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Organized by
SERGIO GRINSTEIN
and
JOSEPH J. FALKE
1. Bacterial Chemotaxis: Using Computer Models to Unravel Mechanism. DENNIS BRAY, Physiology Development and Neuroscience, University of Cambridge, CB2 3DY, UK

The set of biochemical reactions by which an *Escherichia coli* bacterium detects and responds to distant sources of attractant or repellent molecules is probably the simplest and best understood example of a cell signaling pathway. The pathway has been saturated genetically and all of its protein components have been isolated and measured biochemically, and their atomic structures have been determined. We are using detailed computer simulations, tied to experimental data, to find how the pathway works as an integrated unit. Increasingly, we find that the physical location of molecular components within the molecular jungle of the cell interior is crucial for an understanding of their function. Signal amplification, for example, appears to depend on the propagation of activity across clusters of receptors and associated molecules.

Simulation of the swimming behavior of individual bacteria allows us to define the molecular parameters necessary for movement and accumulation of bacteria in defined gradients of attractants. Effective accumulation requires not only high sensitivity but also rapid adaptation, with rates of methylation some ten times higher than indicated by in vitro assays. Responses to conflicting gradients of different attractants suggest a “canalization” mechanism that favors the first attractant encountered.

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2. Dynamics of the Bacterial Chemotaxis Pathway. VICTOR SOURJIK, Zentrum für Molekulare Biologie Heidelberg, University of Heidelberg, D-69120 Heidelberg, Germany

Chemotaxis pathway in *Escherichia coli* is a relatively simple and well-studied model system for signal transduction, noted for its high sensitivity, integration of multiple stimuli, wide dynamic range, and robustness to various sources of perturbation. To better understand functioning of the pathway in vivo, we apply several fluorescence microscopy techniques, including fluorescence resonance energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP), to study spatial and temporal dynamics of the intracellular signal processing. We recently constructed a complete library of YFP and CFP fusions to chemotaxis proteins and tested all 28 possible binary protein interactions in the pathway by FRET. Our assay detected a majority (10 out of 12) of the interactions that were expected based on previous genetic and biochemical studies, but also nine positive FRET pairs that were not predicted. Among FRET-positive interactions, only interactions that involve response regulator CheY and interactions between receptors were found so far to depend on attractant or repellent stimulation. The former reflect binding of CheY to the sensory complex, whereas the latter indicate conformational changes in the complex. We also performed a FRAP-based analysis of protein exchange kinetics at the chemoreceptor cluster. In addition to a rapid recovery of unclustered proteins, we see three classes of protein exchange kinetics: stable core of the sensory complex (receptors, CheA, and CheW) with an exchange half-time of over 10 min; CheR, CheZ, and CheB proteins with intermediate exchange half-times of \( \sim 30 \) s; and rapidly exchanged CheY protein with a half-time of \( \sim 1 \) s. Together, these results suggest that the cluster is stable on the time scale of signaling, with the only exception being CheY that has to diffuse from receptor clusters to flagellar motors. (Supported by Deutsche Forschungsgemeinschaft grants SO 421/3-1 and SO 421/6-1.)
3. Phosphorylation-mediated Regulation of OmpR/PhoB Family Transcription Factors. ANN M. STOCK, PRITI BACHHAWAT, DAVID R. BUCKLER, NATALIA FRIEDLAND, TIMOTHY R. MACK, VICTORIA L. ROBINSON, ALEJANDRO TORO-ROMAN, and TI WU, Center for Advanced Biotechnology and Medicine, Department of Biochemistry, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, Piscataway, NJ 08854 (Sponsor: Joseph J. Falke)

Two-component systems are the most prevalent signal transduction pathways in bacteria, functioning to couple environmental cues to adaptive responses. The conserved core of these pathways consists of a histidine protein kinase that transfers phosphoryl groups to a phosphorylation-activated response regulator protein. Response regulators contain a conserved phosphorylation-regulated switch domain that controls the activity of an associated effector domain that elicits the systemspecific response. The majority of bacterial response regulators are transcription factors and can be classified into subfamilies based on similarities in their DNA-binding domains. The OmpR/PhoB subfamily, characterized by a winged-helix DNA-binding domain, accounts for approximately one third of all bacterial/archaeal response regulators. We have determined structures of four OmpR/PhoB response regulators in their inactive states. Despite structural similarity of the individual regulatory and effector domains, the domain arrangements in each inactive protein are distinct. In contrast, structures of the regulatory domains of nine OmpR/PhoB response regulators in their active states indicate that all form a similar dimer with twofold rotational symmetry. The dimeric surface is formed by the \( \alpha_{4-5-\alpha_{5}} \) surface and consists of a small hydrophobic patch and an extensive network of salt bridges between residues that are highly and exclusively conserved in the OmpR/PhoB subfamily. Based on these structures, together with knowledge of the tandem binding of the winged-helix domains, we propose a common active conformation for all OmpR/PhoB subfamily members. Active regulatory domains, stabilized by phosphorylation, dimerize with rotational symmetry via their \( \alpha_{4-5-\alpha_{5}} \) faces and are tethered by flexible linkers to DNA-binding domains that associate head-to-tail, facilitating interactions with direct repeat DNA half-sites. Biochemical analyses provide evidence that different inactive states may underlie different mechanisms for regulating the transition between inactive and active states, adapting each response regulator to the specific needs of the system in which it functions. (Supported by National Institutes of Health grant R37GM047958 and the Howard Hughes Medical Institute.)

4. Interaction of the Phosphotransfer and Catalytic Domains of the Histidine Autokinase CheA. DAMON HAMEL, HONGJUN ZHOU, and FREDERICK W. DAHLQUIST, Department of Chemistry and Biochemistry, University of California, Santa Barbara, Santa Barbara, CA, 93103 (Sponsor: Joseph J. Falke)

During bacterial chemotaxis, the activity of the histidine autokinase CheA is regulated by a combination of ligand binding and methylation of the chemoreceptors. Activated CheA acts on two proteins, CheB and CheY. CheB acts to remove methyl groups from the receptors and CheY acts at the flagellar basal body to modulate the sense of rotation of the bacterial flagella. The CheA autophosphorylation reaction involves two CheA domains, the catalytic domain P4 and the P1 domain that contains the phospho-accepting histidine residue. The P4 domain binds ATP and catalyzes the transfer of the \( \gamma \)-phosphate of ATP to the reactive histidine on P1. The P4 domain alone is poorly active and requires the presence of the dimerization domain P3 for optimal activity. We have used modern nuclear magnetic resonance methods to monitor the interaction of the P1 and P3P4 domains from the hyperthermophile Thermotoga maritima. The observed chemical shift changes in P1 upon binding suggest that the P1 domain is bound by interactions on the side opposite the histidine that will be phosphorylated. The observed shifts in P3P4 upon P1 binding suggest that P1 is bound at a site distinct from the catalytic site on P4. This binding mode may be regulatory or it may reflect the binding mode needed for effective transfer of the histidyl phosphate of P1 to the substrate proteins CheY and CheB. Additional insights gained from using paramagnetic relaxation enhancement to define the structure of the P1–P3P4 complex will be discussed. (Supported by National Institutes of Health grant GM 059544.)

5. Lipid Signaling, Directional Sensing, and Cell Shape Control. PETER DEVREOTES, MIHO IJIMA, LINNAN TANG, FRANCESCA VAZQUEZ, and LING-FENG CHEN, Department of Cell Biology, Johns Hopkins University, Baltimore, MD 21205

The ability to sense and respond to shallow gradients of extracellular signals is remarkably similar in Dictyostelium discoideum amebas and mammalian leukocytes. Consideration of the features of a chemotactic response presents several fascinating and unique challenges. Shallow external gradients must generate sharply localized internal responses at the leading edges of the cells. Moreover, cells at different points in the gradient sense equally well so there is a powerful mechanism for background subtraction or adaptation. We have suggested that a balance between local excitatory and global inhibitory processes controls the response to chemoattractants. An extensive series of studies in the last several years have indicated that the upstream components and reactions in the signaling pathway are uniformly localized in cells exposed to a chemoattractant gradient. However, downstream responses such as PI (3,4,5)P3 ac-
cumulation and actin polymerization are sharply localized toward the high side of the gradient, suggesting that these responses are selectively activated at the cell’s leading edge. We have found that uniform stimuli transiently recruit and activate PI3Ks while PTEN is released from the plasma membrane. Although chemotactic receptors and G proteins are evenly distributed along the cell surface, gradients of chemotactic cause PI3Ks and PTEN to bind to the membrane at the front and the back of the cell, respectively. This reciprocal regulation provides robust control of PI(3,4,5)P3 and leads to its sharp accumulation at the anterior. Interference with PI3Ks modifies chemotaxis, whereas disruption of PTEN broadens PI localization and actin polymerization in parallel. Thus, counteracting signals from the upstream elements of the pathway converge to regulate the key enzymes of PI metabolism, localize these lipids, and direct pseudopod formation. Recent data indicate that a similar PIP3-based “polarity circuit” plays a key role in cytokinesis. PI3Ks and PTEN move to and function at the poles and furrow, respectively, of the dividing cell. Disruption of local PIP3 signaling blocks cytokinesis resulting in large multinucleate cells. (Supported by RO1GM28007 and RO1GM34933.)

6. Neutrophil Chemotaxis in Autoinflammation. ANNA HUTTENLOCHER and JONATHAN MATHIAS, Department of Pediatrics and Pharmacology, University of Wisconsin, Madison, WI 53706

Neutrophil chemotaxis to sites of inflammation is a critical process during both normal immune responses to tissue injury and infection and pathological immune responses leading to chronic inflammation. A hallmark of the chronic inflammatory disorders, autoinflammatory disease, is aseptic infiltration of neutrophils into tissues including the skin, joints, and central nervous system. Our recent studies suggest that patients with the autoinflammatory disease neonatal onset multisystem inflammatory disease have defects in neutrophil chemotaxis. To further define the molecular mechanisms that regulate neutrophil chemotaxis and contribute to the development of chronic inflammation in vivo, we have developed a novel transgenic zebrafish in which the neutrophils express GFP under control of the myeloperoxidase promoter (zMPO:GFP). Tissue injury induces a robust inflammatory response that is characterized by the directed migration of neutrophils to the wound site. Interestingly, neutrophils subsequently display retrograde migration back toward the vasculature, suggesting a novel mechanism by which inflammation is resolved. The zMPO:GFP zebrafish provides unique insight into the mechanisms of neutrophil-mediated inflammation, and thereby offers opportunities to identify new regulators of the inflammatory response in vivo. (Supported by National Institutes of Health grant R01 GM074827.)

7. Chemotaxis and Motility in Escherichia coli. HOWARD C. BERG, Department of Physics and Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138

Flagellated bacteria swim by rotating long, thin, helical filaments that arise at different points on the cell surface. Each filament is driven at its base by a rotary motor only 45 nm in diameter made from ~20 different kinds of parts. Control of the direction of rotation of such motors is the basis for the chemotactic response, i.e., for the ability of cells to swim up spatial gradients of chemical attractants. I will review the history of this subject, mention some of the physics that Escherichia coli knows, outline the signal transduction pathway that links chemoreceptors to flagella, and describe some of the fluorescence techniques that we have used to probe the behavioral physiology of this remarkable microscopic creature. (Supported by the National Institute of Allergy and Infectious Diseases.)

8. Molecular Mechanisms of Swimming and Tumbling in Bacterial Motility. KEIICHI NAMBA, Graduate School of Frontier Biosciences, Osaka University, and Dynamic Nano-Machine Project, International Cooperative Research Project, Japan Science and Technology Agency, 1-3 Yamadaoka, Suita, Osaka 565-0871 Japan

The bacterial flagellum is made of a rotary motor and a long helical filament by means of which bacteria swim. The flagellar motor drives the rapid rotation of each flagellum to propel cell movements. The long helical filament with a diameter of ~20 nm is made of a single protein flagellin and switches between left- and right-handed helical forms in response to reversal of the motor rotation, allowing bacteria to alternate their swimming pattern between run and tumble for taxis. The flagellum also has a short, curved segment called hook, which connects the motor and the propeller. Its bending flexibility makes it work as a universal joint, whereas the filament is more rigid to function as a propeller. The flagellum is constructed by self-assembly of proteins translocated to the distal end of the growing structure through the narrow central channel. The flagellar proteins are exported by the type III protein export apparatus, which consists of membrane and soluble proteins. One of the proteins is FliF, an ATPase that provides energy for the export process. A few cytosolic chaperones are also involved to facilitate the export process.

We have been looking at their structures by X-ray crystallography, fiber diffraction, and electron cryomicroscopy. Visualization of the structures at work is difficult by X-ray crystallography alone because many components do not form stable complexes. Subunit stoichiometry may also have distributions. Electron cryomicroscopy including single particle image analysis, helical image reconstruction, and tomography would allow us to visualize those structures.
I will describe how we visualized flagellar structures at nearly atomic resolution by complementary use of the two methods, what we learned from them, and how we will proceed further to solve the flagellar structure as a whole for ultimate understanding of the mechanisms of its protein export, self-assembly, and rotation.

9. Facile Production of Live Cell Biosensors to Study Activatin of Endogenous, Underivatized Signaling Proteins. Multiplex Measurements in Individual Cells. LOUIS HODGSON,1, ALEXEI TOUTHCHINE,1 PERIHAN NAL-BANT,1 BRIAN KAY,2 and KLAUS HAHN,1 1Department of Pharmacology and Lineberger Cancer Center, University of North Carolina, Chapel Hill, NC 27599; 2Biology Department, University of Illinois, Chicago, IL 60607

Many proteins are out of the reach of current biosensor technologies, either because modification of the targeted protein is required (i.e., often for FRET) or because relatively low biosensor sensitivity requires use of biosensor concentrations that perturb cell physiology. We recently published a biosensor approach that can report the activation state (conformation and phosphorylation) of endogenous, untagged proteins (Nal-bant, P., L. Hodgson, V. Kraynov, A. Toutchkine, and K.M. Hahn. 2004. Science. 305:1615–1619). An “affinity element” that binds only to the activated state of the target protein is covalently linked to a novel solvent-sensitive dye whose fluorescence is very sensitive to its target in a living cell, the fluorescence change can be used to quantify the changing level and subcellular location of activation. Because bright dyes are directly excited, sensitivity is greatly enhanced. This, combined with study of endogenous protein, provides a low perturbation method to study cell signaling. Use of dye-labeled proteins such as these biosensors has in the past been severely limited by the need to introduce them into the cell via microinjection, electroporation, or other mechanical means, unlike genetically encoded sensors. Recent development of new methods for non-endocytic introduction of dye-labeled molecules into cells, developed by our collaborator Gilles Divita, brings these sensitive biosensors into the practical realm. In this talk, we will also explore use of engineered scaffolds and high throughput screening for rapid generation of biosensors and the use of fluor at different wavelengths for multiplex measurements of two Rho family signaling activities in the same cell. (We thank National Institutes of Health grant GM57464 for financial support.)

10. Scar/WAVE Proteins in Cell Spreading and Membrane Dynamics. LAURA M. MACHESKY, JOHN A. LEGG, OWEN J. MCCARTY, SIMON D. J. CALAMINUS, and STEVE P. WATSON, University of Birmingham School of Biosciences and Centre for Cardiovascular Sciences, Edgbaston, Birmingham B15 2TT, UK

Actin assembly is regulated in response to activation of various receptors, including during cell spreading. We have a long standing interest in how the actin nucleating complex Arp2/3 is controlled by Scar/WAVE proteins and by IRSp53 to regulate the balance between filopodia and lamellipodia assembly in platelets and fibroblasts. These processes also link to membrane-trafficking events, but the mechanisms are not well understood. Scar1 null mouse platelets have specific spreading defects, indicating a role for Scar1 in controlling actin dynamics in response to integrin engagement. However, Scar1 null fibroblasts assemble normal dorsal and peripheral ruffles, in contrast to previously published papers. We postulate that Scar/WAVE proteins are not essential for dorsal ruffle formation, but that the WASP proteins can compensate for their absence. We have also uncovered connections between Scars and the exocytic machinery, which may indicate a membrane-trafficking function for Scars.

11. Role of the Coronin Proteins in Phagocytosis and Chemotaxis. WILLIAM S. TRIMBLE, MING YAN, and SERGIO GRINSTEIN, Program in Cell Biology, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada

Coronins are a family of conserved actin-associated proteins that have been implicated in a variety of cellular processes dependent on actin rearrangements. All coronin proteins have a variable N-terminal domain, followed by WD repeats and a C-terminal coiled-coil oligomerization domain. Using RT-PCR we find that primary human neutrophils express coronins-1–4 and -7. Antibodies to coronin-1 demonstrate its accumulation at the leading edge of migrating neutrophils and at the nascent phagosome. Inhibition of coronin function by transduction of a dominant-negative form of the protein leads to inhibition of chemotaxis and a reduction in neutrophil spreading and adhesion. This inhibition appears to correlate with changes in the distribution of F-actin structures within the cell. In addition, phagocytosis is inhibited, but neither secretion nor activation of the NADPH oxidase appears to be affected. Using RAW 264.7 cells transfected with GFP-coronin-1 we find that coronin-1 rapidly and transiently associates with the phagosome. We show that whereas TAT-WD has no effect on binding of opsonized red blood cells to RAW 264.7 cells, receptor clustering, or several downstream signaling events, lamellipodial extensions and actin accumulation at the base of the bound particle are diminished. Furthermore, Arp2/3 accumulation at the phagosome is impaired after TAT-WD treatment, although recruitment of coronin-1 itself is not affected. Interestingly, although coronin-1 also accumulates at the sites of actin remodeling associated with Salmonella invasion, TAT-WD has no effect on this process. Together, our data demonstrate that coronin-1 is required for an early step in phagosome formation, possibly linking Arp2/3 with sites of actin re-
12. Role of Formin Family Protein mdDia2 in Protrusion of Lamellipodia and Filopodia. CHANGSONG YANG,1 LUBOV A. CZECH,2 SHIN-ICHIRO KOJIMA,2 and TATYANA M. SVITKINA,1 1Department of Biology, University of Pennsylvania, Philadelphia, PA 19104; 2Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL 60611

Actin polymerization–driven protrusion of the cell leading edge depends on two types of dynamic actin filament arrays: dendritic networks in lamellipodia and parallel bundles in filopodia. We previously showed that filopodial bundles are formed by reorganization of the lamellipodial dendritic network, as described by the convergent elongation model (Svitkina, T.M., E.A. Bulanova, O.Y. Chaga, D.M. Vignjevic, S. Kojima, J.M. Vasiliev, and G.G. Borisy. 2003. J. Cell Biol. 160:409–421). Formin family proteins are essential molecular players in this process as they can nucleate actin filaments, protect their barbed ends from capping, and have cross-linking capabilities (Pruyne, D., M. Evangelista, C. Yang, E. Bi, S. Zigmond, A. Bretscher, and C. Boone. 2002. Science. 297:612–615; Sagot, I., A.A. Rodal, J. Moseley, B.L. Goode, and D. Pellman. 2002. Nat. Cell Biol. 4:626–631; Harris, E.S., I. Rouiller, D. Hanein, and H.N. Higgs. 2006. J. Biol. Chem. 281:14383–14392). Here, we investigated roles of a formin family protein, mdDia2, during leading edge protrusion in cultured B16F1 mouse melanoma cells. Constitutively active mdDia2 lacking GTPase-binding domain (GBD) induced unusual club-like filopodia with GBD-mDia2 at their tips. In this process, mdDia2 was specifically targeted to the lamellipodial edge, where it nucleated filaments and protected them from capping. Convergence and bundling of these filaments completed the process of filopodia formation. Arp2/3-nucleated filaments participated in formation of the same filopodia. Leading edge targeting was compromised upon deletion of sequences outside the FH1FH2 module of mdDia2 or depletion of the WAVE–Abi1–Nap1–PIR121 complex from cells. Knockdown of endogenous mdDia2 severely impaired both filopodia and lamellipodia. Thus, Arp2/3 complex and mdDia2 function together to promote protrusion: mdDia2 is necessary for lamellipodia formation by the Arp2/3 complex and targeting of mdDia2 to the lamellipodial leading edge is necessary for efficient filopodia formation. The WAVE–Abi1–Nap1–PIR121 complex is a likely candidate to coordinate activities of two nucleators. (Supported by National Institutes of Health grant GM 070898 to T.M. Svitkina.)

13. Phosphoinositide-dependent Coordination of Small GTPases during Phagocytosis. JOEL A. SWAN-SON, Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109

Fcγ receptor (FcR)–mediated phagocytosis of IgG-coated particles by macrophages entails coordinated movements of membrane and actin cytoskeleton and the localized activation of the phagocyte oxidase. These activities are regulated in part by the small GTPases Cdc42, Rac1, Rac2, Arf1, and Arf6 and by phosphatidylinositol 3′-kinase (PI3K). Inhibitors of PI3K arrest phagocytosis of large particles after the formation of actin-rich phagocytic cups, indicating that the early stages of phagocytosis can be initiated without PI3K and later stages require activation of PI3K. These studies analyzed the roles of 3′ phosphoinositides in the spatial organization of GTPase activation during phagocytosis. Ratiometric fluorescence microscopy of fluorescent chimeras of the lipid phosphatase SHIP-1 and p85, the regulatory subunit of type I PI3K, indicated their transient localization to the phagosome. YFP chimeras of 3′ phosphoinositide-binding PH domains indicated that PI(3,4)P2 (TAPP1PH-YFP) and PI(3,4,5)P3 (BtkPH-YFP) accumulated transiently on forming phagosomes. Fluorescence resonance energy transfer stoichiometry was used to determine where Cdc42, Rac1, Rac2, Arf1, and Arf6 were in their active, GTP-bound forms during phagocytosis. Three patterns of GTPase activation were observed. Cdc42 and Arf6 were active at distal regions of extending pseudopods and less active in basal regions of the phagocytic cups (distal signals). Activation of Rac2 and Arf1 occurred later and was delocalized over the base of the phagocytic cup (proximal signals). Rac1 activation was both distal and proximal. The transition from the distal to the proximal signals coincided with the timing and localization of PI(3,4,5)P3 on forming phagosomes. Unclosed phagocytic cups that formed in the PI3K inhibitor LY294002 showed sustained activation of Cdc42, Rac1, and Arf6, and constant but reduced activation of Rac2 and Arf1. These results indicate a PI3K-dependent signal transition in the forming phagosome, in which PI(3,4,5)P3 generated on phagosomal membranes coordinately deactivates the distal signal GTPases and activates the proximal signal GTPases. In this way, the accumulation and lateral diffusion of PI(3,4,5)P3 could integrate the distinct activities regulated by FcR.

14. Role of the ARF6 Machinery in Cell Invasion. CHIARA RECCHI, GAELLE LE-DEZ, MIKA SAKurai, GUILLAUME MONTAGNAC, ANIKA STEFFEN, and PHILIPPE CHAVRIER, Membrane and Cytoskeleton Dynamics Group, UMR 144 Centre National de la Recherche Scientifique/Institut Curie, 75248 Paris, France

The small GTP-binding protein ARF6 (ADP-ribosylation factor 6) localizes to the plasma membrane, where it regulates endocytic membrane recycling and actin remodeling. ARF6-mediated recycling impinges on cellular functions that require transient cell polar-
ization of the plasma membrane, such as cell migration and cell invasion, by regulating the insertion of plasma membrane proteins at defined sites at the cell periphery. Cell invasion requires remodeling of the extracellular matrix (ECM) through activation of matrix metalloproteinases (MMPs). The membrane-tethered MT1-MMP is crucial for ECM degradation and invasion. In invasive tumor cells, MT1-MMP accumulates at discrete foci called invadopodia, corresponding to actin-based structures that require Rho protein and ARF6 activity for their formation. Endocytosis and recycling of MT1-MMP is essential for localizing its activity at invadopodia.

With the aim of characterizing the function of ARF6 during cell invasion, we are analyzing the function of several ARF6 downstream effector proteins in invasive tumor cells with respect to MT1-MMP trafficking to invadopodia and invasion. In particular, we reported that ARF6 interacts with the Sec10 subunit of the complex, a multiprotein complex involved in docking of recycling vesicles to specific regions of the plasma membrane (Prigent, M., T. Dubois, G. Raposo, V. Derrien, D. Tenza, C. Rosse, J. Camonis, and P. Chavrier. 2003. J. Cell Biol. 163:1111–1121). Small interfering RNA–based knockdown of several subunits of the complex established that the function of the exocyst complex is required for invasion in the human breast carcinoma cell line MDA-MB-231. In addition, we identified a novel pathway for recruiting the exocyst complex to invadopodia, which is controlled by Cdc42 and RhoA, two Rho GTPases previously implicated in invasion. Finally, we will present evidence for a novel role for ARF6 in controlling the function of a family of proteins that interact with dynein and kinesin-1, and which are known to control vesicular trafficking along microtubules. (Supported by grants from Institut Curie, Centre National de la Recherche Scientifique, Ligue Nationale contre le Cancer, and Fondation BNP-Paribas.)
15. Reconstruction of the Bacterial Chemotaxis Receptor-Kinase Assembly with X-ray Crystallography and Pulsed ESR Spectroscopy. ABIOLA M. POLLARD,1 JAYA BHATNAGAR,1 GABRIELA GONZALEZ-BONET,1 SANG-YOUN PARK,1 PETER P. BORBAT,2 JACK H. FREED,2 ALEXANDER M. BILWES,1 and BRIAN R. CRANE,1 1Department of Chemistry and Chemical Biology and 2The Advanced ESR Technology Center, Cornell University, Ithaca, NY 14853 (Sponsor: Joseph J. Falke)

How chemoreceptors (MCPs) regulate the activity of the histidine kinase CheA is a central question in understanding the signaling pathway that controls bacterial chemotaxis. MCPs, CheA, and the coupling protein CheW assemble into a transmembrane complex that processes environmental signals with great sensitivity, gain, and dynamic range. We have applied X-ray crystallography combined with pulsed-ESR measurements to define component interactions within the receptor-kinase assembly of the chemotactic thermophile T. maritima. The dimeric CheA–CheW complex positions two CheWs to form a cleft that is lined with residues important for receptor interactions. CheW residues involved in kinase activation map to interfaces that orient the CheW clamps, whereas CheA residues important for ligand-induced kinase regulation map to regions participating in CheA:CheA self contacts. The structure of a receptor cytoplasmic domain defines CheA interaction regions and charge centers that undergo chemical modification to tune receptor response. Site-specific spin labeling combined with pulsed ESR measurements are currently being used to define interactions between receptor fragments and dimeric CheA–CheW in soluble ternary complexes. Models of assembly consistent with the current structural restraints will be discussed.

16. cAMP Dynamics in Chemotaxing Dictyostelium Cells. ANNA BAGORDA and CAROLE A. PARENT, Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892 (Sponsor: Joseph J. Falke)

The simple ameba Dictyostelium possesses the ability to migrate directionally in chemoattractant gradients, a process referred to as chemotaxis. Upon starvation, Dictyostelium cells produce and secrete cAMP. Although cAMP is mainly known as an intracellular second messenger, in Dictyostelium it also acts as a chemoattractant. The binding of the extracellular cAMP to its specific G protein–coupled receptor triggers a cascade of signaling events giving rise to chemotaxis, the synthesis and secretion of additional cAMP for signal relay, and changes in gene expression. The enzyme responsible for the production of cAMP, adenylyl cyclase A (ACA), is highly enriched at the back of polar, chemotaxing cells. As cells are chemotaxing toward the secreted cAMP, they align in a head to tail fashion forming characteristic chains called streams. Our group has established that the ACA enrichment at the back of cells is essential for streaming and proposed that the back of cells acts as a compartment from which cAMP is locally produced and secreted to relay the chemoattractant signal to neighboring cells. To test our model, we set out to visualize cAMP distribution in live cells. We adapted mammalian FRET-based cAMP sensors for Dictyostelium and performed FRET analyses in either wild type or ACA lacking mutant cells expressing those sensors. The stimulation of cAMP receptors with a saturating dose of chemoattractant provoked an immediate and sustained decrease of FRET efficiency, which reflects a fast cAMP accumulation, in wild-type cells—a response that was absent in ACA-null cells. Furthermore, a subsaturating dose of chemoattractant gave rise to a transient accumulation of cAMP, showing for the first time cAMP dynamics in live cells. These data, together with the spatial analysis of cAMP distribution in chemotaxing cells
phenotype. Our findings suggest a role for SopD in the formation, and we found that SopD also contributes to this ropinosome formation during SigD. SigD has been previously shown to promote macropinosomes (large endocytic vesicles formed during S. Typhimurium invasion). Using live cell imaging we have shown that SopD associated with the Arp2/3 complex. Filopodia, in contrast to branches from existing filaments. This branched actin network forms the basis for lamellipodia, phagocytosis, and the rocketing of various intracellular pathogens.

Lysophosphatidic acid (LPA) is a platelet-derived bioactive lipid that is postulated to regulate a variety of processes, including wound healing. LPA activates G protein-coupled receptors to induce Ca\(^{2+}\) signaling in MC3T3-E1 preosteoblasts and is a potent chemotactic stimulus for these cells. Because bone fracture healing requires the migration of osteoblast progenitors, we postulate that LPA is among the factors that stimulate bone repair. UMR 106-01 cells, which express a more mature osteoblastic phenotype than MC3T3-E1 cells, did not migrate in response to LPA, although they express LPA receptors and exhibit LPA-induced Ca\(^{2+}\) signals. This suggests that LPA differentially induces preosteoblast chemotaxis, consistent with our hypothesis that LPA stimulates the motility of osteoblast progenitors during bone healing. LPA-stimulated MC3T3-E1 cells exhibit striking changes in morphology and F-actin architecture.

We investigated the role of phosphatidylinositol-3 kinase (PI3K), required for motility-associated cytoskeletal rearrangements in other cell types, in LPA-stimulated MC3T3-E1 chemotaxis. We found a dose-dependent reduction in LPA-induced migration and cytoskeletal changes upon treatment with the PI3K inhibitor LY294002. LPA treatment is associated in many cell types with an autocrine/paracrine transactivation of the EGF receptor (EGFR) via shedding of surface-tethered EGFR ligands, a phenomenon often required for LPA-induced chemotaxis. MC3T3-E1 cells express multiple EGFR ligands (epigen, epiregulin, HB-EGF, and amphiregulin) and migrated in response to EGF. The EGFR kinase inhibitor PD153035 blocked EGF-stimulated motility in MC3T3-E1 cells, but did not significantly alter LPA-induced chemotaxis. Activation of MAP kinases is a hallmark of EGFR-mediated signaling, and EGF treatment of MC3T3-E1 cells led to a strong stimulation of ERK1/2 kinase. In contrast, LPA induced only a weak elevation in ERK activity. We conclude that LPA-induced MC3T3-E1 cell chemotaxis requires PI3K-associated cytoskeletal changes, but not transactivation of the EGFR.

19. The Role of the Arp2/3 Complex in Cell Motility and the Transition between Lamellipodia and Filopodia. SIMON A. JOHNSTON and LAURA M. MACHESKY, School of Biosciences, The University of Birmingham, Birmingham B15 2TT, UK (Sponsor: Joseph J. Falke)

The Arp2/3 complex nucleates new actin filaments as branches from existing filaments. This branched actin network forms the basis for lamellipodia, phagocytosis, and the rocketing of various intracellular pathogens. The Arp2/3 complex is directly activated by the WASP family that acts downstream of integrins and the Rho family of GTPases. There are also many other binding partners and signaling pathways that have been associated with the Arp2/3 complex. Filopodia, in contrast to

S. Typhimurium infection, and the SCV. Additionally, we have shown this SopD-GFP recruitment to be dependent on the phosphatase activity of another S. Typhimurium effector, SigD. SigD has been previously shown to promote macropinosome formation during S. Typhimurium invasion, and we found that SopD also contributes to this phenotype. Our findings suggest a role for SopD in the coordination of membrane dynamics by the bacteria beginning early during S. Typhimurium invasion. (Supported by an operating grant from The Canadian Institutes for Health Research.)

18. Lysophosphatidic Acid-Induced Chemotaxis of Bone Cells. SUE A. KARAGIOSIS, LISA M. MASIELLO, NIKKI BOLLINGER, and NORMAN J. KARIN, Cell Biology and Biochemistry Group, Pacific Northwest National Laboratory, Richland, WA 99352
branched structures, consist of parallel bundles of single filaments. The function of filopodia, especially in cell migration, is still unclear. Using a cell spreading model to study the active cytoskeleton, we have examined the localization and biochemical characteristics of Arp2/3 complex under varied conditions. This includes the role of matrix, soluble factors (e.g., PDGF and LPA), and the various proteins that have previously been implicated in the localization and activation of the Arp2/3 complex. Our goal is to understand the central role that the Arp2/3 complex plays in cell motility. We have also investigated the transitions between filopodia and lamellipodia to understand the initiation and reorganization of these actin structures. (S.A. Johnston is funded by the Biotechnology and Biological Sciences Research Council and L.M. Machesky is funded by the Medical Research Council.)

20. Filopodial Sprouting in Neural Progenitor Cells is Triggered by SK3 Channel Activation. OLIVER H. WITTEKINDT,1,2 BIANCA VAIDA,3 TOBIAS BÖCKER,3 PAUL DIETL,1 STEPHAN GRISSMER,2 and STEFAN LIEBAU,3 1Department of General Physiology, 2Department of Applied Physiology, and 3Department of Cell Biology and Anatomy, University of Ulm, 89081 Ulm, Germany

Neural progenitor cells (NPCs) play a key role in the regeneration of the central nervous system. We identified an 1-EBIO–activated and scyllatoxin (ScTX)-sensitive K+ current (IC50 = 1.9 ± 0.3 nM) and observed the highest expression levels for SK3 transcripts in comparison to other SK channel transcripts in NPCs. Furthermore, almost all NPCs were positive for SK3 proteins in immunocytochemical experiments. These results gave evidence for the functional expression of SK3 channels in NPCs. Performing preembedding immunoelectron microscopy, we detected SK3 subunits in short filopodial structures with an approximate length of 0.3 to 1.0 μm within 15 min and were not retracted. ScTX prevented the 1-EBIO–induced sprouting when added at least 10 min before 1-EBIO application. Phalloidin stained the 1-EBIO–induced filopodias. We conclude that SK3 channel activation is a determinant of filopodial sprouting of NPCs, which might involve a massive reorganization of the cytoskeleton. (Supported by 4SC, Martinsried (to S. Grissmer), Medicinische Fakultät, Ulm, P770 (to O.H. Wittekindt), P868 (to S. Liebau), and the Austrian Science Foundation, grants P15742 and P15743.)

21. Phosphoinositide-specific PH Domains: A Comparative Analysis of Electrostatic Contribution to Membrane Docking. KYLE E. LANDGRAF, JOHN A. CORBIN, and JOSEPH J. FALKE, Department of Biochemistry, University of Colorado, Boulder, CO 80309 (Sponsor: Joseph J. Falke)

The Pleckstrin Homology (PH) domain is a conserved signaling motif found in a wide array of signaling proteins that dock to membranes. PH domain–containing proteins play important roles in chemotaxis signaling events that occur at the inner leaflet of the plasma membrane. An important class of PH domains comprises those that dock to membranes in response to phosphatidylinositol phosphate (PIP) lipid second messengers. Structural evidence indicates that this class of PH domains contains an inositol phosphate binding pocket. Aside from specific PH head group coordination little else is known of the molecular basis of their interaction with membranes. To investigate the mechanism of PH domain membrane docking, the membrane-binding parameters of GRP1-PH, AKT-PH, TAPP1-PH, and PLCδ1-PH domains were measured. A protein-to-membrane FRET assay was used to detect membrane binding, enabling measurement of high affinity equilibrium dissociation constants using a competitive binding assay. Additionally, association and dissociation kinetics were investigated using stopped-flow FRET measurements of on- and off-rate constants for docking to target lipid on bilayer surfaces. Equilibrium and kinetic parameters reveal an electrostatic on-rate enhancement mechanism used by these PH domains to rapidly bind target lipids. Interestingly, this mechanism is most pronounced for GRP1-PH, AKT-PH, and TAPP1-PH domains, which bind to rare and transient 3′-phosphoinositide target lipids. In contrast, PLCδ1-PH, which binds the more abundant and constitutively present PI(4,5)P2, does not exhibit significant electrostatic on-rate enhancement. These findings suggest background anionic lipids contribute to affinity tuning through nonspecific electrostatic interactions that enhance productive PIP lipid collisions. The inherent electrostatic surface properties of these domains may be uniquely suited for a two-dimensional search mechanism important in PIP-mediated protein recruitment to membrane sites of phosphoinositide signaling. (Supported by National Institutes of Health grant GM063235.)

22. Localized Changes of Surface Potential during Phagocytosis: Critical Role in Signal Transduction. TONY YEUNG,1 MAURICIO TEREZIBNIK,1 LIMING YU,1 JOHN SILVIUS,3 MARK PHILIPS,3 TIM LEVINE,4 ANDRAS KAPUS,5 and SERGIO GRINSTEIN,1 1Division of Cell Biology, The Hospital for Sick Children, Toronto, M5G
nificance of the inner bilayer surface potential and its
cationic residues or polybasic domain. However, the sig-
anionic liposomes can attract proteins with clusters of
experiments have shown that the surface potential of
the phosphoinositides and phosphatidylserine. These
riched in negatively charged phospholipids including
M5G 1X8, Canada; \( ^2 \)Department of Medicine, McGill Uni-
versity, Montreal, H3G 1Y6, Canada; \( ^3 \)Department of Medicine, New
York University School of Medicine, New York, NY 10016;
\( ^4 \)Division of Cell Biology, University College, London, EC1V
9EL, UK; \( ^5 \)St. Michael’s Hospital Research Institute, Toronto,
M5B 1W8, Canada

The inner bilayer of the plasma membrane is en-
riched in negatively charged phospholipids including
the phosphoinositides and phosphatidylserine. These
phospholipids contribute significantly to the net an-
onic surface charge of the inner leaflet, and in vitro ex-
periments have shown that the surface potential of
anionic liposomes can attract proteins with clusters of
cationic residues or polybasic domain. However, the sig-
nificance of the inner bilayer surface potential and its
role in intracellular signaling are still not fully under-
stood. In FcγR-mediated phagocytosis, de novo genera-
tion of the phosphoinositides PI(4,5)P₂ and PI(3,4,5)P₃ occurs
transiently at the phagocytic cup, followed by their rapid
conversion and degradation. We therefore
decided to explore whether changes in surface poten-
tial occur as a result of lipid metabolism at the phago-
some. We used two types of cationic probes
(RRRRRRARAR-acyl and KKFWKRLRKLRLKKS) that
can be anchored to the plasma membrane to detect
changes in surface potential. In vitro liposome binding
assays showed preferential binding of these probes to
anionic liposomes in a charge-dependent manner.
When expressed in mammalian cells, the cationic
probes were targeted to the plasma membrane and,
more importantly, they responded to changes in surface
potential when the distribution of anionic lipids was al-
tered by various treatments. Interestingly, in RAW
macrophages undergoing phagocytosis, our cationic probes
dissociated from the phagosomal membrane, indicat-
ing substantial changes in the local surface potential at
the phagosome. Using the PH domain of various
phosphoinositide-binding proteins and the phosphatidylser-
ine-binding protein annexin-V, we were able to correlate
the observed changes in surface potential with the re-
moval of these anionic lipids from the phagosome.
We further propose that, as a consequence of the changes
in local surface potential, the targeting of signaling mol-
ecules including Rac1 and K-Ras to the phagosome can
be modulated.

23. Calcium and Phosphoinositide Metabolism during
Phagocytosis in Murine Macrophages. JOHN H. EV-
ANS and JOSEPH J. FALKE, Department of Chemistry and
Biochemistry, University of Colorado, Boulder, CO 80309
(Sponsor: Joseph J. Falke)

Phagocytosis of zymosan (yeast cell wall) particles is
temporally associated with oscillating changes in the in-
tracellular calcium concentration and with changes in
the phosphoinositide (PIPₓ) composition of the cyto-
solic face of the phagosome (Marshall, J.G.; J.W. Booth,
V. Stambolic, T. Mak, T. Balla, A.D. Schreiber, T. Meyer,
and S. Grinstein. 2001. *J. Cell. Biol.* 153:1369–1380; Gi-
rotti, M., J.H. Evans, D. Burke, and C.C. Leslie. 2004. *J.
Biol. Chem.* 279:19113–19121). A host of signaling pro-
teins are able to respond to such Ca²⁺ and PIPₓ signals
using calcium-dependent lipid binding, or C₂, and
pleckstrin homology (PH) domains, respectively.

Using a variety of PIPₓ-specific PH domains and two
different C₂ domains fused to color variants of green
fluorescent protein expressed in the RAW264.7 cell line
(a murine macrophage cell model), we are investigating
the temporal and spatial coordination of Ca²⁺ and PIPₓ
signals during zymosan phagocytosis by multicolor
fluorescence imaging. Progress in this research will be
presented. (Supported by National Institutes of Health
grant GM063235.)

24. Immobilization of Acylated Proteins and Lipids on
the Inner Leaflet of the Plasma Membrane during
Phagocytosis. DAVID MASON, \( ^1 \)ELAINE F. COR-
BETT-NELSON, \( ^1 \) and SERGIO GRINSTEIN, \( ^1 \)
\( ^2 \)Division of Cell Biology, The Hospital for Sick Children, Toronto,
M5G 1X8, Canada; \( ^2 \)Department of Biochemistry, University of
Toronto, Toronto, M5S 1A8, Canada

Phospholipid constituents of the plasma membrane
play an important role in regulating signaling during
Fcγ-mediated phagocytosis, through the generation of
active metabolites and the subsequent recruitment of
effectors. Interestingly, these changes are mostly limited
to the point of engagement, known as the phagocytic
cup, whereas they are not apparent in the neighboring
regions of the plasma membrane. It is unclear how this
asymmetry is maintained, being stable over the course
of a phagocytic event, which can last up to 8 min for
larger particles.

We studied the mobility of lipids and lipid-associated
proteins at the phagocytic cup using fluorescence recov-
ery after photobleaching. Lipid-anchored proteins were
found to be freely mobile in the unengaged plasma
membrane. However, a marked immobilization oc-
curred at sites of phagocytosis. This phenomenon was
limited to acylated probes anchored to the inner leaflet
of the plasma membrane and furthermore was insensi-
tive to depletion of cholesterol or actin.

We found that immobilization required the activity of
tyrosine kinases and therefore hypothesize that a signal-
dependent recruitment of adaptors and lipid-binding
effectors generate an annulus of lipids with reduced
and restricted mobility around the clustered phagocytic
receptors. These cholesterol-independent microdo-
mains likely play a central role in recruiting lipid-associ-
ted signaling proteins to sites of phagocytosis.

25. Coordination of ARF- and Rho-Family GTPases
during Phagocytosis by a PI-3K–mediated Signal
Transition. ADAM D. HOPPE, \( ^1 \)PETER BEEMILLER, \( ^2 \)
and JOEL A. SWANSON,1,2 1Department of Microbiology and Immunology and 2Cellular and Molecular Biology Graduate Program, University of Michigan, Ann Arbor, MI 48109

Phosphatidylinositol-3'-kinase (PI-3K) activity is central to the coordination of Fcγ receptor (FcyR)-mediated phagocytosis. PI-3K function is required for complete pseudopod extension and phagosome closure. By using fluorescence resonance energy transfer (FRET) microscopy of live cells (Hoppe, A., K. Christensen, and J.A. Swanson. 2002. Biophys. J. 83:3652–3664), we previously observed that the ARF1 and ARF6 GTPases are regulated in distinct manners by PI-3K during phagocytosis (Beemiller, P., A.D. Hoppe, and J.A. Swanson. 2006. PLoS Biol. 4:e162) and Rac1, Rac2, and Cdc42 display distinct patterns of activation during phagocytosis (Hoppe, A.D., and J.A. Swanson. 2004. Mol. Biol. Cell. 15:3509–3519). In particular, ARF6 was rapidly activated upon FcyR ligation and was primarily active at the tips of extending pseudopods. The PI-3K inhibitor LY294002 failed to block the activation of ARF6 at nascent phagosomes despite its ability to prevent completion of phagocytosis. Conversely, ARF1 activation at phagosomes was not restricted to the leading edge of pseudopods, but its activation was inhibited by PI-3K inhibitor. This led to the hypothesis that PI-3K activity at the phagosome directs a signal transition that coordinates the deactivation of early stage GTPases and activation of late stage GTPases. To determine whether Cdc42, Rac1, and Rac2 were also regulated via this signal transition, we measured the effects of LY294002 treatment on their activation by FRET microscopy. We found that, like ARF6, Cdc42 and Rac1 were activated immediately upon receptor ligation, and LY294002 pretreatment blocked the deactivation of Cdc42 and Rac1 without affecting their activation. Interestingly, formation of cups in LY294002-treated macrophages displayed a constant but reduced level of Rac2 activation in phagocytic cups. Together with our previous data, these results indicate that PI-3K directs the morphological rearrangements of phagocytosis by simultaneously activating and deactivating different members of the ARF- and Rho-family GTPases. (Supported by National Institutes of Health grants AI35950 and AI64668 to J.A. Swanson.)

26. Actin and Rho-GTPase Dynamics during CR3-mediated Phagocytosis in Macrophages. GABRIELA COSIO AND SERGIO GRINSTEIN, Cell Biology Program, Hospital for Sick Children Research Institute, Toronto, M5G 1X8, Canada

Phagocytosis of pathogens by macrophages is elicited by Fcγ and CR3 receptors through different signaling pathways. Whereas the signaling events triggered by Fcγ receptors are comparatively well characterized, the cascade coupling CR3 engagement to phagocytosis is poorly understood. Rho-family GTPases have a central role in cytoskeletal remodeling during particle internalization. CR3-initiated uptake was reported to be dependent on the activity of RhoA, but not on Cdc42 and Rac1. Conversely, Cdc42 and Rac1 are thought to mediate Fcγ-mediated uptake. We analyzed actin dynamics and reexamined the role of Rho GTPases during CR3-mediated phagocytosis using complement-opsonized sheep red blood cells and RAW 264.7 macrophages stably transfected with mCherry actin. We monitored the dynamics of RhoA, Rac1, Rac2, and Cdc42 during phagocytosis by transient transfection with their respective GFP fusion constructs, while correlating their distribution with that of mCherry actin by confocal imaging of live cells. We found that Rac1 accumulated at the site of CR3-initiated phagocytosis, but failed to detect redistribution of Rac2, Cdc42, or RhoA to the phagocytic cup. We also assessed the activation of the GTPases using binding domains of specific effector proteins. The p21-binding domain of PAK (PBD-PAK1), which interacts with active Rac1, Rac2, and Cdc42, accumulated at sites of internalization and disappeared after phagosome closure, correlating with Rac1 and actin accumulation. Recruitment of PBD-PAK1 accompanied the accumulation of Rac1. Interestingly, we observed two distinct cycles of actin polymerization. The first cycle occurred at the time of internalization, lining the phagocytic cup as pseudopods extended to engulf the opsonized particle. The second cycle took place some time after phagosome sealing and detachment from the plasmalemma. During this second phase, actin transiently surrounded the entire phagosome, and then dissociated asymmetrically, resulting in the appearance of an actin tail that drove short-lived phagosome displacement. This second cycle was preceded by PBD-PAK1 accumulation around the phagosome, suggesting involvement of Rac and/or Cdc42. Additionally, the Arp2/3 complex was recruited to the actin tails. These results suggest that RhoA is not the sole GTPase responsible for CR3-mediated phagocytosis. In addition, they reveal that formed phagosomes display intracellular motility that is driven by actin, a process that had remained undetected, likely due to its transient and highly dynamic nature.

27. An Essential Role for Talin in Mammalian Phagocytosis. JENSON LIM, AGNES WIEDEMANN, GEORGE TZIRCOTIS, DAVID CRITCHLEY, and EMANUELLE CARON, Centre for Molecular Microbiology and Infection, Division of Molecular Cell Biology, Imperial College London, SW7 2AZ London, UK (Sponsor: Joseph J. Falke)

Classically, phagocytosis is a multi-step process that involves receptor-mediated particle recognition; zipper-like, F-actin-driven uptake; phagosome maturation; and particle clearance. Numerous phagocytic receptors exist, which can bind their target directly or indirectly
through opsonins. Ligated receptors induce intracellular signaling cascades that lead to the local organization of signaling complexes and direct cytoskeletal and membrane dynamics underneath bound particles respectively through the activation of Rho- and Arf-family GTP-binding proteins. Many cytoskeletal proteins are recruited to forming phagocytic cups, although their role is not always well defined.

Talin, a cytoskeletal protein of 2,541 amino acids and 270 kD has been repeatedly involved in phagocytosis. Talin transiently accumulates around IgG-opsonized red blood cells, unopsonized zymosan, and Leishmania amastigotes and colocalizes with F-actin during the early stages of uptake (Greenberg, S., K. Burridge, and S.C. Silverstein. 1990. J. Exp. Med. 172:1853–1856; Allen, L.H., and A. Aderem. 1995. J. Exp. Med. 182:829–840; Love, D.C., M. Mentink Kane, and D.M. Mosser. 1998. Exp. Parasitol. 88:161–171). The general role of talin in phagocytosis found a recent confirmation in Dictostelium discoideum, where talin-null mutants have a slower rate of uptake than wild-type cells for both heat-killed yeast particles and latex beads (Niewohner, J., I. Weber, M. Maniak, A. Muller-Taubenberger, and G. Gerisch. 1997. J. Cell Biol. 138:349–361; Gebbie, L., M. Benghezal, S. Cornillon, R. Froquet, N. Cherix, M. Malbouyres, Y. Lefkir, C. Grangeasse, S. Fache, J. Dalous, F. Bruckert, F. Letourneur, and P. Cosson. 2004. Mol. Biol. Cell. 15:3915–3925). Nevertheless, the exact role of talin in mammalian phagocytosis remains elusive.

We will present our latest results on the role of talin in mammalian, opsonin-dependent phagocytosis. We have confirmed that endogenous as well as overexpressed talin can be transiently recruited to IgG-opsonized red blood cells and shown that it was also recruited to particles ligating complement receptor 3 (CR3, Mac-1, CD11b/CD18/αMβ2). Data from a variety ofcell systems (receptor-transfected fibroblasts from wild-type and talin knock-out mice as well as macrophage cell lines) and techniques (mutagenesis and RNA interference) led us to conclude that talin is an essential regulator of phagocytosis in mammalian cells. (Supported by the Wellcome Trust grant GR068556AIA and the Biotechnology and Biological Sciences Research Council grant 28/C18637.)

28. A Measurement of the Electrical Potential Difference Generated Across the Phagosome Membrane in Live Cells. BENJAMIN E. STEINBERG, NICOLAS TOURET, and SERGIO GRINSTEIN, Program in Cell Biology, Hospital for Sick Children, Toronto, M5G 1X8, Canada (Sponsor: Sergio Grinstein)

The phagosome is an intracellular organelle formed upon internalization of pathogens by cells of the immune system, such as macrophages and neutrophils. Once formed, both the membrane and luminal contents of the phagosome are extensively remodeled to generate the highly microbicidal milieu necessary to destroy the internalized pathogen. Ionic changes are thought to accompany this maturation process, but direct measurements of the ionic fluxes and associated changes in membrane potential are lacking. A quantitative description of the electrical properties of the phagosomal membrane would provide valuable information about its ionic permeabilities as well as the kinetics and activity of electrogenic ion pumps. However, to date, no methods exist to monitor the electrical potential generated across the phagosomal membrane. Here, we present a method based on fluorescence resonance energy transfer (FRET) to measure the electrical potential generated across the phagosomal membrane in live macrophages. The assay uses the potentiometric dye bis-(1,3-dibutylbarbituric acid)pentamethine oxonol (DiBAC4(5)) as the FRET acceptor and spatially targets the phagosome by using phagocytic targets (sheep red blood cells) that have been labeled with the FRET donor 7-diethylaminocoumarin-3-carboxylic acid. The degree of FRET within the phagosome is proportional to the membrane potential across the phagosomal membrane and can be converted to an actual voltage measurement using an external calibration to determine the intra-phagosomal DiBAC4(5) concentration and assuming that the acceptor partitions across membranes in a Nernstian fashion. With this approach the phagosome was found to have a transmembrane potential of +13.0 mV relative to the cytoplasm. Application of concannamycin virtually eliminated this potential difference, suggesting involvement of the vacuolar proton pump. Conversely, dissipation of the luminal acidification using permeant weak bases increased the potential difference, confirming the role of the proton pump and implying that, in the steady state, its rate is limited by the development of a transmembrane pH gradient.

29. The Effect of Crystalline Silica on Macrophages: Phagocytosis and Cell Death. RENEE M. GILBERTI and DAVID A. KNECHT, University of Connecticut, Storrs, CT 06269 (Sponsor: Sergio Grinstein)

Silicosis is a chronic lung disease induced by the inhalation of crystalline silica. Some evidence suggests crystalline silica is phagocytosed via the scavenger receptor and that this uptake leads to macrophage cell death. However, it is unclear that crystalline silica is actually internalized by cells. The objective of this study is to determine the molecular mechanisms by which crystalline silica is recognized and internalized by macrophages, and the relationship of uptake to cell death. Time-lapse imaging shows macrophages bind particles that fall onto the apical surface, but also extend filopods and lamellipods to capture particles up to 6.7 μm away. There is little subsequent movement of particles on the surface or inside the cell. Using three-dimensional optical sectioning, TRITC-labeled silica was shown to localize within the cytoplasmic
volume of CMFDA-labeled macrophages, confirming internalization. To quantify uptake, silica was opsonized with ovalbumin so noninternalized particles can be measured by their binding to anti-ovalbumin antibody. Silica uptake is relatively slow; by 5 min, 6 particles are bound per cell, but no particles are internalized (phagocytic index [p.i.] = 0 particles/100 cells). After 1 h, 55% uptake is reached (p.i. = 453). Ovalbumin-opsonized latex beads, regardless of charge, were also slowly internalized (50% uptake by 1 h, p.i. = 140). This is in contrast to Fc-opsonized latex beads (uptake reaches 86.4% within 15 min [p.i. = 323] and 94.6% by 1 h [p.i. = 412]).

70% of crystalline silica–treated macrophages became permeable to propidium iodide within 8 h and 100% by 24 h. Neither ovalbumin-opsonization nor TRITC labeling of silica significantly affects the induction of cell death. No other particle types tested caused significant cell death. Thus, a variety of particles are internalized by macrophages, but only crystalline silica causes cell death. (Supported by Patterson Trust Foundation.)

30. Trojan Horses: Macrophages as Vectors of Fungal Disease. HANSONG MA and ROBIN C. MAY, School of Biosciences, University of Birmingham, Birmingham, B15 2TT, UK

Cryptococcus neoformans is a fungal pathogen of humans and other mammals that causes cryptococcosis, a fatal infection of the central nervous system (CNS). Initial infection occurs by inhalation, but Cryptococcus rapidly spreads to the brain and spinal tissues via an unknown mechanism. Recent work by Tucker and Casadevall (Tucker, S.C., and A. Casadevall. 2002. Proc. Natl. Acad. Sci. USA. 99:3165–3170) has shown that Cryptococcus is able to survive within the phagosome of mammalian macrophages, leading to the suggestion that this pathogen may be transported into the CNS within host phagocytes—a so-called Trojan horse mechanism.

We are interested in two aspects of this concept. First, how is Cryptococcus able to survive within the hostile environment of the phagolysosome and, second, how is this pathogen able to escape from its host cell once it reaches the CNS?

To address these issues, we are exploring the interaction of Cryptococcus with J774 macrophages. Specifically, we are investigating three issues. First, whether intracellular survival of Cryptococcus depends on the route of uptake (e.g., complement or Fc receptor mediated). Second, whether intracellular Cryptococcus react to changes in the host macrophage by switching from latent to proliferative behavior. Third, whether escape from the host macrophage is a signaled process and, if so, what the signals are that induce this.

By understanding the biology of this host–pathogen interaction, we hope to develop strategies to shift the cellular balance in favor of the host and thus improve our ability to treat cryptococcosis.
of dominant-negative Rab5 precluded the formation of giant endosomes, implying that vacuolation is dependent on active Rab5. Fluorescence recovery after photo-bleaching of vacuoles containing GFP-Rab5 was used to deduce the dynamics of Rab5 during alkalinization. Maximal recovery of control and alkalized Rab5 was 0.85 and 0.75, and with t_{1/2} of 34.2 and 73.1 s, respectively, indicating that Rab5 is hyperactivated and remains active for longer times, implicating a role for Rab5-specific GTPase-activating proteins (GAPs).

Collectively, these data show that acidification maintains the size of early endosomes and induces termination of Rab5 signaling via the action of GAPs allowing maturation to proceed.

32. A Novel Interaction between Transcription Repressor of IL-12 p35 Gene GC BP and Hampin. RUSLAN I. DMITRIEV, TATYANA V. KORNEENKO, and MIKHAIL I. SHAKHPARONOV, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow 117871, Russia (Sponsor: Joseph J. Falke)

The clearance of apoptotic cells by macrophages is an evolutionary conserved process important for elimination of potentially cytotoxic, immunogenic, or inflammatory cellular contents. Defects in phagocytosis of apoptotic cells may cause accumulation of self-reactive lymphocytes that follows some autoimmune disorders such as rheumatoid arthritis. This process is finely regulated by expression of cytokines IL-12 and IL-10. Recently it was discovered that protein GC BP plays a pivotal role in expression of IL-12 by phagocytic macrophages. Upon phagocytosis, this protein goes to dephosphorylated state and binds to the p35 promoter of IL-12 gene, thereby repressing its transcription. Despite the obvious physiological role of GC BP action, the details of its functioning are not clear. In search of protein–protein interactions in a yeast two-hybrid system, we observed true positive interaction between GC BP and novel protein hampin. The latter is a nuclear protein that associates with chromatin and is involved mainly in histone modification. Our data suggests that interaction with hampin might be important to dephosphorylation of GC BP or for its nuclear localization. Additionally, the ubiquitous expression of GC BP and hampin points that both proteins can interact in all cell types. So, physiological role of GC BP might be broader than currently accepted. (Supported by Russian Foundation for Basic Research grant 04-04-49413.)

33. Hv1 Is a Mammalian Voltage-sensing Domain Protein with Voltage-gated H⁺ Channel Function. I. SCOTT RAMSEY, MAGDALENE M. MORAN, JAY-HONG A. CHONG, and DAVID E. CLAPHAM, Howard Hughes Medical Institute; Department of Neurobiology, Harvard Medical School; and Department of Cardiovascular Research, Children’s Hospital, Boston, MA 02115

We recently identified and cloned a novel mammalian voltage-sensing domain protein, Hv1, which functions as a voltage-gated H⁺ channel when overexpressed in mammalian cells (Ramsey, I.S., M.M. Moran, J.A. Chong, and D.E. Clapham. 2006. Nature. 440:1213–1216). Hv1 is 272 amino acids long and forms an N-terminal phosphatase-like domain followed by four transmembrane-spanning segments homologous to S1–S4 in archetypal voltage-gated cation channels. However, Hv1 conspicuously lacks a discernable S2–P–S6 pore-forming domain. Hv1 currents are activated at depolarizing voltages, sensitive to the transmembrane pH gradient, H⁺-selective, and inhibited by micromolar concentrations of Zn²⁺. Mutagenesis of Hv1 identified three Arg residues in S4 that regulate channel gating and voltage dependence and two His residues that are required for extracellular inhibition of Hv1 by Zn²⁺. Hv1 exhibits the hallmark biophysical features of the native proton conductance that is required to support the respiratory burst in phagocytic leukocytes (DeCoursey, T.E. 2003. Physiol. Rev. 83:475–579). Hv1 mRNA is enriched in human lymph node and protein is expressed in a variety of human immune tissues and cell lines, suggesting that Hv1 may represent the molecular correlate of native voltage-gated H⁺ channels. We have developed an Hv1 knockout mouse line to determine whether Hv1 is required for H⁺ channel function in vivo. (Supported by the Howard Hughes Medical Institute and the Sandler Program for Asthma Research.)

34. Phospholipase A2 Is Not Required for Activation of Voltage-gated Proton Channels during the Respiratory Burst in Human Eosinophils or Mouse Granulocytes. THOMAS E. DeCOURSEY, DERI MORGAN, MICHAEL H. GELB, and VLADIMIR V. CHERNY, Department of Molecular Biophysics and Physiology, Rush University Medical Center, Chicago, IL 60612

Electrophysiological events are crucial in the phagocyte respiratory burst because NADPH oxidase is electrogenic. Voltage-gated proton channels play an essential role in charge compensation during the respiratory burst. Arachidonic acid (AA) profoundly enhances both NADPH oxidase activity and proton channel gating. It has been proposed that AA generated by phospholipase A2 (PLA2) is the final step in activating both the oxidase and proton flux. For example, PLA2 inhibitors reportedly prevent activation of H⁺ flux, which is restored by exogenous AA. We investigated whether PLA2 activation is a necessary step in the activation of proton currents in granulocytes. Human eosinophils were examined in perforated patch configuration. Stimulation with PMA activated both H⁺ channels (converts them to “enhanced gating mode”) and NADPH oxidase activity (measured directly as electron current). Neither response was affected by pretreatment with the PLA2 inhibitors Pyrrolidine 2 (1–10 μM), Wyeth-1 (10–20 μM), or AACOCF3.
(1–25 μM) for several minutes. In contrast, pretreatment with the PKC inhibitor GF109203X (2 μM) prevented the response to PMA and also reversed the effects of PMA activation when added during the response. Measurements were also done in mice with cPLA2 genetically knocked out. The responses of granulocytes from these mice to PMA or fMetLeuPhe were indistinguishable from those of cells from normal mice. We conclude that PLA2 does not contribute to the activation of H+ channels by PMA, but PKC activation is essential. (Supported in part by the National Heart, Lung, and Blood Institute of the National Institutes of Health, grants HL52671 and HL61437.)

35. A Mechanical Spike during the Action Potential in Mammalian Nerve Terminals Monitored with an Atomic Force Microscope. GI-HO KIM,1 PAUL KOSTERIN,1 RAJ LARTIUS,3 ANA L. OBAID,1 and BRIAN M. SALZBERG,1,2 1Department of Neuroscience and 2Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104; 3Novascan Technologies, Inc., Ames, IA 50010

We have used a specially modified atomic force microscope to monitor mechanical events associated with the plasma membrane of nerve terminals from the mouse neurohypophysis that occur during, and immediately after, an electrically evoked action potential. By disabling the x-y scanning, and sampling z-axis position at up to 10 KHz, we could record mechanical events with a signal/noise ratio better than 25:1 in a single sweep. The AFM probe that we used was an uncoated silicon/tipless cantilever having a sensitivity of ~1,500 nm/volt in mouse Ringer’s solution. The mechanical “spikes,” which represent a rapidly reversible upward movement of the membrane, exhibit a full width at half maximum of 1.4–2.0 ms at 23°C, were eliminated by 1 μM tetrodotoxin, and were insensitive to [Ca2+]o and to the polarity of the electrical stimulation. A slow phase that follows the spike and reflects a decrease in the volume of the nerve terminals lasted more than 100 ms and was eliminated in “0” Ca/0.5 mM EGTA and blocked by 0.5 mM Cd2+. Taking into account the sensitivity of the cantilever in mouse Ringer’s solution, the vertical movement of the neurohypophysis during the action potential was on the order of 10 nm. Both the fast mechanical spikes and the slow phase “shrinkage” of the terminals are closely correlated with light scattering signals recorded from this tissue (Salzberg, B.M., A.L. Obaid, and H. Gainer. 1985. J. Gen. Physiol. 86:395–411). (Supported by National Institutes of Health grant NS40966.)