Gerald Weeks [2]

1Dept. Biological Sciences, University of Iowa, Iowa City, IA 52242 USA and 2Department of Microbiology and Immunology, University of British Columbia, Vancouver B.C. V6 TIZ3, Canada

Cellular translocation is a highly complex process that, even in the absence of chemotactic signals, requires activation of specific pathways that ultimately impinge upon a full repertory of cytoskeletal elements. Moreover, receptor mediated behaviors are modulated by signals transduced from the cell surface cAMP receptors to the cytoskeleton, and these pathways are not necessarily involved in the basic motile behavior. We have designed an experimental protocol to determine if a particular signal transduction or cytoskeletal molecule functions in basic motile behavior, and/or the spatial or temporal component of the natural cAMP wave (1). Here we present work on cells in which the *rasC* gene has been disrupted (2). Six *Dictyostelium* proteins of the Ras subfamily have been previously described, but only one of these, RasC, has been implicated as the Ras protein required for aggregation and chemotaxis (2). Using 2D-DIAS and 3D DIAS, we have investigated the possible role of RasC in basic motile behavior (i.e., motility in the absence of an extracellular cAMP signal), as well as in chemotaxis. We determined, first of all, that this protein is required for normal pseudopod extension and translocation during basic motile behavior. Furthermore, we have found defects in the rate of cellular translocation and cell shape even when cAMP pulses are exogenously applied to suspension cultures of *rasC* null cells. The role of *rasC* in receptor-mediated responses including the behavior in spatial gradients of cAMP and simulated temporal cAMP waves will be described.

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Ca^{2+}/calcineurin-mediated up-regulation of a novel gene family in *Dictyostelium*

Barrie Coukell, Yi Li, and Anne Cameron

Department of Biology, York University, 4700 Keele St., Toronto, Ontario, CANADA, M3J 1P3

Although free intracellular Ca^{2+} is an important regulator in eukaryotic cells, elevated levels of this ion can be toxic. Consequently, cells have evolved a variety of mechanisms to maintain intracellular Ca^{2+} homeostasis in the presence of high extracellular Ca^{2+}. Previously, we cloned and characterized the *Dictyostelium* gene patA that encodes the P-type Ca^{2+} ATPase pump, PAT1. When amoebae are exposed to high external Ca^{2+}, patA expression is up-regulated dramatically via a calcineurin-dependent mechanism and PAT1 becomes associated with the contractile vacuolar membrane (Moniakis *et al.*, J. Cell Sci. 112, 405, 1999). To identify other Ca^{2+} up-regulated genes in *Dictyostelium*, we used suppression subtractive hybridization with RNA extracted from vegetative cells either untreated or treated with 80 mM $CaCl_2$ for 3 h. Of the 10 differentially expressed clones isolated, 5 detected similar mRNAs on Northern blots that were elevated ~ 40-fold in the Ca^{2+}-treated cells. Southern blot analysis as well as cDNA and gDNA databases searches revealed that these transcripts are products of a new gene family with at least 8 members. These genes have been designated *cupA-H* (Ca^{2+} up-regulated) and the corresponding proteins CUP1-8. Western blot analysis of Ca^{2+}-treated cells using polyclonal antibodies raised against the N-terminal half of CUP1 detected the up-regulation of 3-5 proteins with molecular masses of 70-78 kDa. An alignment of the deduced amino acid sequences of the CUP proteins indicated that they are >80 % identical. CUP proteins are acidic (pI = ~4.7) and contain two ricin (carbohydrate-binding) domains in the N-terminal half of the molecules. Results of cell fractionation and immunofluorescence studies suggested that the CUP proteins are largely soluble and cytosolic. BLAST searches failed to reveal closely related genes or proteins in other organisms. Induction experiments indicated that *cup* gene up-regulation by Ca^{2+} is quite specific; no up-regulation was detected by other ions (except weakly by Mn^{2+}), heat shock, osmotic stress or oxidative stress. Moreover, like *patA*, Ca^{2+}-mediated *cup* induction is completely abolished by inhibitors of calcineurin (cyclosporin A or FK506) and protein synthesis. Together, these results suggest that the CUP proteins are members of a family of Ca^{2+}-shock proteins in *Dictyostelium* up-regulated by the same mechanism as PAT1. Experiments are in progress in an attempt to understand the function(s) of these proteins in normal and in Ca^{2+}-stressed cells. (Supported by the NSERC of Canada)

W 7, a calmodulin antagonist, enhanced cAMP oscillations and caused release of stored calcium.

D. Malchow, D.F. Lusche and C. Schlatterer

Faculty of Biology, University of Konstanz, D-78457 Konstanz, FRG

To find out whether Ca^{2+} is involved in the regulation of cAMP oscillations, we applied the calmodulin antagonist W 7 at the onset of cAMP oscillations to cells in suspension. It turned out that W 7, in contrast to calmidazolium, another calmodulin antagonist, enhanced cAMP synthesis. Moreover, W 7 caused alterations in Ca^{2+}- and H^{+}-fluxes that can be explained in part by the inhibition of the V-type H^{+} ATPase activity.

Since the W 7-induced release of stored Ca^{2+} was biphasic we asked whether W 7 acts on both the acidic Ca^{2+}-store and the endoplasmic reticulum. We used 2-APB, a drug known to block the store-operated Ca^{2+} channel I_{CRAC} and to bind non-cooperatively to the $InsP_3$ receptor, thereby reducing $InsP_3$-mediated Ca^{2+}-release. In *D. discoideum* we found that 2-APB blocked the cAMP-induced Ca^{2+}-influx and $[Ca^{2+}]$i-elevation and it reduced W7-induced Ca^{2+} release. Since the action of 2-APB was time dependent and 2-APB caused release of stored Ca^{2+} in vesicle suspensions, we conclude that the target of 2-APB is the $InsP_3$-receptor and not the plasma membrane Ca^{2+} channel I_{CRAC}. When 2-APB was applied to oscillating cell suspensions in the presence of W 7 cAMP oscillations stopped.

These results show that W 7 acts on both types of Ca^{2+} stores and that a functional $InsP_3$-receptor is required to allow for W 7-enhanced cAMP synthesis.

Prolyl oligopeptidase and inositol phosphate metabolism during early development

R.S.B. Williams[*1], E. C Dalton[1], B. Serreck[2], G.W. Mayr[2] and A.J. Harwood[1]

1MRC-LMCB, University College London, Gordon St. LONDON, WC1E 6BT, UK and 2Institute for Physiological Chemistry, University Hospital Eppendorf, University of Hamburg D20246, Germany
*Present address: Wolfson Institute for Biomedical Research, University College London, Gower St London, WC1 E6BT

Inositol (1,4,5) trisphosphate ($InsP_3$) is produced by hydrolysis of phosphatidyl (4,5) bisphosphate (PIP_2) in the plasma membrane through the action of phospholipase C (PLC). $InsP_3$ however is also generated by dephosphorylation of $InsP_6$ via a pathway present in both *Dictyostelium* and mammalian cells. We have previously found that this pathway is up-regulated after loss of the *Dictyostelium* prolyl oligopeptidase (*dpoA*; 1). Such mutants are resistant to the inositol depeleting drugs lithium and valproic acid (2). DpoA inhibition leads to a rapid increase in $InsP_3$, suggesting a direct interaction between peptidase activity and InsP metabolism. Although we can find no evidence for short term regulation of DpoA, we find that *dpoA* transcription and enzyme activity is regulated throughout long term development.

To investigate the interaction between DpoA and InsP metabolism, we have used metal dye detection hplc to compare the InsP content of wild type, $_{dpoA}$ mutant and DpoA overexpressing cells. We find that changes in DpoA activity lead to gross changes in both absolute mass and proportion of all InsP species. Analysis of these results suggests that DpoA may act as a “master regulator” of InsP metabolism.

Lithium and VPA treatment both perturbs InsP metabolism and retards aggregation. To understand the importance of InsP metabolism during aggregation, we have analysed chemotaxis and cell movement in lithium treated cells. We find that lithium treatment does not block movement or chemotaxis, but causes cell rounding and poor persistance when moving to a localised cAMP signal.

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Are there two types of calcium channels in the plasma membrane?

Christina Schlatterer, Ralph Schaloske*, Karen Bezares-Roder, Dieter Malchow

Faculty of Biology, University of Konstanz, 78457 Konstanz, FRG; *Dept. of Chemistry & Biochemistry, University of California at San Diego, La Jolla CA 92093-0601, USA

The *iplA* knockout mutant was observed to form fruiting bodies and displays normal cAMP-activated cAMP and cGMP elevations in the absence of $45Ca^{2+}$-entry or detectable changes in cytosolic free [Ca^{2+}] [1]. These results challenged the view that Ca^{2+} signalling is essential for chemotaxis [2]. It is not clear whether the *iplA* gene product represents a Ca^{2+} channel in the plasma membrane or an IP_3-receptor-like channel located in the membrane of the ER. We analyzed the mutant with respect to its development and its Ca^{2+}-fluxes, both across the plasma membrane and membranes of stores.

Aggregation of mutant cells was delayed as compared to wild type. Single aggregation strands broke up leading to formation of multiple, smaller mounds and fruiting bodies. Incubation with EGTA or Ca^{2+} affected the aggregation pattern of both wild type and mutant. In the presence of EGTA, aggregation was further delayed; by contrast, incubation with Ca^{2+} accelerated the aggregation of the mutant compared to wild type.

We studied Ca^{2+}-fluxes in cell suspensions with a Ca^{2+}-sensitive electrode and in single cells by Mn^{2+}-quenching of Fura2-dextran fluorescence. cAMP evoked influx of Ca^{2+} in cell suspensions. Entry was small as compared to wild type: it amounted to 2-5% of influx of Ax2. Arachidonic acid also evoked Ca^{2+}-entry. In suspensions of permeabilized cells, cAMP or arachidonic acid released stored Ca^{2+}. Similar to electrode recordings, when Mn^{2+} was used to assess Ca^{2+}-influx into intact amoebae, both basal and cAMP-stimulated Mn^{2+}-entry occurred, yet higher doses of Mn^{2+} were required than in wild type. However, when cells were preincubated with EGTA which empties intracellular stores, both basal and cAMP-induced Mn^{2+}-entry were strongly activated. Moreover, entry of Mn^{2+} was effectively suppressed by preincubating amoebae with the IP_3-receptor antagonist 2-aminophenoxyborane (2-APB) which blocks release of Ca^{2+} from stores.

Assaying vesicular Ca^{2+}-fluxes, we found similar Ca^{2+}-uptake in the mutant as in wild type. Addition of both the calmodulin antagonist W7 and of IP3 led to slow sustained release of stored Ca^{2+}.

From our data we conclude that the *iplA* mutant possesses an IP_3-responsive Ca^{2+} storage compartment. cAMP activates release of stored Ca^{2+} and reduced entry of Ca^{2+}. These Ca^{2+}-fluxes regulate chemotaxis and aggregation. Either the coupling of the emptied store to the plasma membrane is disturbed leading to reduced Ca^{2+}-entry or the mutation directly affects a plasma membrane Ca^{2+}-channel which can be compensated by a second type of channel permeable to both Ca^{2+} and Mn^{2+}.

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A single cell-density sensing factor stimulates distinct signal transduction pathways through two different receptors

William J. Deery, Tong Gao, Robin Ammann, and Richard H. Gomer

Howard Hughes Medical Institute, Department of Biochemistry and Cell Biology, MS-140, Rice University, Houston, TX 77005-1892 USA

When *Dictyostelium* cells starve, they begin secreting a glycoprotein called conditioned medium factor (CMF). When there is a high density of starved cells, as indicated by a high concentration of CMF, the cells begin expressing some genes and aggregate using pulses of cAMP as a chemoattractant. CMF regulates gene expression via a G protein-independent pathway, while CMF regulates cAMP signal transduction via a G protein-dependent pathway. To elucidate receptors mediating cell density sensing, we used CMF-sepharose to isolate membrane proteins that bind CMF. We identified a 50 kD protein, CMFR1, that is sensitive to trypsin treatment of whole cells. We obtained partial amino acid sequence of CMFR1 and isolated the cDNA encoding it. The derived amino acid sequence has no significant similarity to known proteins, and has two or three predicted transmembrane domains. Expression of CMFR1 in insect cells caused an increase in CMF binding. Repression of CMFR1 in *Dictyostelium* by gene disruption resulted in a ~50% decrease of the CMF binding, and a loss of CMF-induced G protein-independent gene expression. The G protein dependent CMF signal transduction pathways appear to be functional in *cmfr1* cells, suggesting that cells sense the density-sensing factor CMF using two or more different receptors. Using recombinant fragments of CMF, we find that stimulation of the IP_3 pathway requires amino acids 170 – 180 whereas SP70 accumulation does not, corroborating a two-receptor model. Cells lacking CMFR1 do not aggregate, due to the lack of expression of several important early developmentally regulated genes, including *gp80*. Although many aspects of early developmental cAMP-stimulated signal transduction are mediated by CMF, CMFR1 is not essential for cAMP-stimulated cAMP and cGMP production or Ca^{++} uptake, suggesting the involvement of a second CMF receptor. Exogenous application of antibodies against either the region between a first and second or a second and third possible transmembrane domain of CMFR1 induces SP70 accumulation. Antibody- and CMF-induced gene expression can be inhibited by recombinant CMFR1 corresponding to the region between the first and third potential transmembrane domains, indicating that this region is extracellular and likely contains the CMF binding site. These observations support a model where a one- or two-transmembrane CMFR1 regulates gene expression and a G protein-coupled CMF receptor mediates cAR1 signal transduction.

A Phospholipase D regulates quorum sensing in *Dictyostelium discoideum*

Yi Chen, Vanessa Rodrick, Yi Yan, and Derrick Brazill

Department of Biological Sciences, Hunter College, 695 Park Avenue, New York, NY 10021,USA.

The ability of animal cells to sense the density of the cells around them plays an important role in cellular growth control and differentiation. Without such a quorum sensing ability, a developing embryo would be unable to properly proportion its cells into different tissue types. However, studying this phenomenon in animals is difficult due to their complexity and genetic intractability. Therefore, we examine quorum sensing in *Dictyostelium discoideum*. Starving *Dictyostelium* cells are able to calculate the concentration of starving cells by simultaneously sensing and secreting a glycoprotein called conditioned medium factor (CMF). When the number of starving cells is low, the level of CMF is also low. This prevents signaling through the chemoattractant cAMP receptor cAR1, and aggregation does not occur. When the number of starving cells is high, the level of CMF is also high. This allows signaling through cAR1 to proceed, and aggregation ensues. Binding of cAMP to cAR1 activates a heterotrimeric G protein whose alpha subunit is Gα2. We have shown previously that CMF regulates cAMP signal transduction in part by decreasing the GTPase activity of Gα2. CMF exerts its effect by activating a signaling pathway that involves Gα1, phospholipase C and protein kinase C. We have currently been examining the role of phospholipase D (PLD) in CMF signaling. We have identified the gene *pldB*, which has homology to mammalian PLD1. *pldB* expression can be seen by 8 hours of development and peaks at 16 hours. PldB protein expression parallels gene expression. We have disrupted the pldB gene by homologous recombination and find that the cells are able to chemotax and form fruiting bodies similar to wild type cells. Interestingly, these cells develop rapidly and generate fruiting bodies within 14 hours, suggesting that *pldB* may be involved in controlling the timing of development. In addition, cells lacking *pldB* aggregate at very low cell density, arguing that *pldB* is a negative regulator of quorum sensing.

Genetic modules expressed during early development of *Dictyostelium*

Negin Iranfar, Danny Fuller, and William F. Loomis with the cooperation of the Japanese EST Project

Cell and Developmental Biology, Division of Biology UCSD, La Jolla, CA 92093

We have carried out expression analyses on cells developed either on solid supports or in shaken suspension. Cy5 labelled probes were prepared from RNA isolated at two hour intervals throughout development and compared to Cy3 labelled probes from time averaged RNA prepared by pooling samples from different stages in development as previously described (Iranfar *et al.*, 2001). The samples were simultaneously hybridized to microarrays carrying 690 previously characterized targets from developmental genes and an additional 5,655 cDNA targets from the Japanese EST Project (Morio *et al.*, 1998). When wild type AX4 cells in suspension were given 30 nM pulses of cAMP at 6 minute periods for four hours and then treated with high concentrations of cAMP, the expression profiles were very similar to those seen in cells developed on filters up to the culmination stage. We have focused on a non-redundant set of 125 targets that showed robust developmental signals in pulsed cells.

Nine genes were found to be expressed immediately following the initiation of development in the presence or absence of cAMP pulses. The products of these genes include ACA, CAR1, Gα2 and RegA, that together establish the oscillatory network that generates cAMP pulses. A second module of 17 genes was found to be expressed 2 hours later, but only if the cells were pulsed with cAMP. This module includes *lagC*, *pgmA* and genes encoding several calcium binding proteins. Expression of later genes was dependent on the pulse activated adenylyl cyclase ACA. These 109 genes will be separated into modules on the basis of their profiles in mutant strains.

Cells lacking both adenylyl cyclases ACA and ACR expressed genes of module 1 in the presence or absence of cAMP pulses but did not express genes of module 2 even when pulsed. However, module 2 genes were expressed in the double mutant if the catalytic subunit of PKA was overexpressed. When developed on filters, these $acaA^{-}$ $acrA^{-}$ $pkaC^{over}$ cells expressed all but 13 developmental genes on the microarrays. Most of these late genes are prespore specific and included *acbA* which encodes the signal peptide SDF-2.

Beyond development: transcriptional profiling of the *Dictyostelium* cell cycle, spore germination and de-differentiation

Chad Shaw[1], Nancy Van Driessche[1,2], Miroslava Ibarra[1], Sujata Sharma[1], Ezgi Okyay[1,4], Takahiro Morio[1,5], Mariko Katoh[5], Hideko Urushihara[5], Yoshimasa Tanaka[5], Junji Chida[6], Aiko Amagai[6], Yasuo Maeda[6], Dana Mahadeo[7], David Cotter[7], Adam Kuspa[1,,2,3] and Gad Shaulsky[1,,2,4]

1. Department of Molecular and Human Genetics, 2. Graduate Program in Developmental Biology, 3. Department of Biochemistry and Molecular Biology, 4. Graduate Program in Structural & Computational Biology and Molecular Biophysics; Baylor College of Medicine, Houston, TX, USA; 5. Institute of Biological Sciences, University of Tsukuba, Tsukuba, Japan; 6. Department of Developmental Biology and Neurosciences, Graduate School of Life Sciences, Tohoku University, Aoba, Sendai, Japan; 7. Department of Biological Sciences, University of Windsor, Ontario, Canada.

We have used microarrays to follow the transcriptional profiles of *Dictyostelium* cells as they progress through the cell cycle after cold-shock synchronization and as they starve at various points in the cell cycle. We found a large number of genes that are regulated during the cell cycle, some of which are similar to classical cell cycle genes from other organisms. We also found that most of the cell cycle regulated genes are up-regulated after about 12 hours of development. This finding is consistent with the observation made by G. Chen and A. Kuspa (see abstract) that G2-phase prespore cells undergo mitosis and exhibit G1 phase DNA content at that period.

We followed the transcriptional profiles of *Dictyostelium* during spore germination after activation by heat activation or by DMSO treatment. We found several groups of genes with peaks of expression during the process. Three of the major peaks correspond to the three morphological stages in germination: activation, swelling and emergence of amoebae. The remaining groups suggest the existence of yet unidentified stages in spore germination. The most striking observation we made is a large change in the level of gene expression immediately after the activation event, several hours prior to spore swelling and the emergence of amoebae.

We followed the transcriptional profiles of *Dictyostelium* cells during de-differentiation (erasure) from the aggregation stage, the finger stage and the Mexican hat stage of development. We found a large number of genes that were expressed with specific erasure patterns, regardless of the developmental stage, and that many of the erasure-induced genes were regulated during normal development as well. These results indicate that the process of erasure is regulated by a dedicated pathway. The expression of erasure genes during development may reflect an evolutionary adaptation to the possibility that developing cells may become disaggregated prior to culmination.

Microarray phenotyping of the PKA and YakA pathway

Nancy Van Driessche[1],[2],[*], Chad Shaw[1],[*], Sujata Sharma[1], Miroslava Ibarra[1], Trushar Surang[1], Adam Kuspa[1],[2],[3] and Gad Shaulsky[1],[2*] equal contribution

1. Department of Molecular and Human Genetics, 2. Graduate Program in Developmental Biology, 3. Department of Biochemistry and Molecular Biology; Baylor College of Medicine, Houston, TX, USA

Development in *Dictyostelium* is characterized by a series of highly regular sequential steps that occur with nearly invariant timing and eventually give rise to a fruiting body. We tested whether expression patterns can reflect the developmental physiology of *Dictyostelium* development and found that the temporal relationship between the RNA samples, isolated from developing cells at different developmental stages is conserved, and that transition from unicellularity to multicellularity is accompanied by the biggest change in the expression of the genes in the genome (Van Driessche N. *et al*., 2002). More recently, we defined six expression modes that characterize *Dictyostelium* development, representing events such as cAMP-mediated aggregation, culmination and sporulation. We are defining modes that reflect the dependent sequence of *Dictyostelium* development.

Different mutants may have the same morphology or other common characteristics. To test the idea that expression patterns can be used as a high resolution phenotype, we analyzed several mutants from the PKA and YakA pathway. Cells were collected at two-hour intervals during the 24 hours of the development, RNA was isolated and tested with the micro-array. We found that agg$^-$ mutants like *pkaC*$^-$*, acaA*$^-$ *and yakA*$^-$ do not down-regulate hundreds of genes that are expressed during the growth phase and fail to induce developmental genes. By analyzing the *pkaR*$^-$, *regA*$^-$ and *pufA*$^-$ mutants we found that physiological parameters such as cAMP-dependent or independent aggregation and precocious spore formation can be deduced from the array data. Preliminary results also show that transcriptional profiles reflect epistatic relations between genes. We generated several double mutants such as *yakA*$^-$ *pufA*$^-$*,; pkaR*$^-$ *regA*$^-$; and *pufA*$^-$ *pkaR*$^-$, and compared their transcriptional profiles to those of the single mutants. We found that in general, expression patterns are sufficient to reconstruct epistatic relations.

Van Driessche N. *et al.* (2002) A Transcriptional Profile of Multicellular Development in *Dictyostelium discoideum*. Development 129, 1543-1552.

Prespore cell-cycle arrest during *Dictyostelium* development

Guokai Chen[1], Gad Shaulsky[2] and Adam Kuspa

Departments of 1Biochemistry and Molecular Biology, and 2Molecular and HumanGenetics, Baylor College of Medicine, One Baylor Plaza, Houston TX 77030.

To clarify the cell cycle state of developing *Dictyostelium* cells we followed cell division while monitoring new DNA synthesis and the DNA content of Ax4 cells during development. In the first 6 hours, cell numbers increased ~20%. This increase could be accounted for by the resolution of multi-nucleated cells into mono-nucleated cells by cytokinesis, as the nucleus/cell ratio decreased from 1.28 to 1.08 in the first 6 hours. Between 12 to 20 hours of development the total number of cells increased ~60%, consistent with what has been reported for the axenic strain Ax2 (1). We monitored the synthesis of chromosomal DNA, rDNA and mtDNA by monitoring BrdU incorporation. Cells were allowed to develop on filters layered over cellulose pads saturated with starvation buffer containing BrdU. High molecular weight DNA was harvested from filter-developed cells and from purified spore or stalk cells. DNA synthesis was detected by resolving the three major cellular DNAs by pulsed-field gel electrophoresis and BrdUspecific antibody staining after Southern transfer. Our results are consistent previous BrdU incorporation studies where it was observed that the mtDNA is the only cellular DNA that incorporates BrdU during development (2). The lack of significant incorporation of BrdU into chromosomal DNA or rDNA indicates that cells do not synthesize nuclear DNA at anytime during development. These results suggest that more than half of the cells undergo mitosis between 12-16 hours of development without initiating a new round of DNA synthesis and therefore must complete development as G1-arrested cells. We examined DNA content by flow cytometry on whole cells since our analyses of nuclei led to unreliable and aberrant DNA content profiles. Between 6-20 hours the average whole-cell DNA content decreased ~40%, while rDNA and mtDNA did not decrease during this same time interval as determined by quantitative Southern blots. Spores were found to have ~48% the DNA content of growing cells. We then monitored the DNA content in prespore and prestalk cells by FACS and flow cytometry with the cell-specific reporters *cotB/GFP* and *ecmA/GFP*. The DNA content of *cotB/GFP*-positive prespore cells decreased from 12-20 hours of development while that of *ecmA/GFP*positive prestalk cells did not decrease. To confirm that prespore cells undergo differentiation into spores while in G1, we monitored BrdU incorporation in germinating spores. We observed significant BrdU incorporation into chromosomal DNA after germination but prior to the first cell division indicating that chromosomal synthesis precedes mitosis as emerging spores initiate vegetative growth. Our results indicate that prespore cells divide and arrest in the G1 phase of the cell cycle prior to terminal differentiation.

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A homologue of Cdk8 required for optimum growth, aggregation and spore cell differentiation.

Hsiu-Hsu Lin, Hao-Jen Huang[3], Christine Michaelis[2], Gerry Weeks[2] and Catherine Pears

Biochemistry Department, Oxford University, South Parks Road, Oxford OX1 3QU, England; 2 Department of Microbiology and Immunology, University of British Columbia, 300-6174 University Boulevard, Vancouver, British Columbia, Canada V6T 1Z3;
3 Current address:- Department of Biology, National Cheng-Kung University, 701, Tainan, Taiwan.

The Cdk8 proteins are kinases which can phosphorylate the carboxy terminal domain (CTD) of RNA polymerase II and are, therefore, involved in the regulation of transcription. We have characterised a Cdk8 homologue from *Dictyostelium*. Like its mammalian counterpart, the protein is part of a high molecular weight complex localised in the nucleus and, when immunoprecipitated from *Dictyostelium* cells, is capable of phosphorylating a CTD peptide. Analysis of the null strain revealed that DdCdk8 activity plays a role during both growth and development. *Ddcdk8*$^-$ cells grew slowly and failed to form aggregates on starvation in conditions where parental cells aggregated readily. This was accompanied by altered expression patterns for some, but not all, genes normally expressed in response to starvation. When plated at high cell density, especially after being pulsed with cAMP in starved suspension, *Ddcdk8*$^-$ cells formed some aggregates and some of these aggregates went on to form abnormal terminally differentiated structures. Within these structures the *Ddcdk8*$^-$ cells did not differentiate into spore cells, although stalk cell differentiation appeared unaffected. Examination of the fate of *Ddcdk8*$^-$ cells in chimaeric structures with parental cells confirmed a stalk cell preference. Altered expression of both prestalk and prespore marker genes was apparent. These results suggest that modulation of DdCdk8 activity is used to regulate the changes in gene expression which accompany the transition from growth to aggregation and the determination of cell type.

A transcriptional switch at growth/differentiation transition (GDT) of *Dictyostelium* cells: *cis*- and *trans*- elements of the *dia1* and *fkbp2* genes regulated during the GDT

Shigenori Hirose, Aiko Amagai and Yasuo Maeda

Department of Developmental Biology and Neurosciences, Graduate School of Life Sciences, Tohoku University, Aoba, Sendai, 980-8578, Japan

The precise mechanism of growth/differentiation transition (GDT) is a crucial issue to be solved in cellular and developmental biology. We have already demonstrated that *dia1*, one of genes specifically expressed in response to differentiation from the PS-point (a growth/differentiation checkpoint in the cell cycle), negatively regulates the GDT by suppressing cAMP signaling cascades (Hirose *et al.*, 2000).

Recently, the *Dictyostelium* Genome Sequencing Project has revealed the 5' flanking region of *dia1*. By search of this region, we found that there is another gene upstream of *dia1*, of which the ORF is located in the reverse direction of the dia1 ORF. After BLAST search, the gene (termed *fkbp2* by Tapparo *et al.*, 1999) encodes a homologue of FK506 binding proteins. The *fkbp2* gene is specifically expressed at the vegetative stage, and the expression is rapidly eliminated in response to deprivation of nutrients. Thus, the expression profile of *fkbp2* is inversely co-related to that of *dia1*. In other words, both of the genes hold the promoter region in common, possibly as reciprocally *cis*-acting elements. Analysis of the promoter region must offer us valuable information about how a set of genes involved in the GDT are up-regulated or down-regulated

The intergenic promoter region between the *dia1* and *fkbp2* ORFs is 605 bp long and highly AT-rich. The proximal part of *dia1* has two AGCTCGA sequences with a 67-bases insertion, while that of *fkbp2* has two similar sequences, GT(G/C)A(C/G)TC. DNA retardant assay using the promoter sequence showed the presence of a different set of DNA-binding proteins between vegetative cells and differentiating cells.

We are also attempting to establish a system to visualize the GDT of *Dictyostelium* cells with GFP and RFP.

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Evolutionary and functional implications of the complex glycosylation of Skp1, a cytoplasmic/nuclear glycoprotein associated with polyubiquitination

Christopher M. West[a], Hanke van der Wel[a], Suzanne Z. Fisher[a], Howard Morris[b], Maria Panico[b],Thanai Paxton[b], Anne Dell[b], Lee Kaplan[a], and Eric A. Gaucher[c]

[a]Dept. of Anatomy and Cell Biology, University of Florida College of Medicine, Gainesville, FL32610-0235; [b]Dept. of Biochemistry, Imperial College of Science, Technology and Medicine, LondonSW7 2AY, UK, and [c]Dept. of Chemistry, University of Florida, Gainesville, FL 32611-7200, USA

Protein degradation is regulatory for the cell cycle, gene transcription and other physiological pathways. A critical step in this highly dynamic and selective process is the initial marking of the target protein, resulting in recognition for polyubiquitination by one of a large number of E3-ubiquitin ligases. Both target marking and E3-ubiquitin ligase activity are associated with common as well as unusual posttranslational modifications. Hydroxylation of specific Proresidues marks mammalian HIF-α for rapid polyubiquitination, and also occurs in Skp1, a subunit of the SCF-class of E3-ubiquitin ligases, from *Dictyostelium*. In *Dictyostelium* Skp1, the HyPro is further modified by a chain of five sugars. These sugars are added sequentially by enzymes that reside in the cytoplasmic compartment rather than in the secretory pathway. Two of the glycosyltransferases appear to be positioned in ancient evolutionary lineages that bridge prokaryotes and eukaryotes. The first glycosyltransferase, which attaches GlcNAc to HyPro, is related in sequence and function to animal Golgi-associated mucin-type polypeptide GalNActransferases. GlcNAc is extended by a bifunctional glycosyltransferase that mediates the ordered addition of β–1,3-linked Gal and α–1,2-linked Fuc. The architecture of this enzyme resembles that of certain 2-domain prokaryotic glycosyltransferases. Mutational and pharmacological perturbation of glycosylation alters the subcellular localization of Skp1, growth properties of cells, and dominant negative effects of Skp1 overexpression on terminal morphogenesis associated with a checkpoint for spore coat formation. Complex *O*-glycosylation provides the cell with new options for epigenetic regulation in its cytoplasmic and nuclear compartments.

G protein-coupled cAMP receptor activation is regulated by hydrophobic residues near the cytoplasmic end of the receptor's third transmembrane domain

M. Zhang and D. Hereld

Department of Microbiology & Molecular Genetics, University of Texas Health Science Center, Houston, TX77030, USA

cAMP receptor 1 (cAR1) of *Dictyostelium discoideum*, a seven-transmembrane-spanning G protein-coupled receptor, is required during aggregation for chemotaxis, cAMP wave propagation, and cAMP-induced gene expression. In a genetic screen, we previously identified two dominant negative cAR1 mutants that block aggregation. Interestingly, both mutant receptors appear to be constitutively activated because they are constitutively phosphorylated and have elevated affinities for cAMP. Other evidence suggests that these mutant receptors block development by constantly activating normal adaptation mechanisms. Both mutations (L100H and I104N) alter neighboring hydrophobic residues at the cytoplasmic end of the third transmembrane helix (TM3), suggesting that this region of cAR1 plays an important role in regulating the receptor's activation.

To better understand this region's role in cAR1 activation, we mutated isoleucine-104 to all other possible amino acids and expressed this set of mutants independently in wild-type AX3 cells. These substitutions had varying effects on the receptors' constitutive phosphorylation, dominant negative activity, and cAMP affinity which were all positively correlated with the polarity of the substituting amino acid. In terms of constitutive phosphorylation, which was quantified as the percentage of cAR1 in the low mobility, ~43- kDa form in the absence of cAMP, the I104 mutants were broadly distributed, ranging from a few percent for the wild-type receptor and those with conservative substitutions to over 75% for highly polar substitutions such as I104K and I104R. In terms of their dominant negative activity, the mutant receptors with constitutive phosphorylation in excess of 50% (roughly one-third of the mutants) blocked aggregation, whereas the remainder had little, if any, impact on development. Taken together, these observations suggest that substitution of I104 yielded mutant receptors with varying degrees of constitutive activity but only those exceeding a specific threshold can dictate the outcome of development. This threshold is presumably determined by the competing activities of the expressed mutant and the endogenous wild-type cAR1.

Lastly, systematic I104 substitution had a dramatic impact on receptor affinity. With the exception of a few highly conservative mutations, the majority of I104 substitutions effected 10- to 100-fold elevations in cAMP affinity. The hyperbolic relationship between the apparent Kd's of the I104 mutants and their more broadly distributed extents of constitutive phosphorylation is consistent with a simple two-state model for cAR1 activation in which mutation of I104 merely affects the equilibrium between the receptor's inactive (R) and active (R*) states.

Gα9-mediated inhibition of developmental signal-response

Joseph A. Brzostowski, Carole A. Parent* and Alan R. Kimmel.

LCDB, NIDDK (Bldg. 50/3351), *LCMB, NCI, National Institutes of Health, Bethesda, MD 20892-8028

Seven-transmembrane receptor (7-TMR)-G protein networks cycle through activated (sensitized) and inhibited (de-sensitized) states, and, while many of the molecular components for signal activation have been described, inhibitory mechanisms are not well characterized. In *Dictyostelium*, 7-TM cAMP receptors direct chemotaxis and development, but also regulate the periodic synthesis of their own ligand, the chemoattractant/morphogen cAMP. We now demonstrate through loss-of-function, gain-of-function studies that the novel heterotrimeric Gα9 protein subunit regulates an inhibitory pathway during early development for cAMP signal-response.

ga9-null cells form more cAMP signaling centers, are more resistant to compounds that inhibit cAMP signaling, and complete aggregation sooner and at lower cell densities than wild-type. These phenotypes are consistent with the loss of an inhibitory signaling pathway during development of Gα9-null cells. Cells expressing constitutively activated Gα9 are defective in cAMP signal center formation and development at low cell density and display an increased sensitivity to cAMP signal inhibition that is characteristic of enhanced suppression of cAMP signal-response. In addition, when Gα9-null cells are co-developed with a majority of wild-type cells, Gα9-null cells primarily establish cAMP signaling centers and are able to non-autonomously direct wild-type cells to adopt a Gα9-null-like phenotype. We have analyzed the biochemical response of CAR1-mediated pathways including CRAC translocation, the production of cGMP and cAMP, actin polymerization and chemotaxis in a variety of Gα9-mutant cell lines. The results from these experiments suggest that, mechanistically, Gα9 mediates the adaptation/de-adaptation of the cAMP response in cells. We propose that Gα9functions in an inhibitory-feedback pathway that regulates cAMP signal center formation and propagation. Gα9may be part of the mechanism that regulates lateral signal inhibition or that modulates receptor de-sensitization.

Internalisation of G-protein coupled cAMP receptors upon stimulation followed at the single-molecule level in *Dictyostelium discoideum*.

A. Sergé[1], S. de Keijzer[1,2], M. Zhang[3], D. Hereld[3] , H.P. Spaink[2] , T. Schmidt[1] and B.E. Snaar-Jagalska[2]

1 Department of Biophysics, 2 Institute of Molecular Plant Science, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands and 3 Department of Microbiology and Molecular Genetics, University of Texas, Houston, TX 77030, USA.

Single molecule microscopy is a new approach to monitor the behaviour of single molecules *in vivo*. Our experimental set-up consists of an inverted microscope, laser excitation between 450 and 640 nanometers, and image detection on an ultra-sensitive CCD-camera. This set-up allows images of individual fluorophores within a time-frame of a few milliseconds at a signal-to-background ratio of 30. The technique, as being developed for artificial systems, has recently been extended by us to the auto-fluorescent proteins. This will enable a multitude of cell biological systems to be analysed at the single molecule level.

Here we applied this technique to the chemotactic model of *Dictyostelium discoideum*.

It is the general assumption that G-protein coupled receptors are internalised upon persistent stimulation. In *Dictyostelium discoideum*, for the cAMP receptors, this has not been visualised. We tested the internalisation hypothesis by measuring the behaviour of cAR1 receptors fused to YFP *in vivo* at the single molecule level.

We followed individual cAR1 in real time, after stimulation with 10_M cAMP. The numbers of receptors at the plasma membrane and in the cytosol were simultaneously determined, by focusing into the middle of the cell. We could show that the proportion of cytosolic receptors increases with time after stimulation. For the phosphorylation deficient mutant cm1234-YFP, the proportion of cytosolic receptors remained constant after stimulation, leading to the conclusion that phosphorylation is required for this process. Further, we found that the actin cytoskeleton is not involved in this process. Experiments are under way to determine if receptors are recycled or degraded, and if they are internalised in the ligand-bound state.

A secreted cell-number counting factor regulates metabolic pathways to control group size in *Dictyostelium*

Wonhee Jang[*], and Richard H. Gomer[*,+]

[+]Howard Hughes Medical Institute and *Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77005-1892

It is unclear how an organism can regulate the size of its components during development. *Dictyostelium* cells form relatively evenly sized groups of about $2x10^4$ cells during aggregation. A secreted 450 kDa protein complex called counting factor (CF) helps to regulate group size by inducing stream breakup when there are too many cells in a stream. Stream breakup is induced by repressing cell-cell adhesion and myosin polymerization, and increasing cAMP-stimulated cAMP production, actin polymerization, and cell motility. Countin is a component of CF, and recombinant countin appears to have the same activity as purified CF, with both inducing the formation of small fruiting bodies.

Garrod and Ashworth (1972) showed that wild-type cells grown with a high concentration (86 mM) of glucose formed larger fruiting bodies than control cells. We previously found that CF regulates group size in part by repressing internal glucose levels, which in turn regulate adhesion and motility. We have found that a one-minute exposure of cells to recombinant countin significantly reduces the levels of internal glucose. The addition of glucose does not change CF secretion, and there is not a significant amount of glucose secreted into conditioned media. Adding 1 mM exogenous glucose negates the effect of high levels of extracellular CF and mimics the effect of depleting CF on glucose levels, gp24 expression, cell-cell adhesion, cAMP pulse size, actin polymerization, myosin assembly, and motility. Addition of phosphodiesterase or pulsing cells with cAMP did not have any effect on internal glucose levels, suggesting that glucose is upstream of cAMP with respect to group size. Using computer simulations to model stream breakup, we observed that although changing adhesion alone or motility alone by these amounts only slightly increased group size, changing both adhesion and motility by these amounts increased group size by roughly the amount observed with glucose. In cells, some enzymes that make glucose are glycogen phosphorylase and amylase, which break down glycogen, and fructose 1,6-bisphophatase, which is a key enzyme in gluconegenesis. Using *smlA*$^-$ cells (which oversecrete CF), and *countin*$^-$ cells (which have very little detectable CF activity), we found that in addition to glucose the levels of glycogen, pyruvate, ATP, and oxygen consumption are negatively regulated by CF. CF also repressed the activity of fructose 1,6-bisphosphatase, and increased the activity of glycogen phosphorylase, and had no effect on the activity of amylase. These results suggest that CF uses a fast signal transduction pathway to regulate metabolism which in turn, possibly through glucose levels, regulates group size.

A STAT regulated stress-induced signalling pathway in *Dictyostelium*

Tsuyoshi Araki, Masatsune Tsujioka, Tomoaki Abe, Pauline Schaap, Takahiro Morio[+], Hideko Urushihara[+], Mariko Katoh[+], Mineko Maeda[^], Yoshimasi Tanaka[+], Ikuo Takeuchi[@] and Jeffrey G. WilliamsTsuyoshi Araki and Masatsune Tsujioka contributed equally to this work

School of Life SciencesUniversity of DundeeWellcome Trust BiocentreDow StreetDUNDEE, DD1 5EH, UK; [+] "Institute of Biological Sciences, University of Tsukuba,Tsukuba, Ibaraki 305-8572, JAPAN"; ^Department of Biology,Osaka University,Machikaneyama 1-16, Toyonaka,Osaka 560-0043 JAPAN; @Novartis Foundation (Japan) for thePromotion of Science, Roppongi, Minato-ku, Tokyo 106-0032, JAPAN

The *Dictyostelium* stalk cell inducer DIF directs tyrosine phosphorylation and nuclear accumulation of Dd-STATc. We show that hyper-osmotic shock, and several other stress factors, also activate Dd-STATc. Hyper-osmotic stress elevates intracellular cGMP levels and the membrane permeant analogue 8-bromo-cGMP activates Dd-STATc with the same rapid kinetics as osmotic shock or DIF. 8-bromo-cAMP is a less effective inducer than 8-bromo-cGMP and analysis of mutants in components in the cAMP-mediated stress-response pathway also argue against cAMP involvement. Micro-array analysis identified two genes genes that are induced by hyper-osmotic stress. Neither gene is inducible by DIF. Osmotic stress induction is, however, entirely dependent upon Dd-STATc and both genes are inducible with 8-bromo-cGMP. These data suggest that, in addition to its role as a DIF-regulated repressor of developmental gene expression, Dd-STATc functions as a transcriptional activator in a stress response pathway.

LvsA, a *Dictyostelium* BEACH protein with essential roles in cytokinesis and osmoregulation.

A. De Lozanne, W. Wu, J. Yajnik, & M. Siano

Department of Molecular Cell & Developmental Biology, University of Texas, Austin TX 78712

BEACH-domain containing proteins are a novel family of proteins with important but poorly understood roles in membrane traffic and signal transduction. We have identified six members of this family in *Dictyostelium* (see related poster) and have begun to dissect their functions. Of those, LvsA is a protein that we identified in a screen for proteins essential for cytokinesis. To better understand the function of this 408-kDa protein we tagged LvsA with GFP using recombination techniques. GFP-LvsA is primarily associated with the membranes of the contractile vacuole system and it also has a punctate distribution in the cytoplasm. Two markers of the *Dictyostelium* contractile vacuole, the vacuolar proton pump and calmodulin, show extensive colocalization with GFP-LvsA on contractile vacuole membranes. Interestingly, the association of lvsA with contractile vacuole membranes occurs only during the discharge phase of the vacuole. In LvsA mutants the contractile vacuole becomes disorganized and calmodulin dissociates from the contractile vacuole membranes. Consequently, the contractile vacuole is unable to function normally, it can swell but seems unable to discharge and the LvsA mutants become osmosensitive. These results demonstrate that LvsA can associate transiently with the contractile vacuole membrane compartment and that this association is necessary for the function of the contractile vacuole during osmoregulation.

We are currently mapping the functional contribution of different domains in LvsA to its function *in vivo*. We have also initiated a genetic screen for suppressors of LvsA mutants using D. Robinson's expression library. Interestingly, we have recently isolated an LvsA mutant strain that has been suppressed for its cytokinesis defect but not for its osmoregulation defect. This result indicates that the function of LvsA in these two processes is distinct and separable.

Regulation of *Dictyostelium* stress responses by YakA, PKA and KeaA.

Raquel Bagattini, Luciana Mantzouranis and Glaucia Mendes Souza.

Instituto de Química, Departamento de Bioquímica, University of São Paulo.glmsouza@iq.usp.br

The *Dictyostelium* protein kinase YakA is required for the growth to development transition. During growth YakA controls the cell cycle, regulating the intervals between cell divisions. When starved for nutrients *Dictyostelium* cells arrest growth and undergo changes in gene expression, decreasing vegetative mRNAs and inducing the expression of *pkaC.* YakA is an effector of these changes, being necessary for the decrease of vegetative mRNA expression and the increase of PKA activity that will ultimately regulate expression of adenylyl cyclase, cAMP synthesis and the induction of development. An additional role for this kinase includes the regulation of nitrosoative and oxidative stress responses. Hydrogen peroxide and sodium nitroprusside arrest the growth of cells and trigger cAMP synthesis and activation of PKA in a manner similar to the well established response to nutrient starvation. We have found that *yakA* null cells are hyper sensitive to nitrosoative/oxidative stress and that a second-site mutation in *pkaC* suppresses this sensitivity. Likewise, a second-site mutation in *keaA,* supresses the death induced by SNP. A similar gene has been implicated in mice in the regulation of the defenses against reactive oxygen species. In *Dictyostelium* we have found that KeaA is also a regulator of aggregation, since *keaA* null cells are deficient in this process.

More about the two programmed cell deaths of *Dictyostelium discoideum*

I.Tatischeff

Laboratoire de Physicochimie Biomoléculaire et Cellulaire, CNRS UMR 7033, Université Pierre et Marie Curie, 4, pl. Jussieu, case 138, F- 75252 Paris Cedex 05, France

Two programmed cell deaths (PCD) processes have been shown in *Dictyostelium* cells: a unicellular–specific PCD, mimicking mammalian apoptosis (1,2) and a developmental PCD occuring during the starvation–induced differentiation (3). The unicellular-specific PCD is aimed to eliminate the abnormal amoebae, which are unable to aggregate and further differentiate, whereas the multicellular-specific PCD shapes the whole differentiated organism.

To study the inter-relationship between these two PCDs, the HMX44 mutant used for *in vitro* studying of the developmental PCD was compared with the Ax-2 axenic strain used for showing *Dictyostelium* apoptosis. HMX44 cells were exposed to both experimental conditions for which an apoptotic death was observed with Ax-2 cells, i.e. aging in culture and starvation- induced death in a t22 conditioned buffer. Each cell population (HMX44 or Ax-2) was also induced to die in a t22 conditioned buffer prepared from a previous starvation of the other cell population, Ax-2 or HMX44 respectively. Thus, it was possible to observe both *Dictyostelium* PCDs together. This suggests that the type of PCD is not programmed by the cells themselves but that it is imposed on to them from extracellular signals found in their environment.

On the other hand, the last step of mammalian apoptosis corresponds to the clearing of apoptotic cells. This process is important for the *in vivo* protection of tissues from the toxic contents of dying cells. It is performed by both professional phagocytes, such as macrophages, and by neighbouring cells, which are known as "amateur" phagocytes. For *Dictyostelium*, such clearing of apoptotic cells does also occur. A few neighbouring cells, which are apparently more resistant to induced apoptosis, efficiently engulf their apoptotic congeners. The whole life cycle of the *Dictyostelium* phagocytes (growth and starvation-induced development) is sustained by eating remnants of the dead *Dictyostelium* cells. Thus, apoptotic death of most cells of an Ax-2 cloned *Dictyostelium* population might be a "germ of life" for the few emerging phagocytes surviving the stress of death. Those *Dictyostelium* phagocytes have been isolated and they keep their specific recognition of the same *Dictyostelium* cells although apoptotic. These phagocytes have been compared with the normal cells of the cloned Ax-2 population with regard to growth, aggregation, differentiation and death. Other experiments aimed to specify the nature of the phagocytic death recognition mechanism (s) are in progress. Thus, *Dictyostelium* might be of some help for further studying the less well known step of mammalian apoptosis.

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The role of cAR2 and cAR3 in differentiation and the control of movement.

Nicholas Oswald, Gerti Weijer and Cornelis J Weijer

Wellcome Trust Biocentre, University of Dundee, Dundee DD1 5EH

We are investigating the role of the various cAMP receptors in later development to better understand their role in the reception and transduction of cAMP signals to cell movement and cell differentiation. We have made GFP tagged versions of the cAR2 and cAR3 receptors and show that these constructs are fully functional. We have over expressed these receptors in various wild type and receptor null backgrounds under the control of generic A15 or prespore (psA) and prestalk (ecmA) specific promoters. We show that cAR3 is effectively downregulated in prestalk A cells at the protein level via and endocytic process. We also show that car3 does not affect the initial cell type proportioning, but plays a major role in the regulation of cell types in the slug stage. Over expression of cAR3 in prespore cells results in an increase of pstO cells and a concomitant decrease in prespore cells. This increase in pstO cells, at the expense of prespore cells is not found in the DIF methylase null mutant, suggesting that cAR3 over-expression results in and increased DIF synthesis in prespore cells resulting in their re-differentiation in pstO cells in the slug stage. Furthermore we are re-analysing the role of cAR2 in the control of cell movement and cell movement, since we find that in Ax2 and AX3 backgrounds deletion of cAR2 does not result in a mound arrest phenotype.

Receptor-dependent and tyrosine phosphatase-mediated inhibition of GSK3 regulates cell fate choice

Leung Kim and Alan R. Kimmel

Laboratory of Cellular and Developmental Biology (Bldg. 50/3351), NIDDK, National Institutes of Health, Bethesda, MD 20892-8028, USA

Asymmetric body axis formation is central to metazoan development. *Dictyostelium* establishes an anterior/posterior axis utilizing 7-TM cAMP morphogen receptors (CARs) and GSK3-mediated signal transductions that has a parallel with metazoan Wnt/Frizzled-GSK3 pathways. In *Dictyostelium*, GSK3 promotes posterior cell patterning but inhibits anterior cell differentiation. Tyrosine kinase ZAK1 mediates GSK3 activation. We now show that CAR4 regulates a tyrosine phosphatase that inhibits GSK3 activity. We have also identified essential phospho-tyrosines in GSK3, confirmed their role in activated/de-activated regulation and cell fate decisions, and relate them to the predicted 3D-structure of GSK3β. CARs differentially regulate GSK3 activity by selectively activating a tyrosine phosphatase or kinase for pattern formation. The findings may provide a comparative understanding of CAR-GSK3 and Wnt/Frizzled-GSK3 pathways.

Regulation of GskA and Aardvark during *Dictyostelium* development

J. Ryves, J Reynolds, and A.J. Harwood

MRC-LMCB, University College London, Gordon St. LONDON, WC1E 6BT, UK

The protein kinase GSK-3 and the cytoskeletal protein β-catenin are central components of the "canonical" Wnt signalling. In this signal transduction pathway, Wnt stimulation inhibits GSK-3 phosphorylation of β-catenin leading to its stablisation. Increased concentrations of β-catenin lead to changes in gene expression through its binding to members of the TCF/LEF transcription factor family.

We have isolated homologues of GSK-3 and β-catenin from *Dictyostelium*, known as *gskA* and *aardvark (aar)*. These genes interact to regulate expression of the prepore gene, *psA* (1). We will discuss new results concerning the regulation of GskA and Aar.

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Control of morphogenesis by histidine kinase signaling

Peter Thomason and Ted Cox

Molecular Biology Dept., Princeton University, Princeton, NJ 08544 USA

The first step in *Dictyostelium* culmination is morphogenesis, during which the slug begins to change shape and reorganize itself prior to forming the fruiting body. The front of the slug stops moving, allowing the rear to catch up, resulting in a shortened, fattened slug. The tip of the slug then rises off the substratum and the anterior-posterior axis of the organism changes from horizontal to vertical. This mound-like structure then compacts further to form the Mexican Hat, which normally precedes stalk elongation and culmination. We have identified a histidine kinase gene necessary for Mexican Hat formation, which we call *Sombrero* (*SmbA*). Through the use of time-lapse microscopy we find that *smbA*$^{-}$ mutants form slugs that behave normally until morphogenesis. Slugs migrate and then compact as expected, but can neither form a Mexican Hat nor extend their tip upwards from the compact mound. With no other choice, they switch their axis from vertical back to horizontal, and re-form a migrating slug. The slug tries several times to undergo morphogenesis but, unable, either arrests as a rounded structure or else culminates horizontally on the substratum. The reduced ability of *smbA* to culminate is to some extent dependent on the growth conditions of cells, with axenically grown cells having the most difficulty culminating, and cells grown in association with *Klebsiella* having the least trouble. The developmental phenotype of the *smbA* mutant is reflected in its expression of cell-type specific genes. The mutant shows greatly prolonged expression of the pre-spore gene PsA and conversely a very delayed expression of the spore gene SpiA. Spore production during development is also delayed by several hours.

SmbA encodes a histidine protein kinase predicted to have multiple transmembrane domains and it is likely that it is a receptor for a factor that regulates morphogenesis (and as a consequence, culmination and sporulation). We are currently using an *in vitro* sporulation assay to explore whether SmbA is required for the response of cells to spore differentiation factors (SDFs) and other activities present in wild-type *Dictyostelium*, which regulate morphogenesis and culmination in normal development.

Correlating the slug behavior with its internal three-dimensional cell movement

Kota Miura [1], [2]

1. JSPS fellow, Dept. of Biology, Faculty of Science, Osaka Univ., Japan; 2. Cell Biology & Biophysics, EMBL Heidelberg, Germany

Study of the phototactic behavior of *D. discoideum* slugs led to hypotheses that: a) Bending of the slug body causes the phototactic turning of slug. b) Changes in the relative position of cells bend the slug body (the Asymmetric Cell Accumulation hypothesis). c) Light induced secretion of cAMP affects the cell movement pattern and causes the asymmetric cell accumulation (*1). The second conjecture is currently under examination to know exactly how the cell movement contributes to the slug morphological changes / movement. Analysis of phototaxis mutants with a phenotype of aberrant phototaxis directionality showed that defects in the cell movement pattern does affect the slug movement. Since cells are moving three-dimensionally within slug, cell movement must be followed in the three-dimension to correlate the cell movement to slug turning. Global analysis of the multicellular movement was previously done using 2D video sequences (xy-t *2, 3) by a gradient method (the vector field method). This algorithm was extended to the three-dimension to enable the processing of 3D (xyz-t) sequences. The program was written using IgorPro programming language (Wavemetrics). The program was first tested using artificially generated animations with dots moving in three-dimensional space. Three-dimensional cell movement during slug turning was analyzed using this program. In particular, the correlation between the slug bending movement and cell movement will be discussed in the presentation.

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Differential localization and roles in mitochondrial division of two FtsZs in *Dictyostelium*

P.R. Gilson[1], X.-C. Yu[2], D. Hereld[2], C. Barth[3], A. Savage[1], S. Lay[3], P. R. Fisher[3], W. Margolin[2], and P.L. Beech[1]

1Centre for Cellular and Molecular Biology, School of Biological and Chemical Sciences, Deakin University, 221 Burwood Hwy, Melbourne, 3125, Australia; 2Department of Microbiology and Molecular Genetics, University of Texas- Medical School, 6431 Fannin, Houston, TX 77030, USA; 3Department of Microbiology, La Trobe University, Bundoora, 3083, Australia

In bacteria, FtsZ is the principle component of the Z-ring which constricts the cell at division. Though all mitochondria probably arose through a single, ancient endosymbiosis of an alpha-proteobacterium, the mitochondria of only certain protists appear to have retained FtsZ. FtsZ is absent from fungi, animals and higher-plants, organisms that use dynamin-like proteins to regulate division of their outer mitochondrial membrane. We showed that mitochondria of the chromophyte alga, *Mallomonas splendens*, contain FtsZ (Beech *et al.* 2000, Science 287:1276-1279) and have now investigated the role FtsZ plays in mitochondrial division in the genetically-tractable *Dictyostelium discoideum*. *Dictyostelium* amoebae have two nuclear-encoded FtsZs, FszA and FszB, which, when fused to the green fluorescent protein, are targeted to mitochondria. FszA-GFP (and endogenous FszA) forms puncta or belts at presumptive mitochondrial division sites. FszB-GFP, in constrast, usually localises to a subdomain within mitochondria at only one end of the organelles. Null mutations of *fszA* and/or *fszB* do not prevent mitochondrial division, though they do reduce its efficiency, producing slightly enlarged mitochondria. It is surprising that these FtsZs are not required for mitochondrial division in *Dictyostelium*, because FszA localizes to likely sites of mitochondrial division, and other FtsZs are essential for the division of bacteria and chloroplasts. It may be that dynamin-like proteins (Wienke *et al.* 1999, Mol. Biol. Cell 10: 225-243) are the dominant mitochondrial division effectors in *Dictyostelium*, and that this organism illustrates a transition from a primitive to an advanced type of mitochondrial division.

Developmental interactions of the *Dictyostelium* F-box protein ChtA/FbxA with a histidine kinase signaling pathway

David I. Ratner[1,3], Turgay Tekinay[3], Herbert L. Ennis, Mary Wu, Margaret K. Nelson[2], Jakob Franke, and Richard H. Kessin

Department of Anatomy and Cell Biology, Columbia University, 630 W. 168th St New York, NY, 10032; 1, Department of Biology, Amherst College, Amherst, MA, 01002; and 2, Department of Biology, Allegheny College, Meadville, PA 16335; 3, these authors contributed equally.

Dictyostelium amoebae altered in a gene called *chtA/fbxA*, which is thought to encode a component of an SCF E3 ubiquitin ligase, have defective regulation of cell-type proportionality. In chimeras with wild-type cells, the mutant amoebae (chtA/fbxA) form mainly spores, leaving the construction of stalks to wild-type cells. We have recovered the promoter of *chtA/fbxA* and shown that, when fused to the structural gene, it complements the mutation. The promoter, when fused to the gene coding for green fluorescent protein (GFP), shows a pattern of early expression resembling that of a prestalk-specific gene, but late in development GFP is also expressed in developing spore cells.

Because *chtA/fbxA* cells are developmentally deficient in pure culture, we were able to select suppressor mutations that promote sporulation of the original mutant. One suppressor mutation resides within the gene *regA*, which encodes a cAMP phosphodiesterase linked to an activating "response regulator" domain. RegA appears precociously and accumulates to a high level in *chtA/fbxA* mutants. In another suppressor, there has been a disruption of *dhkA*, a gene encoding a "two-component" histidine kinase known to influence *Dictyostelium* development. RegA appears precociously but is restored to wild-type levels in the *chtA/fbxA* mutant suppressed by a mutation in *dhkA*. The tendency of the *chtA/fbxA* mutant to "cheat," that is to form mainly spores in mixture with wild-type amoebae, is also suppressed by the *regA* and *dhkA* mutations.

Coding-region control of PKA and its inhibition by LRR-bearing DeliriumA

Myriam Adam,[1] Jean-Pierre Levraud,[1] Marie-Françoise Luciani,[1] Fabienne Savoy,[2] Karen Brennan,[3] Christophe Reymond,[2] Matthias Hentze,[3] and Pierre Golstein[1]

1Centre d'Immunologie de Marseille-Luminy, INSERM-CNRS-Univ.Med., Campus de Luminy, Case 906, 13288 Marseille Cedex 9, France; 2Universite de Lausanne,IBCM, Rue du Bugnon 9, Lausanne CH-1005, Switzerland; 3EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

Expression of important biological molecules is tightly regulated in particular at the mRNA level. Protein kinase A (PKA) is a major signal transducer in animal and other cells, such as in *Dictyostelium* where it plays an essential role in controlling development. Regulation of PKA-C (the catalytic subunit of PKA) expression and activity is therefore crucial and achieved at several levels in mammalian and *Dictyostelium* cells.

Insertional mutagenesis and functional screening in *Dictyostelium* led to the identification of a leucine-rich-repeat (LRR)-bearing gene we named DeliriumA (DlrA). Inactivation of DlrA resulted in block of development at an early stage. This was traced to a DlrA requirement for expression of PKA-C mRNA, which involved two components. First, in the absence of DlrA, expression of PKA-C mRNA was negatively controlled by its own coding region. Transfection of exogenous constructs showed that a nucleotide stretch within the PKA-C coding region could down-regulate to undetectable levels the mRNA expression of a cis-linked reporter sequence. Second, in the presence of DlrA, e.g. in wild type conditions, PKA-C mRNA as well as mRNA of an experimentally linked reporter sequence were normally expressed. Thus, expression of DlrA counteracted the down-regulation by the PKA-C coding region. DlrA may be a first example of a molecule counteracting, in an apparently specific manner, coding-region-mediated down-regulation of a given mRNA. Band shift experiments provided an independent demonstration of a link between DlrA and PKA mRNA, and specified a binding oligonucleotide.

These results demonstrate coding-region control of mRNA expression in *Dictyostelium*, beyond the small number of essential molecules and of organisms where it had previously been described ; show that coding-region control in this case can be counteracted by an LRR-bearing molecule, DlrA ; and reveal a powerful, previously unsuspected, LRR-driven, PKA regulation system.

Transcriptional coactivator SnwA/SKIP – integrating diverse functions

P. Folk, R. Blatny, O. Tolde, I. Fukova, K. Martinkova, M. Skruzny, L. Hamplova, A. Blahuskova, and F.Puta

Department of Physiology and Developmental Biology, Faculty of Sciences, Charles University, Vinicna 7, 128 00 Praha 2, Czech Republic; dicty@mbox.cesnet.cz

Coregulators mediate the coupling between transcription initiation and later phases of mRNA production by modifying nucleosomes, interacting with splicing complexes, or targeting nucleoskeletal components. All these features seem to be indispensable for explaining the structural dependence of gene expression regulation in the nuclei of eukaryotic cells (Elgin & Workman, 2001).

We discovered in *Dictyostelium discoideum* and later characterized in other lower eukaryotes (*Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*) a novel type of coregulator - the SNW/SKIP protein. We found the SNW proteins from *D. discoideum* and S. pombe to sequester a hitherto unknown cyclophilin (CypE/Cyp2), suggesting that these coregulators may aid conformational changes of the complexes involved in transcription regulation (Skruzny *et al*., 2001). Human SNW modulates the activity of vitally important signaling pathways, such as those of steroid receptors, Notch receptor, or TGFbeta receptor, its mode of action is however, unknown. Human SNW was found enriched in interchromatin granule clusters (Mintz *et al*., 1999) or reconstituted spliceosomes (Neubauer *et al.*, 1998) and was implicated in nuclear targeting of CBF1 in HeLa cells (Zhou & Haward, 2001). We found the S. *pombe* SNW in contact with the auxiliary splicing factor U2AF35 and also showed that in *S. pombe* SNW is an essential gene (Ambrozkova *et al*., 2001), which, together with other information from lower eukaryotes, suggests that SNW has a role that is ancestral to its functioning as a coregulator.

Here we characterized the intracellular distribution of SNW proteins *in vivo* and their binding properties *in vitro*. *D. discoideum* GFP-SNW was accumulated in distinct, actinomycin D-sensitive foci, which were associated with nucleoli. The accumulation into nuclear foci, which was also apparent in the yeast cells, was in contrast to the reported distribution of the human GFP-SNW, which was found dispersed among multiple interchromatin granule clusters (IGCs; Neubauer *et al.*, 1998). Biochemical analysis of *D. discoideum* SNW revealed its strong association with nuclear matrix fractions. We succeeded in isolating the GFP-SNW-enriched foci as protein complexes amenable for proteomic analyses. The SNW complexes may be exploited to address the differences in nuclear organization between *D. discoideum* and higher eukaryotes.

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SadA, a novel adhesion receptor In *Dictyostelium*

Petra Fey[1], Stephen Stephens[2], Margaret Titus[2], and Rex L. Chisholm[1]

1 Northwestern University, Feinberg School of Medicine, Dept. of Cell & Molecular Biology, 303 E Chicago Ave, Ward 11-100, Chicago, IL 60611; 2University of Minnesota, Department of Genetics, Cell Biology and Development, 321 Church St. S.E., 6-160 Jackson Hall, Minneapolis, MN 55455.

Little is known about cell-substrate adhesion and how motile and adhesive forces work together in moving cells. The ability to rapidly screen a large number of insertional mutants prompted us to perform a genetic screen in *Dictyostelium* to isolate adhesion deficient mutants. The resulting sad (substrate adhesion deficient) mutants grew in plastic dishes without attaching to the substrate. One of these mutants, *sadA*$^-$, completely lacked substrate adhesion in growth medium. The s*adA*$^-$ mutant also showed a slightly impaired cytokinesis, an increased growth rate, and a strong phagocytosis defect. Deletion of the *sadA* gene by homologous recombination resulted in the recreation of the original mutant phenotype. Expression of *sadA-GFP* in s*adA*-null cells restored the wild-type phenotype. In *sadA-GFP* rescued mutant cells, *sadA-GFP* localized to the cell surface, appropriate for an adhesion molecule. These data combined suggest that *sadA* is a novel substrate adhesion receptor in *Dictyostelium*.

SadA contains nine putative transmembrane domains and three conserved EGF-like repeats in a predicted extracellular domain. The EGF repeats are similar to corresponding regions in proteins known to be involved in adhesion, such as tenascins and integrins. Outside this region, sadA has little similarity to any other known proteins. In general, EGF repeats are found in the extracellular domains of membrane - or secreted proteins, and in some cases they have been shown to be directly responsible for protein – protein interactions. We hypothesize that sadA's EGF repeats might be involved in specific extracellular interactions that mediate substrate adhesion, and that one or several of the intracellular domains might link the sadA receptor to the cytoskeleton. Studies to investigate these hypotheses are currently under way.

Synergistic roles of members of the TM9 superfamily of proteins

Mohammed Benghezal[1], Sophie Cornillon[1], Leigh Gebbie[1], Laeticia Alibaud, Franz Brückert[2], François Letourneur[3], Pierre Cosson[1]

1 Université de Genève, Centre Médical Universitaire, Département de Morphologie, 1 rue Michel Servet, CH-1211 Genève 4, Switzerland; 2 Laboratoire de Biochimie et Biophysique des Systèmes Intégrés, UMR314, CNRS, CEA, Grenoble, France; 3 Institut de Biologie et de Chimie des Protéines, UMR 5086 CNRS, Université Lyon I, 7 passage du Vercors, 69367 Lyon Cedex 07, France.

The transmembrane protein 9 superfamily (TM9SF) comprises numerous members in eukaryotes. Whereas their function remains essentially unknown in higher eukaryotes, the *Dictyostelium discoideum* Phg1a TM9SF protein has recently been reported to be essential for cellular adhesion and phagocytosis.

To gain insight into the function of the TM9 family of proteins we analyzed the TM9 family in *Dictyostelium discoideum*. Analysis of the phenotype of *phg1a, phg1b* and *phg1a/phg1b* mutants revealed that Phg1a and Phg1b proteins play synergistic roles in controling cellular adehsion, phagocytosis and development. However complementation experiments are not compatible with two distinct adhesion receptors operating in parallel. Rather they favor a model where Phg1 proteins would act as regulators of cellular adhesion. Consistent with this view, in *phg1* mutants the pattern of cell surface protein expression is altered, suggesting that Phg1 proteins might regulate cellular adhesion by controling the composition of the cell surface.

Autophagy is essential for *Dictyostelium* development

Grant P. Otto, Mary Y. Wu, Nevzat Kazgan, and Richard H. Kessin.

Department of Anatomy and Cell Biology, Columbia University.

Autophagy is a mechanism employed by eukaryotic cells to recycle non-essential cellular components during starvation. Double membrane vesicles enclose cytosol non-selectively, and are targeted to the vacuole/lysosome for degradation. Autophagy is a universal process, occurring in yeast, plants, animals and *Dictyostelium*. Despite its universality, autophagy is a poorly understood process. A number of autophagy mutants have been isolated in yeast, and they define a set of genes required for this process. A number of autophagy genes are required for two conjugation reactions related to ubiquitination that are essential for this process: Apg12p conjugation to Apg5p, and Apg8p conjugation to the lipid phosphatidylethanolamine. These reactions require the action of the E1-like enzyme Apg7p, and the E2-like enzymes, Apg3p and Apg10p.

We have identified *Dictyostelium* orthologues of most yeast autophagy genes from the *Dictyostelium* Genome Sequencing Consortium. To study the function of autophagy in *Dictyostelium*, we generated null mutations in two genes involved in the conjugation reactions, Apg5 and Apg7. Apg5 is the target for Apg12 conjugation, whereas Apg7 is an E1-like enzyme required for the activation of both Apg8 and Apg12 prior to E2-mediated conjugation to their targets. *apg5* and *apg7* do not affect growth, but survival of mutant cells in amino acid-free medium is reduced. These autophagy mutants have a protein turnover defect, and do not show the reduction in whole cell protein levels observed in wild-type cells upon starvation and subsequent development. Both mutants have defective development, forming multi-tipped aggregates that result in aborted fruiting bodies that contain no viable spores. To further characterise autophagy in *Dictyostelium*, we constructed GFP fusions of Apg5, Apg7, Apg8, Apg9 and Apg12, and examined their subcellular distribution.

Autophagy has been implicated in the intracellular multiplication of bacterial pathogens such as *Brucella abortus, Legionella pneumophila* and *Porphyromonas gingivalis,* in macrophages. We demonstrate that *L. pneumophila* intracellular replication in *Dictyostelium* does not require autophagy. The *apg5* and *apg7* mutants support *Legionella* infections, and kill intracellular multiplication mutants of *L. pneumophila,* in a manner similar to wild-type. These results suggest that the presumed role of the autophagolysosomal pathway in *L. pneumophila* multiplication must be reevaluated.

The novel anti-adhesive protein AMPA effects cell type specific differentiation and pattern formation.

Daphne D. Blumberg, Hoa Ho, and Timothy R. Varney.

Department of Biological Sciences, University of Maryland, Baltimore County, 1000 Hilltop Circle Baltimore, Maryland 21250

The *ampA* gene encodes a novel protein that modulates cell-cell and cell-substrate adhesions during development. Developmental expression of the *ampA* gene is localized to the anterior like cells. In mounds, these *ampA* expressing cells are largely localized to the mound periphery and base where they differentiate to form basal disc and lower cup structures. A few *ampA* expressing cells are scattered within the mound and localize to the upper cup. Here we demonstrate that insertional inactivation of the *ampA* gene results in patterning defects that define two distinct roles for the *ampA* gene product during development. Analysis of reporter gene expression during development of *ampA* null strains and chimeras formed with varying percentages of wild type cells mixed with the *ampA* null cells indicates that the AmpA protein is necessary in a non-cell autonomous manner to prevent expression of a prespore gene marker in cells at the mound periphery which will ultimately differentiate into prestalk cells. The use of synthetic oligopeptides has identified a 9 amino acid domain of AmpA that functions to reduce the number of cells that assume the prespore fate when added back to *ampA* null cells. A factor that induces cells to assume a prespore fate accumulates in *ampA* null cells. A model is presented suggesting that extracellular AmpA protein secreted from anterior like cells at the mound periphery functions to inactivate a prespore inducing factor (PIF) allowing cells in at the mound periphery to assume a prestalk fate. A second cell autonomous function of *ampA* is needed to reduce cell-cell adhesion and to enable a subset of *ampA* expressing cells to localize to the interior of the mound. A model is presented for two distinct *ampA* functions during development.

Microarray and mutational analysis of DIF Signaling

Chris Thompson*, Chad Shaw, Mimi Ibarra and Gad Shaulsky

Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, USA 77030

Although DIF signaling has been studied for many years, the isolation of a mutant specifically defective in DIF biosynthesis (DmtA$^-$) has enhanced our understanding of its basic function *in vivo*, leading to the proposal that DIF acts as the prestalkO cell inducer. Despite this, little is known about the signaling pathway utilized by DIF *in vivo*. One problem is that the DIF signaling pathway is not amenable to a simple genetic analysis where one might assume DIF signaling mutants would resemble DmtA$^-$, since the phenotype is practically impossible to distinguish from wild type. Furthermore, there need not necessarily be a simple relationship between DIF and its signaling pathway(s) (e.g. some genes may play other roles), so DIF signaling mutants could actually appear more or less severe than DmtA$^-$.

We have attempted to address this in two ways: (1) To define the DmtA$^-$ microarray phenotype (2) To isolate mutants in DIF signaling.

(1) Microarrays provide a powerful method to assess the expression profile of thousands of genes simultaneously. These profiles can be regarded as the phenotype resulting from the input of many signaling cascades. We therefore sought to determine the microarray phenotype of DmtA$^-$ to generate a "DIF signaling signature". mRNA was extracted from DmtA$^-$, DmtA$^-$+DIF, Ax2 and Ax2+DIF at various developmental stages and hybridized to a 8000 gene microarray. Analysis revealed overall gene expression trajectories to be similar between samples. However, a subset of genes show markedly different levels of expression. These genes fall into smaller groups according to their timing of expression and therefore provide insights into the timing of DIF action *in vivo*. This analysis not only provides a valuable dataset to which potential DIF signaling mutants can be compared but also reveals a set of *in vivo* DIF dependent genes, the nature of which will be discussed.

(2) We devised a screen to identify mutants unable to respond to DIF based on the simple idea that cells (either pkaR$^-$ or Ax4) incubated at low density in buffered salts with cAMP (pkaR$^-$) or 8-Br-cAMP (Ax4) will normally be diverted away from the spore cell fate (detergent resistant/alive) by DIF to remain amoeboid (detergent sensitive) or become stalk cells (dead). However DIF insensitive mutants (DIMs) or DIF undersensitive mutants (DUMs) will remain as viable spores. We found that this resulted in an ~1000x enrichment for such mutants. From ~10,000 mutants generated by REMI in Ax4 or pkaR$^-$, 1 DIM was isolated in each background. Southern blots revealed these to be different alleles of the same mutation (*DIMa*). In addition, we have isolated 3 DUMs from the Ax4 screen. Although Br-cAMP treatment results in levels of spore induction indistinguishable to Ax4, when 100nM DIF-1 is added, *DIMa* shows no response whatsoever whereas *DUMa*, *b* and *c* exhibit <25% response compared to Ax4. Strikingly, the morphological phenotype of *DIMa* is indistinguishable from DmtA$^-$, supporting the idea that it represents the first key regulator of DIF signaling *in vivo* to be identified. Although DUMa-c do not phenocopy DmtA$^-$, they also exhibit stalked slug migration, raising the possibility of partially overlapping function or that they may act in specific elements of the DIF response. The gene disrupted in *DIMa* has been cloned out. Sequencing reveals it is likely to encode a previously uncharacterized transcription factor of the basic region leucine zipper family. It is developmentally regulated and expressed at highest levels in prestalk cells after the finger stage of development. The implications of these findings will be discussed with reference to previously identified DIF responsive sequences.

Regulated expression of the MADS-box transcription factor SrfA mediates activation of gene expression by PKA during *Dictyostelium* sporulation

Ricardo Escalante and Leandro Sastre

Instituto de Investigaciones Biomédicas C.S.I.C/U.A.M. C/Arturo Duperier, 4, 28029Madrid, Spain.

Cell differentiation and morphogenesis are tightly regulated during sporulation in the lower eukaryote *Dictyostelium discoideum*. The control of the cAMP-dependent protein kinase (PKA) is essential to coordinate this process. Several signal transduction pathways are being recognized that lead to the regulation of intracellular cAMP levels. However, very little is known about the events laying downstream of PKA that are essential to activate late gene expression and terminal differentiation of the spores. We have studied the relationship between PKA and the MADS-box transcription factor SrfA, essential for spore differentiation. The constitutive activation of PKA was not able to rescue sporulation in a strain that lack *srfA* suggesting the possibility that *srfA* function downstream of PKA in a signal transduction pathway leading to spore maturation. A distal promoter region regulates the induction of *srfA* expression in the prespore region during culmination. We found that this promoter can be induced precociously by activating PKA with 8-Br-cAMP suggesting a transcriptional regulation by PKA. Moreover, precocious sporulation and expression of the spore marker *spiA* in the strain that over-expresses PKA correlates with a precocious induction of *srfA* expression. The temporal and spatial pattern of expression was also studied in a mutant strain lacking the main adenylyl cyclase that functions during culmination, ACR. This strain is expected to have lower PKA activity and consistently, the *srfA* level of expression was reduced. Moreover, the temporal induction of *srfA* in the prespore region was also delayed during culmination. Our results strongly suggest that PKA activation during culmination lead to the induction of the expression of *srfA*. SrfA is, in turn, essential for the expression of a set of spore specific genes that have been identified by a differential screening. The correct temporal and spatial pattern of *srfA* expression appears to be part of a mechanism that ensures the addecuate coordination of gene expression and morphogenesis.

Microarray-assisted *in situ* hybridization of *Dictyostelium* prestalk genes.

Mineko Maeda[1], Keiko Nishio[1], Haruyo Sakamoto[1], Takahiro Morio[2], Hideko Urushihara[2], Yoshimasa Tanaka[2], Negin Iranfar[3], Danny Fuller[3] and William F. Loomis[3]

1 Osaka University, Osaka, Japan; 2 University of Tsukuba, Tsukuba, Japan; 3 University of California San Diego, La Jolla, CA,USA

Gene expression analyses of prespore and prestalk cells separated on percol gradients have been carried out using genome-wide cDNA microarrays (Iranfar *et al.* 2001; Van Driessche *et al.*, 2002; our unpublished data). mRNAs from several hundred genes were found to be more than two fold enriched in prestalk cells. However, this population of prestalk cells is heterogeneous and can be subdivided into PstA, PstO ,and PstAB cells based on position within slugs. Therefore, we have used *in situ* hybridization to localize mRNA abundance of a large number of prestalk genes at various stages of development.

We were able to show strong anterior (prestalk) localization at the slug stage for 29 out of 30 genes that gave at least 5 fold higher signals with prestalk RNA than with prespore RNA on the microarrays. Among 90 genes that were recognized by microarray analyses to be enriched 2.5 to 5 fold, 57 showed anterior localization. Only 11 of the 30 genes apparently enriched 2.25 to 2.5 fold in prestalk cells were found to give strong anterior specific *in situ* hybridization. These results help establish the reliability of microarray analyses.

The spatial patterns of *in situ* hybridization were determined for prestalk genes at the tipped mound, slug, Mexican hat, and culmination stages. At the slug stage we found 56 genes expressed throughout the anterior domain (PST-AO) or preferentially in the most anterior cells (PST-A); 38 genes expressed only at the back of the anterior domain (PST-O); and 13 genes expressed in a funnel of axial cells at the anterior (PST-AB). At later stages, these genes were found to be expressed at the positions expected from their cell type specificity.

A few genes were found to be expressed in all cell types at the slug stage but preferentially expressed in prestalk cells at the Mexican Hat stage and during culmination. Recognizing such dynamic changes in gene expression is essential for a holistic understanding of development in *Dictyostelium*.

Primary sequence homology and structural inferences will be used for putative functional annotation of these prestalk genes and posted in a publically available database. Molecular genetic approaches will be used to verify these predictions.

The *Dictyostelium* homolog of the DBI neuropeptide is the precursor of the spore differentiation factor SDF-2

Christophe Anjard and William F. Loomis

Center for Molecular Genetics, Cell and Developmental Biology, Division of Biology, UCSD. La Jolla, CA 92093

Prespore and prestalk cells differentiate and sort out from one another during the mound stage of development in *Dictyostelium* but do not form spores or stalk cells until culmination about10 hours later. Terminal differentiation is temporally coordinated among the cell types by a signal peptide, SDF-2, that induces rapid encapsulation of prespore cells. We have previously shown that release of SDF-2 is dependent on the prestalk specific ABC transporter TagC and that responses to SDF-2 are dependent on the receptor histidine kinase, DhkA. Phenotypic and epistatic analyses suggest that DhkA inhibits RegA phosphodiesterase such that cAMP can accumulate in the cell and lead to an increase in PKA activity. In prestalk cells PKA activity leads to further release of SDF-2 while in presore cells it leads to rapid encapsulation.

During gene expression analyses using microarrays we noticed that a prespore specific gene, *acbA*, which is expressed late in development, encodes a homolog of the mammalian neuropeptide DBI. DBI is cleaved to generate various peptides one of which counteracts the effects of benzodiazapims (such as Valium) in the brain by affecting the GABA B receptor. Others are involved in cell differentiation and mitochondrial function. The neuroactive peptides are predicted to have many of the biochemical properties expected for SDF-2 such as low pI and a high content of hydrophobic residues. We synthesized two peptides expected to be generated by trypsin cleavage of the product of *acbA* and found that they mimicked SDF-2 and were able to induce rapid encapsulation of sporogenous cells of the K-P strain when added at concentrations as low a 0.1 pM. The human counterpart of these peptides was also able to induce encapsulation but required concentrations greater than 10 nM.

We found that strains in which we had disrupted *acbA* have reduced spore viability and form long stalks reminiscent of the phenotype of *dhkA*$^{-}$ strains which lack the SDF-2 receptor. Spore viability of *acbA*$^{-}$ cells was rescued when they were developed synergistically with 10% wild-type cells as expected for mutants unable to generate an intercellular signal. Measurements of SDF-2 using specific antibodies raised to the peptides will be reported.

Analysis of slug cell polarisation

Dirk Dormann, Gerti Weijer and Cornelis J. Weijer

Wellcome Trust Biocentre, University of Dundee, Dundee DD1 5EH, UK

Cell movement in slugs is coordinated by periodic cAMP signals, which emanate from the slug tip and propagate backwards through the entire slug in a wave-like fashion. We have previously shown that the GFP tagged CRAC PH domain, which is required for the activation of adenylyl cyclase ACA and cAMP relay, translocates transiently to the leading edge of aggregating cells in response to cAMP induced activation of PI_3-Kinases. However cells in slugs are constantly polarised with the strongest binding of CRAC-PH-GFP at the leading edge, without signs of periodic translocation. CRAC-PH-GFP localisation does not show adaptation to high levels of cAMP as occurs in the aggregation and mound stages. Furthermore dissociated slug cells do not show CRAC-PH-GFP membrane localisation, while slug cells in direct contact with other cells do show localised CRAC- PH binding at the site of cell-cell contact implying that cell-cell contact might activate PI_3-Kinase signalling. Using various cAMP receptor and cell adhesion mutants that express PH-domain and Actin binding-domain GFP fusion proteins we are currently investigating the molecular basis of cell polarisation in slugs.

A rapid, generally applicable method to produce gene-disruption constructs based on *in vitro* transposition

Tomoaki Abe and Jeff Williams

Wellcome Trust Biocentre, University of Dundee, Dundee DD1 5EH

Production of gene-disruption constructs with traditional ‘cut and insert’ methods involving restriction digests and ligations can often be troublesome and laborious. We have therefore employed an *in vitro* transposon reaction in order to rapidly produce disruption constructs. The target gene is first isolated from genomic DNA via PCR and inserted into a "topo" vector, with amp and kan resistance cassettes, in a simple one-step cloning reaction. Then a transposon containing the bacterial tetr resistance cassettes and *Dictyostelium* Actin15-bsr cassette, linked in tandem, is randomly inserted into the topo plasmid containing the target gene in an *in vitro* reaction. Transformants are selected with tetracycline, ampicillin and kanamycin. Bacteria that carry plasmids lacking the transposon insertion and bacteria that contain plasmids with the transposon inserted in a ‘wrong’ location, (e.g., in the selection marker-resistance cassettes or the origin of replication) are eliminated by the selection process. In our hands, roughly 1 in 4 of clones selected in the process carry the transposon in the target gene and these clones are suitable to use for *Dictyostelium* gene-disruption. We describe the use of this technique to search for the tyrosine kinases that are responsible for activating Dd STATs.

How to observe "jumping" tRNA-gene targeted retrotransposons in the *Dictyostelium* genome

Peter Beck, Theodor Dingermann and Thomas Winckler

Johann Wolfgang Goethe-Universität (Biozentrum), Institut für Pharmazeutische Biologie, Marie-Curie Strasse 9, D-60439 Frankfurt am Main, Germany

About 10% of the *D. discoideum* genome is composed of mobile elements. The TREs (tRNA gene-targeted retroelements) are a family of seven retrotransposons that integrate at precise positions upstream or downstream of tRNA genes. Are the TREs of the modern *D. discoideum* genome still actively moving? To address this question we developed a genetic screening protocol that allowed us to isolate new integration events upstream of a "bait" tRNA gene with known flanking sequences. We positioned a tRNA gene within an artificial intron that was placed into the *D. discoideum* UMP synthase gene. This construct was inserted into the *D. discoideum* genome and presented as a potential site of *de novo* insertions of mobile TREs. We found that the tRNA gene-tagged UMP synthase gene was frequently disrupted by *de novo* insertions of endogenous TRE5-A copies, resulting in mutants that survived 5-fluoro orotic acid (5-FOA) selection. Approximately 96% of all isolated 5-FOA-resistent clones contained TRE5-A insertions, whereas the remaining 4% resulted from transposition-independent mutations. The newly inserted TRE5-As showed complex structural variations and were found about 50 bp upstream of the reporter tRNA gene similar to previously analysed genomic copies of TRE5-A. tRNA gene-targeted retrotransposition in modern *D. discoideum* cells may be limited to TRE5-A since no integrations into the UMP synthase reporter gene by other members of the TRE family were observed.

Sequencing and finishing strategies for *D. discoideum* at the Sanger Institute

Nancy Hamlin, Paul Davis, David Saunders

Pathogen Sequencing Unit, The Wellcome Trust Sanger Institute, Genome Campus, Hinxton, Cambridge, CB10 1SA, UK

The Pathogen Sequencing Unit (PSU) at the Wellcome Trust Sanger Institute is one of the members of an international collaboration set up to sequence the genome of the social amoeba *Dictyostelium discoideum*. In addition to providing clones and library resources, the PSU has performed shotgun sequencing on chromosomes 4/5 and 6. The finishing of chromosome 6 is divided amongst several of the members of the EC funded EUDICT consortium and Baylor College of Medicine, Houston, USA. The PSU is responsible for finishing nearly all of a 1.5Mb section on the right hand end of chromosome 6 and all of chromosome 5. The latter is being done with funding from the MRC. The unique way in which these *D. discoideum* chromosomes have been sequenced has shaped the approach to finishing them, whilst also drawing on experience gained in finishing other genomes of similar composition (e.g. *Plasmodium falciparum* chromosomes 1, 3-9, 13). Additional mapping information in the form of Happy Mapping, RFLP information and YAC skims were instrumental in piecing together the chromosomes. Key stages of the finishing process and specific problems encountered will be covered.

Transcription and transcript processing in *Dictyostelium* mitochondria

C. Barth[1], B. Jayawardena[1], U. Greferath[2], P. Le[1] and P. R. Fisher[1]

1Department of Microbiology, La Trobe University, Melbourne, Victoria, Australia; 2Department of Anatomy and Cell Biology, University of Melbourne, Melbourne, Victoria, Australia.

The circular mitochondrial genome of *Dictyostelium discoideum* is transcribed into eight major, polycistronic transcripts encoding polypeptides, ribosomal RNAs and interspersed transfer RNAs. Most of these polycistronic transcripts are subsequently processed into smaller mono-, di- or tricistronic RNAs. The processing mechanisms appear to be similar to those reported in mammalian mitochondria, where the maturation of the polycistronic transcripts involves cleavage using tRNA as excision signals. A comparison of the cDNA sequence for the small ribosomal subunit RNA (*rns*) with the mitochondrial DNA sequence of the *rns* gene revealed C-to-U substitutional editing of the *rns* ribosomal RNA at a single site. It is suggested that the editing may be required for normal pseudoknot formation in the 530 loop of the RNA and thus important for efficient, accurate translation in the mitochondria.

The 5' ends of the polycistronic transcripts have been mapped in primer extension experiments. Based on sequence alignments of these potential transcription start sites a short oligonucleotide consensus initiation sequence (GAGGNTKA) has been identified which did not reveal any significant sequence homologies to known promoter regions from other organisms. To verify and to further characterize the putative promoter regions in foot printing experiments, the nuclear gene of the *Dictyostelium* mitochondrial RNA polymerase has been cloned in order to express the gene in *E. coli* cells and to purify the protein. The gene exhibits a high sequence homology to RNA polymerases of T3/T7 bacteriophages and to mitochondrial RNA polymerases of *Chenopodium album, Arabidopsis thaliana* and of various yeasts. A helical wheel plot of the first 18 amino acids of the RNA polymerase sequence suggests the presence of an N-terminal mitochondrial targeting sequence and the targeting of the protein to the mitochondria.

Generation of *D. discoideum* mutants disrupted in gene for transcription factors by the PCR-generated gene disruption construct

Hidekazu Kuwayama[1], Shinji Obara[1], Takahiro Morio[1], Mariko Katoh[1], Hideko Urushihara[1], Satoru Kuhara[2], and Yoshimasa Tanaka[1]

1. Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan. and 2. Graduate School of Genetic Resources Technology, Kyushu University, Fukuoka 812-8581, Japan

A large-scale analysis of gene-expression in *Dictyostelium* development revealed that many developmentally regulated genes are classified into several groups judged by their expression pattern. This observation indicates that genes showing a similar expression pattern are governed by a common transcription factor. Furthermore, orchestration of the regulatory mechanisms of the factors is considered to play a central role in regulating transcriptional profile in development. In order to understand which transcription factor concerns the developmental gene-expression of a gene group, how it regulates the developmental program and, thus to draw the genetic network for the developmental gene-expression by DNA array technology, we identified a number of genes potentially encoding transcription factors from our cDNA and *Dictyostelium* genome database and tried to make all KO transformants of the factors.

The strategy for the identification of transcription-regulating genes from the cDNA database was 1) to obtain amino acid sequences categorized in ‘transcriptional control’ or ‘transcription factor’ from the databases, TRANSFAC, MIPS for *Arabidopsis thaliana* and MIPS for *Saccharomyces cerevisiae*, 2) to run blastx homology search with the cDNA contigs against the amino acid sequences, and then 3) to sort out the contigs which gave e-value>0.01 and significant sequence homology in important domains for the function of transcription. On the other hand, from the genome database, we 1) obtained amino acid sequences of conserved domains for transcription factors from the CDD (Conserved Domain Database and Search Service), using the key-words, ‘transcription factor’, and ‘DNA-bind’. 2) run the tblastn homology search with the amino acid sequences against the presumed 8,017 ORF sequences discovered by Dr. Loomis group. 3) selected ORFs which gave e-value>0.001 and significant sequence homology in key domains for the function of transcription.

By careful examination of these contigs and ORFs, we have identified 40 clones and 64 ORFs and obtained 54 independent genes as transcription factor candidates. Generation of gene disruption constructs for almost all these candidates was performed speedily by the PCR-based preparation of gene disruption construct, which we have presented in the last international *Dictyostelium* conference. Subsequently, effective isolation of some KO transformants was succeeded. The latest data including phenotypic characterization of the mutants will be presented.

Functional studies on two nuclear encoded mitochondrial proteins, SdhA and Cpn60, in *Dictyostelium discoideum*.

S. T. Lay[1], M. Kotsifas[1], C. Barth[1], P. R. Gilson[2] and P. R. Fisher[1]

1 Department of Microbiology, La Trobe University, Bundoora, VIC, Australia , 3086; 2 Centre for Cellular and Molecular Biology, School of Biological and Chemical Sciences, Deakin University, VIC, Australia, 3125.

Here we describe the cloning, sequencing and functional characterization of the *Dictyostelium discoideum* genes for the nuclear encoded mitochondrial proteins, the flavoprotein subunit (SdhA) of succinate dehydrogenase and chaperonin 60 (HspA). Succinate dehydrogenase is an essential enzyme of the tricarboxylic acid (TCA) cycle that is responsible for the oxidation of succinate to fumarate. It is a heterotetrameric enzyme composed of four subunits, a flavoprotein subunit containing the active site of the enzyme, an iron sulfur protein and two hydrophobic membrane anchoring subunits. Together they form Complex II of the electron transport chain which is located in the inner mitochondrial membrane of eukaryotes. Chaperonin 60 (Cpn60), together with chaperonin 10 (Cpn10), forms a protein folding machine that is ubiquitous in eubacteria and in the mitochondria and chloroplasts of eukaryotic cells where it assists in the folding (or refolding) of nascent (or denatured) proteins.

Sequence comparisons of the *hspA* and *sdhA* genes revealed that both gene sequences have been highly conserved throughout evolution. Transcription of both genes is down regulated during early *Dictyostelium* development in response to starvation, while the levels of the chaperonin 60 protein and flavoprotein subunit remained constant throughout the life cycle. The *hspA* gene contains two introns and, unusually for a gene belonging to one of the major heat shock gene families, is not stress-inducible in response to heat, cold or cadmium ions. The *sdhA* gene contains no introns and encodes a protein of about 64 kDa that is targeted to the mitochondria. This localization was supported by the expression and observation of SdhA-GFP fusion protein in D. dicoideum cells. In an attempt to understand how mitochondrial dysfunction affects phototactic signal transduction, targeted disruption, gene knockout and antisense inhibition experiments were performed using the cloned gene sequences.

Consistent with the essential role of chaperonin 60 in mitochondrial biogenesis and succinate dehydrogenase in cellular energetics, we were unable to isolate mutants in which the *hspA* gene or the *sdhA* gene had been disrupted. Transformants were however isolated that exhibited differing levels of antisense inhibition of chaperonin 60 expression, depending upon the number of copies of the antisense-expressing plasmid in the genome. Orientation in phototaxis (and thermotaxis) was severely impaired in *hspA* antisense transformants, while growth and morphogenesis were markedly defective only in transformants with higher levels of antisense inhibition. In contrast, transformants containing an antisense RNA-expressing plasmid did not show decreased levels of *sdhA* mRNA or protein, nor was any apparent effect on phototactic accuracy or growth observed. To investigate the possible impact of decreased SdhA activity in cells, an *sdhA* construct carrying a point mutation (His77Ser) at a conserved histidine residue was introduced. His77 is necessary for covalent binding of FAD and enzyme activity of Complex II. Northern and Western blot analysis and measurement of the energy status of resultant transformants were then conducted.

Isolation of genes whose expression is dependent on the MADS-box transcription factor SRFA.

N. Moreno, J.J. Vicente, L. Sastre and R. Escalante

Instituto de Investigaciones Biomedicas CSIC/UAM. Arturo Duperier, 4. 28029-Madrid. Spain

The MADS-box transcription factor SRFA is involved in terminal spore differentiation and morphogenesis. Strains where the *srfA* gene has been interrupted present developmental delay, defects in slug migration and rounded spores that are not resistant to heat or detergent treatments (Escalante and Sastre, 1998; Escalante *et al.* 2001). Since SRFA is a transcription factor, this phenotype must be the consequence of alteration in the expression of SRFA-dependent genes in these strains. Therefore, we are interested in the isolation of SRFA-dependent genes to study their function in spore differentiation and morphogenesis. A differential screening, looking for genes expressed in terminal structures of Wild Type, but not in the same strain with the *srfA* gene interrupted, has allowed the isolation of four SRFA-dependent genes. One of these genes codes for the homologue of the NADP-dependent malic enzyme. This gene is expressed in vegetative cells and throughout development although a large induction is observed after 20 h of development. The induction is significantly impaired in *srfA*$^-$ strains. The malic enzyme mRNA is greatly enriched in the sorus of culminant structures.

The second gene isolated codes for a protein homologue to the hMP70 family of human proteins, of unknown function. These proteins present a putative signal peptide and nine predicted transmembrane fragments. This gene is transcribed into two different mRNAs, originated by differential splicing of two internal exons, that are only present in the larger mRNA. The exclusion of the two exons originates a protein with a 173 aminoacid long deletion, including one predicted transmembrane fragment, which could drastically affect the functionality of the protein. The larger mRNA is induced after 20 h of development in wild type but not in *srfA*$^-$ strains. Another of the isolated genes codes for a 445 aminoacids long protein with more than 25 % identity with *D. discoideum* Spore Coat proteins SP87, SP70 and SP60. The corresponding mRNA is only expressed at late culmination (24h) in the sorus of Wild Type but not *srfA*$^-$ strains.

The fourth gene isolated codes for the previously described GP63 protein. This gene is only expressed after 20 h of development in the sorus of Wild Type strains, but not in *srfA*$^-$ strains. Louhgran et al (2000) have shown that GP63 expression is dependent on the presence of the GATA transcription factor Stalky. The expression of other Stalkydependent gene, that codes for a RNP homologue, is also absent in *srfA*$^-$ strains. These results could indicate partial overlapping in the regulation of gene expression by SRFA and Stalky transcription factors at late developmental stages. The expression of the mRNA coding for the RNP homologue can be induced by 8-Br-cAMP treatment in *srfA*$^-$ strains, as previously described for spiA (Escalante and Sastre, 1998). However, the expression of the other four SRFA-dependent genes can not be induced by 8-Br-cAMP in *srfA*$^-$ strains, although it is induced in Wild Type strains. These data could indicate that the expression of some genes can be regulated by PKA through SRFA-dependent and -independent pathways. Other genes, however, seems to require SRFA for their expression Escalante, R. and Sastre, L. (1998) Development, 125, 3801-3808.

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Distribution of transposable elements in the genome of *D. discoideum*

K. Szafranski[1], A.A. Noegel[2], G. Glöckner[1]

1 IMB Jena, Dept of Genome Analysis, Beutenbergstr. 11, 07745 Jena, Germany; 2 Center for Biochemistry, Medical Faculty, University of Cologne, Joseph-Stelzmann-Str. 52, 50931 Köln, Germany

A proportion of 10 % of the genome of *D. discoideum* is contributed by transposon (Tn) sequences. A total of 18 different Tn species grouping to 7 separate families has been identified in a systematic, genome-wide search for Tns (1). Tn copies are inhomogenously distributed over the *D. discoideum* genome. This is partly due to the Tn's insertion behaviour. A well-defined family of non-LTR retrotransposons is found strictly associated with tRNA genes, therefore named "tRNA-associated retroelements" (TREs) (2). A similar insertion-site specificity is found for an LTR-retrotransposon, DGLT-A.

Current studies in our group focus on the genomic distribution of the remaining Tns. According to results of large-scale sequencing of particular Tn loci and on statistical analysis of Tn sequence association, these Tn species form large copy clusters in the vicinity of the centromeres. But, since the spatial relations of the Tn copies in these clusters does not follow detectable rules, this picture of distribution cannot be explained by an hypothesized sequence-specific insertion mechanism of all these Tn species. We postulate global, phenotype-mediated selection as a shaping force for the distribution pattern.

Futher analysis of these phenomena should help to understand *D. discoideum*'s genome organisation and fluctuation.

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(2) K. Szafranski *et al.* Mol. Gen. Genet 262, 772-780 (1999).

A series of Gateway expression vectors for use in *Dictyostelium*

Peter Thomason

Molecular Biology Dept., Princeton University, Princeton, NJ 08544 USA

The Gateway cloning technology developed by Invitrogen is based on bacteriophage λ recombination, and allows for restriction enzyme-free cloning of PCR products into any vector bearing the appropriate phage λ recombination sites. The usefulness of this system is that any gene region of interest (coding sequence, promoter, combinations thereof, or anything else) can be easily cloned using proof-reading PCR into a so-called entry vector, and from there recombined into any suitable expression vector of choice. Expression vectors must contain phage λ attR1 and attR2 sites, and I have converted several useful *Dictyostelium* vectors so that they contain these recombination sites. Since all cloning is carried out without restriction enzymes, there are no limitations imposed by the nucleotide sequence of either the insert of interest or the expression vector to be used. Furthermore, both the 5'-3' directionality of the insert and its translational reading frame are set by the initial PCR cloning reaction, and are compatible with all of the expression vectors. This allows all expression systems to be tried with all inserts, literally without any restrictions. Two examples of the use of this new system are:

1) A coding region of interest can be PCR cloned and then recombined into *Dictyostelium* expression vectors that will drive the expression of the coding region from the Actin15 promoter. Vectors are available that will express just the native protein, or a fusion of the protein with GFP at either the N- or C-terminus. It is not necessary to choose which vector is most appropriate – they can all be tried at the same time.
2) A promoter of interest (or just as easily, a complete gene including promoter and coding region) can be recombined into expression vectors that will express reporter genes (GFP or β-gal) under the control of the inserted promoter. This is ideal for the analysis of novel promoters, and may offer a technique that is much more sensitive than *in situ* hybridization, but with equivalent fidelity. For expression vectors including GFP, there is also the potential to investigate subcellular localization of a protein by driving its expression (from its own promoter) with a C-terminal GFP fusion.

I have tested several of these vectors using the promoters for known cell type marker genes and verified their use. I am now using them

The *Dictyostelium* family of BEACH-domain containing proteins.

A. De Lozanne, N. Wang and W. Wu

Department of Molecular Cell & Developmental Biology, University of Texas, Austin TX 78712

The BEACH-domain containing proteins constitute a new family of proteins found in all eukaryotes. The function of these proteins, which include the Chediak-Higashi syndrome protein, Neurobeachin, LvsA and FAN, is still poorly understood. To understand the diversity of this novel protein family, we analyzed a large array of BEACH-family protein sequences from several organisms. Comparison of all these sequences suggests that they can be classified into five distinct groups that may represent five distinct functional classes. In *Dictyostelium* we identified six proteins in this family, named LvsA-F, which belong to four of those classes. To test the function of these proteins in *Dictyostelium* we created disruption mutants in each of the *lvs* genes. Phenotypic analyses of these mutants indicate that LvsA is required for cytokinesis and osmoregulation and LvsB functions in lysosomal traffic. The LvsC-F proteins are not required for these or other processes such as growth and development. These results strongly support the concept that BEACH proteins from different classes have distinct cellular functions. Having six distinct BEACH proteins, *Dictyostelium* should be an excellent model system to dissect the molecular function of this interesting family of proteins.

The development of monoclonal antibodies against phosphorylation state-specific proteins in *Dictyostelium* in the developmental studies hybridoma bank

Karla J. Daniels and David R. Soll

Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, Iowa 52242 USA.

The Developmental Studies Hybridoma Bank at Iowa is run under the auspices of the Institutes of Child Health and Human Development at NIH. The bank now has a collection of close to 500 hybridoma lines and filled close to 6000 orders in 2001. Collection and sales have expanded at approximately 20% per year for the past 6 years. The only developmental system not represented in the bank is *Dictyostelium.* The Bank has now begun to make monoclonals against *Dictyostelium* molecules involved in motility and chemotaxis. Monoclonals can play a major role in defining changes in the localization of molecules, especially those changes due to phosphorylation. The first monoclonals are now being developed against components of the RegA pathway and the phosphorylated and unphosphorylated states of the myosin II heavy chain (MHC) and the myosin II regulatory light chain (RLC). Phosphorylation of these latter molecules has been implicated in the basic motile behavior of a cell and in chemotaxis. The phosphorylation sites in myosin II have been mapped to a single serine residue (Ser13) at the N-terminus of RLC and three threonine residues in the tail region of MHC. These identified sequences were the targets for generation of phosphorylation-specific reporter monoclonal antibodies. Amino acid sequences were synthesized as peptides (unphosphorylated) and phosphopeptides with a cysteine residue added to the C-terminal end to permit conjugation to a carrier protein. The target peptide for the RLC (STKRRLNREESSVVLGEEC) extends from serine 3 at the N-terminus. Ser14 is not phosphorylated by MLCK and was not modified in the phosphopeptide. The target sequence for MHC was a linear 18-mer peptide containing Thr1823 and Thr1833 (DLKDTKYKLN DEAATKTQ). Inclusion of Thr1823 and Thr1833 as a single peptide was chosen because of the close proximity to each other and prospect of generation of contiguous epitopes. Peptides were prepared by solid phase peptide synthesis using 9-fluorenyl-methoxycarbonyl (FMOC) protected amino acids. Phosphoserine residues were incorporated as FMOC-Ser (PO_3HBzl)-OH and phosphothreonine residues as FMOC-Thr- (PO (OBzl) OH)-OH. These modified phosphoamino acids are protected from phosphatase activity. Four sets of peptides were prepared (phosphorylated and non-phosphorylated MRLC peptides and phosphorylated and non-phosphorylated MHC peptides). The peptides and phosphopeptides conjugated to KLH and 50 μg of immunostimulatory unmethylated CpG oligodeoxynucleotides were injected into Balb/c mice. Following standard protocols for boosting immunization and peptide blot analysis of tail bleeds, hybridomas were formed by fusion of splenocytes with myeloma cells. Positive hybridomas were identified in a series of selections including HAT media, peptide dot blots and immunostaining of fixed cells. Myosin II phosphorylation-deficient strains S13A (MRLC mutant) and 3XALA (MHC mutant) were used as negative controls for staining. The staining pattern will be described of phosphorylated and nonphosphorylated MHC and MRLC during cellular translocation in buffer, in a spatial gradient of cAMP, and in the different phases of a simulated temporal wave.

Expression of the transcription regulator CbfA (CMBF) is required for growth-development transition

Monika Baik, Peter Beck, Theodor Dingermann and Thomas Winckler

Johann Wolfgang Goethe-Universität (Biozentrum), Institut für Pharmazeutische Biologie, Marie-Curie Strasse 9, D-60439 Frankfurt am Main, Germany

C-module-binding factor (CMBF) is a DNA-binding activity specific for a DNA sequence within the *D. discoideum* retrotransposon TRE5-A (the C-module). The CMBF-encoding gene *cbfA* predicts a 115 kDa protein that is detectable in whole-cell and nuclear extracts by means of monoclonal antibodies. By comparison of amino acid motifs the CMBF protein can be divided into three functional domains. A jumonji-C (JmjC) domain is located at the amino-terminus of CMBF. JmjC domains are found in proteins from a wide range of organisms and are discussed to possess metalloenzyme functions in chromatin remodelling complexes. The central part of the CMBF protein contains three zinc finger-like motifs that may either serve as interaction interfaces for other proteins or function in DNA binding. A 25 kDa carboxy-terminal domain, which is separated from the zinc fingers by a ca. 200 amino acids asparagine-rich spacer, contains an AT-hook that we showed to mediate the binding of CMBF to A+T-rich DNA *in vitro*. Searching the *Dictyostelium* genome databases has identified a second JmjC-containing *Dictyostelium* protein that is 23% identical with CMBF in the JmjC and zinc finger regions. Due to the apparent common origin of the two proteins we propose to use *cbfB* as the name for the newly identified gene (genef01955), whereas CMBF will be renamed *CbfA*.

The *cbfA* gene could not be inactivated by homologous recombination, suggesting that it may be essential for growth. We therefore produced viable mutant cells that expressed <5% of wildtype CbfA levels by introducing an *amber* (TAG) translation stop codon into the chromosomal *cbfA* copy and expressing a suppressor tRNA gene in the same cell. The resulting mutant cells grew slowly on bacterial lawns prepared on nutrient-rich (SM) agar plates and formed aberrant aggregates that did not proceed to multicellular development. When plated on nutrient-free agar only a minor fraction of mutant cells managed to enter multicellular development after a prolonged period of starvation (72 hours). CbfA-depleted cells plated on buffer-supported filter pads failed to activate genes required for cyclic AMP pulsing and aggregation, e.g. *carA, acaA,* and *csaA.* Addition of pulsatile nanomolar cAMP to mutant cells developing in shaking culture partially rescued the expression of cAMP pulse-induced genes and speeded up multicellular development of the mutant cells. Mutant cells expressed discoidin in a cell density-dependent manner similar to wildtype cells, demonstrating normal expression of prestarvation factor (PSF). Thus CbfA-depleted cells may be blocked in a pathway downstream of PSF that mediates the onset of cAMP pulsing at the growth-to-development transition.

DdNek2, the first non-vertebrate homologue of human Nek2, is involved in the formation of microtubule-organizing centers

R. Gräf

Adolf-Butenandt-Institut / Zellbiologie, Universität München, Schillerstr. 42, D-80336 München, Germany, Email: ralph.graef@lrz.uni-muenchen.de

Dictyostelium Nek2 (DdNek2) is the first structural and functional non-vertebrate homologue of human Nek2, a NIMA-related serine/threonine kinase required for centrosome splitting in early mitosis. DdNek2 shares 43% overall amino-acid identity with its human counterpart and even 54% within the catalytic domain. Both proteins can be subdivided in an N-terminal catalytic domain, a leucine zipper, and a C-terminal domain. Kinase assays with bacterially expressed DdNek2 and C-terminal deletion mutants revealed that catalytic activity requires the presence of the leucine zipper and that autophosphorylation occurs at the C-terminus. Microscopic analyses with DdNek2 antibodies and expression of a GFP-DdNek2 fusion protein in *Dictyostelium* showed that DdNek2 is a permanent centrosomal resident and suggested that it is a component of the centrosome core. The GFP-DdNek2 overexpressing mutants frequently exhibit supernumerary microtubule-organizing centers (MTOCs) most likely representing bona fide centrosomes. This phenotype did not require catalytic activity because it was also observed in cells expressing inactive GFP-K33R. However, it was caused by overexpression of the C-terminal domain since it also occurred in GFP-mutants expressing only the C-terminus or a leucine zipper/C-terminus construct but not in those mutants expressing only the catalytic domain or a catalytic domain/leucine zipper construct. These results suggest that DdNek2 is involved in the formation of MTOCs. Furthermore, the localization of the GFP-fusion proteins revealed two independent centrosomal targeting domains of DdNek2, one within the catalytic or leucine zipper domain and one in the C-terminal domain.

The genetic structure of *D. discoideum* populations

Joan E. Strassmann, David Queller, Kevin R. Foster, Lorenzo A. Santorelli, Angelo Fortunato, Monica Landi

Department of Ecology and Evolutionary Biology, MS 170, Rice University, 6100 Main St., Houston TX 77005-1892 USA

D. discoideum has great potential as a model system for social evolution. This promise is based on the extensive work already done on this species as a model system for development; many genes that influence aggregation and specialization within resulting slug and fruiting body are known. In one crucial respect, *D. discoideum* fruiting resembles sociality more than development: the sorocarp forms by aggregation of numerous independent individuals. Thus, aggregations may not be clonal, and we have confirmed that different clones will coaggregate. We have also demonstrated that different clones co-occur on a very small scale in nature, making chimera formation likely. Thus, stalk cells may be viewed as analogous to social insect workers, giving up their lives for others who may or may not share the same genes. As expected in this situation, there is competition within chimeric mixtures, with some clones obtaining a selective advantage by being better at getting into the spores. Possibly because of this competition, chimeras suffer a cost of decreased slug migration. This cost is overcome by a benefit; chimeras can form larger slugs and larger slugs move farther. This benefit probably accounts for chimera formation in the wild where cells available for aggregation are much scarcer. Future work will investigate costs and benefits under different conditions, test wild populations for sexual recombination, and identify genes involved in recognition and cheating. *D. discoideum* gives students of social evolution a system in which processes of social organization understood at non-molecular levels in other organisms may be understood at the molecular level. This perspective also benefits developmental studies by highlighting the fact that the function of some developmental genes may be missed unless we consider competition within chimeras.

Clathrin coated vesicle formation in *Dictyostelium* controlled by actin dynamics

John E. Heuser* and Terry O'Halloran#

*Washington University, St. Louis and #University of Texas at Austin

Despite many recent discoveries of a number of proteins that interact with both actin and clathrin, it has remained very difficult to demonstrate a clear-cut *functional* link between actin dynamics and clathrin dynamics in the living cell. Here, we will show such a link, by showing that in Dicty treated with drugs that block actin turnover (latrunculin, mycalolide, or jasplakinolide; henceforth abbreviated "LMorJ"), clathrin coated pits stop pinching off and accumulate in concentrated "patches" right under the plasma membrane. This drug induced arrest of coated vesicle formation we will show both by interference-reflection light microscopy (whereby the ventral surfaces of glass-attached Dicty are seen to become "dotted" with small clear spots the size of coated vesicles) and will also show by fluorescence-LM (whereby GFP/clathrin expressing Dicty show a complete cessation of the normal "blinking" of bright dots that reflects their normal clathrin coated vesicle dynamics, and in addition, show a *dramatic* accumulation of static GFP-stained spots in the plasma membrane after treatment with LM&J). Finally, we will show by "deep-etch" electron microscopy that these accumulations of dots or spots do in fact represent clathrin lattices, and that these lattices are arrested by the LMorJ in a variety of stages, including everything from totally flat lattices (the presumed starting -points of coated vesicle formation) to completely spherical clathrin lattices (apparently surrounding membrane compartments that represent early endosomes, since these compartments accumulate HRP when this extracellular tracer is applied right along with the LM&J). The accumulation of the latter structures -- endosomes unable to free themselves from the plasma membrane in the absence of actin dynamics -- is particularly striking. It demonstrates that regardless of any other effect on endocytosis, actin dynamics is at least required to separate early endosomes from the plasma membrane. Importantly, this drug-induced accumulation of clathrin-coated early endosomes is completely *reversible*, because when the LMorJ is washed off of Dicty amoebae and they are given time to resume their normal actin dynamics, all of the stuck endosomes disappear from the plasma membrane. The mechanism of this removal is also particularly dramatic and informative. The very first ruffle that each amoeba manages to form after LMorJ washout simply appears to "sweep away" all of the trapped endosomes as it passes over them. Thus, the coordinated wave of actin polymerization that creates a ruffle also appears to be able to "amputate" all the coated vesicles and endosomes that are trapped in its wake. This "amputation" appears more dramatic and distinct in Dicty than in any other type of cell, amoeboid or not. Hence, Dicty has again proved to be an ideal model system for resolving a fundamental issue in cell biology: in this case, the functional link between actin dynamics and endocytosis.

Identification and characterization of a member of importin-β-like nuclear transport receptors in *D. discoideum.*

Andrea Mai[1], Enrico Bracco[1], Alessandra Balbo[1], Barbara Peracino[1], Günther Gerisch[2] and Salvatore Bozzaro[1]

1Department of Clinical and Biological Sciences, University of Turin Ospedale S. Luigi 10043-Orbassano-To, Italy; 2Max Planck Institute of Biochemistry, Am Klopferspitz 18a D-82152 Martinsried, Germany

Macromolecule shuttling between cytoplasm and nucleus is primarily mediated by three components: nuclear pore complex (NPC), nuclear transport receptors and Ran GTPase. In the classical import pathway importin-β-like proteins form complexes with cargo proteins and promote their translocation through the NPC. Dissociation in the nucleus depends on the action of GTP-loaded Ran. High levels of GTP-bound Ran in the nucleus and GDP-bound Ran in the cytoplasm are maintained by the selective localization of Ran-GEF and Ran-GAP in the nucleus and the cytoplasm, respectively. Export from the nucleus, instead, requires a complex of cargo protein with importin-β-like proteins loaded with GTP-bound Ran.

The importin-β-like superfamily includes 14 members in the yeast *S. cerevisiae* and at least 22 members in human cells. The relative molecular masses of these proteins vary between 90 kDa and 130 kDa, but all are characterized by an acidic isoelectric point. The overall sequence similarity is low and is mainly restricted to the Ran-binding domain, usually located in the amino-terminal region.

Here we report for the first time the cloning and partial characterization of an importin-β-like protein in *D. discoideum*.

From a REMI mutant (V26) defective in phagocytosis the tagged region was rescued and sequenced. The BLAST searching (Genome Sequencing Centre Jena web site) allowed us to identify a genomic clone (JC3a10e08) for further sequence. The disrupted locus encompasses a 3.8 kb putative coding sequence organized in 9 exons and 8 short introns and is interrupted by the REMI plasmid within the ninth exon. The gene codes for a putative member of importin-b-like proteins that we temporary named Dd-importin-β-A (Dd-impβA).

The predicted product of *Dd-impβA* is a 113 kDa protein which has a calculated pI of 5.12 and 35% of homology versus Hs-importin-13.

Preliminary data suggest that the *Dd-impβA* specific mRNA expression is low in vegetative cells and regulated during development.

The V26 mutant is characterized by reduced growth on both fresh or heat-inactivated bacterial lawns. During development, the mutant forms larger aggregates and slugs which persist for hours in the migration stage before undergoing fruiting. This phenotype can be partially reproduced in Ax2 cells disrupting *Ddtrn* within exon 4 by homologous recombination: KO mutants grow slowly on bacterial lawn and present the “slugger”-mutant-like phenotype, though they form normal aggregates.

Full length *Ddtrn* cDNA was amplified via RT-PCR and cloned in expression vectors for both N-terminal and C-terminal GFP-tagging. Stable clones expressing both the GFP-chimeras were obtained. In cells at both vegetative and aggregative stages, fluorescence microscopy showed a predominantly nuclear localization.

Copper resistance and copper pumps in the slime mold *Dictyostelium discoideum*

B. Burlando[1], F. Dondero[1], J. Camakaris[2] and A.Viarengo[1]

1Dipartimento di Scienze e Tecnologie Avanzate, Università del Piemonte Orientale “Amedeo Avogadro”, 15100 Alessandria, Italy; 2Department of Genetics, University of Melbourne, Victoria 3010, Australia

We found that amebae of the slime mold *Dictyostelium discoideum* are resistant to copper. Exposure to Cu^{2+} for 5 days, followed by cell growth rate determination and use of the neutral red cytotoxicity assay, allowed to estimate EC_{50} values of 469 ± 30 µM and 334 ± 45 µM, respectively. Moreover, SDS-PAGE separation of cytosolic proteins -labelled with the thiol dye monobromobimane- showed no metallothionein in controls and no metallothionein induction in cells exposed to 100 µM Cu^{2+} or to 5 µM Cd^{2+} for seven days. Conversely, the use of a cytochemical technique revealed the presence of Cu-ATPase activity that is copper-inducible and sensitive to the Cu chelator TPEN, to the P-type pump inhibitor vanadate, and to heat, suggesting that it is related to a P-type Cu-ATPase pump. Confocal immunofluorescence analysis using an antibody against human MNK Cu-ATPase indicated the occurrence of Cu-ATPase on both the plasma membrane and internal vacuoles. Accordingly, Western blot analysis, performed using the same anti-MNK antibody, detected the presence of a copper-inducible peptide. Further confirmation derived from gene expression analysis using real-time quantitative PCR. Primers were designed for the EST sequence *ssc204* available at the Dicty cDNA Project website (http://www.csm.biol.tsukuba.ac.jp) and showing high homology with the human Wilson and Menkes Cu-ATPases. Data showed dose-dependent gene induction by copper at 30 min, while prolonged exposures showed a recovery towards basal expression levels. In conclusion, copper tolerance and homeostasis in *Dictyostelium* seem to depend mainly on Cu-ATPase activity, whereas failure to detect basal or inducible metallothioneins suggests that these proteins may not be present or play a negligible role.

The adenylyl cyclase ACA is localized at the uropod of chemotaxing cells

P.W. Kriebel, and C.A. Parent

Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda MD 20892-4255

In *Dictyostelium discoideum* cAMP is important in signal relay and development. Upon starvation, *D. discoideum* amoebae secrete cAMP, which is detected by G protein-coupled receptors (cARs) that specifically bind cAMP. In this system, cAMP acts as a chemoattractant and the binding of cAMP to cARs activates a multitude of signaling pathways giving rise to chemotaxis, the synthesis and secretion of additional cAMP via the activation of ACA (signal relay), and changes in gene expression. In an attempt to gain insight into the role of cAMP in chemotaxis, we sought to visualize the cellular distribution of ACA in live chemotaxing cells. We expressed the ACA-YFP fusion protein in *aca*$^-$ cells (ACA-YFP/*aca*$^-$) and found that it rescued the developmental defect of the *aca*$^-$ cells and retained wild type biochemical properties. Surprisingly, microscopic imaging of ACA-YFP/*aca*$^-$ cells revealed plasma membrane labeling that was highly enriched at the uropod of chemotaxing cells. Moreover, this polarized cellular distribution was dependent on the actin cytoskeleton since the addition of inhibitors of actin polymerization led to a uniform distribution of ACA-YFP. Closer examination of ACA-YFP/*aca*$^-$ cells using confocal imaging, revealed that ACA-YFP also labeled intracellular vesicles, which appeared to traffic during chemotaxis. To further explore ACA's role in chemotaxis, the capacity of *aca*$^-$ cells to respond to chemoattractant gradients was studied. We found that *aca*$^-$ cells responded well to the gradient but failed to produce streams, which are generated when cells align in a head to tail fashion. The observations that ACA is essential for streaming and that ACA-YFP is enriched at the uropod, suggest a mechanism for streaming. In this model the release of cAMP from the posterior of the cell attracts and orients other cells so that the cells align head to tail. To further explore this hypothesis, cell mixing experiments were performed where cells expressing ACA-YFP at the posterior were mixed with *aca*$^-$ cells. We found that although both ACA-YFP/*aca*$^-$ and *aca*$^-$ cells consistently streamed to the uropods of cells expressing ACA-YFP, under no circumstances did ACA-YFP/*aca*$^-$ cells align to the back of *aca*$^-$ cells. These results support the theory that cAMP is released from the uropod during chemotaxis and streaming. Our findings show that the polarized distribution of adenylyl cyclases has impact on the biology of *D. discoideum* development. We propose that a similar distribution of adenylyl cyclases could be present in other cell types since compartmentalization of cAMP metabolism has been suggested in a variety of mammalian cells.

The AP-1 clathrin adaptor complex is required for efficient pinocytosis and phagocytosis.

B. de Chassey, A. Dubois Y. Lefkir, and F. Letourneur

Institut de Biologie et Chimie des Protéines, UMR5086 - CNRS/Université Lyon I7, Passage du Vercors, 69367 Lyon cedex 07, France

Adaptor protein (AP) complexes are major components of the cytoplasmic coat found on clathrin-coated vesicles. Here, we analyze in *Dictyostelium discoideum* the function of the AP-1 complex, which in mammalian cells, participates mainly in the budding of clathrin-coated vesicles from the trans-Golgi network (TGN). Targeted disruption of the AP-1 medium chain in *Dictyostelium* results in viable cells displaying a massive growth defect and a delayed developmental cycle as compared to parental cells. Lysosomal enzymes are constitutively secreted as precursors, suggesting that protein transport between the TGN and lysosomes is defective. While the internalization of a cell surface membrane protein is not affected, fluid-phase endocytosis and phagocytosis drop by 63.5 and 60%, respectively. We observe that AP-1 is enriched in the phagocytic cups of murine macrophages or *Dictyostelium* cells, a localization which rapidly disappears during phagosome maturation. The dynamic recruitment of AP-1 to phagosomal membranes further suggests that AP-1 plays a role in the early steps of phagosome formation. Therefore AP-1 might function in more than one intracellular transport pathway.

Mutations in the relay loop region result in dominant-negative inhibition of myosin function

Georgios Tsiavaliaris, Setsuko Fujita-Becker, Renu Batra, Dmitrii I. Levitsky [1], F. Jon Kull [2], Michael A. Geeves [3] & Dietmar J. Manstein [+]

Max-Planck-Institut für medizinische Forschung, Jahnstrasse 29, D-69120 Heidelberg, Germany; 1 A.N.Bach Institute of Biochemistry, Russian Academy of Science, Moscow 119071, Russia; 2 Dartmouth College, 6128 Burke Laboratory, Hanover, NH 03755, USA; 3 Department of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, UK

Dominant-negative inhibition is a powerful genetic tool for the characterization of gene function *in vivo*, based on the specific impairment of a gene product by the coexpression of a mutant version of the same gene product. Here we describe the detailed characterization of two myosin constructs containing either point mutations F487A or F506G in the relay region. *Dictyostelium* cells transformed with F487A or F506G myosin are unable to undergo processes that require myosin II function including fruiting-body formation, normal cytokinesis and growth in suspension. Our results show that the dominant-negative inhibition of myosin function is caused by disruption of the communication between active site and lever arm, which blocks motor activity completely, and perturbation of the communication between active site and actin-binding site, leading to an approximately 100-fold increase in the mutants' affinity for actin in the presence of ATP.

Investigating the role of CRAC in chemotaxis.

D.C. Mahadeo[1], F.I. Comer[1,2] and C.A.Parent[1]

1. Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda MD, U.S.A; 2. PRAT research fellowship program, National Institute of General Medical Science, National Institutes of Health, Bethesda, Maryland, USA

The Cytosolic Regulator of Adenylyl Cyclase (CRAC) was first identified as a required factor for the G protein-mediated activation of the adenylyl cyclase ACA. More recently, it was shown to specifically translocate to the leading edge of cells chemotaxing towards a cAMP source. We now show that although *crac*$^-$ cells exhibit normal random motility and express chemoattractant receptors properly, they are defective in chemotaxis. When placed in a gradient of chemoattractant, *crac*$^-$ cells did not migrate towards the tip of the needle effectively. Further analysis using under agarose assays showed that they are unresponse to a wide range of cAMP concentrations.

Other than the recognized PH (Pleckstrin Homology) domain contained within the N-terminus of the protein, which mediates the translocation event, the remainder of the sequence does not conform to any known conserved protein domains. Conventional searches through genetic databases have not revealed any cognate CRAC homologues in other species. Nevertheless, there is biochemical evidence that the functional equivalents to CRAC are required in other systems. In an attempt to further characterize CRAC, as well as identify possible conserved domains responsible for its function, we have carried out a screen of other closely related species including *P. pallidum*, *D. mucoroides*, and *D. purpureum.* Identification and cloning of CRAC homologues from these organisms may provide a better understanding of the role of this protein in adenylyl cyclase activation and chemotaxis.

Role of *Dictyostelium discoideum* Rho-related proteins RacG and RacH in cytoskeleton-dependent processes

Baggavalli P. Somesh, Heidrun Dislich and Francisco Rivero

Institut für Biochemie I, Medizinische Fakultät, Universität zu Köln, Joseph-Stelzmann-Str. 52, 50931 Köln, Germany

Rho family proteins have been implicated in regulating various cellular processes, including actin cytoskeleton organisation, endocytosis, cell cycle and gene expression. Up to 15 genes coding for Rho related proteins have been identified in *Dictyostelium discoideum*, a eukaryotic microorganism whose cells exhibit actin based cell motility comparable to cells of higher eukaryotes like the leukocytes, are chemotactically active and are able to efficiently perform phagocytosis. Except for Rac1a/b/c, RacC, RacE and RacF1, the data available to date are insufficient to assign a functional role for each of the *Dictyostelium* Rac proteins. The evidence available to date indicates that many signal transduction pathways regulated by Rho GTPases in animal cells are also present in *Dictyostelium*. In particular, components like PI3K, PAK, WASP, Scar, diverse RhoGAPs and RhoGEFs as well as RhoGDI have been identified in this organism.

In this study, we are investigating the function of two *Dictyostelium* Rho family proteins, RacG and RacH, whose genes are constitutively expressed throughout the *Dictyostelium* life cycle. To investigate the role of these proteins in cytoskeleton-dependent processes, we have fused them at their amino-terminus with green fluorescent protein (GFP) and studied the dynamics of subcellular redistribution using confocal laser-scanning microscopy. GFP-RacG is distributed predominantly in the plasma membrane, whereas GFP-RacH associates to intracellular membrane compartments, especially the Golgi apparatus and the nuclear envelope. This was confirmed by fractionation experiments. RacG is also detected in the Triton-insoluble pellet. In *in vivo* experiments we have observed that GFP-RacG accumulates at the margins of phagocytic cups; localization around the phagosome is transient. Furthermore, we have generated cell lines that overexpress constitutively active or dominant negative RacG or RacH using a tetracycline-regulated expression system. Our results demonstrate that RacG controls motility and *in vitro* actin polymerization upon cAMP stimulation. RacG is also involved in phagocytosis, whereas RacH controls both phagocytosis and pinocytosis. RacG, but apparently not RacH, also controls the developmental cycle.

A study on a pinocytosis-deficient REMI mutant isolated from *Dictyostelium discoideum*

Kazunori Sasaki, Hitomi Yamaguchi, Aiko Amagai and Yasuo Maeda

Department of Developmental Biology and Neurosciences, Graduate School of Life Sciences, Tohoku University, Sendai 980-8578, Japan

Using REMI mutagenesis, we have isolated and characterized a pinocytosis-deficient mutant (referred to as Pinocytosis- deficient 1; PD1) derived from *Dictyostelium discoideum* Ax-2 cells. PD1 cells are able to grow almost normally in bacterial suspension by phagocytosing external bacteria such as *E. coli*, but never grow axenically in liquid growth medium, thus suggesting a complete defect of pinocytosis. In fact, the measurements of the pinocytic activity using FITC-dextran as a fluid phase marker have shown that PD1 cells are entirely devoid of pinocytic activity. Here, it is of interest to note that PD1 cells are capable of entering the differentiation phase to aggregate even in the presence of extracellular fluid nutrients, possibly coupled with the complete defect of pinocytic nutrient uptake. Moreover, when PD1 cells were starved and incubated on nonnutrient agar, they exhibited delayed aggregation compared to parental Ax-2 cells, and their development was arrested at the mound stage. Such a developmental defect was not nullified by a mixed culture with parental Ax-2 cells at a ratio of 9:1, and therefore the developmental phenotype of PD1 cells is cell- autonomous. Taken together, these results indicate that the gene disrupted in PD1 cells is entirely responsible for multicellular development beyond the mound stage as well as for pinocytic ability. Identification and characterization of this interesting gene are now under investigation.

Cryptococcus neoformans virulence is enhanced by intracellular growth in *Dictyostelium discoideum*

J. N. Steenbergen, J. D. Nosanchuk, and A. Casadevall

Albert Einstein College of Medicine, Bronx, NY

C. neoformans is an encapsulated, environmental fungus that can cause life-threatening meningitis. *Acanthamoeba castellanii* is a host cell for *C. neoformans* infection. We speculate that the pathogeneicity of *C. neoformans* for macrophages and vertebrate hosts originally evolved as a mechanism for protection against environmental, amoeboid predators. In this study, we investigated whether *C. neoformans* infection of *D. discoideum* simulates *C. neoformans* infection of macrophages and whether *C. neoformans* infection of *D. discoideum* affects the fungal virulence in a mouse model. *D. discoideum* provides advantages for the study of fungus-amoeba interactions. Defined, avirulent mutants of *C. neoformans* were unable to replicate in *D. discoideum*, as demonstrated by killing assays, while *D. discoideum* was killed when infected with virulent C. neoformans cells. Transmission electron microscopy demonstrated that *C. neoformans* replicates inside *D. discoideum* and that the *C. neoformans* cells were engulfed into a membrane enclosed vacuole. Passage of *C. neoformans* through *D. discoideum* resulted in a significant increase in the virulence of *C. neoformans* in mice, as determined by mouse survival studies (14 d vs 21 d, $p \leq 0.001$). The passaged *C. neoformans* cells also killed *D. discoideum* more rapidly. These results suggest that *D. discoideum*, a genetically malleable host, can be exploited as a model system for *C. neoformans* infection and that passage of *C. neoformans* through *D. discoideum* dramatically increases *C. neoformans'* virulence. These results add to other evidence implicating soil amoebae as important factors for the maintenance of *C. neoformans* virulence in the environment.

Single-molecule imaging of signaling molecules in living *Dictyostelium* cells.

Masahiro Ueda[1,2], Yasushi. Sako[2], Toshiki Tanaka[3], Peter.N. Devreotes[4] and ToshioYanagida[2]

1PRESTO, JST and 2Soft Biosystem Group, Graduate School of Frontier Bioscience, Osaka University, Osaka 565-0871, Japan; 3Department of Applied Chemistry, Nagoya Institute of Technology, Showa-ku, Nagoya 466-8555, JAPAN; 4Dpartment of Cell Biology, Johns Hopkins University, School of Medicine, 725N. Wolfe Street, Baltimore MD, 21205, USA.

Recent progress in single molecule detection techniques has allowed us to directly monitor signaling molecules in living cells (1-3). We have extended this technique to real-time imaging of single fluorescent-labeled cAMP molecules bound to their receptors on living *Dictyostelium* cells (4). To achieve the single molecule imaging, we used an objective-type total internal reflection fluorescence microscope (TIRFM). The binding sites are uniformly distributed and diffuse rapidly in the plane of the membrane. The kinetic analysis of Cy3-cAMP binding to the receptors revealed that the probabilities of individual association and dissociation events were greater for receptors at the anterior end of the cell. The differences in cAMP dissociation kinetics were retained in a mutant cell expressing a receptor lacking the phosphorylation residues but not in a mutant cell lacking G-protein subunit. These observations illustrate the dynamic properties of receptors involved in gradient sensing and suggest that these may be polarized in chemotactic cells.

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2D, 3D DIAS and DIAS 4.0: Existing and emergin software packages for analyzing the motility and shape of *Dictyostelium* amoebae and other cell types

E.R. Voss* and D.R. Soll

Department of Biological Sciences and the W. M. Keck Dynamic Image Analysis Facility, The University of Iowa, Iowa City, IA 52242 USA; *presenter.

The 2D Dynamic Image Analysis System (2D-DIAS – developed for Macintosh in 1988) and the 3D Dynamic Analysis System (3D-DIAS – developed for Macintosh in 1995) are playing a fundamental role in understanding how a *Dictyostelium* amoeba crawls and in particular in identifying abnormal behavior in mutants, providing an extended and precise motility phenotype. The system has been continually modified to incorporte new measurements and to greatly extend the resolution and length of an experiment, and now has reached a size exceeding one million lines of C code that has become extremely unwieldy. Moreover, the DIAS system has diverged into separate systems, each geared to a specific need. Some of these are 3D-DIAScon (for live confocal microscopy), 3D-DIASfl (for fluorescent stains), 3D-DIASpar (for tracking 3D particles within a cell), 3D-DIASemb (for multicellular development within an embryo) and DEAS (Dynamic Echocardiogram Analysis System for the human heart). While these systems are functional, they suffer from 1) lack of integration; 2) a linear locked-in modal design that requires users to restart when something goes wrong or when the input data is altered; and 3) a lack of ability to incorporate new technologies such as multithreading, object-oriented design, hardware for 3D rendering and virtual reality, and the internet. A new integrated system DIAS 4.0 is being developed using Java. It takes advantage of Java's 'Swing' graphical interface to implement a novel 'notebook' concept in which every aspect of an experiment along with all processes (partial and completed) are saved as an integrated unit. The user is able to intervene in any step of the processing of an experiment with a minimal amount of computing overhead. All of the above-mentioned DIAS spin-offs (and more) will be integrated into DIAS 4.0. DIAS 4.0 is about 30% completed and runs on either PC or Macintosh without change. We will demonstrate both the old and new technologies not only as they apply to *Dictyostelium*, but also to embryos, human leukocytes and the heart.

The calcineurin inhibitor Gossypol impairs growth, cell signaling and development in *Dictyostelium discoideum*

Barbara Weissenmayer, Katrina Böckeler, Rupert Mutzel

Fachbereich Biologie, Chemie, Pharmazie, Institut für Biologie - Mikrobiologie, Freie Universität Berlin, Königin-Luise-Strasse 12-16, D-14195 Berlin, Germany

Calcineurin, a conserved Ca^{2+}/calmodulin-regulated protein phosphatase, plays a crucial role in Ca^{2+} signaling in a wide variety of eucaryotic cell types. Recently Gossypol was found to efficiently inhibit human calcineurin *in vitro* and *in vivo* (Baumgrass *et al.*, J. Biol. Chem, 276, 47914-47921, 2001).

In *Dictyostelium discoideum* cell growth was clearly slowed when Gossypol was added to the culture media in micromolar concentrations. In light scattering experiments the application of Gossypol reduced cAMP-stimulated cell responses around 50 per cent in comparison to untreated cells. Since Gossypol was reported to also effect other enzymes (protein kinase A and C, phospholipase A and lactate dehydrogenase), we tested its effects on cellular signaling in a cell line which overproduces the catalytic A subunit of calcineurin. 5 time higher concentrations were necessary to reach the same effect in the calcineurin A overexpressing mutant. Likewise, autonomous light scattering oscillations could be blocked by 10 µM Gossypol in the wildtype and 50 µM in the calcineurin A overexpressing mutant.

Differentiation assays performed on HABP filters showed significantly delayed aggregation and development of Gossypol-treated cells. In contrast to untreated cells, which completed morphogenesis within 24 hours, 25µM Gossypol was sufficient to delay fruiting body formation by more than 24 hours.

Constitutive expression of PKA disrupts the shape of chemotactically responsive amoebae

Deborah Wessels[a], Karla Daniels[a], Hui Zhang[a], Paul Heida[*], William F. Loomis[b] and David R. Soll[a]

a Department of Biological Sciences and the W. M. Keck Dynamic Image Analysis Facility, The University of Iowa, Iowa City, IA 52242 USA; bDepartment of Biology, University of California, La Jolla, CA 92037 USA;

We show in our first poster on PKA that constitutive expression in the mutant *pkaR*$^-$ causes defects in basic motile behavior, the efficiency of chemotaxis in a spatial gradient of cAMP, and in the suppression of lateral pseudopod formation in the front of a simulated temporal wave. 2D-DIAS analyses revealed that *pkaR*$^-$ cells were rounder than parental wild type cells. We, therefore, used 3D-DIAS to reconstruct cells and pseudopods in 3D and confocal imaging to assess the distribution of F-actin, myosin II, REGA and microtubules in 3D. Our results demonstrate that 1) *pkaR*$^-$ cells are ovoid in buffer, in a spatial gradient of cAMP and in all phases of a temporal and natural wave; 2) that the dominant pseudopod of *pkaR*$^-$ cells is usually off the substratum and frequently atop the cell, rather than positioned anterior and on the substratum. The constitutive, abnormal shape of the cell may be the basis for all of the behavioral abnormalities during motility and chemotaxis documented in our first poster on PKA. An analysis of the distribution of F-actin, myosin II and microtubules in cells in buffer and in the front of a simulated temporal wave of cAMP revealed that all of these cytoskeletal elements localized normally, but conformed to the ovoid shape of the *pkaR*$^-$ cell. These results indicate that PKA activity must be suppressed in order for a cell to elongate along a 2D surface. This elongate shape is necessary for normal locomotion and efficient chemotaxis. Interestingly, when PKA is maximally activated at the peak of a natural wave, cells do round up in the normal dismantling of cell polarity, suggesting that PKA plays a role in this necessary behavioral component of chemotaxis.

Ras-GEFM, a novel putative Ras-GEF required for *Dictyostelium* development

M.Arigoni, E. Bracco and S. Bozzaro

Dipartimento di Scienze Cliniche e BiologicheUniversità degli Studi di Torino - Italy

Small GTPases are simple molecular switches regulated mainly by two sets of proteins: a) guanine-nucleotide-exchange factors (GEFs) causing their activation by catalysing the exchange of bound GDP with GTP, and b) GTPase-activating proteins (GAPs) which inactivate them by increasing the rate of hydrolysis.
We have identified a novel putative Ras-GEF from *Dictyostelium*, temporarily named *D.d. ras-GEFM. D.d.ras-GEFM* sequence analysis of the catalytic domain of the protein revealed higher homology with murine Cdc25 and *D.melanogaster* Sos. Its mRNA expression is developmentally and G-protein beta subunit modulated.
Inactivation of the gene results in cells unable to develop normally. The knock-out cells (MA11) produce very loose aggregates and fail to develop further. This phenotype remind that observed in cells lacking a ras interacting protein (*rip*3 null cells). Co-culture experiments, showed that MA11 cannot be rescued by AX2 cells.
The knock-out cells produce cAMP during the first hours of development but at reduced level compared to the parental strain. The appearance of EDTA-resistant adhesion is delayed of several hours. Pulsing with cAMP accelerates expression of EDTA-stable adhesion. When stimulated with cAMP pulses, Adenylyl Cyclase (AC) is transiently activated in MA11 cells but to a lower extent compared to the parental strain. In contrast Guanylyl Cyclase (GC) peaks two to six fold higher than wild-type.
When stimulated with cAMP diffusing from a microcapillary, the *D.d. ras-GEFM* minus cells extend several thin pseudopodia, but they are unable to properly polarize and to form an organized leading front. As a consequence cell motility toward the capillary is highly impaired.
Stimulation of *Dictyostelium* with chemoattractants causes a rapid polymerization of G-actin, which has been correlated with the extension of new pseudopods during chemotaxis. We therefore investigated the levels of F-actin in MA11 cells following treatment with cAMP. Wild type cells shows the expected biphasic response, whereas in MA11 cells the time course of the response appears to be roughly flat.
These results are consistent with D.d. *ras-GEFM* being involved both in regulation of the actin cytoskeleton, and thus motility, and in regulation of development. We propose that *D.d. ras-GEFM* is an important component of a ras/rac regulated pathway involved in integrating signals acting as positive regulator for AC and negative regulator of GC.

Basic motility and chemotaxis defects in a mutant with constitutively active PKA activity

Hui Zhang[a*], Paul Heid[a*], Deborah Wessels[a], Karla Daniels[a], Bill F. Loomis[b] and David R. Soll[a]

a Department of Biological Sciences and the W. M. Keck Dynamic Image Analysis Facility, The University of Iowa, Iowa City, IA 52242 USA; b Department of Biology, University of California, La Jolla, CA 92037 USA;

The cAMP-activated protein kinase PKA is involved in the intracellular transduction of signals emanating from the surface cAMP receptor cARI. It has been implicated in developmental gene regulation, developmental timing and cellular regulation. Here, we investigate its role in basic cell motility and chemotaxis, employing a deletion mutant of the PKA regulatory unit (PKA-R) that results in constitutive PKA activity
(mutant *pkaR*$^-$). Using 2D-DIAS, we have analyzed the motile behavior of *pkaR*$^-$ cells 1) in buffer ("basic motile behavior"); 2) in a spatial gradient of cAMP (the deduced mechanism for assessing direction at the beginning of the front of a natural wave); 3) in response to the temporal and concentration components of simulated, temporal waves; 4) after the rapid addition of a high concentration of cAMP; and 5) in natural waves generated by wild type cells. Our results demonstrate that *pkaR*$^-$ cells are highly defective in basic motile behavior, can chemotax, but with less efficiency than parental wild type cells, do not suppress lateral pseudopod formation to the same level as wild type cells in a spatial gradient of cAMP and do not suppress lateral pseudopods in the front of the natural wave. Our results reveal, however, that *pkaR*$^-$ cells respond normally to the peak and back of a temporal wave. Together, these results indicate that PKA activity must be suppressed for a cell to locomote in a normal fashion in the absence of an extracellular cAMP signal, in a spatial gradient of cAMP and in the front of a natural wave. However, PKA may play a role in dismantling cell polarity, as argued in the second of our two posters. Because PKA functions downstream in a pathway that includes ERK2 _ REGA _ [cAMP] $\rightarrow$ PKA, we compare the behavioral phenotype of *pkaR*$^-$ and the *regA*$^-$ mutant in considering models incorporating both REGA and PKA in the regulation of cell motility and chemotaxis.

The NRAMP1 protein in *Dictyostelium*.

Alessandra Balest, Carina Skriwan*, Barbara Pergolizzi, Michael Steinert* Barbara Peracino & Salvatore Bozzaro

Dipartimento di Scienze Cliniche e Biologiche, Ospedale S. Luigi, 10043-Orbassano, Italy and *Institut fuer Molekulare Infektionsbiologie, Universitaet Wuerzburg, Roentgenring 11, D-97070 Wuerzburg, Germany

We have cloned the *Dictyostelium* homologue of NRAMP1, a lysosomal membrane protein expressed mostly in macrophages, which is responsible for resistance to infection by intracellular bacteria or protozoa, such as *Legionella*, *Mycobacteria*, *Salmonella* and *Leishmania*. NRAMP1 is highly conserved during evolution and is supposed to act as a metal transporter protein that regulates metal ion concentration in the lysosome and contributes to the pH homeostasis and, possibly, to free oxygen radical formation. The mechanism of action of NRAMP1 remains, however, to be defined.

The *Dictyostelium Nramp1* gene encodes for a 53 Kda protein with 11 putative TM domains and wich share a 55% homology with human NRAMP1. The *Nramp1* mRNA is expressed mostly during vegetative stage, its expression declines rapidly within 2 hours from the beginning of starvation. To characterize Dd NRAMP1, we have generated a GFP fusion protein and we have observed that the GFP is localized to the membranes of intracellular vesicular compartments. These vescicle are in part identified as lysosomes, phagosomes and endosomes. Cells expressing an *Nramp1* antisense gene showed a reduced rate of *E. coli*, *L. pneumophila* and *M. avium* phagocytosis, but supported better than control cells intracellular growth of *L. pneumophyla* and *M. avium.* In order to confirm these data and to understand the mechanism of NRAMP1 action in *Dictyostelium*, we have recently generated a Knock-out mutant. Evidence of the Knock-out is supported by southern and nothren experiments which show respectly the band shift due to homologue recombination and the mRNA absence in the vegetative stage. The phenotype characterization of KO mutant is in progress.

Characterizing the components of CF

Debra A. Brock and Richard H. Gomer

Howard Hughes Medical Institute and Department of Biochemistry and Cell Biology, Rice University, Houston, Texas, 77005-1892, USA

Dictyostelium cells use a secreted signal named counting factor (CF) to sense the number of cells in an aggregation stream. This information is used to regulate group size and hence fruiting body size, with a high concentration of CF causing streams to break up. We partially purified CF and found that it behaves as a complex of at least 5 polypeptides. Disruption of the genes encoding two of the polypeptides, countin and CF50, greatly decreased the activity of CF, resulting in streams not breaking up and huge groups forming as a consequence. We are now characterizing additional components of CF.

CF60. This is a resolvable doublet. CF60T contains an ATP/GTP-binding site motif and has 28% identity to a bacterial protein of unknown function; we are currently trying to disrupt *cf60T*. CF60B has 57% identity to histidine acid phosphatase. Several attempts have been made to disrupt this gene with initial recovery of transformants that form slightly larger fruiting bodies, but the cells invariably soon die.

CF50. Although *cf50*$^{-}$ cells form large fruiting bodies like *countin*$^{-}$ cells, *cf50*$^{-}$ cells have an increased cAMP-stimulated cAMP pulse while countin^{-} cells have a decreased cAMP pulse. In addition, cf50^{-} cells have an abnormal initial cell-type differentiation while that of *countin*$^{-}$ cells is normal. This indicates that countin and CF50 have overlapping but distinct functions.

CF45. Like CF 60, this appears to be two proteins. CF45-1 shares 64.2% identity with CF50. Also, like CF50, CF45-1 is 30% identical to lysozyme and contains distinctive serine-glycine motifs. Transformants with a disrupted *cf45-1* gene form abnormally large groups, and addition of recombinant CF45-1 rescues their phenotype by decreasing group size. The absence of CF45-1 also caused an increase in *countin* and *cf50* mRNA's and the corresponding proteins. CF45-2 is the CprF cysteine protease.

CF40 (countin). Disruption of countin results in the formation of large fruiting bodies.

CF32. There are two very similar genes encoding CF32-1 (85% identity) that are 304 bp apart. Neither CF32-1A nor B has any identity or motifs compared with other known proteins. *cf32-1A*$^{-}$ cells form large groups and have an altered initial cell-type differentiation. CF32-2 appears to encode a novel protein.

The interplay between Aardvark and cell movement.

E.C. Dalton[1], D. Wessels[2], D.R. Soll[2], and A.J. Harwood[1]

1MRC-LMCB, University College London, Gordon St. LONDON, WC1E 6BT, UK and 2WM Keck Dynamic Image Analysis Facility, Dept of Biological Sciences, University of Iowa, Iowa City, IA 52242, USA.

Aardvark (Aar) is a *Dictyostelium* homologue of β-catenin that is present in adherens junctions during culmination and is required for expression of the prespore gene, *psA* (1). We find that growth phase *aar* null mutant cells have an unusual morphology and *aar* mutants exhibit slow aggregation. In addition, we have previously found that a VE to GR substitution at residues 267,268 of GskA causes aberrant cell morphology and movement (2), which is also seen in cells that over-express Aar. Our observations suggest a role of Aar in cell movement during aggregation. Here, we present the results of 2-D and 3-D dynamic image analysis (3) of *aar* mutants. To investigate the basis of the abnormal cell shape and defective motility exhibited by these mutants, we examine the effects of *aar* loss on the cytoskeleton.

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Coding- region control of PKA-C : defining the target sequence

Myriam Adam [1], Karen Brennan [2], Matthias Hentze [2], Pierre Golstein [1].

1 Centre d'Immunologie de Marseille Luminy, INSERM-CNRS-Univ.Med., Campus de Luminy, case 906, 13288 Marseille cedex 9, France; 2 EMBL, Meyerhostrasse 1, D-69117 Heidelberg, Germany.

In a REMI screen for cell death resistant mutants, we isolated a novel gene that we named *dlrA* (*Dictyostelium* LRR Molecule A). Cells knocked out for this gene are aggregation minus mutants.

Expression analysis of early developmental markers showed that the PKA-C messager is destabilized in the *dlrA*- mutant. In order to better understand the relationship between *pka-C* and *dlrA*, we examined the coding sequence of *pka-C* : using reporter constructs, a 371 nt fragment was shown to down-regulate the expression of PKA-C in *dlrA*$^-$ cells ; this destabilization was inhibited by dlrA.

So PKA-C exerts a control on its own messager through its coding region. There are other examples of coding region control of mRNA stability (c-myc, c-fos, …), usually for very specific and tight control (Lavenu, 1995 ; Grosset, 2000).

We employed electrophoretic mobility shift assays (EMSA) with several labeled RNA fragments (within the 371 nt fragment mentioned above), together with total extracts from *pka-C* null or *dlrA*$^-$ cells. A complex pattern was observed with only one of these fragments. Moreover, the pattern differed as a function of the cells used for preparing the extracts.

We thus present the first detection and analysis of a coding control region in the slime mold *Dictyostelium*.

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intercellular communication genes in *Dictyostelium* development

K. Kibler[1,3], T. Nguyen[2,3], J. Svetz[3], N. VanDriessche[1,3], M. Ibarra[3], C. Shaw[3],and G. Shaulsky[1,3]

1Program in Developmental Biology, 2SMART Program, and 3Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, Texas, 77030

Integration of individual cells into a multicellular organism requires intercellular communication. Developmental aberrations that result from mutations in signal-production genes can be overcome by generating chimerae of mutant and wild-type cells. We have performed a large scale mutagenesis screen and identified nine mutant strains that are unable to generate spores when developed in a pure population but capable of bearing spores when developed in chimerae with wild-type cells. We postulate that the wild-type cells supply these mutant cells with a non cell-autonomous factor that rescues the mutant's defect in spore production. We cloned and sequenced the mutated genes and grouped the mutant strains based on their ability to sporulate when mixed with each other. Our assumption is that a pair of mutants which fail to form spores in chimerae are members of one signaling pathway.

Our efforts are directed towards characterizing a putative secreted signaling pathway mediated through the novel *comA* and *comB* gene products. Co-development with other strains rescues the sporulation defect of *comA*$^-$ or *comB*$^-$ mutant cells, but chimeric mixtures of *comA*$^-$ with *comB*$^-$ fail to sporulate. This finding implicates *comA* and *comB* as players in one signaling pathway. The developmentally regulated *comA* and *comB* transcripts are prestalk enriched and are detected earlier in development then the morphological arrests and cell-type specific defects of the respective mutant strains. Although the late developmental defects of *comA*$^-$ and *comB*$^-$ are dissimilar, several lines of evidence support the hypothesis that ComA and ComB function in a common signaling pathway, including similar expression array transcriptional profiles and a common genetic suppressor, *pkaR*$^-$. We propose that ComA and ComB perform similar functions early in development and divergent functions later in development.

Actin tyrosine phosphorylation patterns during spore aging in wild type and mutants of *Dictyostelium discoideum*

Dana Mahadeo[1], Tracy Marr[1], Keith Gale[1], Yoshiro Kishi[2], Kashif Kirmani[1], Masazumi Sameshima[2] and David Cotter[1].

1 Department of Biological Sciences, University of Windsor, Windsor, Ontario, Canada; 2 Department of Cell Biology, The Tokyo Metropolitan Organization for Medical Research, Tokyo, Japan.

The phosphorylation of a tyrosine residue of actin begins 22 hours after development is initiated during mid culmination in nascent spores of wild type *Dictyostelium discoideum* strain NC4 [2-4].Spores of strain NC4 are not fully dormant, however, until after 36 hours of development when mitochandria are crenated, 50% of spore actin is phosphorylated in cytoplasmic and nuclear rods, and resistance to a heat-shock of 45oC for 30 minutes is detected [2-6]. The same pattern of development was observed for strains V12, SG1 and SG2. When the ambient humidity of the fruiting body was low, the phosphorylation level peaked in the above spores from 3 to 5 days and then declined slightly during 14 days of aging. SpiA null spores in fruiting bodies at low humidity contained normal levels of phosphorylated actin by 36 hours of development suggesting that the regulation of actin tyrosine phosphorylation is a very late sporulation event unlinked to the earlier function of SpiA.

Axenic strains AX2 and AX3 did not begin to accumulate phosphorylated actin until 24 hours of development, but then accumulated normal levels which persisted for up to 10 days. The levels of phosphorylated actin then abruptly fell with a loss in spore viability. The loss in phosphorylated actin in spores of AX2 and AX3 between 5 to 10 days of aging was not temperature dependent since fruiting body formation at 22oC or 15oC resulted in similar declines during aging. The RegA null mutation suppressed the AX3 phenotype extending high levels of actin phosphorylation and spore viability past 10 days of aging.

As previously shown at these proceedings (*Dictyostelium* 2000) the SplA null strain formed spherical spores with very low levels of phosphorylated actin. Overexpression of PKA in *splA*$^{-}$/PKACAT^{+} suppressed the SplA null phenotype restoring tyrosine phosphorylation in capsule shaped spores.

Overall the past and present results support the hypothesis that normal sporulation and dormancy maintenance are regulated by numerous genes which control phosphorylation of actin through cAMP cascades [1].

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The *Dictyostelium* stalk cell inducer DIF regulates nuclear accumulation of a STATc protein by controlling its export from the nucleus

Masashi Fukuzawa, Tomoaki Abe and Jeffrey G. Williams

School of Life Sciences,University of Dundee,MSI/WTB Complex,Dow Street,Dundee DD1 5EHUK

Dd-STATc becomes tyrosine phosphorylated, dimerises and accumulates in the nuclei of Dictyostelium cells exposed to DIF, the chlorinated hexaphenone that directs prestalk cell differentiation. By performing cytoplasmic photobleaching of living cells, we show that DIF inhibits the nuclear export of Dd-STATc. Within Dd-STATc there is a 50 amino acid region containing several consensus CRM1 (exportin 1)-dependent nuclear export signals (NESs). Deletion of this region causes Dd-STATc to accumulate in the nucleus constitutively and, when coupled to GFP, the same region directs nuclear export. We show that the N terminal-proximal 46 amino acids are necessary for nuclear accumulation of Dd-STATc and sufficient to direct constitutive nuclear accumulation when fused to GFP. Combining the photobleaching and molecular analyses, we suggest that DIF-induced dimerisation of Dd-STATc functionally masks the NES-containing region and that this leads to its nett nuclear accumulation, directed by the N terminal-proximal import signals. These results show that that the regulated nuclear accumulation of a STAT protein can be controlled at the level of nuclear export and they also provide a better understanding of the precise mechanism whereby DIF directs cell type divergence.

The evolution of SH2 signalling: a biologically functional *Dictyostelium* STAT protein with an aberrant SH2 domain

N. V. Zhukovskaya[*], M. Fukuzawa[*], K. A. Jermyn, M. Tsujioka, T. Abe T. Kawata[+]and J. G. Williams[+]
[*]These authors contributed equally to this work

School of Life Sciences,University of Dundee,MSI/WTB Complex,Dow Street,Dundee DD1 5EHUK

Dictyostelium is the only non-metazoan organism thus far shown to utilise SH2 domain:phosphotyrosine interactions in intracellular sigalling, so a study of *Dictyostelium* SH2 domain containing proteins may give insights into their functional evolution. We describe a novel *Dictyostelium* STAT, Dd-STATb, that is nuclear-enriched in all cells during growth and early development but that is progressively lost from prestalk cells. We can detect no developmental defect in Dd-STATb null cells but it has a subtle role in cell growth; when Dd-STATb null cells are co-cultured with parental cells through several growth cycles, the proportion of mutant cells falls. Also, micro-array analyses show that the discoidin 1a gene is over-expressed in growing Dd-STATb null cells. Thus Dd-STATb is a regulator of gene expression that is needed for optimal cell growth. However, Dd-STATb contains a 21 amino acid insertion in its SH2 domain and the conserved and essential arginine residue, that interacts with phosphotyrosine in all known SH2 domains, is replaced by a leucine residue. In vitro binding reactions confirm that the Dd-STATb SH2 domain is defective in binding to its own tyrosine phosphorylation site. These data suggest a mode of STAT signalling that functions independently of tyrosine phosphorylation and dimerisation.

A novel prespore-inducing factor with low molecular weight in *Dictyostelium*: its partial purification and characterization

A.A. Oohata[1], and Y.Takaya[2]

1Biological Laboratory, Kansai Medical University, Hirakata, Osaka, 573-1136, Japan and 2Faculty of Pharmacy, Meijo University, Yagoto-yama, Tempaku, Nagoya, 468-8503, Japan

Previously, we have reported that conditioned medium (CM) of *D. discoideum* contains two types of prespore inducing factors which function synergistically in prespore cell induction (1). One is a 106 kDa glycoprotein termed psi (ψ) factor (prespore-inducing-factor) which induces not only prespore cell differentiation but also induces cell division of prespore cells. We cloned the corresponding cDNA that encodes a novel protein. The other is a heat-stable dialyzable factor. We are temporarily calling it "small factor". In this study, we established a simple bioassay system for the measurement of prespore-cell-inducing activity of small factor. As the tester cell, we use a sporogenous mutant, HTY506 (rdeC) which is known to differentiate into spore or stalk cell in a cell-density dependent manner under submerged monolayers in the presence of cAMP. However, at very low cell densities, the cells do not differentiate. In our submerged monolayer system, at a cell density of 5×10^2 cells/cm^2, most cells do not differentiate but cells efficiently differentiate into spore cells if small factor is provided. In this system, spore differentiation factors (SDFs) appear to be released from cells enough for spore formation to occur at this density. Because prespore cells are not detected whether small factor is provided or not. This finding suggests that all of the cells induced prespore cells differentiate into spore cells, and prespore inducing factor(s) are the limiting factors to spore formation. SDFs are also low-molecular-weight factors but they are not small factor (2). Moreover, the addition of ψ factor had no effect on prespore cell induction in this mutant, unlike wild type strains such as V12M2 and AX-2. Taken together in this system, one can estimate the prespore inducing activities of small factor by measuring the increase of spore cells by the addition of it. Surprisingly, 5000- to 7000-fold diluted CM is enough for a half maximal spore induction. Using this bioassay system, we are purifying the factor from CM of strain V12M2. Its characterization is also now in progress.

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Low molecular weight factors secreted by wild-type and DIF-less mutant of *Dictyostelium*, that are involved in the restoration of a mutant *erkB*$^-$

Masatsune Tsujioka[1,2], Tatsuyoshi Yamamoto[1], Christopher R. Thompson[2], Robert R. Kay[2] and Mineko Maeda[1]

1Osaka University,Osaka, Japan; 2MRC, Cambridge, UK

MAP-kinase ERK2 is essential for the multicellular development of *Dictyostelium*. Although the mutant *erkB*-null missing functional ERK2 fails to undergo morphogenesis (Segall *et al.*, 1995), but such defect can be restored by DRFs (Development-Rescuing Factors) that are secreted by developing wild type (Maeda and Kuwayama, 2000). We revealed that DIF-1 (Differentiation-Inducing Factor 1 for stalk cells) mimics the role of DRFs, that is a newly discovered role of DIF-1 (Kuwayama *et al.*, 2000). Here we report in this study that a *dmtA*-null mutant lacking methyltransferase essential for the DIF-1 synthesis also secretes a strong DRF activity, indicating the existence of novel DRFs other than DIF-1. We presumed that DIF-2 and DIF-3, analogues of DIF-1, might act as DRF instead of DIF-1 in the *dmtA*-null mutant, and tested whether these reagents rescue the development of the *erkB*-null. Both reagents rescued the mutant development but with much less activities than DIF-1. Then we expected that DRFs secreted by the *dmtA*-null mutant might differ from these DIFs, and decided to resolve DRFs secreted by the developing *dmtA*-null mutant and wild-type cells with HPLC. Consequently, two novel DRF activities were identified in conditioned medium prepared from these strains. These novel DRFs share some common properties with DIFs, however, they do not show any stalk inducing activity. Base on these results, we conclude that these DRFs are novel low molecular factors functioning in the restoration from the developmental defect of the *erkB*-null mutant. Further analysis of these factors would bring the insight into the mechanism how the developmental defect of *erkB*-null mutant is restored and how these DRFs act in the normal development of *Dictyostelium*.

Regulation and function of adenosine kinase in *Dictyostelium discoideum*

Elisa Alvarez-Curto and Pauline Schaap.

Department of Biochemistry, Wellcome Trust Building, School of Life Sciences, University of Dundee, Dundee, DD1 5EH, Scotland.

Degradation of cAMP by the extracellular PDE produces 5'AMP that will be later degraded to adenosine by the activity of the 5'nucleotidase. This adenosine is transformed into 5'AMP again by an adenosine kinase that is present in the extracellular medium.

Adenosine has been considered to play an important role in tip dominance and cell proportioning. This made us interested in the role and regulation of the enzyme adenosine kinase during development.

Screening the database we found a gene in *Dictyostelium*, which shares homology with the yeast, rat and human adenosine kinases. Adenosine kinase enzymatic activity appears to be present during the entire course of *Dictyostelium* development. Northern blot analysis shows an increase in adenosine kinase mRNA levels between 0 and 8 hours of starvation and a second increase at 18 h that reaches a peak during culmination.

We used two regions of this gene to create a knock-out construct that after transformation and blasticidin selection produced two Adok knock-out lines. Biochemical assays on knockout slugs showed that the extracellular adenosine kinase activity is lost.

Further analysis on other aspects of the phenotype such as cell proportioning and slug size is in progress.

Structure and function of ACG

Shweta Saran and Pauline Schaap,

MSI/WTB Complex, Dow Street, University of Dundee, DD1 5EH, Scotland

Adenyl cyclase germination specific (ACG) gene is expressed only during germination and is involved in the maintenance of spore dormancy and inhibition of spore germination. It encodes a single pass membrane protein expressed only in spores and is now known to act as an osmosensor.

In the first line of experimentation we are investigating if osmosensing is intrinsic to ACG. To understand this phenomenon we have expressed ACG in a heterologous system like *Saccharomyces cerevisiae* which are deficient in adenylyl cyclase activity. At present we are measuring the cAMP levels before and after osmotic stimulation.

In a different line of experimentation, our studies on the mechanism of activation of the adenyl cyclase have demonstrated that this enzyme is activated by dimerization. Results show inhibition of spore germination was obliterated when dominant interfering mutants were made. *In vitro* assays also showed that the mutated protein reduced the ACG activity when coexpressed with the wild type ACG. We are currently investigating the domains of the proteins involved in these interactions.

Clusters of gene expression in dicty time course data

Chad Shaw[1], Nancy Van Driessche[1], Mariko Katoh[2], Mimi Ibarra[1], Gad Shaulsky[1]

1Department of Molecular and Human Genetics, Baylor College of Medicine, Texas USA; 2 Institute of Biological Sciences, Tsukuba University, Tsukuba , Ibaraki, Japan

Microarray time course data is highly specialized data in which to search for co-expressed groups of genes. The time axis can be used explicitly to determine time-ordered trajectories for each gene. These time curves provide rich feature information for clustering genes, and the time ordered samples have more information than a collection of unordered values.

Time ordered curves can be estimated for each gene, and these curves can be introduced to a number of clustering techniques in order to recover group assignments for genes. Such techniques include mixture model and likelihood based clustering as well as Bayesian methods. We have used the time-trajectory approach to uncover at least 6 major modalities of gene expression in WT Dicty development. In addition, we have expanded this time-trajectory technique to incorporate multiple time course data from mutant genotypes.

Opposite activity patterns of the aggregative and late pdsa promoters in *Dictyostelium* slugs. Requirement for novel cell-cell interactions.

Karin E. Weening[1], Irene Verkerke-Van Wijk[2], Christopher R. Thompson[3], Richard H. Kessin[4], Gregory J. Podgorski[5] and Pauline Schaap[1]

1School of Life Sciences, University of Dundee, Dundee DD1 5EH, UK; 2Department of Biology, University of Leiden, 2333 AL Leiden, The Netherlands; 3MRC Laboratory of Molecular Biology, Cambridge, CB2 2QH, UK; 4Department of Anatomy and Cell Biology, College of Physicians and Surgeons of Columbia University, New York, NY 10032, USA; 5Department of Biology, Utah State University, Logan UT 84322-5305, USA

cAMP and DIF are considered to be the major determinants for cell type specification in *Dictyostelium* slugs. We studied the regulation of the aggregative (*PdA*) and late (*PdL*) promoters of the *PdsA* gene in slugs using labile _-galactosidase (gal) reporter enzymes. *PdL* was active in prestalk cells as was also found with stable gal. *PdA* activity decreased strongly in slugs from all cells except those at the rear. This is almost opposite to *PdA* activity traced with stable gal, where slugs showed sustained activity with highest levels at the front. After aggregation, *PdA* was down-regulated irrespective of stimulation with any of factors known to control gene expression in *Dictyostelium*. *PdL* activity was induced in cell suspension by cAMP and DIF acting in synergy. However, a DIF-less mutant showed normal *PdL* activity during development, indicating that DIF may not control *PdL in vivo*. Dissection of the *PdL* promoter showed that all sequences essential for correct spatio-temporal control of promoter activity are downstream of the transcription start site in a region between -383 and -19 nucleotides relative to the start codon. Removal of nucleotides to position -364 eliminated responsiveness to DIF and cAMP, but normal *PdL* activity in prestalk cells in slugs was retained. Further 5' deletions abolished all promoter activity. This result also indicates that the induction by DIF and cAMP as seen *in vitro* is not essential for *PdL* activity *in vivo*. We conclude that cell-type specification in *Dictyostelium* requires other intercellular signals than cAMP and DIF.

The Genetics of valproic acid resistance

K. Gregory and R.S.B.Williams

Wolfson Institute for Biomedical Research, Cruciform Building, University College London, Gower St London, WC1 E6BT, UK.

Bipolar or manic depression is a major health problem world-wide causing a substantial reduction in quality of life, with current treatments still giving a poor prognosis for recovery. Biological predisposition is a major factor in the occurrence of this disorder, yet no specific molecular abnormalities have been identified as giving rise to this disorder. However, previous work in *Dictyostelium* has defined genes involved in the response to lithium – a commonly used bipolar treatment - and has provided insight into how specific enzymes found to be elevated in bipolar patients may function in this disorder (1,2).

We are now using a pharmacogenetics approach to define mechanisms of valproic acid (VPA) action, a drug increasingly being used in the treatment of bipolar depression. This drug was originally identified as an anti-epileptic treatment and is also teratogenic, presenting difficulties in treatment during pregnancy. The mechanism of its function in these processes remains unknown, although it has been associated with inositol depletion in regard to bipolar depression efficacy (3,4) and inhibition of histone deacetylase in regard to teratogenicity (5,6). We have isolated three independent pools of *Dictyostelium* mutants resistant to VPA during growth, and are currently characterizing genes giving rise to these mutants.

We will present preliminary results concerning the effect of VPA on *Dictyostelium*, and the isolation and characterization of the genes inferring VPA-resistance. The overall aim of this work is to define novel genes involved in manic depression, epilepsy and in VPA teratogenicity and to help define its cellular mechanism of action.

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Induction of sexual development in *Dictyostelium mucoroides* by the overexpression of ACC-oxidase

Shousaku Soramoto, ÅõSoichi Yagura, Yasuo Maeda, and Aiko Amagai

Department of Developmental Biology and Neurosciences, Graduate Schoolof Life Sciences, Tohoku University, Aoba, Sendai, 980-8578, Japan

The cellular slime mold *Dictyostelium* mucoroides-7(Dm7) exhibits clear dimorphism in development depending upon environmental conditions such as light and water: macrocyst formation occurs as the sexual process, and sorocarp formation as the asexual process. Ethylene known as a potent plant hormone induces sexual development (macrocyst formation) by inducing zygotes which are formed by cell fusion and subsequent nuclear fusion. In *Dictyostelium* cells, ethylene was found to be biosynthesized from methionine through S-adenosylmethionine (SAM) and
1-aminocyclopropnane-1-carboxylic acid (ACC), as the case for higher plants. ACC-synthase and ACC-oxidase catalyze the conversion from SAM to ACC and ACC to ethylene, respectively. To analyse more directly the ethylene function, we prepared transformants (ACC^{OE}cells) overexpressing a homologue of ACC oxidase (Dd-aco) in *Dictyostelium*. ACC^{OE} cells actually produced a more amount of ethylene compared to parental Dm7 cells, and exerted macrocyst formation even under the conditions favorable to asexual development. Moreover, the overproduction of ethylene was found to enhance the expression of *zyg1*, a novel gene involved in zygote induction. Thus ethylene induces macrocyst formation by inducing the *zyg1* expression.

Transcriptional profiling of the dedifferenciation process in *Dictyostelium*

Mariko Katoh[1], Nancy Van Driessche[2], Chad Shaw[2], Miroslava Ibarra[2], Takahiro Morio[1], Hidekazu Kuwayama[1], Shinji Obara[1], Hideko Urushihara[1], Adam Kuspa[2], Gad Shaulsky[2] and Yoshimasa Tanaka[1]

1 Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan and 2 Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA

Upon nutrient depletion, *Dictyostelium discoideum* cells initiate their developmental program to form multicellular structure comprising of two differentiated cell types. On the way to completion of development, cells can be made to dedifferentiate and return to the growth phase by mechanical disaggregation and subsequent incubation in a fresh nutritional source. Previous studies have suggested the possibility that the dedifferentiation process may be regulated by a dedicated genetic program. We have tested that possibility using transcriptional profiling.

We first developed cells to three stages: aggregation, fingers, or mexican hat, disaggregated them, and incubated in growth medium. We prepared RNA from the cells and analyzed the relative mRNA abundance of about 7,000 genes throughout the time-course of dedifferentiation using *Dictyostelium* cDNA microarray. The timing of re-entry into the growth phase was monitored by the amount of BrdU incorporation into DNA and increases in cell number. Re-differentiation experiments were performed to assess the totipotency of the erasing cells. We found that the timing to revert to vegetative growth was directly proportional to the developmental stage. Aggregation stage cells entered growth phase faster than slug stage cells and the Mexican hat stage cells were the slowest. We also found that the process consists of 3 transcriptional phases and that the expression of about 300 genes was up-regulated during the erasure process, regardless of the initial developmental stage of the cells. Many of these genes were also regulated during normal development. Therefore, the transcriptional analysis of the erasing cells revealed a profile that is common to all three stages, indicating that *Dictyostelium* cells utilize a dedicated physiological pathway during the process of erasure.

A *Dictyostelium* gene encoding a disintegrin domain protein has pleiotrophic effects on cell-cell and cell substrate adhesion, and developmental progression.

Daphne D. Blumberg, Chere' Petty, Hoa N. Ho, and Timothy R. Varney.

Department of Biological Sciences, University of Maryland, Baltimore County, 1000 Hilltop Circle Baltimore, Maryland 21250

The *Dictyostelium* protein AmpA (Adhesion modulation protein A) contains repeated domains homologous to proteins that influence cell adhesion including the Disintegrins. The protein accumulates during development, reaching a maximal level at the finger stage. It is also detectable in growing cells as they reach high density. Much of the AmpA protein is found extracellularly during development but it is not secreted from growing cells. While it is expressed in about 50% of growing cells, during development its expression is restricted to the Anterior-Like Cells that comprise less than 10% of the total cells in the fruiting body. The migration of these cells plays a role in morphogenesis of the fruiting body. Characterization of an *ampA*$^-$ strain generated by gene replacement reveals a significant increase in cell-cell clumping both in growing cells and when cells are developed by starving in non-nutrient buffer suspensions. Developing *ampA*$^-$ cells are also more adhesive to the underlying substrate. The increase in cell-substratum adhesion appears to cause a delay in developmental progression and morphogenesis. Reintroduction of the ampA gene rescues the developmental defects of *ampA*$^-$ cells. However, expression of additional copies of the gene in wild type cells results in more severe developmental delays and decreased clumping in suspension culture. We propose that the AmpA protein functions as an anti-adhesive to limit cell-cell and cell-substrate adhesion during development and thus facilitate cell migration during morphogenesis.

Kinetics of intracellular localization of ERK2 during the multicellular development of *Dictyostelium*

Daisuke Ikeno, Tatsuyoshi Yamamoto, and M. Maeda

Department of Biology, Graduate School of Science, Osaka University Toyonaka, Osaka 560—0043, Japan

ERK2 is essential for the multicellular development of *Dictyostelium* (Segall *et al.*, 1995). The mutant *erkB*-null lacking functional ERK2 is unable to aggregate and show impaired response involved in the extracellular cAMP-induced cAMP increase. However, the molecular mechanism how ERK2 functions in the cAMP response is unknown. In order to get some insights into the mechanism, intracellular localization of ERK2 was analyzed by monitoring ERK2-DsRed1 in the *erkB*-null mutant.

Expression of ERK2-DsRed1 restored the phenotype of the *erkB*-null and ERK2-DsRed1 was activated by cAMP with a similar kinetics seen in wild-type Ax2, indicating ERK2-DsRed1 is functional. We found that ERK2 is distributed uniformly in whole cytoplasm at the vegetative stage, but several hours after starvation but before aggregation, ERK2 strongly accumulated in a single spot at the periphery of a nucleus. Immunohistochemistry with anti-α-tubulin suggests that the spot is unlikely to be a centrosome. Interestingly, at the aggregation stage, ERK2-DsRed1 became accumulated in a nucleus. However, such nuclear translocation of ERK2-DsRed1 did not occur at the mound stage. These results suggest that ERK2 may regulate gene expression at the aggregation stage. Next we examined whether the nuclear translocation of ERK2 is regulated through the activation of ERK2. We found that ERK2 activation occurred prior to the nuclear translocation, suggesting that ERK2 activation might be required for the nuclear translocation of ERK2. Currently, we are examining whether a spatial gradient or global increment of cAMP affects the nuclear translocation of ERK2 and a dominant negative form of ERK2 is able to translocate into a nucleus. Our results will contribute to the further understanding of ERK2 function in *Dictyostelium* development.

Control of cAMP pulse propagation during *Dictyostelium discoideum* aggregation by external d.c. electric fields.

Elena Slamova, Lenka Forstova, Tomas Godula and Hana Sevcikova

Center for Nonlinear Dynamics of Chemical and Biological Systems, Prague Institute of Chemical Technology, Technicka 5, 166 28 Prague 6, Czech Republic.

From the point of view of the scientific field called nonlinear dynamics the layer of starving *Dictyostelium* cells in the early stage of aggregation is perceived as an excitable medium through which the pulses of increased cAMP concentration propagate by reaction-diffusion mechanism. Biochemical reactions with the positive feedback (the production of cAMP in cells) lead to the fast local increase of concentration of cAMP which then diffuses to the neighbouring cells triggering there further production of cAMP. The velocity of the propagation of cAMP pulses depends on the parameters of both the biochemical production and diffusive transport. Since cAMP is present as a negatively charged ion an electric field, imposed on the layer of DD cells, can enhance the transport of cAMP between cells. The electromigration flow can either add to the diffusive transport or can act against the diffusive transport. As a result, the changes in the velocity of propagation of cAMP pulses can occur.

The poster will present the results of experimental investigations of the effects of d.c. electric fields imposed on the layer of DD cells on the nutrition-free agar on Petri dish. We will show that relatively week electric fields can substantially change the propagation velocity of cAMP pulses. In the electric field 5V/cm the average velocity was found to increase from 0.28 mm/min (when no electric field was imposed) to 0.37 mm/min when cAMP pulses propagated towards the positive electrode and to decrease to 0.22 mm/min when the cAMP pulses propagated towards the negative electrode. These experimental findings support the previous results of the simulation study [1] of reaction-diffusion-migration mathematical model based on 3-variable Martiel-Goldbeter kinetics of cAMP production in DD cells [2]. The results of simulations will also be briefly reviewed in the poster presented.

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Myosin fusions as tool for protein expression and purification in the lower eukaryote *Dictyostelium discoideum*

Hartmut H. Niemann, Georgios Tsiavaliaris, Menno L.W. Knetsch, F. Jon Kull and Dietmar J. Manstein

Max-Planck-Institut für medizinische Forschung, Abteilung Biophysik, Jahnstr. 29, 69120 Heidelberg, Germany: Hartmut.Niemann@mpimf-heidelberg.mpg.de, Georgios.Tsiavaliaris@mpimf-heidelberg.mpg.de, Menno.Knetsch@BIOCH.UNIMAAS.NL, F.Jon.Kull@Dartmouth.edu Manstein@mpimf-heidelberg.mpg.de

Dictyostelium discoideum is well a established expression system [1]. These lower eukaroytic cells carry out mammalian-like posttranslational modifications. Large amounts can be grown in shaking culture using simple and inexpensive media. Here, we show that the myosin catalytic domain can be used as a versatile tag. Proteins or domains fused to myosin are usually expressed at a high level in *D. discoideum*, even if only low amounts of the untagged protein are expressed. In addition to the expression of endogenous genes, good expression was achieved with hybrid fusions employing rat, mouse, and *Plasmodium falciparum* genes. Myosin's nucleotide dependent affinity for actin provides a means for a fast and efficient initial purification step by centrifugation. After this initial step, a single metal chelate column is sufficient to purify the fusion protein to homogeneity using an N-terminal His-tag. The target protein can be proteolytically cleaved from the tag if a protease cleavage site is included. Alternatively the whole fusion protein can be used for structure determination by X-ray crystallography. So far, the crystal structures of two protein domains have been solved using myosin fusions. This approach proved successful in the case of repeats 1 and 2 of □-actinin [2] and in the case of a dynamin GTPase domain [3]. The fusion proteins crystallized under conditions similar to those of the untagged myosin. Myosin also served as a phasing tag. The structures were solved by molecular replacement with the known structure of the myosin catalytic domain as search model. Using myosin fusions structures of new proteins can be solved with a single native data set. Hence, the myosin motor domain is an ideal expression tag for eukaryotic cells like *D. discoideum*. Furthermore it serves as a purification tag and in combination with a His-tag allows proteins to be purified to homogeneity in a rapid two-step procedure. In several cases, the myosin motor domain has successfully been used as a crystallization tag and a phasing tag for molecular replacement.

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Functional Characterization of Members of the Myosin I Family from *Dictyostelium discoideum*

Ulrike Dürrwang[1], Setsuko Fujita-Becker[1], Muriel Erent[1], Michael A. Geeves[2], and Dietmar J. Manstein[1,3]

[1]*Max-Planck Institut für medizinische Forschung, Jahnstr. 29, D-69120 Heidelberg, Germany*
[2]University of Kent, Canterbury CT2 7NJ, United Kingdom

Dictyostelium discoideum MyoB, MyoD and MyoE are members of the class I myosins, single-headed molecular motors, that play important roles in actin-based cellular processes, such as membrane traffic and cell locomotion. The functional properties of these myosins were investigated using a combination of direct functional assays, kinetic measurements and localization studies. The linking of an engineered lever arm to the motor domains of MyoB, MyoD, and MyoE produced motors that move actin with similar or up to 6-fold faster velocity than a similar myosin II construct. Transient kinetic results indicate that the ATP-induced dissociation of actomyosin is fast. Values of 1.11 $\mu M^{-1}s^{-1}$, 0.49 $\mu M^{-1}s^{-1}$, and 0.40 $\mu M^{-1}s^{-1}$ were measured for $\mathbf{K_1k_{+2}}$ with motor domain constructs B698, D692 and E698, respectively. In comparison to *Dictyostelium* myosin II (K_D = 14 μM) the ADP affinity of MyoB, MyoD, and MyoE is increased. K_D values are 1 μM for B698, 2 μM for D692, and 7 μM for E698. In the case of MyoB and MyoD actin binding strongly affects the affinity for ADP, while coupling between the actin- and nucleotide-binding sites appears to be much weaker for MyoE. $\mathbf{K_{AD}}$ values of 62 μM for B698, 105 μM for D692, and 12 μM for E698 were obtained. The regulation of the *Dictyostelium* class I myosins by heavy-chain phosphorylation was analyzed using TEDS-site mutants and protein constructs treated with a kinase or phosphatase, respectively. TEDS phosphorylation affects actin affinity mainly by decreasing the rate of dissociation of the actomyosin complex.
Visualization of Yellow Fluorescent Protein-fusions with MyoD and MyoE indicated that the proteins are localized throughout the cytoplasm and at the plasma membrane, where they are accumulated in pseudopodia, cell protrusions, and macropinocytic cups.

Abe, Tomoaki
Dundee University
Wellcome Trust Biocentre, Dow Street,
DDI 5EH Dundee
Scotland
t.abe@dundee.ac.uk

Alexander, Hannah
University of Missouri
Division of Biological Sciences
303 Tucker Hall
65211 Columbia,Missouri
USA
Alexanderh@missouri.edu

Alexander, Stephen
University of Missouri
Division of Biological Sciences
303 Tucker Hall
65211 Columbia,Missouri
USA
Alexanderst@missouri.edu

Alvarez-Curto, Elisa
WTB, University of Dundee
Dow Street DD1 5EH
Dundee,
Scotland
curtos@hotmail.com

Amagai, Aiko
Dept. of Developmental Biology and Neuroscience
Graduate School of Life Science, Tohoku University,
Sendai 980-8578
Japan
aiamagai@mail.cc.tohoku.ac.jp

Anjard, Christophe
UCSD
9500 Gilman Drive Dept. Biology 0368
La Jolla, CA 92093,
USA
canjard@biomail.ucsd.edu

Annesley, Sarah
Microbiology Dept.
La Trobe University
Plenty Rd. Bundoora
3086 Victoria,
Australia
Sarahannesley@hotmail.com

Araki, Tsuyoshi
Dundee University
Wellcome Trust Biocentre, Dow Street, DDI 5EH
Dundee
Scotland
t.araki@dundee.ac.uk

Arigoni, Maddalena
Dip. Scienze Cliniche e Biologiche
Ospedale S.Luigi
10043 Orbassano
Italy
maddalena.arigoni@unito.it

Baik, Unha
J.-W.- Goethe Universitat Frankfürt, Germany
Institut Pharm. Biologie;AK Dingermann
Marie-Curie- Strasse 9
Frankfurt
Germany
um.baik@web.de

Balbo, Alessandra
Dip. Scienze Cliniche e Biologiche
Ospedale S.Luigi
10043 Orbassano
Italy
alessandra.balbo@unito.it

Balest, Alessandra
Dip. Scienze Cliniche e Biologiche
Ospedale S.Luigi
10043 Orbassano
Italy
maddalena.arigoni@unito.it

Barth, Christian
MicrobiologyDept. , La Trobe University,
Plenty Rd, Bundoora,
306 Victoria
Australia
C.Barth@latrobe.edu.au

Beck, Peter
J.-W.- Goethe Universitat Frankfurt, Germany
Institut Pharm. Biologie;AK Dingermann
Marie-Curie- Strasse 9
Frankfurt
Germany
Peter_Beck@t-online.de

Beech, Peter
School of Biological and Chemical Sciences, Deakin University
221 Burwood HWY
3125 Melbourne
Australia
plbeech@deakin.edu.au

Benghezal, Mohammed
Dept. Morphology CMU Geneva
Centre Medical Universitaire, Morphologie
1 Rue Michel Servet, 1211 Geneve 4,
Switzerland
MOHAMMED.BENGHEZAL@MEDICINE.UNIGE.CH

Blaauw, Mieke
University of Groningen, the Netherlands
Dept. of Biochemistry, Nijenborh, 4
9747 AG Groningen
The Netherlands
Blaauw@chem.rug.nl

Blumberg, Daphne
Dept. Biological Sciences, University of Maryland
1000 Hilltop Circle
Baltimore, MD 21250
USA
blumberg@umbc.edu

Bozzaro, Salvatore
Dip. Scienze Cliniche e Biologiche
Ospedale S.Luigi
10043 Orbassano
Italy
sbozzaro@polito.it

Bracco , Enrico
Dip. Scienze Cliniche e Biologiche
Ospedale S.Luigi
10043 Orbassano
Italy
enrico.bracco@unito.it

Brazill, Derrick
Hunter College, Dept of Biological Sciences,
Rm 927 North Blag, 695 Park Eve
10021 New York,NY
USA
brazill@genectr.hunter.cuny.edu

Brock, Debbie
Harward Hughes Medical lstitute /RICE University
6100 S Main Biochem MS-140
Rice University Houston
77005-1892 Houston Texas
USA
dbrock@bioc.rice.edu

Brzostowski, Joseph
National Institute of Health, LCDB/NIDDK
50 Center Drive, Rm 3347
20892-8028 Bethesda
USA
jb363a@nih.gov

Ceccarelli, Adriano
Dip. Scienze Cliniche e Biologiche
Ospedale S.Luigi
10043 Orbassano
Italy
adriano.ceccarelli@unito.it

Chida, Junji
Graduate School of Life Science, Tohoku university
Aoba,
Sendai 980-8578
Japan
j-chida@biology.tohoku.ac.jp

Chisholm, Rex
Northwestern University
Cell and molecular Biology
303 E. Chicago Ave.
Chicago,IL 60611
USA
R-CHISHOLM@NORTHWESTERN.EDU.

Clarke, Margaret
Oklahoma Medical Research Foundation
825 N.E. 13th Street
73104 Oklahoma city, Oklahoma
USA
clarkem@omrf.ouhsc.edu

Cotter, David
Dept. Biological Sciences, University of Windsor
401 Sunset Avenue
N9B3P4 Windsor,
Canada
cotter1@uwindsor.ca

Cox, Edward C.
Princeton University
Department of Molecular Biology
333 Moffett Lab
Princeton, NJ 08544
USA
ecox@molbio.princeton.edu

Dalton, Emma C.
Laboratory for Molecular Cell Biology and CBU
University College London
Gordon street
London WC1E 6BT
UK
e.dalton@ucl.ac.uk

Daniels, Karla
Dept. of Biological Sciences, 14 BBE,
The University of Iowa
52242 Iowa City
USA
karla-daniels@uiowa.edu

Davies, Rob
Wellcome Trust Sanger Institute
Genome Campus, Hinxton, Cambridge, UK
Cambridge CB10 1SA
UK
rmd@sanger.ac.uk

De Lozanne, Arturo
Dept. Molecular Cell & Developmental Biology
241 Patterson Bldg, Campus mail Code C-0930
University of Texas,
Austin, TX 78712,
USA
a.delozanne@mail.utexas.edu

Dondero, Francesco
Department Science and Advanced Technology
University of Piemonte Orientale Amedeo Avogadro
Corso Borsalino 54 Alessandria
Italy
fdondero@unipmn.it

Dormann, Dirk
Division of Cell and Developmental Biology
WTB, School of Life Sciences,
University of Dundee
DD1 5EH Dundee,
Scotland
d.dormann@dundee.ac.uk

Eichinger, Ludwig
Institute fuer Biochemie I
Joseph-Stelzmann-Str.52
50931 Koeln
Germany
Ludwig.eichinger@uni-koeln.de

Escalante, Ricardo
Instituto de Investigaciones Biomédicas CSIC/UAM
Aurto Duperier 4
28029 Madrid,
Spain
rescalante@iib.uam.es

Faix, Jan
LMV-Munich, Inst. for Cell Biology
Schiller str. 42
80336 Muenchen
Germany
hfaix@bio.med.uni-muenchen.de

Fey, Petra
Northwestern University
303 E Chicago Ave,
CMB,Ward 11-100
60611 Chicago
USA
pfey@northwestern.edu

Firtel, Richard
University of California, San Diego
Section of Cell and Developmental Biology
Center for Molecular Genetics
9500 Gilman Drive
La Jolla CA 92093-0634
USA
rafirtel@ucsd.edu

Fisher, Paul
Microbiology Dept. , La Trobe University
Plenty Rd., Bundoora
3086, Victoria
Australia
fisher@lumi.latrobe.edu.au

Folk, Peter
Dept. Physiology and Developmental Biology,
Charles University Prague
Vinicna 7
Praha 2, 128 00
Czech Republic
dicty@mbox.cesnet.cz

Foster, Kevin
Rice University
EEB,MS170,6100 Main Street
77005 Houston Texas
USA
KRFOSTER@RICE.EDU

Franke, Jakob
Columbia University
630 w.168th Street, P&S 12-517
10032 New York NY
USA
jf31@columbia.edu

Fukuzawa, Masashi
Dundee University
Wellcome Trust Biocentre, Dow Street,
DDI 5EH Dundee
Scotland
m.fukuzawa@dundee.ac.uk

Fuller, Danny
UCSD Division of Biology
Mailcode 0368
La Jolla, CA 92093
USA
dfuller@ucsd.edu

Gerisch, Guenther
MPI fuer Biochemie
Am Klopferspitz 18A
82152 Martinsried b.M.,
Germany
gerisch@biochem.mpg.de

Gloeckner, Gernot
Dept. Genome Analysis, IMB Beutenbergstr. 11
D-07745 Jena
Germany
gernot@imb-jena.de

Golstein, Pierre
Centre d'Immulogie de Marseille-Luminy
Case 906,
13288 Marseille Cedex 9
France
golstein@ciml.univ-mrs.fr

Gomer, Richard
Harward Hughes Medical lstitute /RICE University
6100 S Main Biochem MS-140
Rice University Houston
77005-1892 Houston Texas
USA
richard@rice.edu

Graef, Ralph
Adolf-Butenandt-Institute/Cell Biology, University Munich
Schillerstr. 42
Munich D-80336
Germany
Ralph.Graef@Lrz.uni-muenchen.de

Hamlin, Nancy
Wellcome Trust Sanger Institute
Genome Campus, Hinxton,
Cambridge CB10 1SA
UK
neh@sanger.ac.uk

Harwood, Adrian J.
Laboratory for Molecular Cell Biology and CBU
University College London
Gordon street
London WC1E 6BT
UK
a.harwood@ucl.ac.uk

Heid, Paul
Dept. of Biological Sciences, 14 BBE,
The University of Iowa
52242 Iowa City
USA
paul-heid@uiowa.edu

Hereld, Dale
University of Texas-Houston Medical School
6431 Fannin Street, JFB 1.765
77030 Houston Texas
USA
dale.hereld@uth.tmc.edu

Heuser, John
Dept. of Cell Biology
University School of Medicine.
60 South Euclid Ave.
St Louis, MO. 63110
jheuser@cellbio.wustl.edu

Hirose, Shigenori
Graduate School of Life Science, Tohoku University
Aoba,
Sendai 980-8578
Japan
sigenori@biology.tohoku.ac.jp

Hsu, Duen-Wei
Department of Biochemistry,
Oxford University
Oxford OX1 4BH
UK
dwhsu@bioch.ox.ac.uk

Ibarra, Miroslava
Baylor College of Medicine
One Baylor Plaza, S430
Houston TX 77030
USA
mibarra@bcm.tmc.edu

Iijima, Miho
Johns Hopkins University School of Medicine
Wood Basic Science 116, 725N. Wolfe st.
21205 Baltimore, MD
USA
mshimizu@jhmi.edu

Ikeno, Daisuke
Dept. of Biology, Graduate School of Science, Osaka University
Machikaneyama 1-16,
560-0043 Osaka
Japan
ikeno@bio.sci.osaka-u.ac.jp

Insall, Robert
Dept. of Biosciences, University of Birmingham
EDGBASTON , Birmingham
Birmingham B15 2ATT
UK
R.H.Insall@bham.ac.uk

Iranfar, Negin
UCSD Division of Biology
9500 Gilman Dr.
MC 0368, LA JOLLA, CA,
USA
niranfar@ucsd.edu

Jang, Wonhee
Rice University
Dept. of Biochemistry and Cell Biology
6100 S Main Street
77005-1892 Houston Texas
USA
wany@rice.edu

Jin, Tian
Lab. of Immunogenetics, National Institute of Allergy and Infectious Diseases,
NIAID/NIH, 12441 Parklawn Dr. Twinbrook II,
MD 20878 Rockville
USA
tjin@niaid.nih.gov

Katoh, Mariko
Institute of Biological Sciences, University of Tsukuba
I-I-I Tennodai,
3058572 Tsukuba,
Japan
katoh@sakura.cc.tsukuba.ac.jp

Katz, Eugene
Dept. of Microbiology
Stony Brook University
11794 Stony Brook, NY
USA

Kay, Robert
MRC Laboratory of Molecular Biology
Hills RD,
Cambridge CB2 2QH
UK
rrk@mrc-lmb.cam.ac.uk

Kessin, Richard H.
Columbia University
630 w.168th Street, P&S 12-517
10032 New York NY
USA
rhk2@columbia.edu

Kibler, Kirsten
Baylor College of Medicine
One Baylor Plaza
77030 Houston,TX
USA
KKO38386@bcm.tmc.edu

Kimmel, Alan
NIH
50/3351 50 South Drive
Bethesda, MD 20892,
USA
ark1@helix.nih.gov

King, Jason
Dept. of Biosciences, University of Birmingham
EDGBASTON , Birmingham
B15 2ATT
Birmingham
UK
KINGJ@PUGH.BIP.BHAM.AC.uk

Korn, Edward
Laboratory Cell Biology, NHLBI, NIH
Building 50 Room 2517
9000 Rockville Pike
20892 Bethesda,md
USA
edk@nih.gov

Kriebel, Paul
National Cancer Institute
Bldg 37, Rm 1E24
9000 Rockville Pike
Bethesda, MD 20892,
USA
kpaul@helix.nih.gov

Kuspa, Adam
Department of Biochemistry and Molecular Biology.
Mail station 356A
Baylor College of Medicine
Houston TX77030-3498
USA
akuspa@bcm.tmc.edu

Kuwayama, Hidekazu
Institute of Biological Sciences, University of Tsukuba
Tennodai 1-1-1,
305 8572 Tsukuba,
Japan
kuwayama@sakura.cc.tsukuba.ac.jp

Lay, Sui
Dept. Microbiology, La Trobe University
Plenty Road, Bandoora
3086 Victoria,
Australia
sui@lumi.latrobe.edu.au

Letourneur, Francois
IBCP/CNRS
Institut de Biologie et chemie des Proteines
7, passage du vercors
69367 Lyon
France
f.letourneur@ibcp.fr

Lin, Sophia
Department of biochemistry, Oxford University
South Parks Rd.
Oxford OX1 3QU
U.K.
hhlin@bioch.ox.ac.uk

Loomis, William
Division of Biology, UCSD
0368, UCSD
La Jolla, CA 92093
USA
wloomis@ucsd.edu

Loovers, Harriet
University of Groningen, the Netherlands
Dept. of Biochemistry, Nijenborh, 4
9747 AG Groningen
The Netherlands
Loovers@chem.rug.nl

Luo, Hongbo
Johns Hopkins Medical School
725 N Wolfe street, Dept. of Neuroscience,
Johns Hopkins Medical School
ND21205 Baltimora
USA
hluoa@jhmi.edu

MacWilliams, Harry
University of Munich
Luisenstrasse 14
80333 Munich
Germany
macw@zi.biologie.uni-muenchen.de

Maeda, Yasuo
Dept. of Developmental Biology and Neuroscience
Graduate School of Life Science, Tohoku University,
Sendai 980-8578
Japan
ymaeda@mail.cc.tohoku.ac.jp

Maeda, Mineko
Dept. Biology, Graduate School of Science, Osaka University
Machikakeyama 1-16, Toyonaka
5600043 Osaka,
Japan
mmaeda@bio.sci.osaka-u.ac.jp

Mahadeo, Dana
Lab. Cellular & Molecular Biology, NCI, NIH
37 Convent Drive
Bethesda, MD 20892-4255,
USA
mahadeod@mail.nih.gov

Mai, Andrea
Dip. Scienze Cliniche e Biologiche
Ospedale S.Luigi
10043 Orbassano
Italy
andrea.mai@unito.it

Malchow, Dieter
Fachbereich Biologie, Post fach M646
Universitaet Konstanz
78457 Konstanz
Germany
Christina.Schlatterer@uni-konstanz.de

Maniak, Markus
ZellBiologie, Univ.Kassel, Germany
Heinrich-Plett-Str 40
34132 Kassel
Germany
maniak@uni-kassel.de

Manstein, Dietmar
Max Planck Inst. for medical research
Dept of Biophisics, Jahnstr. 29
69120 HEIDELBERG
Germany
MANSTEIN@MPIMF-HEIDELBERG.MPG.DE

Meima, Marcel
MSI/WTB complex, University od Dundee
Dow Street DDI 5EH
Dundee,
Scotland
m.e.z.meima@dundee.ac.uk

Miura, Kota
Cell Biology & Biophysics, EMBL
Meyerhof Str. 1
69117 Heidelberg
Germany
miura@embl-heidelberg.de

Mueller-Taubenberger, Annette
Max Planck Institut fuer Biochemie
Am Klopferspitz 18A
82152 Martinsried
Germany
amueller@biochem.mpg.de

Muramoto, Tetsuya
Institute of Biological Sciences, University of Tsukuba
Tennodai 1-1-1
305-8572 Tsukuba
Japan
s015478@ipe.tsukuba.ac.jp

Mutzel, Rupert
Institut fuer Biologie-Mikrobiologie, Freie Universitaet Berlin
Koenigin-Luise Str. 12-16
14195 Berlin
Germany
rmutzel@zedat.fu-berlin.de

Nathke, Inke
Dundee University
Wellcome Trust Biocentre, Dow Street, Dundee
DDI 5EH
Dundee
Scotland
i.s.nathke@dundee.ac.uk

Newell, Peter
University of Oxford
2 South Parks Road
Oxford OX1 3UB
UK
NEWELL@BIOCH.OX.AC.UK

Noegel, Angelika
Institute of Biochemistry, Med.Fac, University of Cologne
Institute of Biochemistry Joseph-Stelzmann Str.52
50931 Koeln
Germany
noegel@uni-Koeln.de

Obara, Shinji
Institute of Biological Sciences, University of Tsukuba
Tennodai 1-1-1,
3058572 Tsukuba,
Japan
obara@sakura.cc.tsukuba.ac.jp

Oohata, Akiko
Biological Lab, Kansai Medical University
Hinaka, Osaka 573-1136
5731136 Hinaka
Japan
oohata@makino.kmu.ac.jp

Otto, Grant
Columbia University
630 w.168th Street, P&S 12-517
10032 New York NY
USA
go25@columbia.edu

Pachebat, Justin
LMB, MRC Centre
RM 5010, PNAC Biotech, Laboratory of Molecular Biology,
Addenbrockes Hospital Site Hills Road
Cambridge CB2 2QZ
UK
jpacheb@mrc-lmb.cam.ac.uk

Parent, Carole
Lab. Cellular & Molecular Biology, NCI, NIH
37 Convent Drive, Bldg 37/Rm 1E24, MSC 4255
Bethesda, MD20892-4255,
USA
parentc@helix.nih.gov

Pears, Catherine
Bichemistry Dept, Oxford University
South Parks Road, Oxford
Oxford OXI 3QH
U.K.
pears@bioch.ox.ac.uk

Peracino, Barbara
Dip. Scienze Cliniche e Biologiche
Ospedale S.Luigi
10043 Orbassano
Italy
barbara.peracino@unito.it

Pergolizzi, Barbara
Dip. Scienze Cliniche e Biologiche
Ospedale S.Luigi
10043 Orbassano
Italy
barbara.pergolizzi@unito.it

Postma, Marten
University of Groningen, the Netherlands
Dept. of Biochemistry, Nijenborh, 4
9747 AG Groningen
The Netherlands
Postma@chem.rug.nl

Queller, David
Dept. of ecology and evolutionary Biology MS-170
Rice University, P.O. Box 1892
77251-1892 Houston
Texas,
U.S.A
Queller@rice.edu

Rajandream, Marie-Adèle
Wellcome Trust Sanger Institute
Genome Campus, Hinxton, Cambridge, UK
Cambridge CB10 1SA
UK
mar@sanger.ac.uk

Ratner, David
Amherst College
Dept. Biology
Amherst, MA01002,
USA
diratner@amherst.edu

Rivero , Francisco
Institute of Biochemistry,
Med.Fac, University of Cologne
Joseph-Stelzmann Str.52
50931 Koeln
Germany
Francisco.Rivero@UNI-KOELN.DE

Roper , Janine
University of Dundee MSI/WTB
DDI 5EH
Dundee
Scotland
j.r.roper@dundee.ac.uk

Ryves, Jonathan
Laboratory for Molecular Cell Biology and CBU
University College London
Gordon street
London WC1E 6BT
UK
dmcbwjr@ucl.ac.uk

Saito, Tamao
MRC lab. of Molecular Biology, Hills Road
CB2 2QH
Cambridge
UK
tasaito@mrc-lmb.cam.ac.uk

Sameshima, Masazumi
EM Center, The Tokyo Metropolitan Institute of Medical Science
Honkomagome 3-18-22, Bunkyo-ku
1138613 Tokyo,
Japan
msameshi@rinshoken.or.jp

Santorelli, Lorenzo Andrea
RICE University
EEB DEPT
6100 main Street 77005 Houston (TX)
USA
LASANTO@RICE.EDU

Saran, Shweta
MSI/WTB Complex, University of Dundee
Dow Street
DD1 5EH Dundee,
Scotland
shweta_saran@hotmail.com

Sasaki, Kazunori
Graduate School of Life Science,
Tohoku University Aoba, Sendai 980-8578
Japan
kazu@biology.tohoku.ac.jp

Sastre, Leandro
Instituto de Investigaciones Biomédicas
Arturo Duperier 4
28029 Madrid,
Spain
lsastre@iib.uam.es

Schaap, Pauline
MSI/WTB complex, University of Dundee
DD1 5EW Dundee,
Scotland
p.schaap@dundee.ac.uk

Schilde, Christina
Dundee University
Wellcome Trust Biocentre,
DDI 5EH Dundee
Scotland
c.schilde@dundee.ac.uk

Schlatterer, Christina
Fachbereich Biologie, Post fach M646
Universitaet Konstanz
78457 Konstanz
Germany
Christina.Schlatterer@uni-konstanz.de

Schleicher, Michael
Institute for Cell Biology LMU
University of Muenchen
80336 Muenchen
Germany
schleicher@lrz.uni-muenchen.de

Sevcikova, Hana
Center for Nonlinear Dynamics,
Prague Institute of Chemical Technology
Prague,Czech Republic
Technicka 5
166 28 Prague 6
Czech Republic
Hana.Sevcikova@vscht.cz

Shaulsky, Gad
Baylor College of Medicine
One Baylor Plaza, S430
Houston TX 77030
USA
gadi@bcm.tmc.edu

Silverman, Philip
Oklahoma Medical Research Foundation
825 N.E. 13th Street
73104 Oklahoma City,
Oklahoma
USA
silvermanp@omrf.ouhsc.edu

Snaar-Jagalska, Boguslava, Ewa
Clusius Lab, Cell Biology
Wassenaarseweg 64
2333 AL Leiden,
The Netherlands
jagalska@rulbim.leidenuniv.nl

Soll, David R.
Dept. of Biological Sciences, 14 BBE,
The University of Iowa
52242 Iowa City
USA
david-soll@uiowa.edu

Souza, Glaucia
Instituto de Quimica
University of Sao Paulo
Av. Prof. Lineu Prestes 748, B12, Sala 1208
05508-900 Sao Paulo, SP
Brazil
glmsouza@quim.iq.usp.br

Steenbergen, Judith
Albert Einstein College of Medicine
1300 Morris Park Avenue,
Goldin 701
10461 Bronx,NY
USA
jupie1115@yahoo.com

Strassmann, Joan
Dept. of ecology and evolutionary Biology MS-170
Rice University, P.O. Box 1892
77251-1892 Houston Texas,
U.S.A
Strassm@rice.edu

Sugden, Chris
Dundee University
Wellcome Trust Biocentre, Dow Street, Dundee
DDI 5EH Dundee
Scotland
c.sugden@dundee.ac.uk

Szafransky, Karol
Dept. Genome Analysis, IMB Beutenbergstr. 11
D-07745 Jena
Germany
Szafrans@imb-jena.de

Tanaka, Yoshimasa
Institute of Biological Sciences, University of Tsukuba
Tennodai 1-1-1
305-8572 Tsukuba
Japan
ytanaka@biol.tsukuba.ac.jp

Tatischeff, Iréne
LPBC (CNRS UMR 7033) Université Pierre et Marie Curie
4, place Jussieu (case 138)
F75252 Paris Cedex 05
France
tati@lpbc.jussieu.fr

Thomason, Peter
Molecular Biology Dept. Princeton University
NJ 08544 Princeton
USA
THOMASON@MOLBIO.PRINCETON.EDU

Thompson, Christopher
Baylor College of Medicine, Dept Of Molecular+ Human Genetics
One Baylor Plaza,
77025 Houston
USA
crt@bcm.tmc.edu

Tsang, Adrian
Dept. Biology, Concordia University
1455 de Maisonneuve BLVD, W.
H3G 1M8, Montreal ,
Canada
tsang@vax2.concordia.ca

Tsujioka, Masatsune
Dundee University
Wellcome Trust Biocentre, Dow Street, Dundee
DDI 5EH Dundee
Scotland
m.tsujioka@dundee.ac.uk

Ueda, Masahiro
Dept. Physiology and Biosignaling, Graduate School of Medicine - Osaka University
2-2 Yamadaoka
565-0871, Osaka,
Japan
ueda@phys1.med.osaka-u.ac.jp

Urushihara, Hideko
Institute of Biological Sciences
University of Tsukuba
1-1-1 Tennoudai,
Tsukuba-shi 305-8572
Japan
hideko@biol.tsukuba.ac.jp

Van Driessche, Nancy
Baylor College of Medicine
One Baylor Plaza, S430
Houston TX 77030
USA
nv993804@bcm.tmc.edu

Van Haastert, Peter
University of Groningen, the Netherlands
Dept. of Biochemistry, Nijenborh, 4
9747 AG Groningen
The Netherlands
Haastert@chem.rug.nl

Vicente, Juan Jesus
Instituto de Investigaciones Biomedicas
Arturo Duperier 4
28029 Madrid,
Spain
jjvicente@iib.uam.es

Vines, Maurey
University of California San Diego
Mail Code U368, University of California San Diego,
9500 Gilman Dr.
92093 La Jolla, CA
USA
mvines@ucsd.edu

Voss, Edward
Dept. of Biological Sciences, 14 BBE,
The University of Iowa
52242 Iowa City
USA
edward-voss@uiowa.edu

Weeks, Gerald
Dept. of Micribiology and Immunology
University of British Columbia
300-6174 University Boulevard
V6T 1Z3 Vancouver
Canada
gweeks@interchange.ubc.ca

Weening, Karin
University of Dundee
Dow Street
DD1 5EH Dundee,
Scotland
k.e.weening@dundee.ac.uk

Weijer, Kees
Division of Cell and Developmental Biology
WTB, School of Life Sciences
University of Dundee
DD1 5EH Dundee,
Scotland
C.J.Weijer@dundee.ac.uk

Weissenmayer, Barbara
Institut fuer Biologie-Mikrobiologie, Freie Universitaet Berlin
Koenigin-Luise Str. 12-16
14195 Berlin
Germany
bweiss@zedat.fu-berlin.de

Wessels, Deborah
Dept. of Biological Sciences, 14 BBE,
The University of Iowa
52242 Iowa City
USA
deborah-wessels@uiowa.edu

West, Christopher
Dept. of Anatomy and Cell biology
University of Florida
College of Medicine
Gainesville, FL 32610-0235
USA
westcm@ufl.edu

Williams, Jeffrey
Dundee University
Wellcome Trust Biocentre, Dow Street,
Dundee DDI 5EH
Scotland
j.g.williams@dundee.ac.uk

Williams, Robin
Wolfson institute for BiomedicalResearch
University College London
Gower St.
WC1 E6BT London
UK
robin.williams@ucl.ac.uk

Winckler, Thomas
Institut fuer Pharmazeutische Biologie
Biozetrum
Marie-Curie-Strabe 9
D-60439 Frankfurt am Main
Germany
Winckler@em.uni-frankfurt.de

Wu, Mary
Columbia University
630 w.168th Street, P&S 12-517
10032 New York NY
USA
myw5@columbia.edu

Yagura, Soichi
Graduate School of Life Science,
Tohoku University Aoba,
Sendai 980-8578
Japan
yaguyagu@biology.tohoku.ac.jp

Yamaguchi, Hitomi
Graduate School of Life Science,
Tohoku University Aoba,
980-8578 Sendai
Japan
o-hitomi@biology.tohoku.ac.jp

Zhang, Hui
Dept. of Biological Sciences, 14 BBE,
The University of Iowa
52242 Iowa City
USA
hui-zhang@uiowa.edu

Zhukovskaya, Natasha
Dundee University
Wellcome Trust Biocentre, Dow Street,
Dundee DDI 5EH
Scotland
n.zhukovskaya@dundee.ac.uk

Williams, Robin
Wolfson institute for BiomedicalResearch
University College London
Gower St.
WC1 E6BT London
UK
robin.williams@ucl.ac.uk

Winckler, Thomas
Institut fuer Pharmazeutische Biologie
Biozetrum
Marie-Curie-Strabe 9
D-60439 Frankfurt am Main
Germany
Winckler@em.uni-frankfurt.de

Wu, Mary
Columbia University
630 w.168th Street, P&S 12-517
10032 New York NY
USA
myw5@columbia.edu

Yagura, Soichi
Graduate School of Life Science,
Tohoku university Aoba,
Sendai 980-8578
Japan
yaguyagu@biology.tohoku.ac.jp

Yamaguchi, Hitomi
Graduate School of Life Science,
Tohoku University Aoba,
980-8578 Japan
o-hitomi@biology.tohoku.ac.jp

Zhang, Hui
Dept. of Biological Sciences, 14 BBE,
The University of Iowa
52242 Iowa City
USA
hui-zhang@uiowa.edu

Zhukovskaya, Natasha
Dundee University
Wellcome Trust Biocentre, Dow Street,
Dundee DDI 5EH
Scotland
n.zhukovskaya@dundee.ac.uk