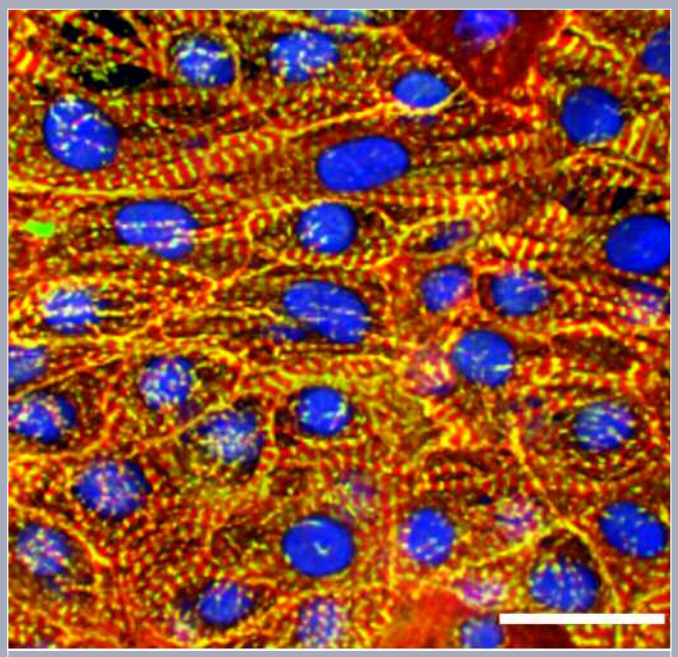
Cell Biology



Confocal optical section of the outer wall of the heart of a mouse at 8.5 days of development shows cardiomyocytes stained for actin (red), α-actinin (green), and DNA (blue). Scale bar = 20 μm. Image by Roberta Nowak, research assistant, in the laboratory of Velia Fowler, Ph.D., professor.

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Chairman's Overview

The mission statement of The Scripps Research Institute is "to make basic discoveries in science that enable medical innovations of tomorrow and to train scientists in the chemical and biological sciences." Most research efforts in the Department of Cell Biology and at Scripps Research are funded by the National Institutes of Health (NIH). During the economic boom of the late 1990s, Congress committed to investing in basic research by doubling the NIH budget. This doubling ended in 2003; since then funding levels have flatlined. Naturally, during the doubling, many young scientists were encouraged to train in basic research, and fruitful new areas of research were launched. The temporal convergence of this increase in research effort and decrease in funding levels has led to the "perfect storm": an acute drop in funding levels. Moreover, the leading researchers at Scripps Research often hold several NIH grants used to support their talented students and postdoctoral fellows in tackling several important biological questions. In an effort to spread the limited funds around, there is much discussion about limiting the number of grants a single investigator can hold. Thus, the need for private investment in the long-term basic research enterprise is urgent.

Sandra Schmid, Ph.D.

Basic research does not "sell" as well as discovering a new drug or curing a disease does. As a society we have grown accustomed to short-term goals and expectations: our corporations must set and meet quarterly projections, lest their stock prices fall; our politicians must demonstrate accomplishments to ensure their reelection every few years; sports utility vehicles sell like hotcakes, so why invest in designing fuel-efficient cars? Fundamental research is akin to the value-investing strategies that have made Warren Buffet a rich man. The financial and intellectual investment in basic cell biological research will pay huge dividends and build the knowledge assets and equity needed to generate the innovative medical treatments of tomorrow. I hit a personal milestone this year in turning 50. Will my research efforts or those of many of my outstanding colleagues have an impact on my own longevity and health? Probably not. But they will almost certainly affect the health and well-being of my children and their children, and without a doubt they will plant the seeds for generations of future medical discoveries. If we consider long-term impact, supporting basic research is a wise investment.

With that preamble, the following are a few highlights from today's cell biology research and hints of how the results might affect medical treatment for future generations.

Elizabeth Winzeler continues her research on the malaria parasite. Her use of genomic technology to identify the proteins expressed at key stages in the malaria life-cycle and of invariant surface proteins is providing essential targets for development of antimalaria therapies and vaccines.

William Balch, together with John Yates, Department of Chemical Physiology, and Jeff Kelly, Department of Chemistry, is identifying the cohort of folding chaperones and other factors that maintain the tenuous balance between correctly folded and misfolded proteins, a condition termed proteostasis. An imbalance in these pathways leads to protein folding diseases such as Parkinson's disease, Alzheimer's disease, cystic fibrosis, and many others. Understanding these fundamental pathways is providing insights into how future physicians might restore balance and correct the disease state.

Larry Gerace's basic research to identify new proteins that localize to the nuclear envelope that confines the genetic material has revealed several proteins that when mutated are associated with human muscular dystrophies. Analysis of the function of these new proteins will provide information on the underlying mechanisms that lead to disease and hence identify new targets for future therapeutic intervention.

Lisa Stower has completed the first purification of a mammalian pheromone and is using the purified molecule in studies of the simple neuronal circuitry that triggers instinctive behavioral responses. Her find-

ings will provide the foundations for understanding complex human behavior and ultimately for more effectively treating schizophrenia, depression, and, perhaps, autism.

Dynamin-2, a protein that I and the members of my group study, has recently been linked to a common human neuropathy, Charcott-Marie-Tooth syndrome. As a result of our long-standing basic research and understanding of this protein in its physiologic context, we have a 15-year head start and the tools to quickly determine how mutations in this protein lead to human disease.

Finally, some important transitions to note: Ardem Patapoutian was promoted to full professor, Elizabeth Winzeler to tenured associate professor, and Lisa Stowers to associate professor. Clare Waterman-Storer has left to assume a position at the NIH. Most notably, Mark Yeager, one of the founding members of the Department of Cell Biology, will assume the position of the Andrew P. Somlyo Professor and Chair of the Department of Physiology and Biological Physics at the University of Virginia. He will be greatly missed as a colleague, but he leaves behind a core laboratory allowing us to maintain strong interactions. The University of Virginia will greatly benefit from his leadership.

INVESTIGATORS' REPORTS

Structural Characterization of Macromolecular Machines

F.J. Asturias, Y. Chaban, E. Brignole, G. Cai, F. Zhang, J. Chittuluru, F. Xu

We use state-of-the-art electron microscopy and image analysis to determine the 3-dimensional structures of macromolecular complexes involved in a variety of cellular processes, including DNA transcription, chromatin modification and remodeling, and fatty acid synthesis. Macromolecular electron microscopy is ideal for these studies because it requires only a small amount of material, and the conditions for preparing samples are physiologically relevant. Images of individual macromolecules are recorded and then computationally combined to obtain low- to moderate-resolution (25–10 Å) structures. These structures are often interpreted by docking atomic resolution structures of component subunits in the lower resolution map of an entire complex. Our ultimate goal is to use a combination of biochemical and structural information to reveal the mechanism by which a macromolecular complex carries out its function.

In our current studies on DNA transcription and its regulation, we are analyzing the structure of complexes involved in the regulation of transcription during initiation and in earlier steps in which the structure of chromatin is altered to control access to DNA. We are particularly interested in the structure and function of Mediator, a multiprotein complex that is well conserved across eukaryotic organisms from yeast to humans and that plays a central role in the regulation of transcription initiation in eukaryotes. We have developed an efficient protocol for purifying Mediator and have been able to engineer molecular "tags" into specific subunits of the complex. This breakthrough has enabled us to determine a detailed structure of Mediator and to localize individual Mediator components. Our next goal is to improve the current understanding of the interaction between Mediator and components of the basal transcription machinery.

We also are continuing the structural characterization of the chromatin remodeling complex RSC and its interaction with the nucleosome. The structure of an RSC-nucleosome complex revealed that interaction with RSC results in extensive changes in the structure of the nucleosome. This finding provides essential clues for the mechanism of ATP-dependent nucleosome remodeling.

Finally, we continue to investigate the role that conformational changes play in the function of mammalian fatty acid synthase (FAS), the enzyme responsible for the synthesis of long-chain fatty acids. In a true "macromolecular assembly line," the different enzymes involved in fatty acid synthesis are fused into a single polypeptide chain that includes 6 catalytic and 1 acyl carrier protein domains. Detailed classification of electron microscopy images of wild-type and catalytic FAS mutants in the absence and presence of substrates revealed a variety of conformational states (Fig. 1). Correlated movement of specific domains makes possible domain interactions essential for catalysis and fatty acid synthesis.

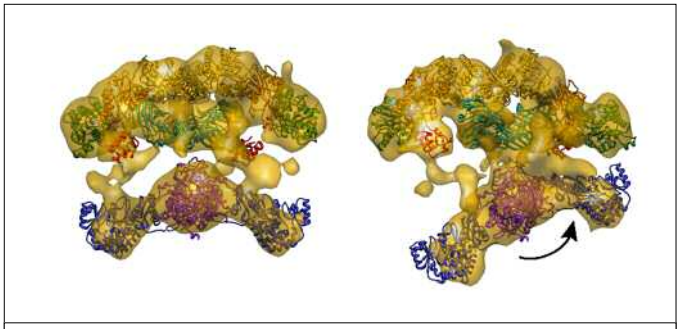

Fig. 1. Structure and conformational variability of mammalian FAS. Electron microscopy and single-particle image analysis were used to calculate 3-dimensional reconstructions of the synthase in different conformations. High-resolution structures of individual FAS domain homologs were docked into the electron microscopy reconstructions by using information from an x-ray model of FAS. A single conformation of the FAS polypeptide cannot account for all of the interdomain contacts required for fatty acid synthesis. The different structures revealed by macromolecular electron microscopy analysis illustrate concerted domain movements that enable fatty acid synthesis by FAS.

PUBLICATIONS

Brignole, E.J., Smith, S., Asturias, F.J. Conformational flexibility of metazoan fatty acid synthase enables catalysis. Nat. Struct. Mol. Biol., *in press*.

Chaban, Y.L., Ezeokonkwo, C., Chung, W.H., Zhang, F., Kornberg, R.D., Maier-Davis, B., Lorch, Y., Asturias, F.J. Structure of an RSC-nucleosome complex and insights into chromatin remodeling. Nat. Struct. Mol. Biol., *in press*.

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Structural and Functional Basis for Membrane Traffic and Misfolding Diseases

W.E. Balch, S. Becker, J. Coppinger, V. Gupta, J. Hulleman, D. Hutt, A.V. Koulov, P. LaPointe, J. Matteson, A. Murray, A. Nauli, L. Page, S. Pankow, H. Plutner, A. Pottekat, A. Razvi, L. Ryno, K. Subramanian, P. Szajner, I. Yonemoto

A major challenge in human health is to understand and treat the many conformational diseases that affect protein homeostasis (proteostasis) during development and aging. These diseases are due to an imbalance between the energetics of the protein fold and the properties of the local folding environment. Such diseases include cystic fibrosis, childhood emphysema, type 2 diabetes, and amyloidosis. Many of these are broadly classified as membrane-trafficking diseases because the defect in protein folding during transit through the mammalian exocytic pathway leads to loss of normal function and/or a gain of toxic function. Our key goals are to define the basic operation of these trafficking pathways, determine the cause of the underlying folding disorders, and learn how these events can be altered to rescue the ability of a protein to function in a cell.

STRUCTURAL AND FUNCTIONAL BASIS FOR MEMBRANE TRAFFIC

Eukaryotic cells are highly compartmentalized; each compartment of the exocytic and endocytic pathways provides a unique chemical and biological environment in which protein folding and function can be modulated to maintain cellular proteostasis. Movement between these compartments involves the activity of both anterograde and retrograde transport tubules and vesicles. During export from the first compartment of the exocytic pathway, the endoplasmic reticulum, assembly of vesicle-budding sites involves assembly of the coatomer complex II (COPII) coat. In collaboration with C. Potter and B. Carragher, Department of Cell Biology, we have solved the 2-dimensional electron cryomicroscopy structure of the COPII cage, a self-assembling polymeric scaffold. To collect cargo, this scaffold interacts with an adaptor protein complex that binds nascent cargo. Using electron cryomicroscopy, we recently solved the structure of the intact COPII coat containing both the cage scaffold and the adaptor complexes (Fig. 1). The structural organization of this striking COPII coat system led to a new model that now describes the basis for cargo capture and trafficking through the early secretory pathway.

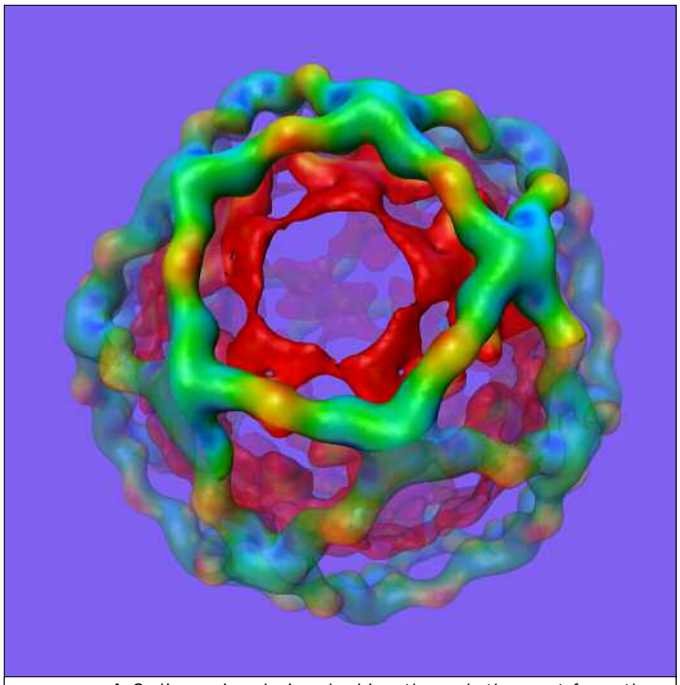

Fig. 1. A 3-dimensional view looking through the coat from the pentagonal cage of the intact COPII coat directing export of cargo from the endoplasmic reticulum. The self-assembling nanoparticle contains the outer layer (green-yellow) scaffold composed of Sec13-31 (the cage) and the inner layer adaptor protein complex (red) consisting of Sec23-24. The COPII coat structure was solved by using electron cryomicroscopy. Reprinted from Mu, T.W., Ong, D.S., Wang, Y.J., Balch, W.E., Yates, J.R. III, Segatori, L., Kelly, J.W. Chemical and biological approaches synergize to ameliorate protein-folding diseases. Cell 134:769, 2008.

COPII vesicles also recruit GTPases, tethers, and fusion components to promote vesicle fusion with downstream compartments. The x-ray structure of a complex composed of Rab GTPase interacting with the p115 tether, solved in collaboration with I.A. Wilson, Department of Molecular Biology, revealed a superhelical platform that directs function. We have shown that the assembly of this superhelical platform with other components of the tether-fusion system is likely regulated by the activity of the Hsp90 family of chaperone-cochaperone components. Using bioinformatics and systems biology to build interaction networks, we are beginning to define how such systems likely integrate the folding and trafficking component networks with the overall structure and function of membrane compartments.

BIOLOGICAL AND STRUCTURAL BASIS FOR MISFOLDING DISEASE

Many mutations disrupt cargo traffic from the endoplasmic reticulum by preventing proper protein folding during synthesis, resulting in loss of recognition by the COPII machinery. In collaboration with J.W. Kelly and E. Powers, Department of Chemistry,

we are studying the underlying basis for these events. Using biophysical modeling approaches, we have developed a rigorous quantitative framework to describe in a global way the adaptable role of proteostasis and the chaperone environment in membrane trafficking and disease. In addition, in collaboration with J.R. Yates, Department of Chemical Physiology, we used mass spectrometry to analyze the proteome that regulates the trafficking and function of the wild-type and mutant cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel that when defective is responsible for the disease cystic fibrosis. We have discovered a cohort of chaperones involving the Hsp90 chaperone system that dictates the CFTR folding environment (referred to as the chaperome). In disease, the Hsp90 system becomes trapped in an intermediate folding complex, blocking export and triggering degradation. In a system-wide analysis of the role of folding pathway components, using small interfering RNA to alter the steady-state distribution of chaperones led to restoration of the function of the chloride channel at the cell surface. These results, in combination with those of recent studies on the role of transcriptional regulatory circuits that control the expression of the components of the entire set of molecular interactions in cells in cystic fibrosis, revealed an extensive proteostatic network necessary for CFTR function in health and disease (referred to as the CFTR "interactome"). This environment is likely responsive to numerous environmental factors, such as caloric intake, that also influence the onset of other conformational diseases, such as type 2 diabetes and neurodegenerative amyloid diseases of aging, which we are studying in collaboration with Dr. Kelly, J. Buxbaum, Department of Experimental Medicine, and A. Dillin, Salk Institute for Biological Studies, La Jolla, California, and R. Morimoto, Northwestern University, Evanston, Illinois.

Through such a multidisciplinary approach, we hope to gain critical insight into the fundamental principles of protein folding and trafficking and a new understanding of a variety of inherited diseases sensitive to the proteostasis environment that controls human health and aging. We anticipate that knowledge of the function of proteostatic pathways will enable the development of general, corrective small-molecule proteostasis regulators and chemical modulators of specific protein folds that will provide increased stability and thereby enhance the delivery and function of misfolded proteins in downstream environments, leading to alleviation of disease.

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Smells and Stem Cells: Understanding How Genes Build the Brain

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WIRING THE OLFACTORY SYSTEM

The sense of smell influences many intriguing aspects of human and animal behavior. Scents elicit innate responses of attraction and revulsion and evoke enduring emotional memories. These features suggest that behaviorally important olfactory neural circuits may be hardwired. We are interested in learning how genes build specific neural circuits. We use gene targeting in embryonic stem cells, molecular biology, and imaging to visualize and modify the neurons that recognize odors in the periphery and the brain. These studies are critical first steps toward understand-

ing how neural circuits produce sensory perception and how genetic alterations may contribute to neuronal dysfunction and cognitive disorders.

A major goal of our research is to understand how the distributed representation of olfactory information in the olfactory bulb is integrated in the cortex. This endeavor has been hindered by the lack of specific promoters for mitral cells, the output neurons of the bulb. We identified a gene expressed specifically in mitral cells and have engineered mice in which subsets of mitral cells are genetically tagged. We have developed novel viral tools that allow us to label individual mitral cells and trace their projections into the brain. We will now assemble a map of olfactory information in cortical regions. We have also begun to identify genes differentially expressed in mitral cells that may regulate formation of specific neural circuits.

One such family of genes is the large genomic cluster of approximately 60 protocadherin genes. We have shown that stochastic expression of several protocadherins per neuron endows each neuron with a unique cell-surface code. We have generated mice that lack protocadherin expression in defined subsets of neurons. These mice have behavioral abnormalities consistent with defects in neuronal function. We are investigating the cellular and physiologic consequences of loss of protocadherin diversity.

GENERATING CELL LINES FROM NEURONS

Scientists have postulated that in addition to reversible genetic changes, irreversible chromosomal alterations occur in neurons during development, aging, and disease. Currently, no specific de novo DNA changes in neurons have been identified. The diversity and postmitotic state of neurons have hindered these studies. In addition, no method to survey the genome of an individual neuron exists. We are using somatic cell nuclear transfer technology to develop a method to generate cloned mice or embryonic stem cell lines from defined subsets of neurons. These studies will determine, for the first time, whether it is possible to clone mice from adult cortical neurons and will provide a novel means to understand how chromosomal alterations in neurons may contribute to neuronal differentiation or neurodegenerative diseases.

Automated Molecular Imaging

B. Carragher, C.S. Potter, A. Cheng, D. Fellmann, C. Irving, G. Lander, P. Lau, A. Mulder, M. Palmer, T. Ajero, E. Rodriguez, J. Pulokas, J. Quispe, S. Stagg, N. Voss, C. Yoshioka

During the past decade, electron cryomicroscopy has become a powerful approach for determining the structure of large macromolecular complexes. Elucidating the structure and mechanism of action of these "molecular machines" is an emerging frontier in understanding how the information in the genome is transformed into cellular activities. Examples include ribosomes, transcription complexes, track-motor complexes, and membrane-embedded pumps and channels.

In electron cryomicroscopy, the macromolecular specimen is preserved in a thin layer of vitreous (glassy) ice and imaged in an electron microscope by using low doses of electrons. The low signal-to-noise ratio of the resulting images means that averaging is required to recover the signal and reconstruct a 3-dimensional map of the structure.

In 2002, we established the National Resource for Automated Molecular Microscopy (NRAMM) to develop, test, and apply technology for automating the processes involved in using electron cryomicroscopy to solve macromolecular structures. Current core technology research and development projects focus on 4 areas: improving grid substrates and specimen preparation, further automating and optimizing image acquisition, developing an integrated single-particle analysis and processing pipeline to investigate parameters that limit resolution, and developing automated high-throughput electron microscopy screening. The tools and technologies developed at NRAMM have not only facilitated the process of molecular microscopy but also expanded the scope of accessible problems and pushed experimental frontiers by making possible investigations deemed too difficult or high risk because of the considerable effort involved in using manual methods. Automation has contributed to higher throughput of data, enabling us to compute electron density maps of large macromolecules at resolutions of approximately 6 Å by using completely automated methods. We have also taken advantage of the high throughput to investigate the factors that limit the resolution of the final computed 3-dimensional electron density map.

The current focus of NRAMM is to develop new techniques and technologies that allow us to explore

the structure of small or conformationally heterogeneous macromolecules. The ability to determine the structure of macromolecular complexes in a variety of conformational states is the next big challenge for electron cryomicroscopy, representing a unique opportunity to address interesting biological questions.

These technological developments at NRAMM are driven by, and in turn enable, collaborative research projects, including reconstruction of coatomer complex II cages and coats, the structural understanding of DNA-packaging mechanisms in bacteriophages, and the development of a more detailed understanding of the microRNA gene-silencing pathway. NRAMM also supports training activities, including individual and group training, small workshops on various aspects of automation, and a large international biennial electron cryomicroscopy training course.

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Regulation of Cytomechanochemical Systems

G. Danuser, K. Applegate, A. Bacconi, S. Chen, H. Elliott, P.-S. Hsu, K. Jaqaman, J. Lim, A. Liu, D. Loerke, Z. Maffeo, A. Matov, D. Nunez, M. Sabouri, G. Yang

We study the spatial and temporal integration of chemical signals and mechanical forces in pathways that mediate cell migration, division, and vesicular transport.

Cell migration requires accurate balancing of propulsive forces generated by assembly of actin filaments at the protruding edge of a cell, contraction forces generated by myosin motors, and adhesion forces generated by transmembrane linking of actin filaments to the extracellular matrix. The force balance is continually modulated by adaptation of the cytoskeleton structure in response to force feedback and regulatory chemical signals. Thus, understanding the relationship between force generation and the structural dynamics of cytoskeleton components at a systems level is central to elucidating the mechanisms of cell migration.

A key aspect of cell division is the accurate segregation of replicated DNA from the mother cell into the 2 daughter cells. This segregation is achieved by the mitotic spindle, a bipolar array of microtubule polymers that symmetrically pull sister chromatids to opposite poles. Chromosome movement is mediated by forces from many microtubule-associated motors and by proteins that regulate the structural dynamics of the spindle via localized microtubule assembly and disassembly. We are studying the relationship between structural dynamics and the generation of mitotic forces at the level of the entire spindle.

We have expanded our research to an analysis of the mechanisms by which clathrin-coated structures assemble in the plasma membrane and alter the underlying cortex of actin filaments to shuttle molecular cargo from the extracellular domain into the cytoplasm. This process, referred to as endocytosis, is critical to all aspects of cellular homeostasis in a continually changing environment.

To probe these various molecular systems, we develop high-resolution imaging and computational methods to track the dynamics of relevant system components in live cells. We also develop mathematical models to predict how the action of individual molecules in a sys-

tem relates to the cellular output. In a particularly important effort, we are focusing on new numerical methods to link such models to experimental data, enabling rigorous validation of the models and calibration of unknown model parameters.

For example, we have been able to determine the length distribution of microtubules in meiotic spindles of *Xenopus laevis* extracts. We used the synchronized motion of single fluorophores incorporated in the lattices of spindle microtubules to identify pairs of fluorophores associated with the same microtubule. By mathematical modeling of the random association of fluorophores with microtubules, on the basis of the distribution of fluorophore distances, we were able to predict the distribution of microtubule lengths. Hitherto, this information had been inaccessible because conventional light microscopy lacks the resolution to detect individual microtubule polymers in the spindle, and techniques such as electron microscopy, which have a much higher resolution, cannot depict the architecture of densely packed, 3-dimensional structures on micrometer length scales.

Much to our surprise, we found that 70% of the microtubules are shorter than half the spindle. Textbook models of the spindle architecture show 2 polar arrays of microtubules interlaced in the spindle midzone to form a stable structure. Our data suggest that the spindle consists of a tiled array of thousands of short microtubules in which mechanical stability is mediated by dynamic cross-linking. This finding has major implications for the regulation of mitosis. One of the most important implications is that in a tiled array, no long-range communication is needed between spindle poles and spindle midzone; the mechanisms of such communication were enigmatic in previous models of the spindle. Both force generation and structural adaptation required throughout chromosome segregation can be locally regulated at length scales of molecular signals.

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Synthetic Protein Chemistry

P.E. Dawson, J.B. Blanco-Canosa, F. Brunel, M. Churchill, A. Dirksen, S. Ingale, E. Lempens, N. Metanis, C. Schroeder, T. Shekhter, T. Tiefenbrunn

We have developed a set of highly selective chemical reactions that allow peptide, nucleic acid, or carbohydrate fragments to be synthesized and assembled selectively into functional macromolecules. We are using these chemical ligation methods to incorporate unnatural amino acids to probe fundamental questions about protein folding and stability and the catalytic mechanism of oxidoreductases such as glutaredoxin. In addition, the efficiency and chemoselectivity of these reactions make it possible to specifically label proteins with probes for detailed spectroscopic analysis of proteins. For example, in studies with F.E. Romesberg, Department of Chemistry, we are incorporating specific deuterium probes for the vibrational analysis of proteins by infrared, and with S. Yegneswaran, Department of Molecular and Experimental Medicine, we have developed an approach to transfer fluorescent labels to specific cofactor-binding exosites on the surface of thrombin. We are also part of a collaborative project in which diverse techniques, including synthetic protein chemistry, are used to mimic HIV conformational epitopes; the goal is to develop HIV vaccines.

SYNTHESIS OF POSTTRANSLATIONALLY MODIFIED PROTEINS

The ability to chemically modify biological macromolecules in a specific manner underlies many of the methods and technologies used in modern research. This specific tailoring of macromolecules has been enabled by the development of highly chemoselective ligation (conjugation) chemistries that are characterized by their chemoselectivity, reactivity, and compatibility with neutral aqueous buffers. However, the growing demands of research in analytical biochemistry, chemical biology, protein chemistry, and nanotechnology have pushed the limits of currently available ligation methods.

We recently reported new methods for synthesizing complex *N*-linked glycoproteins. Large quantities

of the amino acid asparagine linked to an intact complex-type human sialyloligosaccharaide can be isolated from eggs in pure form. In collaboration with Y. Kajihara, Yokohama City University, Yokohama, Japan, we have used our ligation methods to build a glycoprotein, the chemokine monocyte chemoattractant protein 3, from these unprotected sugar precursors (Fig. 1A). In addition, we recently described a new method for synthesizing C-terminally modified peptides that are essential for protein synthesis by chemical ligation. As shown in Figure 1B, this approach efficiently generates a reactive *N*-acylurea functional group that can be used directly in native chemical ligation and is compatible with chemical methods for introducing posttranslational modifications. Further development of these approaches for glycoprotein synthesis will enable deciphering of the biological functions of individual protein glycoforms.

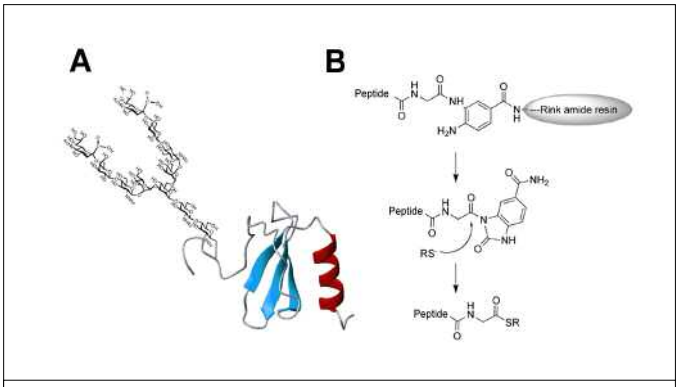

Fig. 1. Chemical protein synthesis. A, MCP-3, a synthetic glycoprotein with a homogeneous *N*-linked carbohydrate. B, An acylurea approach for the C-terminal modifications of peptides.

CONJUGATION METHODS FOR IMAGING AND NANOTECHNOLOGY

We recently found that the aromatic amine aniline is a potent nucleophilic catalyst for imine ligations, the reaction of an aldehyde or a ketone with amines such as hydrazides. We have used these reactions to efficiently label fluorescent quantum dots with synthetic peptides. These peptide–quantum dot conjugates can be tailored to specifically recognize specific protein targets or to act as chromogenic substrates for the sensitive analysis of enzymes such as proteases. We have also used hydrazone ligations to assemble viral nanoparticles with multiple functional groups attached. For example, in collaboration with M. Manchester, Department of Cell Biology, and H. Stuhlmann, Weill Medical College of Cornell University, New York, New York, we engineered a virus to display multiple copies of a tumor-directing peptide, fluorophores, and a solubilizing polyethylene glycol polymer. These nanoparticles selectively localized to H-29 (human colon carcinoma) tumors in mice.

In addition to providing rapid and selective reaction rates, this catalytic approach enables the reversible labeling of molecules. Using rapid reversible covalent chemistry, we anticipate that we will be able to select molecules on the basis of binding affinity or target selectivity. We think that these reactions will enable us to develop reversible tagging strategies compatible with complex biological systems.

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Regulation of Actin Dynamics in Morphogenesis and Development

V.M. Fowler, R. Chow, R.A. Lewis, C. McKeown, R. Nowak

Regulation of actin dynamics at filament ends determines the assembly and organization of actin cytoskeletal structures and is crucial for morphogenetic processes in development and tissue function. We focus on tropomodulins, proteins that cap the pointed ends of actin filaments, inhibiting monomer association and dissociation and thereby stabilizing the filaments and regulating their lengths.

Disassembly of actin at the pointed ends of filaments is the rate-limiting step in the turnover of actin filaments, yet relatively little is known about the regulation of pointed ends. Vertebrates have 4 tropomodulin isoforms; each isoform is approximately 40 kD and has a homolog in flies and worms. Unique among all actin-capping proteins, tropomodulins also bind to tropomyosin to tightly cap pointed ends of tropomyosin-coated actin filaments with affinities more than 1000-fold greater than the affinities for pure actin filaments. Despite the high level of sequence conservation (~70%) among vertebrate tropomodulins, the actin monomer-polymer regulatory properties of tropomodulins are distinct, and tropomodulins are further distinguished by different binding affinities for the many vertebrate tropomyosin isoforms. Tropomodulins are the predominant known capping proteins of pointed ends and thus are expected to have critical roles in actin-based morphogenetic processes.

In the past year, we focused on tropomodulin 1, which caps the pointed ends of the long thin filaments in muscle sarcomeres and the short actin filaments in the membrane skeleton of erythrocytes. Previous research showed that targeted deletion of tropomodulin 1 in mice leads to defects in the assembly and development of cardiac myofibrils, fragility of primitive erythroid cells, and an absence of yolk sac vasculogenesis; embryos die at 9.5 days of development. To test whether the embryonic death of mice lacking tropomodulin 1 is due to defects in cardiac myofibrillogenesis and development or to fragility of erythroid cells and defects in yolk sac vasculogenesis, we expressed tropomodulin 1 specifically in the myocardium of mice lacking the gene for tropomodulin 1 under the control of the α-myosin heavy-chain promoter, *Tg(αMHC-Tmod1)*. In contrast to embryos without the gene for tropomodulin 1 (null embryos), which do not undergo cardiac looping and have defects in yolk sac vasculogenesis, $Tmod1^{-/-Tg(\alpha MHC\text{-}Tmod1)}$ embryos have normal cardiac and yolk sac morphology. Furthermore, the embryos develop into viable and fertile mice, indicating that expression of tropomodulin 1 in the heart is sufficient to prevent the embryonic defects in mice lacking the gene for this isoform. Thus, the primary defect in null embryos is in the myocardium where tropomodulin is essential for myofibril assembly, cardiac function, and development. Moreover, the fragility of primitive erythroid cells in null embryos does not affect yolk sac vasculogenesis, cardiac development, or viability in mice. These $Tmod1^{-/-Tg(\alpha MHC\text{-}Tmod1)}$ mice are a powerful tool for studies of the in vivo function of tropomodulin 1 in the more experimentally accessible erythrocytes and other tissues from adult mice.

A second area of research is the function of tropomodulin 1 in skeletal muscle. In contrast to the heart, which does not assemble myofibrils and does not develop in the absence of tropomodulin 1, skeletal muscles from $Tmod1^{-/-Tg(\alpha MHC\text{-}Tmod1)}$ mice develop relatively normally with striated myofibrils. This situation may be due to isoform compensation by tropomodulin 4, which now caps the pointed ends of thin filaments. Interestingly, thin filaments in the muscles of mice lacking the gene for tropomodulin 1 appear to be longer than those in the muscles of wild-type mice. Preliminary data indicate that tropomodulin 1 binds more strongly to striated muscle tropomyosins than does tropomodulin 4, suggesting that the length of thin filaments is regulated by affinity modulation of tropomodulin-tropomyosin actin capping.

We have also shown that tropomodulin 1 binds with different relative affinities to striated muscle tropomyosins produced from the genes for α-, β-, and γ-tropomyosin and does not bind at all to the slow skeletal muscle γ-tropomyosin with a Met9Arg mutation that occurs in patients with nemaline myopathy. We hypothesize that inability of the mutant Met9Arg γ-tropomyosin to bind to tropomodulin 1 may lead to altered regulation and stability of the length of thin filaments, contributing to muscle weakness and atrophy in patients with nemaline myopathy. Studies in transgenic mouse models are in progress to investigate the consequences of tropomodulin isoform switching for the development, structure, and function of skeletal muscle.

A third area of research is the eye lens, a specialized epithelial tissue composed of radially arranged layers of long, thin fiber cells tightly packed in a regular hexagonal pattern that is essential for lens optical function. Tropomodulin 1 is associated with a short γ-tropomyosin isoform in the actin cytoskeleton on lateral membranes of lens fiber cells. To investigate the function of tropomodulin 1 in the lens, we used the $Tmod1^{-/-Tg(\alpha MHC\text{-}Tmod1)}$ mice, which have no tropomodulin 1 in any tissues except the heart. In the absence of tropomodulin 1, the lens develops normally, but as it grows after birth, maturation of fiber cells is aberrant, resulting in abnormal cell shapes and sizes and disordered packing organization. Levels of the membrane-associated short γ-tropomyosin are reduced dramatically, and the normal polarity of actin filament distribution on lateral membranes is lost.

Tropomodulin 1 stabilization of tropomyosin-actin filaments on lateral membranes is thus required to maintain uniform fiber cell shapes and hexagonal packing symmetry during lens growth and aging.

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Genetics of Metabolic Disorders

N. Gekakis, J.J. Wilkes, J.L. Pitman, M.C. Wheeler

We use forward genetics in mice to identify genes associated with metabolic disorders. To date we have identified 8 genes that lead to metabolic abnormalities when mutated. Mutation in the gene for glucokinase (*Gck*) or *Sec61a1* leads to diabetes; mutation in *Pc1* leads to obesity; mutation in the gene for aquaporin 2 (*Aqp2*) leads to diabetes insipidus; mutation in the gene for myostatin (*Mstn*) prevents obesity and insulin resistance induced by high-fat diets; and mutations in the genes for lecithin cholesterol acyltransferase (*Lcat*), diacylglycerol acyltransferase 2 (*Dgat2*), or *Cpto* lead to low levels of total and high-density lipoprotein (HDL) cholesterol.

Cpto is a wholly novel gene that we recently cloned from mice with abnormally low levels of cholesterol and triglyceride (Fig. 1A). Mice with a homozygous point mutation in *Cpto* (substitution of phenylalanine with serine at position 25, F25S) are viable and fertile and are born at the expected rate of 25% from heterozygous mutant parents. The most prominent defect in homozygous mutant mice is a dramatic reduction in both HDL and very low-density lipoprotein VLDL (Fig. 1B). Although the liver, the major site of *Cpto* expression, is centrally important to the metabolism of both HDL and VLDL, the biogenesis of the 2 lipoproteins differs; VLDL particles are secreted from the liver, and HDL is formed in the bloodstream. This difference does not appear to be a generalized defect in secretion from the liver; the levels of other serum proteins secreted by the liver (e.g., albumin) are normal.

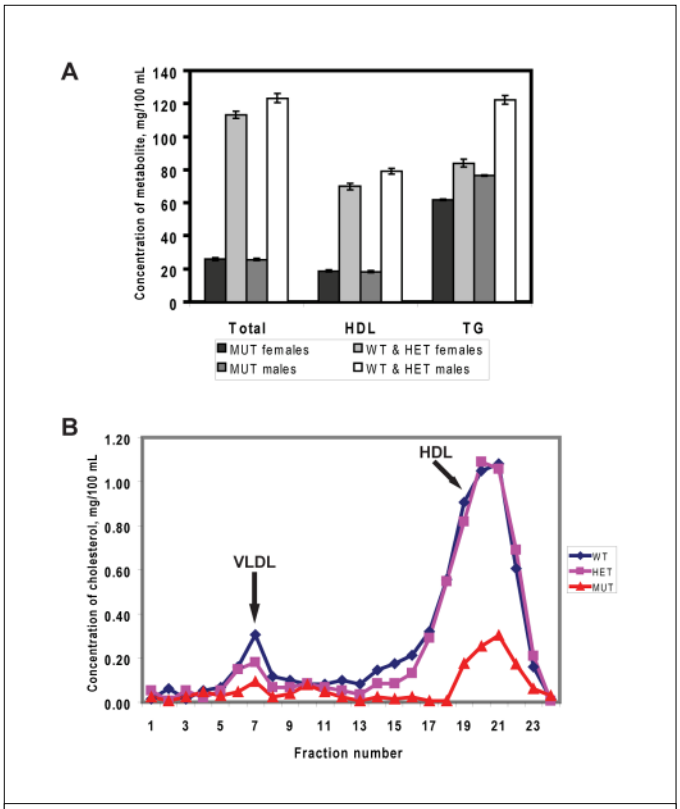

Fig. 1. Low levels of cholesterol and triglycerides in *Cpto* mutant mice. A, Metabolites were measured in plasma samples from homozygous and heterozygous mutant and wild-type mice. Total, total cholesterol; HDL, cholesterol associated with HDL particles; TG, total triglycerides; MUT, homozygous mutant animals; WT & HET, combination of heterozygous mutant and wild-type animals. B, Plasma samples from homozygous and heterozygous mutant and wild-type female mice were size fractionated, and the individual fractions were assayed for cholesterol content.

Currently, we are investigating the mechanism by which a mutation in *Cpto* leads to low levels of cholesterol and triglycerides. A hint comes from experiments in fruit flies; a *Drosophila* homolog of *Cpto* (*Tango1*) plays a role in the secretory pathway. Further, *Tango1* was localized to the golgi apparatus, an organelle responsible for sorting and processing secreted proteins.

Also via forward genetics, we discovered a new gene (*Sec61a1*) required for beta-cell function that highlights the susceptibility of beta cells to "ER stress." Mutation in *Sec61a1* leads to a loss of the insulin-secreting beta cells in the pancreas and to diabetes. The endoplasmic reticulum is the site of synthesis of secreted proteins and as such plays an important role in quality control, detecting and eliminating those proteins that are abnormal. ER stress is the accumulation of abnormal proteins in the endoplasmic reticulum

and can lead to cellular dysfunction or cellular death. *Sec61a1* is clearly important in this quality control function, and the beta cells seem especially vulnerable to ER stress–induced cell death. Because beta cells are central to the development of diabetes and are prone to ER stress, such stress may be a fundamental contributor to type 2 diabetes in humans.

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The Nuclear Envelope in the Regulation of Cell Function

L. Gerace, G. Ambrus-Aikelin, A. Cassany, C. Cher, E. Choi, K. Datta, T. Guan, M. Huber, E. Singer

The functional activities of the eukaryotic nucleus are spatially and temporally separated from the cytoplasm by the nuclear envelope, a specialized domain of the endoplasmic reticulum that forms the nuclear boundary. The nuclear envelope contains a double membrane perforated by nuclear pore complexes (NPCs), massive protein assemblies that mediate the transport of proteins and RNAs between the nucleus and the cytoplasm. A second distinctive component of the nuclear envelope is the nuclear lamina, a protein scaffold lining the inner nuclear membrane that is important for nuclear and chromosomal architecture. In addition to its structural role, the lamina helps regulate signal transduction and gene expression in the nucleus. Mutations in lamina proteins give rise to more than 15 human genetic disorders, including muscular dystrophies, lipodystrophies, bone diseases, and premature aging diseases. We are studying the role of the lamina in nuclear functions and the basis for its link to human diseases. In addition, we are investigating the molecular mechanisms of nucleocytoplasmic trafficking through NPCs.

NUCLEAR LAMINA SIGNALING, GENE EXPRESSION, AND HUMAN DISEASE

The nuclear lamina contains a polymer of 2–4 related intermediate filament proteins called lamins, which are associated with transmembrane proteins of the inner nuclear membrane. Lamins and other inner nuclear membrane proteins interact with numerous nuclear proteins, including chromatin structural components, transcriptional regulators, and signaling components. To obtain a blueprint for more detailed study of the functions of the nuclear envelope, in collaboration with J.R. Yates, Department of Cell Biology, we analyzed the proteome of the envelope. We identified more than 50 nuclear envelope–enriched transmembrane proteins (NETs) that had not been described previously.

We are focusing on functional analysis of these NETs in muscle cells and adipocytes, which are among the cell types most commonly affected by disease-causing mutations in lamina proteins. Using transcriptional profiling of cultured myoblasts, we found that 6 of the NETs are strongly upregulated in myoblast differentiation and are highly expressed in muscle, suggesting a role in muscle biology. On the basis of their homologies to known proteins, 4 of the NETs in this group have potential roles in signaling. Using RNA interference, we found that all 4 are important in myoblast differentiation or maintenance in culture. Of these 4, 1 (NET25) is required for attenuation of MAP kinase signaling during myoblast differentiation; the other 3 have putative roles in lipid signaling. We are developing mouse models to further analyze the functions of these NETs and are working with human geneticists to explore the potential involvement of mutations in these proteins in muscular dystrophies.

NUCLEOCYTOPLASMIC TRANSPORT MECHANISMS

Transport of protein and RNA through NPCs is an energy-dependent process mediated by nucleocytoplasmic shuttling receptors of the karyopherin β family. Karyopherins bind to transport signals on protein or RNA cargo molecules, and the receptor-cargo complexes are translocated through the NPC by receptor binding to a group of NPC proteins (nucleoporins) that contain phenylalanine-glycine amino acid motifs. We are using in vitro assays with digitonin-permeabilized cells to analyze the molecular events that specify translocation of cargo-receptor complexes through NPCs. A major goal is to determine how the conformational dynamics of importin β are linked to discrete transport steps. To this end, we are complementing structure-function studies of importin β with analysis involving small-molecule inhibitors of nuclear import, identified in collaboration with D. Boger, Department of Chemistry.

In a related project, we are analyzing nuclear import of the adenovirus genome, which consists of a 36-kb double-stranded DNA molecule. Results from

our in vitro transport studies indicate that transport of adenovirus DNA is driven by import signals on the adenovirus DNA-associated protein VII. We found that the nucleoporin Nup214 provides an essential docking site at the cytoplasmic surface of the NPC for the adenoviral nucleocapsid via the major capsid protein hexon. Docking of the nucleocapsid at Nup214 positions the virus for subsequent uncoating and viral DNA import.

We also are analyzing nuclear export of unspliced HIV type 1 mRNA, which requires the viral regulatory protein Rev. Rev polymerizes on a *cis*-acting sequence of viral mRNAs, termed the Rev response element, providing a platform for assembly of nuclear export factors. In collaboration with Dr. Yates, we used proteomics analysis of cellular extracts to characterize the proteins that specifically assemble on the complex formed by Rev and the Rev response element. These proteins may define the transport and metabolism of HIV type 1 transcripts. Using RNA interference to screen cells, we identified a group of these new proteins that are selectively involved in HIV mRNA export and not in export of cellular mRNAs. More detailed analysis of these proteins will enhance understanding of HIV replication and could identify new potential drug targets.

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Potassium Channel Function in the Nervous System

T. Jegla, X. Zhang, S. Clancy, F. Bertaso, B. Chen

A major goal of our research is to understand the fundamental mechanisms through which ion channels regulate neuronal signaling. Previously, we studied the role of transient-receptor potential channels in sensory responses, including circadian phototransduction. Recently, we shifted our focus to defining how a diverse set of conserved potassium channels shape the behavior of neurons. These channels set cellular resting potentials, regulate subthreshold excitability, repolarize action potentials, and set neuronal firing patterns. Recent genetic advances have provided a strong link between abnormal activity of potassium channels and diseases of the human nervous system, including epilepsy, ataxia, and retinopathies.

Basic protein structures of potassium channels arose in prokaryotes but have undergone extensive diversification in concert with the evolution of fast electrical signaling in animals. Our understanding of how this molecular diversity of potassium channels corresponds to the rich physiologic diversity of potassium currents in neurons is still rudimentary. Furthermore, large gaps in our knowledge of channel protein structures and gating movements limit the ability to effectively exploit potassium channels as therapeutic targets.

We are using a combination of chemical genomics, mouse genetics, biochemistry, and electrophysiology to understand how orphan classes of potassium channels function in neurons. We are focusing on 2 major families of genes that encode potassium channels: Elk, or Kv12, channels and the so-called silent Kv channels. Elk channels contribute to subthreshold conductance and are highly expressed within many important circuits in the CNS. Silent Kv channels contribute to the functional diversity of the classic delayed rectifiers that repolarize action potentials in most neurons. Key outstanding questions about these 2 types of channels include subcellular localization, modulation during neuronal signaling, and contributions to circuit activity.

We are also using a variety of approaches to gain insight into the structure, gating motions, and trafficking of potassium channels. Our goal is to figure out how phenotypic diversity of potassium channel gating is generated on a structural level. Questions include how are fast- and slow-gating phenotypes derived from structure, how is the threshold for voltage gating determined, how is voltage gating modulated by regulatory domains, and how is protein localization controlled by the composition of subunits? We have made significant progress in defining the closed structure of Kv12 channels. Our findings promise to elucidate how subthreshold voltage gating is generated in potassium channels of the EAG superfamily.

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Structural and Functional Proteomics for Novel Therapeutics and Diagnostics

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DETECTING RARE CELLS IN CIRCULATION

Circulating tumor cells are associated with numerous epithelial malignant tumors and correlate with survival in metastatic breast and colon cancers. Malignant cells from solid tumors begin to circulate at the earliest stages in cancer formation. The circulating cells are quite rare, occurring at a frequency of less than 1 malignant cell for every 1 million normal nucleated blood cells. Current detection methods depend on immunomagnetic separation and immunofluorescence, which allow enumeration of the circulating tumor cells, but detailed morphologic studies cannot be performed. With our technology, we can both enumerate circulating tumor cells and study individual cells by using additional staining. Single cells can then be compared with previous histopathologic and cytologic specimens of a patient's primary or metastatic tumor.

Analysis of circulating tumor cells could provide a valuable tool for identifying patients with lower risk of recurrence, determining prognosis, and aiding clinicians with individualized treatment strategies. Most recently, we compared the morphology of tumor cells in circulation with that of the primary tumor cells in patients with breast, colon, or lung cancer. Now we will analyze the protein expression profile and correlate it with response to treatment.

STRUCTURAL AND FUNCTIONAL PROTEOMICS ANALYSIS OF THE SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS

Four years ago, we began to generate a structure-function-interaction map of the severe acute respiratory syndrome coronavirus (SARS-CoV) proteome and the interactions of the virus with the host cell to define a comprehensive set of targets for rational, structure-based drug and vaccine design. We have made tremendous progress and have gained an unprecedented view of the disease from a systems biology perspective. We use bioinformatics, structural biology, genetic methods, and functional assays. So far, we have determined the structures of 11 SARS-CoV proteins. We used crystallography for 5 structures: the nonstructural proteins nsp3b, nsp3d, and nsp10; a truncated form of nsp15; and the N-terminal domain of the nucleocapsid protein. Another 5 structures, nsp1, nsp3a, nsp3c, nsp3e, and nsp7, were determined by using nuclear magnetic resonance spectroscopy, and the structure of a large surface glycoprotein spike was delineated by using electron cryomicroscopy. These studies have provided important information on formation of the replicase complex, transcription, and maturation of genomic and subgenomic RNA. Several proteins have been successfully expressed in soluble, folded forms for structural studies. Long-standing problems with classical recombinant heterologous expression were overcome by using whole-gene synthesis methods involving codon optimization and maximizing nucleotide distance from the wild-type sequence.

We have expanded this approach to determine similar characteristics of the replicase enzymes of other coronaviruses. Our goal is to delineate aspects of replication conserved across the different members of the family Coronaviridae and aspects unique to a specific virus. We are also using this integrated platform to address other viral infectious diseases such as influenza and dengue fever.

ACCELERATED TECHNOLOGIES CENTER FOR GENE TO 3D STRUCTURE

Scientists at the Accelerated Technologies Center for Gene to 3D Structure are simultaneously developing, operating, and deploying technologies to improve costs of using x-ray crystallography to determine the structure of experimental proteins. Automated processes for visualizing crystallization and diffraction data have been deployed. In addition, data sets consisting of heterogeneous data structures have been integrated within familiar contexts. These approaches are being applied to track work flow throughout the protein structure determination pipeline, from the design of constructs to the acquisition of diffraction data.

We have automated the statistical analysis of data sets from a variety of experiments, including fluores-

cence recovery after photobleaching in a lipid cubic phase medium for membrane proteins. Although initial single experiments were conducted and analyzed manually, our goal is to make the analysis amenable to high-throughput approaches. Significant reuse of existing data structures and experiment metadata has been leveraged to minimize consolidating and associating heterogenous data. Graphical user interfaces to manage such output have also been developed.

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Molecular Imaging and Tumor Targeting With Virus-Based Nanoparticles

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Current treatment of cancer typically involves chemotherapies that have severe adverse effects. The requirement that patients must withstand the toxic effects of treatment often limits the effectiveness of the therapy. Further, many promising anticancer compounds that are highly effective in vitro are too toxic to be used in vivo.

The ability to specifically target therapies to the site of a developing tumor while avoiding healthy tissue is an important goal for cancer research. Similarly, a tremendous need exists to identify, image, and monitor tumors, particularly at early stages and during treatment. Recently, "smart" nanoparticles, which combine these multiple targeting, imaging, and drug delivery functions, have been developed. Therapies based on nanoparticles have tremendous potential to increase the sensitivity and specificity of diagnostic imaging and treatment. Many different classes of nanoparticles are currently in development, including dendrimers, liposomes, paramagnetic nanoparticles, and quantum dots. We focus on virus-based nanoparticles as platforms for the development of tissue-specific targeting and imaging agents in vivo, primarily focusing on cowpea mosaic virus (CPMV).

CPMV AND SENSITIVE IN VIVO IMAGING OF MAMMALIAN ENDOTHELIAL CELLS

CPMV is an icosahedral, 31-nm particle that is produced easily and inexpensively in black-eyed pea plants. In contrast to the structure of most other nanomaterials, the structure of the CPMV capsid is defined and can be engineered to display peptides or proteins in controlled orientations on particle surfaces via either genetic manipulation of the viral genome or by chemical attachment to the particle surface. CPMV is bioavailable and nontoxic, and the capsids are highly stable to temperature, pH, and the conditions required for chemical reactions.

By conjugation to surface lysine residues, CPMV can be labeled with fluorophores at high densities, resulting in an extremely bright, nontoxic material that is an outstanding tool for imaging vasculature in live animals. We previously showed that CPMV can be used to image the complete vasculature in the embryos of several species and that it is superior to other imaging particles such as lectins, fluorescent dextrans, and polystyrene microspheres.

CPMV particles have also been highly useful in highlighting angiogenesis in developing tumors. Uptake of particles into endothelial cells occurs, yielding a bright imaging signal that can be used to differentiate between arterial and venous vessels. This characteristic is rather surprising because CPMV infects plants,

not vertebrates. Nevertheless, we recently showed that endothelial uptake is mediated by a 53-kD, cellular membrane protein, which we characterized as a surface-exposed intermediate filament. Further studies will determine how this virus-filament interaction mediates endothelial targeting.

ENDOTHELIAL AND TUMOR TARGETING WITH VIRUS-BASED NANOPARTICLES

We have designed virus-based nanoparticles in which the natural specificities are masked; the masked particles are then specifically targeted to tumors in vivo. Bioconjugation of CPMV to tumor ligands, whose receptors are upregulated on metabolically active tumor cells, facilitates specific particle-tumor interactions. We showed specific targeting to the folic acid receptor upregulated on tumor cells, and recently we examined the in vivo targeting of CPMV particles directed to the vascular endothelial growth factor receptor 1 expressed on tumors and tumor endothelium. These studies indicated for the first time that tumor-specific targeting with CPMV is feasible in vivo. The results will allow the further design of antitumor agents that can provide localized, highly specific imaging and therapy in vivo. Use of virus-based nanoparticles may help us visualize and eliminate small tumors before the tumors can metastasize. In addition, the ability of the particles to focus toxic effects to the site of the malignant cells, thereby expanding the range of effective therapies that can be used in vivo, holds great promise for reducing cancer-related morbidity and mortality.

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Molecular Mechanisms Underlying Synaptic Plasticity

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A major goal of our research is to dissect the cellular and molecular mechanisms that underlie synaptic development and function in the CNS in mammals. Currently, we are investigating the neuronal membrane-trafficking pathways that govern fast release of chemical neurotransmitters and activity-dependent secretion of soluble factors that regulate synaptic strength. We use a multidisciplinary approach that combines mouse genetics, biochemistry, high-resolution imaging, and electrophysiologic analyses of synaptic transmission.

PRESYNAPTIC PLASTICITY

At the nerve terminals, release of neurotransmitters is mediated by synaptic vesicles, membrane organelles in which calcium-triggered exocytosis is promoted by assembled soluble *N*-ethylmaleimide-sensitive–factor attachment protein receptor (SNARE) complexes. Intriguingly, almost all neuronal cell types have several distinct modes of transmitter release. Complexins are small synaptic proteins that regulate vesicle exocytosis by interacting with SNAREs and by competing for SNARE-complex binding with the calcium sensor synaptotagmin-1. We recently showed that complexins play a central role in controlling the balance between the fast synchronous, slow asynchronous, and miniature modes of synaptic vesicle exocytosis. On the basis of these findings, we designed novel genetic tools that enable us to selectively manipulate presynaptic activity in vivo. We are using these tools to determine how individual neurons and specific neural circuits in the brain respond to brief or prolonged modifications of presynaptic properties.

POSTSYNAPTIC SECRETION

In addition to the classical neurotransmitters, several types of secreted factors, including the neurotrophin family, Wnts, and transforming growth factor β, relay information across chemical synapses. Evidence has accumulated that these factors may be secreted by specialized membrane organelles that undergo activity-dependent exocytosis at dendritic shafts and postsynaptic spines. Remarkably, the molecular mechanisms that control postsynaptic secretion remain largely unknown. To elucidate these mechanisms, we are using forward genetics and unbiased proteomics to systematically examine postsynaptic secretory organelles.

Engineering Eukaryotic Algae for Production of Human Therapeutic Proteins and of Biofuels

S.P. Mayfield, A. Manuell, J. Marín-Navarro, M. Muto, M. Tran, P. Pettersson, P. Lee, B. Rasala

Eukaryotic algae are an excellent system for biotechnology applications, including the production of human therapeutic proteins, industrial enzymes, and biofuels. Algae can produce biomass at more than 10 times the rate of terrestrial plants on a unit-area basis, making the microorganisms a potentially important and economically practical source of both proteins and biofuel molecules. Algal biomass contains starches, lipids, and other hydrocarbon-rich molecules, all of which are fuel precursors. Along with producing biofuel molecules, algae are extremely efficient at expressing proteins and can be used to produce proteins at a fraction of the cost of traditional expression systems and at a scale not achievable with traditional fermentation methods.

To realize the potential of algae, we must understand and control gene expression to optimize the production of proteins and biofuel molecules. In algae, a number of proteins and most biofuel molecules are produced in chloroplasts, and understanding chloroplast translation is essential for understanding chloroplast gene expression. The core translational apparatus of chloroplasts is highly conserved with that of bacteria. However, chloroplasts have incorporated novel protein components that allow for complex regulatory mechanisms. Some of these novel components are found on the plastid ribosomes; others are translation factors that are not found in bacterial systems. Chloroplast mRNAs also contain unique regulatory elements that interact with plastid ribosomes and translation factors to enable the complex regulation.

To better understand translation in algae, we are characterizing the structure of both chloroplasts and cytoplasmic ribosomes from *Chlamydomonas reinhardtii*, a unicellular photosynthetic alga. Using electron cryomicroscopy and single-particle reconstruction, we determined the structure of the *C reinhardtii* cytoplasmic 80S ribosome and found that it is nearly identical to 80S ribosomes from animals. We also determined the structure of the chloroplast ribosome to 15 Å and found that although it is conserved with bacterial 70S ribosomes, it has large unique structural domains. These domains likely are involved in unique aspects of chloroplast translation, including light-activated translation, which occurs in all photosynthetic organisms.

Light-activated translation in chloroplasts is achieved through reducing potential, derived from photosynthesis, which is used to activate the binding of a protein complex to plastid mRNAs. Binding of this protein complex to the 5′ untranslated region of mRNAs enables ribosome association of the mRNA and hence increased translation. How the proteins involved in light activation interact with the unique chloroplast ribosomal proteins is unknown, and understanding these interactions will be an important aspect of our research in the coming years.

In addition to these basic studies on translation, we have developed a system for the expression of recombinant proteins, including human therapeutic agents and enzymes involved in biofuel production in *C reinhardtii* chloroplasts. We have expressed a number of mammalian proteins, including monoclonal antibodies and mammalian growth factors, and have shown that this alga-based system can produce human therapeutic proteins at high levels. Most recently, we have focused on producing antibody-toxin fusion proteins in which a targeting antibody domain is linked to a cell-killing toxin. Using this technology, we have produced an antibody-toxin fusion protein that binds and kills human B-cell lymphomas, and cell-based assays have shown the usefulness and specificity of this molecule. These proteins have great potential for the treatment of cancers and infectious diseases, and chloroplasts offer perhaps the only system in which these types of proteins can be produced.

We have also begun to engineer algae for the production of hydrocarbon molecules that can be used as biofuels. Introducing enzymes from other organisms that can increase the accumulation of isoprenoids and fatty acids should allow for the use of microalgae as a biological source of these fuel precursors. Because algae can be grown by using sunlight and carbon dioxide as primary inputs, the potential of algae as a sustainable energy source is obvious. We have shown the tremendous potential of eukaryotic algae for the expression of recombinant human therapeutic proteins and for the production of biofuels. Our continued genetic, biochemical, and structural studies should lead to a greater understanding of the mechanism of chloroplast translation. With this understanding, we should be able to

design appropriate transgenes to affect higher levels of expression of therapeutic proteins and allow algae to become a practical source for sustainable production of biofuels.

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Molecular Basis of Cognitive Function and Dysfunction

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The ability to remember is perhaps the most significant and distinctive feature of our cognitive life. We are who we are in large part because of what we have learned and what we remember. Impairments in learning and memory are a component of disorders that affect human beings throughout life, from childhood forms of autism to psychiatric disorders such as schizophrenia with onsets in late adolescence and early adulthood to diseases of aging such as Alzheimer's. We use genetic manipulation in mice to investigate the cellular and molecular events involved in learning and memory.

CALCIUM SIGNALING AND MEMORY

We know relatively little at a molecular level about how the brain stores new information. One hypothesis, which we have tested, is that calcium-regulated changes in the strength of synaptic connections between nerve cells can store information. The calcium/calmodulin-dependent protein kinase is abundant at synapses and when activated by calcium can strengthen synaptic connections. We used genetic manipulations in mice to indiscriminately activate this kinase at all synapses in the entorhinal cortex, a part of the brain important for memory that is affected in the earliest stages of Alzheimer's disease in humans. We found not only that the formation of new memories is impaired but also that previously established memories could be erased. If memories are stored as precise patterns of synaptic weights, then the indiscriminate strengthening of synapses might be expected to erase memories in a manner similar to the way writing all *1*'s in computer memory will erase previously stored information.

MOLECULAR ANATOMY OF MEMORY

When humans learn new information, they use only a tiny fraction of the neurons in the brain. One difficulty in studying memory is an inability to identify and specifically manipulate those neurons that participate in a particular memory trace. We developed a genetic technique for use in mice that enables us to specifically introduce genetic changes into neurons that are activated by behavioral stimuli. By introducing a visible marker protein, we can permanently tag activated subsets of neurons, creating a precise record of the activity pattern at a specific time. We used this approach to address a basic unanswered question in neuroscience: Do the same neurons that are activated during learning become reactivated during recall of the memory? Using fear conditioning in mice, a behavioral model of posttraumatic stress disorder in humans, we found that the same neurons activated during learning were reactivated when an animal recalled the fearful event. We also used this approach to study extinction, a process used in the treatment of phobias by which memories are weakened by repeated exposure to a relevant stimulus. We found the neurons that were originally activated by a fearful stimulus were no longer activated after extinction. This finding suggests that extinction training actually erases or interferes with some component of the original memory trace.

FROM THE NUCLEUS TO THE SYNAPSE

Memories are initially formed by changes in the strength of specific synaptic connections. However, activation of new gene expression in the nucleus is required for those memories to become stable for long periods. A fundamental question in memory research has been how this nuclear-to-synaptic communication occurs. We recently found that the receptors that control synaptic communication are specifically targeted to synapses that are altered with learning. That is, learning produces a sort of molecular tag at certain synapses that allows the synapses to capture the newly synthesized receptors arriving from the nucleus hours after the learning event. Thus, the synapses that are altered in strength to produce a short-term memory must be primed, or tagged, to receive new receptors to maintain that memory long-term.

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Regulation of the Plasminogen Activation System

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Activation of plasminogen, the zymogen of the primary thrombolytic enzyme plasmin, is markedly promoted when plasminogen is bound to cell surfaces, arming cells with the broad-spectrum proteolytic activity of plasmin. Besides thrombolysis, cell-surface plasmin facilitates an array of physiologic and pathologic processes that require cell migration, including macrophage recruitment during inflammation, wound healing, tissue remodeling, tumor cell invasion and metastasis, skeletal myogenesis, and neurite outgrowth. Carboxypeptidase B–sensitive plasminogen-binding sites promote plasminogen activation on eukaryotic cells. However, no integral membrane plasminogen receptors exposing C-terminal basic residues on cell surfaces have been identified.

We used the exquisite sensitivity of multidimensional protein identification technology and an inducible progenitor cell line to search for integral membrane plasminogen receptors that expose a C-terminal lysine on the cell surface. We identified Plg-R_{KT}, a novel transmembrane plasminogen receptor expressing a C-terminal lysine, that partitioned to the detergent phase of membrane preparations. Cells stably overexpressing Plg-R_{KT} stimulated plasminogen activation 4-fold more efficiently than did control transfectants. Database searching revealed that Plg-R_{KT} mRNA is broadly expressed by migratory cells, including leukocytes and breast cancer, leukemic, and neuronal cells. Plg-R_{KT}, with its unique structure, is a novel control point for regulating cell-surface proteolysis.

Structural and Functional Analysis of Molecular Assemblies by Electron Cryomicroscopy

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STUDIES ON MEMBRANE PROTEINS

Although membrane proteins account for roughly 30% of the human genome, determination of their structure has lagged far behind that of their soluble counterparts. This situation is largely due to the complex nature of membrane proteins and the constant need to shield their hydrophobic membrane-spanning regions. These characteristics have made membrane proteins extremely difficult to crystallize by using traditional 3-dimensional techniques.

Electron cryomicroscopy is a valuable tool for examining membrane proteins in their native environment, the lipid bilayer. Recent structures solved by using 2-dimensional crystallography rival the resolution of conventional x-ray crystallography. These new structures show how important the interaction of membrane proteins with their lipid surroundings can be in accurately interpreting the proteins' mechanism of action. We are using electron cryomicroscopy to determine the 3-dimensional structure of several membrane proteins, including the ATP-binding cassette transporter MsbA, the multidrug resistance transporter MDR1, the sugar transporter FucP, and the clostridial neurotoxin botulinum.

THE ATP-BINDING CASSETTE TRANSPORTER MSBA

MsbA is a membrane protein that uses ATP hydrolysis to transport lipid A and lipopolysaccharide from the cytoplasmic leaflet to the periplasmic leaflet in gram-negative bacteria. MsbA also can transport a wide spectrum of drug molecules and shares a high sequence homology with human MDR1. X-ray structures of MsbA have indicated the transport pathway through which lipid is transported, but nothing is known about how lipid substrates interact with the protein during transport. Electron crystallography of MsbA reconstituted into lipid bilayers has the potential to enable visualization of these important protein-lipid interactions. We have produced 2-dimensional crystals of MsbA in a lipid bilayer in a nucleotide-free state at a preliminary resolution of approximately 20 Å and helical crystals in a nucleotide-bound state at a preliminary resolution of approximately 12 Å. These data will lead to an interpretation of the structure of MsbA within the bilayer and its potential conformational changes upon substrate binding and nucleotide hydrolysis.

THE MULTIDRUG RESISTANCE TRANSPORTER MDR1

In eukaryotes, the protein MDR1 is responsible for transporting a wide variety of structurally unrelated compounds across the cell membrane. These proteins are active in human tumor cells and contribute to resistance to conventional chemotherapy drugs. Unlike the bacterial ATP-binding cassette transporter MsbA, which is a homodimer, MDR1 is a monomer composed of 2 transmembrane domains and 2 ATP-binding cassette

motifs. The involvement of MDR1 in drug resistance makes it an extremely interesting and useful target for structural and functional studies. During the past year, we successfully crystallized MDR1 in the presence of AMPPNP, a nonhydrolyzable ATP analog, within the lipid bilayer. This initial success gives us great hope of being able to capture MDR1 in multiple conformations in as native an environment as possible.

STRUCTURAL STUDIES OF MICROTUBULE-BINDING PROTEINS

In collaboration with A. Desai, University of California, San Diego, and I. Cheeseman, Massachusetts Institute of Technology, Cambridge, Massachusetts, we have studied the 4-subunit Ndc80 complex, a rod-shaped protein complex composed of Ndc80, Nuf2, Spc24 and Spc25 polypeptides, that localizes at the kinetochore-microtubule interface and plays an important role in chromosome segregation. In previous studies, we observed that microtubule binding of the complex is localized to the Ndc80-Nuf2 heterodimer. We defined the orientation of Ndc80-Nuf2 on microtubules by decorating microtubules emanating with known polarity from centrosomes. To visualize the interaction of the Ndc80 complex with microtubules, we used electron cryomicroscopy of microtubules decorated with Ndc8-Nuf2. The nature of microtubules allowed us to use helical image analysis to obtain a 3-dimensional map of Ndc80-Nuf2 bound to microtubules.

In the map, the 2 asymmetric densities protruding from the protofilament form a horseshoe-like arrangement. The larger density binds strongly to the protofilament, whereas the smaller density is only weakly connected. We were able to dock the recently published crystal structure of the Ndc80 complex into our map. In the best fit, the head domains and part of the coil coiled domains fit well into the large density of the map (Fig. 1). On the basis of these results, we propose a model in which Ndc80-Nuf2 binds strongly to the intertubulin dimer interface and more weakly to the intratubulin dimer interface along the protoflament axis. The strong and weak interactions of the Ndc80 complex may be important for the activity of this complex in forming a dynamic attachment site at kinetochores.

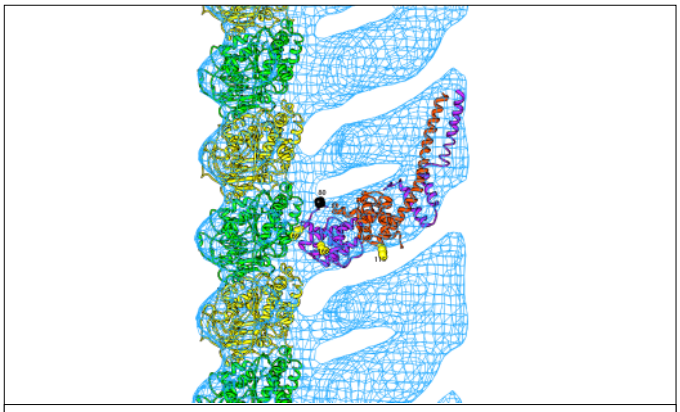

Fig. 1. Fitting of the Ndc80 crystal structure into an electron microscopy map. In order to observe the contact points between the densitites corresponding to the Ndc80-Nuf2 subunits and the microtubule lattice, a model of a microtubule protofilament made from the tubulin dimer was docked into a single protofilament cut from the electron microscopy map of the Ndc80-Nuf2–microtubule complex. Docking of the head dimer domain of the crystal structure of the Ndc80 complex shows the position of Ndc80 (purple) and Nuf2 (red), with amino acids 69 and 166 in Ndc80 and 115 in Nuf2 (yellow spheres). Mutation of these amino acids strongly affects the binding of the complex to the protofilament.

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Auditory Perception and Neuronal Circuit Formation: From Mouse Models to Human Genetic Disease

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A fundamental unresolved question in biology is how the nervous system in humans creates an internal representation of the external world. Sense

organs convert signals such as light and sound into electrical impulses that are processed by the nervous system to create a reflection of the surroundings and to elicit appropriate behavioral responses. Selective pressures during evolution have shaped the human genome and optimized sense organs and neuronal circuits for their tasks. Of all the sensory systems in humans, the auditory system is the least well understood at the molecular level. We identify and study genes that control the function of the auditory sense organ of mammals. We also analyze the mechanisms that establish neuronal connections between the auditory sense organs and the cerebral cortex and the formation of cell layers and neuronal circuits within the cortex.

AUDITORY PERCEPTION

The ability to perceive sound is critically dependent on mechanoelectrical transduction (MET), the conversion of mechanical force into electrical signals. The auditory mechanoreceptor cells in mammals are the hair cells of the cochlea. The architectural features of the cochlea and the properties of the hair cells are essential for encoding time-variant frequency components of sound as spatiotemporal arrays of neural discharge that provide the sense of hearing. The mechanically sensitive organelle of a hair cell is the hair bundle, which consists of dozens of stereocilia that project from the apical cell surface (Fig. 1). MET channels are localized close to

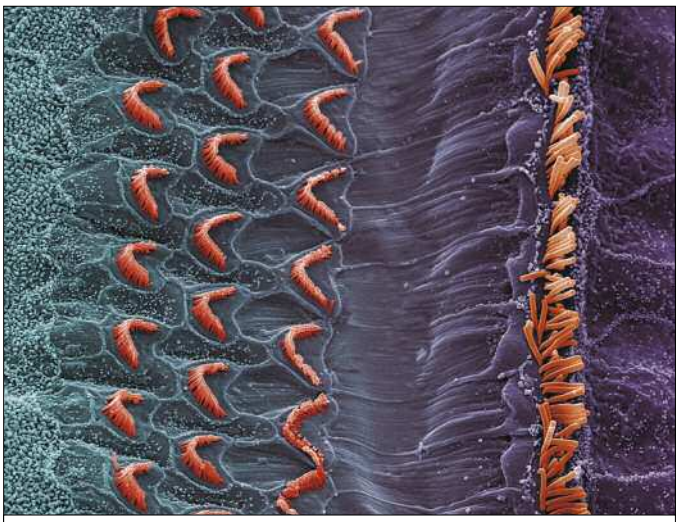

Fig. 1. Scanning electron microscopy image of the mammalian cochlea. Hair cells are arranged in 3 rows of outer hair cells (to the left) and 1 row of inner hair cells (to the right). Each hair cell contains a bundle of stereocilia (orange) at the apical surface that form the mechanically sensitive organelle of the cell.

the tips of stereocilia. Tip-links, extracellular filaments that connect the tips of neighboring stereocilia and are visible by electron microscopy, are thought to transmit sound-induced tension force onto the MET channel. The molecular identity of most components of the MET complex is still unknown.

We are identifying genes that control hair cell function, such as those for the MET channel and the tip-links. Approximately 1 child in 1000 children is born deaf, and a large part of the human population experiences age-related hearing loss. Many forms of hearing loss are of genetic origin, and mutations in more than 400 genes cause deafness. Some of the affected genes have been identified and may encode components of the MET complex in hair cells. Two of the genes linked to deafness encode cadherin 23 and protocadherin 15, members of the cadherin superfamily of cell adhesion molecules. Both genes are expressed in hair cells, and our studies indicate that cadherin 23 and protocadherin 15 interact to form tip-link filaments. Thus, we have defined the first components of the MET complex in hair cells at the molecular level. Our findings provide tools for identifying additional components of the MET complex that likely interact with cadherin 23 and protocadherin 15.

In an alternative approach to studies of auditory perception, we carried out a genetic screen in mice. Using *N*-ethyl-*N*-nitrosourea, we introduced point mutations in the germ line of mice. Using phenotypic screens, we identified 19 mouse lines in which the mice inherit hearing defects as recessive traits. We have mapped many of the mutations to chromosomal intervals and have used DNA sequencing to identify mutations in single genes that cause some of the hearing defects.

All of the genes that we have identified so far are expressed in hair cells. Some of the genes encode proteins with known functions, such as myosin motor proteins. Others belong to entirely new gene families that have not been studied previously. Intriguingly, all the genes identified in our screen are also linked to deafness in humans. Therefore, the screen is powerful not only for identifying genes that control the function of hair cells but also for providing animal models for the human disease.

NEURONAL CIRCUIT FORMATION

Sensory information is ultimately relayed to specific areas of the CNS such as the auditory and visual cortex. Although the cortex is divided into functional domains, the overall organization of all cortical structures is similar and consists of cell layers that connect to each other to form neuronal circuits. The mechanisms that lead to the establishment of neuronal circuits in the cerebral cortex are mostly unknown.

Using genetic tracing studies, we are visualizing neuronal connections that are essential for the processing of auditory signals. In addition, we are defining the genes and mechanisms that lead to the formation of neuronal cell layers. Our studies have already shown that extracellular matrix receptors of the integrin family have important functions in the formation of cell layers and the control of synaptic function. We are currently using genomic approaches to search for novel genes that specify cortical layers and lead to neuronal circuit formation. We are also participating in a large-scale effort by the National Institutes of Health to generate a panel of mice useful for the perturbation of gene function in defined areas of the CNS, including defined neuronal subtypes in cortical cell layers.

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Molecular Mechanisms of Thermosensation

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We are interested in the molecular description of the function of sensory neurons. Of the 5 popularly characterized senses—sight, hearing, taste, smell, and touch—touch is among the most varied and least understood. Within this modality is the ability to sense mechanical forces, chemical stimuli, and temperature, and the molecules that mediate this ability have been a long-standing mystery. Temperature sensation in particular has received relatively little attention from biologists and yet is critical for interaction with the environment.

We recently discovered proteins that enable sensory neurons to convey temperature information. These proteins are ion channels activated by specific changes in temperature; thus, they act as the molecular thermometers of the body. Specifically, our results have led to the identification and characterization of a novel warm-activated transient-receptor potential (TRP) channel, TRPV3 (33°C threshold), and 2 novel cold-activated TRP channels, TRPM8 (25°C threshold) and TRPA1 (ANKTM1, 17°C threshold). We found that TRPM8 is also the receptor for the compound menthol, providing a molecular explanation of why mint flavors are typically perceived as cooling. Furthermore, we discovered that TRPA1 is activated by cinnamaldehyde, allicin (garlic), and other compounds with a burning sensory quality, consistent with a role of TRPA1 in pain sensation. Together these temperature-activated channels represent a new subfamily of TRP channels that we have dubbed thermoTRPs.

Increasing evidence from in vivo studies indicates that thermoTRPs are required for temperature sensation. For example, we recently showed that mice lacking TRPM8 have severe behavioral deficits in response to cold stimuli. In thermotaxis assays of temperature gradient and 2-choice preference tests, TRPM8-deficient mice had strikingly reduced avoidance of cold temperatures. TRPM8-deficient mice also lack behavioral responses to cold-inducing icilin and have an attenuated response to acetone, an unpleasant cooling stimulus. Finally, we showed that TRPM8 mediates the analgesic effect of moderate cooling after administration of formalin, a painful stimulus. Therefore, depending on context, TRPM8 contributes to sensing unpleasant cold stimuli or mediating the effects of cold analgesia.

All organisms have a need for sensing the environment. Because some invertebrate species are more amenable to genetic studies than mammals are, we asked whether nonvertebrates also use thermoTRPs. In collaboration with W. Schafer, MRC Laboratory of Molecular Biology, Cambridge, England, we recently showed that the *Caenorhabditis elegans* ortholog of TRPA1 is an

ion channel activated by mechanical forces and is required for mechanosensation, suggesting an evolutionary conserved role of TRP channels in somatosensation.

Another key area of research is the mechanism of activation of thermoTRPs. TRPA1 is activated by a variety of noxious stimuli, including cold temperatures, pungent natural compounds, and environmental irritants. How such diverse stimuli activate TRPA1 is unknown. In collaboration with B.F. Cravatt, Department of Cell Biology, and P.G. Schultz, Department of Chemistry, we showed that covalent modification of reactive cysteines within TRPA1 can cause channel activation, rapidly signaling potential tissue damage via the pain pathway. This mechanism of activation is novel and raises many important questions about the in vivo role of TRPA1 in pain transduction.

Our long-term goal is to synthesize an integrated picture of sensory neuron function. By identifying the proteins that initiate the molecular cascade leading to temperature perception, we have provided the basis for probing the basic foundation of the sense of temperature. We now have the opportunity to extend these insights into important areas of human health, such as pain pathophysiology. For example, TRPA1 is a potential target for treating pain, and we are identifying small-molecule inhibitors of TRPA1, in collaboration with scientists at the Genomics Institute of the Novartis Research Foundation, San Diego, California. Therefore, the approaches we are using will yield insights into the basic biology of the peripheral nervous system and may also have an effect on novel treatments for pain.

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Functional Proteins in Tumor Metastasis and Angiogenesis

J.P. Quigley, E.I. Deryugina, J.P. Partridge, T. Kupriyanova, V. Ardi, E. Conn, B. Schweighofer

We have established a number of in vivo model systems that can recapitulate the major cellular and tissue events that occur during tumor metastasis and angiogenesis. The model systems readily allow for quantitative measurements, microscopic analysis in real time, biochemical and immunologic probing, and direct molecular and therapeutic intervention.

The use of short interfering RNA molecules directed against specific expressed genes and applied directly into the models has provided insights into the contributory role of select gene products in tumor dissemination and neovascularization. In addition, using activity-based protein profiling, in collaboration with B.F. Cravatt, Department of Cell Biology, has enabled us to detect, isolate, and identify active proteolytic enzymes that are differentially activated during metastasis and angiogenesis. Finally, application of subtractive immunization, which is used to generate unique function-blocking monoclonal antibodies, in combination with immunoproteomics enables us to identify specific antigenic molecules that functionally contribute to metastasis and angiogenesis.

METASTASIS

Selected human tumor cells inoculated onto the chorioallantoic membrane of developing chick embryos form primary tumors on the membrane in 3–6 days. During this period, aggressive cells escape from the primary tumor, disseminate through the vasculature, and arrest and proliferate in secondary organs of the embryo. Measuring a small number of early-arriving metastatic cells (<200) in the secondary organ has always been technically difficult. We use an approach in which unique regions of human DNA, known as *alu* repeat sequences, are amplified by polymerase chain reaction from the total DNA extracted from various organs of the tumor-bearing chick embryo. Chicken DNA contains no *alu* sequences, so any product generated by the polymerase chain reaction indicates that viable human tumor cells are present in the chick embryo organ and would have arrived there via the known sequential steps in metastasis. We can now detect as few as 25–50 human tumor cells present in the entire chick embryo lung, liver, or brain and can monitor the expansion of these metastatic cells by using real-time polymerase chain reaction.

We are using various screening procedures in this model system to identify molecules that enhance, or conversely inhibit, the appearance of metastatic human tumor cells in organs of chick embryos. The screening procedures include direct inoculation of primary tumor cells that have been transfected with various short interfering RNA constructs to silence specific genes that

might contribute to metastatic dissemination. Inoculating monoclonal antibodies directly into the tumor-bearing embryo and monitoring the influence of the antibodies on metastasis are also part of our screening procedures.

We are also monitoring tumor expansion and metastasis in specific immunodeficient mice. In this method, human tumor cells are transplanted to various sites in a mouse and allowed to expand. However, compared with our chick embryo tumor dissemination assay, this method is less quantitative, requires more time (3–6 weeks instead of 3–6 days), and is more difficult to use for inhibitor screening and molecular intervention. We are mainly using the mouse metastasis assay to take advantage of mouse genetics and to confirm the efficacy of various effector molecules and inhibitors that initially are identified in the chick embryo metastasis assay.

ANGIOGENESIS

One established in vivo assay for angiogenesis is the chick embryo chorioallantoic membrane assay. We developed a quantitative variation of this assay that enables us to detect and measure newly sprouting blood vessels responding to an angiogenic stimulus such as a specific growth factor or a growing tumor. A highly specific metalloproteinase, MMP-13, has been implicated in the tissue remodeling that occurs during the formation of the new blood vessels. We recently determined that another metalloproteinase, MMP-9 (gelatinase B), also is necessary for a full angiogenic response, and purified MMP-9 added into the model dramatically induces angiogenesis.

Interestingly, these 2 critical enzymes (MMP-13 and MMP-9) are actively imported into the vascular/stromal tissue by distinct inflammatory cells responding to the angiogenic stimulation. Neutrophil-like heterophils rapidly import MMP-9 into the tissue, and monocytes/macrophages actively deliver MMP-13. Thus, physiologic angiogenesis and tumor angiogenesis are closely linked to an accompanying host inflammatory response that contributes critical functional molecules to the angiogenic process. We are dissecting out and identifying the specific molecules and responding cells that link the inflammatory response to the angiogenic process and to the progression of malignant neoplasms.

INTRAVASATION

We are also investigating intravasation, the entry of primary tumor cells into the host vasculature, including the vasculature that is newly formed during tumor angiogenesis. Intravasation appears to be the least-studied process in the metastatic cascade but likely is the rate-limiting step in tumor dissemination. We isolated 2 congenic variants of a human fibrosarcoma cell line (HT1080) that differ 100-fold in their ability to enter the vasculature in vivo and in their ability to metastasize. We have also selected a congenic pair of human prostate carcinoma variants that manifest a substantial difference in their intravasation capabilities in vivo. We are using array technology, proteomic approaches, activity-based protein profiling, and intravital microscopy in the cellular and molecular analysis of these 2 pairs of human tumor variants. With these approaches, we have identified specific molecules that are functionally important in tumor cell/vascular interactions and that likely contribute to tumor cell intravasation.

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A Dual Role for Dynamin in Clathrin-Mediated Endocytosis

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The plasma membrane is the conduit for communication among cells and between cells and their environment. Thus, vesicular trafficking from the plasma membrane is essential for obtaining nutrients and for controlling signaling by cell-surface receptors. Clathrin-mediated endocytosis (CME) is the major endocytic pathway in mammalian cells and occurs at specialized regions of the plasma membrane called clathrin-coated pits (CCPs). CCPs are formed upon assembly of an endocytic coat machinery composed of clathrin, adaptors, and other accessory factors that concentrate receptor-ligand complexes, deform, invaginate the membrane, and eventually pinch off to form small clathrin-coated vesicles, which carry cargo into the cell. Critical cellular processes are often regulated by members of the GTPase family, and the GTPase dynamin is essential for CME.

Dynamin is a 100-kD multidomain tetramer that unlike most GTPases has a high basal rate of GTP hydrolysis and can self-assemble into rings and helical stacks of rings. Self-assembly stimulates the GTPase activity of dynamin about 100-fold. Previous research in the laboratory suggested 2 different models for dynamin function. On the basis of our discovery that dynamin self-assembles into a collarlike structure at the necks of invaginated CCPs, we first proposed that dynamin collars function mechanochemically at late stages of CME to drive membrane fission and vesicle detachment. However, subsequent findings suggested that the basal GTPase activity of unassembled dynamin regulates early stages of CME. During the past year, using biochemistry, cell biology, live-cell microscopy, and biophysics, we have collected strong evidence that both models are correct and that dynamin plays a dual role in CME.

We used total internal reflection fluorescence microscopy to follow the dynamics of the assembly of coated pits and the formation of coated vesicles by tracking fluorescently labeled clathrin and adaptors in live cells. Our collaborators, G. Danuser and his group, Department of Cell Biology, have developed sophisticated tracking software and statistical analyses to detect 3 kinetically distinct subpopulations of CCPs: 2 short-lived abortive populations and 1 longer-lived productive population. We found that basal GTPase–defective mutants of dynamin regulate the turnover of nonproductive CCPs and the rate of formation of productive clathrin-coated vesicles. These and other data provide direct evidence that dynamin regulates maturation of CCPs and functions as part of an endocytic checkpoint to control and monitor coat assembly and cargo recruitment during early stages of CME.

Using biophysical methods, we have also recently provided direct evidence that dynamin is a component of the fission apparatus. We developed real-time fluorescence-based assays for dynamin-liposome interactions, dynamin self-assembly, and nucleotide-dependent conformational changes. Using these assays, we showed that GTP hydrolysis elicits a major conformational rearrangement in self-assembled dynamin that immediately precedes dynamin disassembly. Interestingly, the dynamin binding partner SNX9 transiently stabilizes dynamin-membrane association during GTP hydrolysis, presumably localizing dynamin's mechanochemical conformational rearrangements to the underlying membrane. These data suggest a direct role for dynamin in membrane fission, and we have developed a novel model membrane system of supported bilayers on silica beads that allows us to visualize and reconstitute dynamin-mediated membrane fission. Unlike conventional supported lipid bilayers, this system retains a substantial excess membrane reservoir and thus is an excellent substrate for vesicle formation. In the absence of GTP, dynamin self-assembles onto these templates to draw out long dynamin-coated tubules. However, when added in the constant presence of GTP, dynamin alone is sufficient to generate membrane vesicles. Dynamin can be seen to assemble at the necks of large constricted buds into short collarlike structures that drive membrane fission and vesicle release. These results establish that dynamin constitutes the minimum cellular fission machinery.

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Molecular Biology of Innate Behavior

L. Stowers, P. Chamero, K. Flanagan, D. Logan, T. Marton, F. Papes

Every breath samples the environment for olfactory chemical information, determining the quality of food, warning of danger, and confirming safety. The neurons that mediate olfaction are of 2 types: those that mediate an evocative perception that varies with an individual's experience and those that regulate stereotyped innate social behaviors such as aggression and mating in response to pheromones. The biochemical nature of the pheromone code that induces innate behavior in mammals is not known, and the sensory system responsible for the detection of pheromones is a matter of controversy. We expect that elucidating the stimulating ligands and responsive neurons will enable us to activate, study, and identify the mechanisms underlying neural information coding of defined behaviors.

We recently isolated novel mouse pheromones that mediate aggressive behavior and determined the identity of the aggression-promoting chemosensory neurons. We have taken a unique approach to decode olfaction; we are working forward from the behavioral activity to identify novel genetically encoded pheromones. These pheromone proteins reproducibly activate a subset of accessory olfactory neurons, enabling the molecular identification of those neurons that mediate the aggression-promoting behavior. This step is a fundamental one for understanding intraspecific communication in mammals and characterizing the neuronal circuit that regulates behavior.

The response of an individual to pheromones varies with the individual's age, sex, and dominance. We are using the purified aggression-promoting pheromone of mice to understand this plasticity. Interestingly, we found that whereas males have sensory neurons that respond to the pheromone, the sensory neurons in females are not activated by this cue. This lack of response suggests that males and females are sampling different features of the environment, a difference that in turn leads to different behavioral outcomes.

Our progress in discovering the ligands and sensory neurons that promote innate behaviors is enabling us to identify mechanisms of neural information coding. We have compiled a unique set of resources that will allow us to answer some of the fundamental unanswered questions about both specific and more general aspects of the neural coding of innate behavior.

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Nicotinic Acetylcholine Receptor

N. Unwin, C. Arthur

The nicotinic acetylcholine receptor is a member of a superfamily of transmitter-gated ion channels, which includes the receptors for serotonin, γ-aminobutyric acid, and glycine. It has a cation-selective pore, delineated by a ring of 5 similar subunits, that opens upon binding of acetylcholine. We are interested in determining how this channel mediates fast synaptic transmission. We use postsynaptic membranes isolated from the (muscle-derived) electric organ of the *Torpedo* ray. Electron tomography is used to view this membrane in its cellular context, and electron crystallography is used to analyze the structure of the receptor trapped in different physiologic states.

Recently, we derived an atomic model of the acetylcholine receptor in the closed-channel form. We found that the individual subunits in the N-terminal ligand-binding domain are organized around 2 sets of β-sheets packed in a curled β-sandwich, as in the related soluble pentameric acetylcholine-binding protein. Each subunit in the membrane-spanning domain is made from 4 α-helical segments. The helical segments arrange symmetrically, forming an inner ring of helices that shape a water-filled pore and an outer shell of helices that coil around each other and shield the inner ring from the lipids. In the closed channel, the helices in the inner ring come together near the middle of the membrane and make a constricting hydrophobic girdle. This girdle, which is about 50 Å from the acetylcholine-binding sites, constitutes an energetic barrier to ion permeation and functions as the gate of the channel.

The structure of the closed channel and the details obtained earlier from the receptor trapped in the open-channel form have enabled us to understand in outline the conformational change triggered by acetylcholine that opens the gate of the channel. Improvements in resolution of the structure, in both the closed- and the open-channel forms, are now being attempted so that the gating mechanism can be described in atomic detail. These studies are yielding crucial insight into the nature of a number of neuromuscular disorders, including several well-characterized congenital myasthenic syndromes. They are also providing important 3-dimensional information about the binding sites for drugs that affect the brain by modulating the functions of the related γ-aminobutyric-acid, serotonin, glycine, and neuronal acetylcholine receptors.

Systems Biology and Malaria

E.A. Winzeler, C. Kidgell, V. Ramachandran, N. Kato, K. Henson, J. Young, J. Johnson

Despite the widespread impact of malaria on the world's health and economies, relatively little is known about the function of the majority of the 5300 genes in the genome of *Plasmodium falciparum*,

the causative agent of the most severe form of malaria in humans. This lack of knowledge retards the development of drugs and vaccines against the parasite. We use systematic discovery-based approaches to predict the function of uncharacterized *Plasmodium* genes; our goal is to facilitate the discovery of new treatments. We are using mRNA and protein expression to reveal genetic regulatory networks and to suggest protein-protein interactions. We are also developing new methods that can be used in systems biology research.

In addition to our past work on blood-stage parasites, we have characterized the expression program of the sexual stages of malarial parasites. These stages, which are essential for the mosquito transmission of the disease, are the focus of the development of drugs and vaccines that block transmission. To better understand genes important to sexual development, we used a full-genome high-density oligonucleotide microarray to profile the transcriptomes of *P falciparum* gametocytes. To interpret this transcriptional data, we developed and used a novel knowledge-based data-mining algorithm termed ontology-based pattern identification. With this algorithm, published or custom gene classifications are used to optimize normalization methods and cluster boundaries so that the largest number of any given gene type is found in the smallest cluster size.

This analysis resulted in the identification of a sexual development cluster containing 246 genes, of which approximately 75% were unclassified and which contained most known sexual-stage genes. These genes had highly correlated, gametocyte-specific expression patterns. Statistical analysis of the upstream promoter regions of these 246 genes revealed putative *cis* regulatory elements. In addition, we extended the ontology-based pattern identification by using current annotations provided by the Gene Ontology Consortium to identify 380 statistically significant clusters containing genes with expression patterns characteristic of various biological processes, cellular components, and molecular functions.

We are also studying genetic diversity by using hybridization-based approaches to further characterize parasite genes. By performing a full genome scan of allelic variability of 14 field and laboratory strains of *P falciparum*, we showed that 10% of the genome has higher than neutral rates of diversity at tens of thousands of loci. We found that whereas many genes are exceptionally well conserved across parasite isolates, paralog genes (i.e., genes related by duplication within a genome that have different functions), genes near the ends of chromosomes, genes that encode proteins trafficked to the surface of infected red cells, and genes that encode known and potential drug targets are exceptionally diverse. These data suggest that rates of mitotic recombination are elevated among genes with paralogs and that selection pressure on those without paralogs is strong.

We also revealed gene amplification events, including one associated with *pfmdr1*, the gene for multidrug resistance in *P falciparum*, and a previously uncharacterized amplification centered on the gene for GTP cyclohydrolase, the first enzyme in the folate biosynthesis pathway. Although GTP cyclohydrolase is not the known target of any current drugs, downstream members of the pathway are targeted by several widely used antimalarial agents. We propose that amplification of the GTP cyclohydrolase enzyme in the folate biosynthesis pathway may facilitate increased flux through this pathway and increase resistance to antifolate drugs.

These data and indications that 90% of a small eukaryote's genetic variation can be captured in a single microarray hybridization suggest that population genomics will be a fruitful approach for discovering new determinants of drug resistance in a variety of infectious agents.

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Membrane Proteins and Viruses Visualized by Electron Cryomicroscopy and Image Analysis

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The ultimate goal of our studies is to gain a deeper understanding of the molecular basis of important human diseases, such as sudden death, heart attacks, and HIV infection, that cause substantial mortality and suffering. The structural details revealed by our research may provide clues for the design of more effective and safer medicines.

At the basic science level, we are intrigued by questions at the interface between cell biology and structural biology: How do membrane proteins fold? How do membrane channels open and close? How are signals transmitted across a cellular membrane when an extracellular ligand binds to a membrane receptor? How do viruses attach to and enter host cells, replicate, and assemble infectious particles? To explore such problems, we use high-resolution electron cryomicroscopy and computer image processing. With this approach, we can examine the molecular architecture of supramolecular assemblies such as membrane proteins and viruses.

In electron cryomicroscopy, biological specimens are quick frozen in a physiologic state to preserve their native structure and functional properties. A special advantage of this method is that we can capture dynamic states of functioning macromolecular assemblies, such as open and closed states of membrane channels and viruses actively transcribing RNA. Three-dimensional density maps are obtained by digital image processing of the high-resolution electron micrographs. The rich detail in the density maps exemplifies the power of this approach to reveal the structural organization of complex biological systems that can be related to the functional properties of such assemblies.

Ongoing research projects include the structure analysis of (1) membrane proteins involved in cell-to-cell communication (gap junctions), water transport (aquaporins), ion transport (potassium channels), and transmembrane signaling; (2) viruses responsible for significant human diseases (HIV type 1 [HIV-1], hepatitis B virus, rotavirus, astrovirus); and (3) viruses used as model systems to understand mechanisms of pathogenesis (arenaviruses, reoviruses, nodaviruses, tetraviruses, sobemoviruses). The following sections summarize selected projects that exemplify the themes of our research program.

GAP JUNCTION MEMBRANE CHANNELS

Gap junction channels connect the cytoplasms of adjacent cells by means of an intercellular conduit formed by the end-to-end docking of 2 hexameric hemichannels called connexons. Gap junctions play an essential functional role by mediating metabolic and electrical communication within tissues. For instance, in the heart, gap junction channels organize the pattern of current flow to allow a coordinated contraction of the muscle.

We expressed a recombinant cardiac gap junction protein, termed connexin 43, and produced 2-dimen-

sional crystals suitable for electron cryocrystallography. Our previous findings indicated that each hexameric connexon is formed by 24 closely packed α-helices. We extended this analysis to 5.7-Å in-plane and 19.8-Å vertical resolution, a step that enabled us to identify the positions and tilt angles for the 24 α-helices within each hemichannel (Fig. 1). The 4 hydrophobic segments

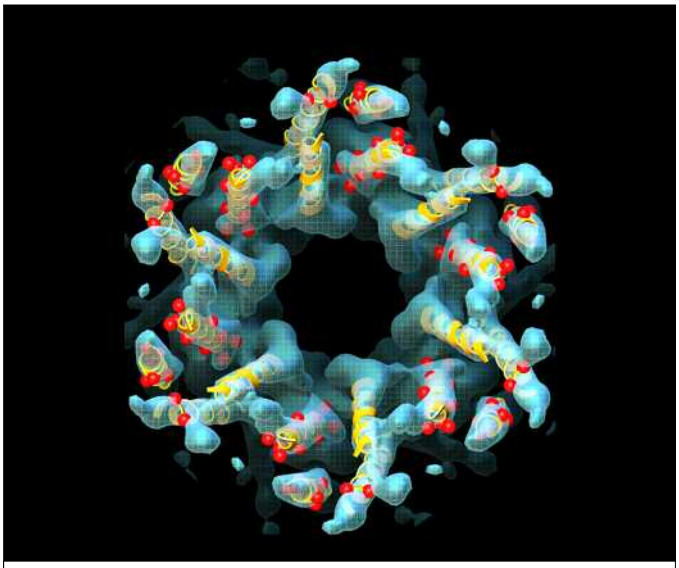

Fig. 1. Intercellular gap junction channels have a diameter of about 65 Å and are formed by the end-to-end docking of 2 hemichannels, each of which is composed of a hexamer of connexin subunits. A C^{α} model (ribbons) for the membrane-spanning α-helices of the hemichannels was derived by combining the information from a computational analysis of connexin sequences, the results of more than a decade of biochemical studies, and the constraints provided by a 3-dimensional map derived by electron cryocrystallography. Although individually none of these approaches provided high-resolution information, their sum yielded an atomic model that predicts how connexin mutations (spheres), which result in diseases such as nonsyndromic deafness and Charcot-Marie-Tooth disease, may interfere with formation of functional channels by disrupting helix-helix packing.

in connexin sequences were assigned to the α-helices in the map on the basis of biochemical and phylogenetic data. Evolutionary conservation and an analysis of compensatory mutations in connexin evolution were used to identify the packing interfaces between the helices. The final model, which specifies the coordinates of C^{α} atoms in the transmembrane domain, provides a structural basis for understanding the different physiologic effects of almost 30 mutations and polymorphisms in terms of structural deformations at the interfaces between helices, revealing an intimate connection between molecular structure and disease.

INTEGRINS

Integrins are a large family of heterodimeric transmembrane receptor proteins that modulate important biological processes such as development, cell adhesion, angiogenesis, wound healing, and neoplastic transformation. The ectodomain of the integrin $\alpha_V\beta_3$ crystallizes in a bent conformation, which is considered to be inactive (i.e., unable to bind physiologic ligands in solution) unless it is fully extended by activating stimuli. To assess whether the bent integrin can bind physiologic ligands, we collaborated with M.A. Arnaout, Harvard Medical School, to generate a stable, soluble complex of the manganese-bound $\alpha_V\beta_3$ ectodomain with a fragment of fibronectin containing type III domains 7–10 and the EDB domain. Electron microscopy and single-particle image analysis were used to determine the 3-dimensional structure of this complex (Fig. 2).

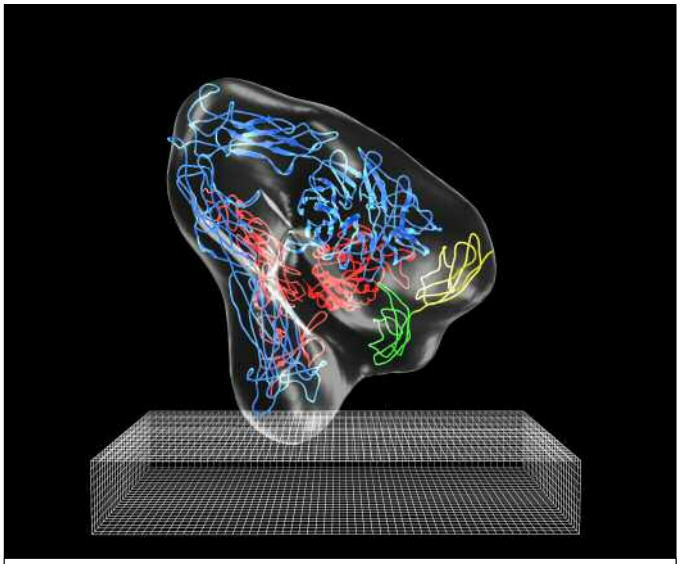

Fig. 2. The 3-dimensional density map (gray-scale transparency) of the integrin $\alpha_V\beta_3$ in a complex with fibronectin was determined by using electron microscopy and image analysis. The x-ray structures of the α_V and β_3 proteins have been docked into the electron microscopy density envelope. Additional density (lower right) can accommodate fibronectin domain 10 adjacent to the ligand-binding site as well as domain 9 at the synergy site. The complex is shown adjacent to the white box, which represents the 30-Å-thick hydrophobic part of the cellular membrane across which signals are transmitted.

Most $\alpha_V\beta_3$ particles, whether ligand-free or bound to fibronectin, had compact, triangular shapes. A difference map comparing ligand-free and fibronectin-bound integrin revealed density that could accommodate the fibronectin type III domain 10 containing arginine–glycine–aspartic acid in proximity to the ligand-binding site of β_3, with domain 9 just adjacent to the synergy site, binding region of α_V.

This study suggests that the ectodomain of $\alpha_V\beta_3$ has a bent conformation that can stably bind a physiologic ligand in solution. These results are relevant for under-

standing how binding of ligands to the extracellular domain leads to conformational changes that transmit signals across the plasma membranes of cells, culminating in changes in gene transcription in the nucleus.

STRUCTURE OF THE HIV-1 CAPSID

Since 1981, more than 25 million people have died of AIDS. HIV assembly and replication proceed through formation of morphologically distinct immature and mature viral capsids that are organized by the Gag polyprotein (immature) and by the fully processed CA protein (mature). The Gag polyprotein is composed of 3 folded polypeptides (MA, CA, and NC) and 3 smaller peptides (SP1, SP2, and p6) that function together to coordinate membrane binding and Gag-Gag lattice interactions in immature virions. After budding, HIV maturation is initiated by proteolytic processing of Gag, which induces conformational changes in the CA domain and results in assembly of the distinctive conical capsid (Fig. 3). The mature capsid can be modeled as fullerene structures composed of closed hexameric arrays of the viral CA protein, but a high-resolution structure of the lattice has remained elusive.

We used electron cryomicroscopy and image analysis to derive a 3-dimensional density map of 2-dimensional crystals of full-length HIV-1 CA. The docking of high-resolution domain structures into the map yielded a pseudoatomic model that (1) provides a structural model for the CA hexameric lattice that will probably apply to all retroviruses, (2) provides a structural basis for understanding a wide variety of structural, biochemical, and genetic studies, (3) defines 3 types of interactions that are required for capsid assembly, and perhaps most significantly, (4) defines a critical interface between the N- and C-terminal domains of adjacent subunits that was not visualized in low-resolution studies and is a potential therapeutic target.

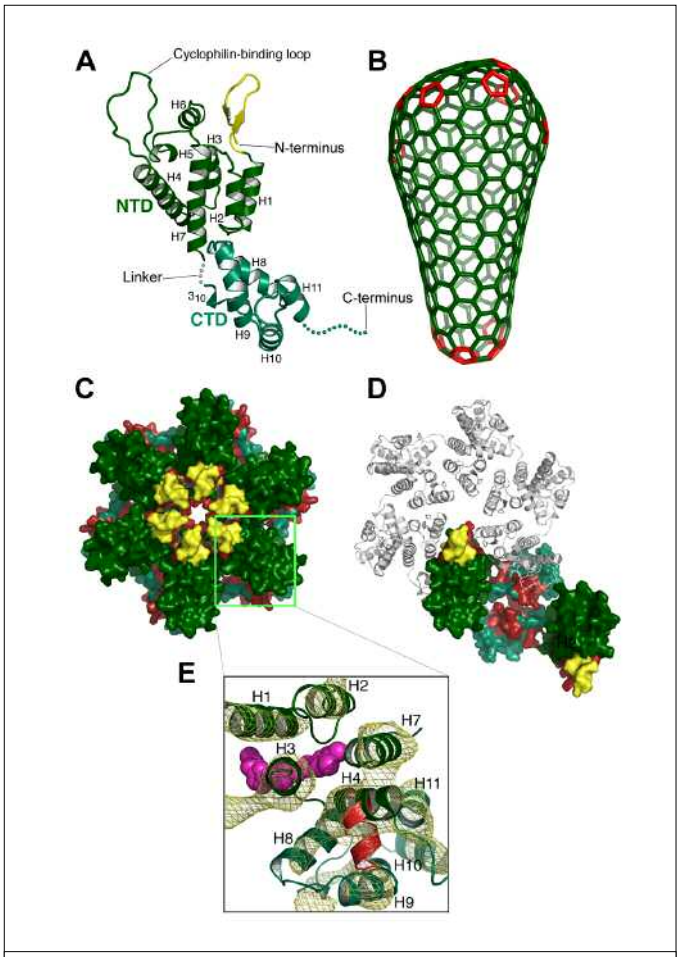

Fig. 3. Structure of the mature HIV-1 capsid. A, Tertiary structure of the mature, processed CA protein, with the N-terminal domain (NTD) in dark green and the C-terminal domain (CTD) in blue-green. The β-hairpin is yellow. The final 11 residues of CA, indicated by the dashed line are typically disordered in crystal structures. B, Fullerene model for the conical capsid, with CA hexamers (green) and pentameric declinations (red). Surface representation of the pseudoatomic model of the mature CA hexamer, emphasizing the CA_{NTD} hexamer (C) and CA_{CTD} dimer (D), viewed from the outer surface of the capsid. Color coding is the same as in A except that CA residues are red to denote sites of significant protection from deuterium exchange in the hexagonal CA lattice. The exposed red patch in D corresponds to the CA_{NTD}-binding site on the CA_{CTD}, which is exposed when the neighboring CACTD domain is removed. E, Top view of the CA_{CTD}-CA_{NTD} intermolecular interface. The pseudoatomic model is shown in ribbon representation, fitted to the experimental density map derived by using electron cryocrystallography (gold mesh). Binding sites of the CA-I (red) and CAP-1 (magenta) maturation inhibitors are indicated.

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