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Section Cover for the Department of Cell Biology: Neurons in infancy: first steps on the road to brain development. Neurite initiation involves a coordinated rearrangement of the microtubule and actin-based cytoskeleton. Shown here are 2 nerve cells stained for actin filaments before (right) and after (left) the initial induction of neurites, the long, thin projections that later become the neuron’s axons and dendrites. Neurite initiation is the first step in a series of complex shape changes required to “wire up” synaptic circuits in the brain and spinal cord. Image by Leif Dehmelt, Ph.D., and Shelley Halpain, Ph.D., Department of Cell Biology and Institute for Childhood and Neglected Diseases.
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Chairman’s Overview

New live cell imaging methods have revealed that ornate and complex cellular assemblies, such as adhesion plaques, adhesion junctions, muscle fibers, and clathrin-coated pits, which were once thought to be static structures, are in fact in continuous and rapid flux. Rapid flux enables these structures to quickly adapt, to respond to change, to break down in some areas and rebuild in others as needed. Similarly, it has been a year of flux in the Department of Cell Biology. Several faculty members left Scripps Research this year and moved their programs to other institutes. Each of these moves means new opportunities for the investigator and, in many instances, career advancements. Thus, Klaus Hahn, an associate professor here, was named the Thurman Professor of Pharmacology at the University of North Carolina in Chapel Hill. Jeff Harper, another associate professor here, accepted a full professorship at the University of Nevada, Reno. Sandy Shattil left Scripps Research for the University of California, San Diego, where he is a professor of medicine and division chief of hematology/oncology. Last, but not east, Mark Ginsburg, who did his postdoctoral studies at Scripps in 1975 and rose through the ranks to full professor in 1990 became a professor of medicine at the University of California, San Diego. Thankfully, Sandy and Mark remain in the neighborhood, and strong scientific interactions will continue. Indeed, science is a global endeavor, and the valuable collaborations initiated at Scripps Research will undoubtedly continue.

Examples of positive flux include the academic promotion of many of our distinguished faculty members, reflecting their success and the respect that they have earned among their local, national, and international colleagues. Congratulations to Mari Manchester who was promoted to associate professor in recognition of her work on the mechanisms of immunosuppression by measles virus and her recent research on engineering viral particles for vaccine development and other nanotechnology applications. Shelley Halpain was promoted to associate professor with tenure. Shelley is a recognized leader in the regulation and function of cytoskeletal dynamics during early neurite development and in the establishment and maintenance of postsynaptic dendritic spines. Finally, completing his meteoric rise from graduate student at Scripps Research, Ben Cravatt was promoted this year to full professor. Ben’s work spans both cell biology and chemistry. Attesting to his outstanding contributions in both areas of research, Ben received 2 prestigious awards: the Eli Lilly Award in Biological Chemistry and the Cope Scholar Award for Organic Chemistry.

Other researchers in the department were recognized this year for their outstanding contributions. John Yates was awarded the Biemann Medal from the American Society for Mass Spectrometry in recognition of his achievements and contributions to protein sequence analysis and tandem mass spectrometry. Clare Waterman-Storer was named a Keith Porter Fellow by the American Society for Cell Biology and a Nikon Instruments Partner in Science, in recognition of her innovation in the development and application of molecular microscopy.

I was named editor-in-chief of Molecular Biology of the Cell, a leading cell biology journal owned and operated by the American Society for Cell Biology. Our young scientists are also being recognized for their past accomplishments and exciting potential. Lisa Stowers was named a Pew Scholar in the Biomedical Sciences, and Elizabeth Winzeler was named a W.M. Keck Foundation Distinguished Young Scholar in Medical Research.

In the following pages, you will read about the impressive research accomplishments. Please note the breadth and quality of the work. Significant advances were made.
in the genomics and proteomics of malaria (John Yates and Elizabeth Winzeler); in high-resolution electron microscopy of transcription factors (Francisco Asturias) and new classes of molecular motors (Ron Milligan); in the development of the inner ear and mechanotransduction in hearing (Ulrich Müller); in the discovery of temperature-sensing ion channels (Ardem Patapoutian); in understanding complex behaviors, such as circadian rhythms (Steve Kay) and learning and memory (Mark Mayford); in diseases of protein folding, such as cystic fibrosis (Bill Balch); in the regulation of cytoskeletal dynamics in migrating cells (Clare Waterman-Storer, Gaudenz Danuser, and Velia Fowler); and in the mechanisms of intracellular protein transport and sorting (Larry Gerace and me). Work in these and other areas continues.
REGULATION OF GENE EXPRESSION IS OF UTMOST SIGNIFICANCE IN CELL DIFFERENTIATION AND DEVELOPMENT AND IN THE RESPONSES OF ALL LIVING CELLS TO THE ENVIRONMENT. THE FIRST STEP IN EXPRESSION OF A PROTEIN-CODING GENE IS TRANSCRIPTION OF THE GENE TO MESSENGER RNA, WHICH IS CARRIED OUT BY RNA POLYMERASE II (RNAPII) AND A NUMBER OF ACCESSORY PROTEINS. OUR GOAL IS TO REVEAL THE MOLECULAR MECHANISMS OF TRANSCRIPTION AND ITS REGULATION BY STUDYING THE STRUCTURES OF THE DIFFERENT COMPONENTS OF THE TRANSCRIPTION MACHINERY AND THE COMPLEXES THAT THE COMPONENTS FORM. WE USE ELECTRON MICROSCOPY TO RECORD IMAGES OF INDIVIDUAL COMPLEXES AS THE COMPLEXES APPEAR UNDER CONDITIONS SIMILAR TO THOSE IN A LIVING CELL. STATE-OF-THE-ART TECHNIQUES ARE THEN USED TO RECONSTRUCT THE 3-DIMENSIONAL STRUCTURES OF THE MOLECULAR COMPLEX.

BECAUSE MOST TECHNIQUES FOR STRUCTURAL ANALYSIS REQUIRE RELATIVELY LARGE QUANTITIES OF MATERIAL, STRUCTURAL STUDIES OF THE TRANSCRIPTION MACHINERY SO FAR HAVE BEEN FOUSED ALMOST EXCLUSIVELY ON MODEL ORGANISMS. IMAGING OF INDIVIDUAL PARTICLES BY ELECTRON MICROSCOPY REQUIRES VERY LITTLE MATERIAL, AND THIS ADVANTAGE ENABLED US TO CALCULATE THE STRUCTURE OF HUMAN RNAPII, THE CENTERPIECE OF THE HUMAN TRANSCRIPTION MACHINERY. NEARLY 50,000 IMAGES OF INDIVIDUAL HUMAN RNAPII PARTICLES WERE COMPUTATIONALLY COMBINED TO CALCULATE A 10-Å RESOLUTION STRUCTURE OF THE ENZYME (FIG. 1).


WE ARE USING THE SAME IMAGE ANALYSIS TECHNIQUES TO STUDY THE STRUCTURE OF DNA POLYMERASE ε, A EUKARYOTIC DNA POLYMERASE INVOLVED IN DNA REPLICATION AND REPAIR. MOST DNA POLYMERASES REQUIRE THE ASSISTANCE OF AN ACCESSORY “CLAMP” THAT ALLOWS THEM TO REMAIN COMMITTED TO THEIR DNA TEMPLATES. IN CONTRAST, POLYMERASE ε HAS MARKED AFFINITY FOR DOUBLE-STRANDED DNA, WHICH ALLOWS THE POLYMERASE TO FUNCTION EFFICIENTLY WITHOUT THE ASSISTANCE OF A DNA CLAMP.

IMAGE ANALYSIS REVEALED THAT POLYMERASE ε ALSO HAS AN UNUSUAL STRUCTURE. THIS FIRST STRUCTURE OF A MULTISUBUNIT DNA POLYMERASE SHOWED THAT 2 SUBUNITS INVOLVED IN INTERACTION WITH DOUBLE-STRANDED DNA FORM AN EXTENDED “HOOK,” WHICH IS FLEXIBLY ATTACHED TO A GLOBULAR DOMAIN THAT CONTAINS THE ACTIVE SITE (FIG. 2). INTERESTINGLY, THE
The structure of the globular domain suggests a mode of interaction with DNA that might be analogous to that of multisubunit RNA polymerases, in which the active site is located deep inside a dead-end cleft. Results from primer extension assays support the notion that the extended hook structure is involved in mediating interaction of polymerase ε with DNA, because a clear correlation is evident between the minimal required length of the DNA primer and the dimensions of the hook domain.

Structural characterization of complexes consisting of polymerase ε and DNA will provide information to either validate or revise our current model for interaction of the enzyme with nucleic acid. The complexity of the DNA replication machinery rivals that of its transcription counterpart, and macromolecular electron microscopy is an ideal technique to provide structural information that will increase our understanding of DNA replication and repair.

**PUBLICATIONS**


**Biology and Chemistry of Protein-Trafficking Conformational Diseases**


A major challenge is to understand and treat the many protein-misfolding diseases that affect human health, including cystic fibrosis, emphysema, type 2 diabetes, and amyloidosis. These abnormalities are classified as membrane-trafficking conformational diseases because a defect in protein folding at some stage of the eukaryotic secretory pathway results in loss of activity or protein aggregation. A key concern is to determine the underlying folding defect and how that defect affects the ability of the protein to function normally within the context of the cell’s intracellular transport machinery or in the extracellular environment of the host.

Our broad objective is to define the molecular basis for the trafficking of normal and misfolded proteins through the secretory pathway of eukaryotic cells. We use chemical, structural, and biological approaches.

The eukaryotic cell is highly compartmentalized; each compartment of the exocytic and endocytic pathways provides a unique chemical landscape in which protein function and folding may be modulated. Movement between these compartments involves the activity of both anterograde and retrograde transport tubules and vesicles. Many conformational diseases are a consequence of dysfunction at different stages of this transport pathway or outside the cell.

Transport through the secretory pathway involves a selective mechanism in which cargo molecules are concentrated into carrier vesicles. Vesicle-mediated transport is regulated by a diverse group of small GTPases belonging to the Ras superfamily. Each of these molecules acts as a “molecular sensor” to regulate different steps in the reversible assembly of vesicle coats and targeting-fusion complexes. During export from the first compartment of the secretory pathway, the endoplasmic reticulum, coat recruitment to budding sites involves activation of the GTPase Sar1, a protein whose structure we solved at 1.7 Å by using x-ray crystallography in collaboration with I.A. Wilson, Department of Molecular Biology.

After activation, the cytosolic coat components Sec23/24 and Sec13/31 form the coatamer complex II coat that polymerizes to promote budding from the surface of the endoplasmic reticulum. We found that cargo selection by the assembling polymer involves “exit codes” on the cytoplasmic domains of cargo and cargo receptors (Fig. 1). These exit codes bind to a pocket in the Sec24 coat component. We are using shotgun proteomics (multidimensional protein identification technology or MudPIT) to identify unknown components involved in cargo selection.

After vesicles separate from the endoplasmic reticulum, targeting and fusion of coatamer complex II transport vesicles to the next step of the secretory pathway, the Golgi apparatus, require a different class of Ras-like GTPases that belong to the Rab family. Members of the large Rab family (63 members) act as molecular switches that assemble complexes involved in vesicle tethering and fusion. Each Rab protein contains 2 prenyl...
(geranylgeranyl) groups at the C terminal that are critical for membrane association and function. Using a bioinformatics approach, in collaboration with J. Hogenesch, Genomics Institute of the Novartis Research Foundation, San Diego, California, we found that each Rab GTPase executes targeting and fusion decisions at a distinct step in the exocytic or endocytic pathway. By coordinating the function of multiple distinct effectors at each step, Rab GTPases define the function and highly distinctive membrane architecture of eukaryotic cells found in different tissues.

An important common effector for all Rab GTPases is the protein GDP-dissociation inhibitor (GDI). GDI extracts Rabs from membranes after vesicle fusion, thereby forming a cytosolic complex that sequesters the prenyl groups for redelivery to the membrane during budding. We used x-ray crystallography to solve the structure of the brain α-isofrom of GDI at 1.04-Å resolution. Combined biochemical studies and molecular genetic studies revealed critical residues that are involved in Rab binding and in retrieval of prenyl groups from the lipid bilayer. Remarkably, extraction of Rab from the bilayer requires the classical chaperone complex Hsp90-70-40. The role of Hsp90 in GDI function and lipid extraction most likely is the same as its role in the steroid hormone receptor pathway, where the Hsp90 complex participates in altering the protein fold of the receptor to accommodate the bulky hydrophobic steroid.

Although traffic from the endoplasmic reticulum can be disrupted by mutations that prevent proper protein folding during synthesis, other protein conformational diseases are associated with mutations that disrupt function at later steps of the secretory pathway and outside the cell in new chemical environments that can alter the protein fold. In collaboration with J. Kelly, Department of Chemistry, we are studying the link between trafficking defects and the protein-folding energetics of a number of conformational diseases, including cystic fibrosis, hereditary childhood emphysema, Gaucher disease, and familial amyloidosis of the Finnish type. Through this multidisciplinary approach that combines the tools of structure, chemistry, and biology, we hope to gain critical insight into the basis for a variety of inherited transport diseases. Knowledge of the function of these cargo selection pathways will enable the development of small-molecule chemical chaperones to encourage export and stability of misfolded proteins, leading to restoration of normal cellular function.

**PUBLICATIONS**


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**Automated Molecular Imaging**


Elucidating the structure and mechanism of action of molecular machines is an emerging frontier in understanding how the information in the genome is transformed into cellular activities. Molecular machines are associations of individual components (e.g., pro-
teins, nucleic acids, lipids) in the form of large complexes; examples include ribosomes, transcription complexes, track-motor complexes, and membrane-embedded pumps and channels. These machines are large and may also be conformationally and compositionally dynamic or present in comparatively low numbers, factors that make them extremely challenging (or impossible) objects for study by x-ray crystallography and nuclear magnetic resonance methods. Molecular microscopy, however, holds great promise for routinely and efficiently providing structural information at a resolution sufficient to resolve the secondary structure in these large molecular machines. This method could then be used in conjunction with high-resolution x-ray structures of individual proteins to interpret very large complexes to near atomic resolution.

Unfortunately, the methods generally used in molecular microscopy are both time-consuming and labor intensive. These include the preparation of suitable specimens, the acquisition of the required very large numbers of electron micrographs, and the supervision of the sometimes-complex software needed for analysis and reconstruction of the 3-dimensional electron density maps.

The challenge then is to transform structure determination via electron microscopy into a high-throughput method. Success in this endeavor will not only facilitate the process of molecular microscopy but also expand the scope of accessible problems and make possible investigations that are currently deemed too high risk because of the inordinate effort involved. To this end, we are developing technologies to address automation for specimen handling, image acquisition, data processing, and integration of data information. Several years ago, we began to develop a system, called Leginon, that automatically collects electron micrographs of macromolecular structures (Fig. 1). This system has been integrated with automated particle-selection algorithms and analysis and processing software.

In 2002, we established the National Resource for Automated Molecular Microscopy (NRAMM). This new biotechnology resource center is funded by the National Center for Research Resources, National Institutes of Health. The overall mission is to develop, test, and apply technology to completely automate the processes involved in using electron cryomicroscopy to solve macromolecular structures. The current focus of NRAMM is the development of new approaches to specimen handling, automated acquisition, automated processing, and information handling.

Projects in specimen handling are all related to improving the handling and monitoring of specimens by developing innovative new engineering devices. The projects include developing methods for high-throughput screening of negatively stained ordered arrays and for controlling the stability of cryostage specimen holders. In the past year, we installed a robotic grid-loading system that is integrated with the Leginon data acquisition system; with the robotic system, we can screen 96 grids at a time.

The fundamental core technology of the NRAMM is the development of a generalized system for automated image acquisition. The goals are to continue developing the Leginon system and to export this system to other laboratories. In the area of automated processing, we are focusing on the tasks required after data acquisition to reconstruct a 3-dimensional map of the macromolecular structure of interest.

The storage, organization, distribution, and archiving of information is a critical aspect of the overall goal of automated data acquisition and processing, and the success of every other project in the NRAMM depends on this core project. An object-oriented database develop-


We are interested in understanding complex physiology and behavior at the level of chemistry and molecules. At the center of cross-physiology and molecules. At the center of cross-chemical messages termed the fatty acid amides, which affect many physiologic functions, including sleep and pain. In particular, one member of this family, oleamide, accumulates selectively in the cerebrospinal fluid of tired animals. This finding suggests that oleamide may function as a molecular indicator of an organism’s need for sleep. Another fatty acid amide, anandamide, may be an endogenous ligand for the cannabinoid receptor in the brain.

The in vivo levels of chemical messengers such as the fatty acid amides must be tightly regulated to maintain proper control over the influence of the messengers on brain and body physiology. We are characterizing a mechanism by which the level of fatty acid amides can be regulated in vivo. Fatty acid amide hydrolase degrades the fatty acid amides to inactive metabolites. Thus, the hydrolase effectively terminates the signaling messages conveyed by fatty acid amides, possibly ensuring that these molecules do not generate physiologic responses in excess of their intended purpose.

We are using transgenic and synthetic chemistry techniques to study the role of the hydrolase in the regulation of fatty acid amide levels in vivo. In collaboration with R.C. Stevens, Department of Molecular Biology, we solved the first 3-dimensional structure of fatty acid amide hydrolase. We are using this information to explore the molecular mechanism of action of the enzyme and to design inhibitors of the hydrolase. We are also interested in proteins responsible for the biosynthesis of fatty acid amides.

Another area of interest is the design and use of chemical probes for the global analysis of protein function. The evolving field of proteomics, defined as the simultaneous analysis of the complete protein content of given cell or tissue, encompasses considerable conceptual and technical challenges. We hope to enhance the quality of information obtained from proteomics experiments by using chemical probes that indicate the collective catalytic activities of entire classes of enzymes. These activity-based probes could be used to record variations in protein function independent of alter-
ations in protein abundance and would be a powerful and complimentary set of tools for proteome analysis.

We succeeded in generating one such class of chemical reagents, biotin- and fluorophore-tagged fluorophosphonates, that target the serine hydrolases, a large family of enzymes composed of numerous proteases, lipases, esterases, and amidases. We are using the probes to explore the roles that serine hydrolases play in a variety of physiologic and pathologic processes, especially cancer progression. Additionally, using both directed and nondirected methods, we designed activity-based probes for many additional enzyme classes, including metalloproteases, glutathione S-transferases, and oxidoreductases.

**PUBLICATIONS**


**Cytoskeleton Mechanics of Cell Migration and Chromosome Segregation**

G. Danuser, A. Ponti, J. Dorn, A. Matov, A. Kerstens, K. Jaqaman, L. Ji, M. Machacek, G. Yang, D. Loerke

Every movement in a cell is the complex kinematic output of molecularly generated forces. We are interested in understanding how the machinery that generates the force is controlled at the molecular level to result in coordinated movement at the cellular level. Specifically, we investigate how assembly and disassembly of the actin cytoskeleton and the dynamic structural interactions of actin filaments with other cytoskeletal components mediate directed cell migration. We also study how assembly and disassembly of microtubules during cell division are orchestrated with the action of kinetochore and chromosome cohesive proteins to mediate symmetric segregation of replicated DNA.

To examine these molecular systems, we develop computational models of actin and microtubule mechanics in which we propagate the effect of molecular cytoskeletal dynamics on motility as it occurs at the level of the cell. We then deduce molecular mechanisms by matching predictions (obtained by using the model) with accurate measurements of dynamic behaviors of cells. Technologically, the 2 main challenges of such data-driven, scale-integrating modeling are the acquisition of high-resolution measurements of processes at the level of the cell and the implementation of sensitive numerical tools for fitting cellular level data to models with molecular resolution.

In the past year, we made great strides in both of these aspects. In collaboration with C. Waterman-Storer, Department of Cell Biology, we completed the first version of a software package for the analysis of fluorescent speckle microscopy movies. Using this package, we can obtain rate maps of the flow and turnover of the actin cytoskeleton at high temporal and spatial resolution and can correlate these events with protrusive responses of migrating cells (Fig. 1). We will use this novel technique to study the integration and spatiotem-
poral regulation of adhesion-, contraction-, and polymerization-induced forces in promoting F-actin and protrusion dynamics. Moreover, we will begin to disseminate the package to support other groups in the quantitative, molecular characterization of these dynamics.

In a similar effort, we are collaborating with P. Sorger, Massachusetts Institute of Technology, Cambridge, Massachusetts, to develop quantitative and automated light microscopy for measuring 3-dimensional chromosome dynamics in yeast. We are using this assay in a functional screen of proteins localized in the kinetochore and are analyzing these data in the framework of a molecular model of the yeast spindle apparatus.

PUBLICATIONS


**Bioorganic Chemistry of Proteins**


Our focus is the development and use of methods to incorporate unnatural chemical groups into proteins. We developed a chemical approach for producing the large polypeptide chains that make up protein molecules, enabling us to change the structure of a protein in ways impossible by natural means. This chemical ligation approach greatly facilitates the synthesis of proteins of moderate size and has opened the world of proteins to the synthetic tools of organic chemistry. Chemical ligation can be extended to biologically expressed proteins, enabling the semisynthesis of proteins of unlimited size that contain fluorophores or cross-linking agents at defined positions. Our goal is to introduce noncoded amino acids and other chemical groups into proteins to better understand the molecular basis of protein function.

**PHOTOLABILE LIGATION AUXILIARY**

We developed several simple chemical auxiliaries that can be attached directly to the N terminus of a peptide. One auxiliary mimics the chemical properties of an N-terminal cysteine and facilitates a sulfur-to-nitrogen acyl transfer, forming a peptide bond at the ligation site. In our original approaches, a strong acid treatment was necessary to remove the auxiliary after acyl transfer. Recently, we developed an o-nitrobenzyl–based auxiliary that can be removed by ultraviolet light, leaving a native peptide as the final product. This approach will facilitate the synthesis of proteins with acid-sensitive labels and posttranslational modifications.

**COVALENT MODIFICATION OF COAGULATION FACTORS**

Multifunctional enzymes such as thrombin are composed of several structurally distinct regions distal to the active-site groove. Some of these sites bind differ-
ent effectors that dictate enzyme specificity, whereas other sites are associated with substrate binding. These distinct binding sites are termed exosites. In the serine protease thrombin, at least 2 distinct positively charged exosites have been identified. Exosite I binds some thrombin substrates such as fibrinogen and protease-activated receptor-1. However, this site also binds thrombomodulin, a thrombin receptor, which changes the substrate specificity of thrombin. The thrombin-thrombomodulin complex cleaves and activates protein C, which initiates a series of anticoagulant events. Thus, thrombomodulin changes the procoagulant thrombin into an anticoagulant as part of a feedback loop.

From both pharmaceutical and scientific points of view, it is useful to generate variants of multifunctional enzymes that largely retain some of their functions but are deficient in other functions. In collaboration with J. Griffin, Department of Molecular and Experimental Medicine, we developed an approach to covalently block exosite I of thrombin while leaving the catalytic site and exosite II unmodified. To achieve this goal, we synthesized an exosite I inhibitor called hirugen and appended a photocross-linking group and a fluorescein label. This reagent was designed to physically attach the inhibitor to the enzyme and to block interactions with exosite I substrates. After cross-linking induced by ultraviolet light and purification of the modified enzyme, the resulting thrombin analog was completely inactive with respect to exosite I–dependent functions. However, this thrombin variant retained its amidolytic activity toward small substrates. We plan to generalize this approach to engineer coagulation factors with specifically tailored activities.

PUBLICATIONS

Actin Dynamics in Cell Morphogenesis and Function
V.M. Fowler, T. Fath, C. Ferreira-Mota, R.S. Fischer, K.L. Fritz-Six, J. Moyer, R. Nowak, K. Weber

Regulation of actin dynamics at the ends of filaments determines the organization and turnover of actin cytoskeletal structures and is critical for cell motility and cell architecture. For example, when cells change shape or crawl, new actin filaments are rapidly assembled by polymerization from the barbed ends and disassembled by depolymerization from the pointed ends during extension of lamellipodia or filopodia. In contrast, in striated muscle, actin filaments are organized into antiparallel, cross-linked arrays that interdigitate with myosin thick filaments, termed sarcomeres; the arrays persist for days, and their precise organization is required for muscle contraction.

Our goal is to elucidate and compare the distinct regulatory mechanisms that control actin polymerization at barbed and pointed ends in rapidly turning over actin filaments of motile cells and the regulatory mechanisms that control the length and stability of actin filaments in sarcomeres of striated muscle. During the past year, we focused principally on the function of tropomodulin, the actin-capping protein for the pointed ends of thin filaments in sarcomeres in striated muscle.

One of our goals is to establish a molecular mechanism for tropomodulin capping of the pointed ends of actin filaments and its regulation by tropomyosin. Previously, we showed that the affinity of tropomodulin-1 for capping tropomyosin-actin filaments is several thousand-fold greater than its affinity for capping pure actin filaments and that the tropomyosin-binding region of tropomodulin-1 is in the N-terminal part between residues 1 and 130. Recently, using pyrene-actin polymerization assays, we identified 2 distinct actin-capping regions on tropomodulin-1 by testing a series of recombinant tropomodulin-1 fragments for their ability to inhibit actin elongation from gelsolin-actin seeds. The C-terminal part of tropomodulin-1 (residues 160–359) contains the principal actin-capping activity, which requires the residues between 323 and 359. The N-terminal part of tropomodulin-1 (residues 1–130) contains a second, weaker actin-capping activity. The actin-capping activity of the N-terminal, but not the C-terminal, part of tropomodulin-1 is enhanced sev-
eral thousand-fold in the presence of tropomyosin. We conclude that the C-terminal capping domain of tropomodulin-1 contains a tropomyosin-independent actin-capping activity for pointed ends, whereas the N-terminal domain contains a tropomyosin-regulated capping activity.

To investigate the significance of interactions between tropomodulin-1 and tropomyosin for the length and stability of thin filaments, we developed monoclonal antibodies that bind to epitopes in the N-terminal part of tropomodulin-1 and block the ability of tropomodulin-1 to bind to tropomyosin without affecting actin-capping activity by the C-terminal part of tropomodulin-1. In collaboration with C. Gregorio, University of Arizona, Tucson, Arizona, we showed that disruption of tropomodulin-1 binding to tropomyosin by microinjection of these antibodies into chick cardiac myocytes caused a dramatic loss of thin filaments, as revealed by immunofluorescence deconvolution microscopy. Strikingly, when live cardiac myocytes expressing α-tropomyosin labeled with green fluorescent protein were microinjected with the antibodies, real-time imaging revealed that depolymerization of thin filaments occurred from the pointed ends of the filaments. This finding contrasts with the findings of previous experiments in which disruption of C-terminal domain actin-capping activity resulted in actin elongation from the pointed ends of thin filaments without affecting tropomyosin association or the integrity of thin filaments. The more recent finding implies that the N-terminal tropomyosin-regulated actin-capping domain and the C-terminal actin-capping domain of tropomodulin have distinct functions in restricting the elongation and depolymerization, respectively, of the pointed ends of actin filaments.

To establish the in vivo function of tropomodulin-1 in muscle, in collaboration with H. Zoghbi, Baylor College of Medicine, Houston, Texas, we analyzed myofibril assembly and cardiac morphogenesis during embryonic development in mice that lack the gene for tropomodulin-1. These mutant animals die on days 9.5–10.5 of embryogenesis because of aborted cardiac development and inability of the heart to beat. The cardiac abnormality is due to a primary defect in myofibril assembly evident at days 8–8.5 of embryogenesis, a stage when embryonic morphology and cardiac development appear indistinguishable from those in wild-type embryos.

Confocal microscopy of hearts of mutant embryos obtained on days 8–8.5 of embryogenesis revealed structures resembling nascent myofibrils with continuous F-actin staining and periodic dots of α-actinin, indicating that I-Z-I complexes assembled in the absence of tropomodulin-1. Myomesin, a component of thick filaments, was also assembled normally along these structures, indicating that assembly of thick filaments is independent of tropomodulin-1. However, myofibrils did not become striated, and gaps in F-actin staining (H zones) were never detected. We conclude that tropomodulin-1 is required for regulation of the length of actin filaments and maturation of myofibrils; thus, the function of tropomodulin-1 is critical for heart morphogenesis during embryonic development.

Tropomodulin-1 is the first sarcomeric component shown to be essential for a specific, discrete step of de novo assembly of myofibrils in the developing heart in mice, namely, restriction of the length of actin filaments at the H zone. Furthermore, although formation of M lines and formation of H zones are both relatively late events in the assembly of myofibrils, our results indicate that these events most likely proceed by independent, parallel pathways. We are further dissecting the pathways and intermediates during the assembly of cardiac myofibrils by analyzing other sarcomeric components in embryos of mice that lack the gene for tropomodulin-1 and by analyzing the assembly of myofibrils in mice that lack the gene for α-tropomyosin.

PUBLICATIONS


Angiogenesis-Dependent Disease


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Most diseases that cause catastrophic loss of vision do so as a result of abnormal growth of blood vessels. Similarly, tumors depend on a blood supply for their growth and use these new vessels as an avenue for metastasis. Blood vessels themselves can generate tumors (e.g., hemangiomas) when the growth and organization of vascular endothelial cells are not properly controlled. Our goal is to understand the mechanisms of ocular neovascularization in normal and pathologic situations.

We use a neonatal mouse retina model to identify regulators of developmental angiogenesis and understand endothelial guidance mechanisms. In addition, in a long-standing collaboration with D.A. Cheresh, Department of Immunology, we are using this system to evaluate the role of integrins in these processes. In collaboration with P.R. Schimmel, Department of Molecular Biology, we found that fragments of tryptophan tRNA synthetase are potent angiostatics, and we showed that a form of cell-based delivery of the fragments resulted in significantly reduced retinal vascularization.

We also explored the potential usefulness of lineage-negative hematopoietic stem cells derived from the bone marrow of adult mice for cell-based delivery of angiostatic and neurotrophic substances and for the trophic actions of the cells themselves in vascular and neuronal degenerative diseases. We discovered that the stem cells contain endothelial precursor cells and that these precursor cells specifically target retinal astrocytes, incorporate into new vessels, and, in a mouse model of retinal degeneration, rescue and stabilize a degenerating retinal vasculature. Most recently, we showed that endothelial precursor cells have a profound neurotrophic effect when injected into eyes of mice with inherited retinal degeneration; not only is the vasculature rescued in these mice, but photoreceptors and visual function are also preserved. The stem cells can also rescue retinal vasculature subject to hypoxic stress and may be useful for the treatment of ischemic retinopathies such as diabetes retinopathy and retinopathy of prematurity.

Glioblastoma multiforme is an incurable brain tumor that is usually fatal within 1 year after diagnosis. We are using gene therapy and a rat model of this disease to study the efficacy of an antiangiogenic approach in treating these tumors. Hemangiomas are endothelial tumors that proliferate rapidly and later involute spontaneously. We are using DNA microarrays to study changes in gene expression as hemangiomas progress. Our goal is to identify (1) new targets for therapy for these tumors and (2) novel regulators of angiogenesis. In collaboration with G.R. Nemerow, Department of Immunology, we used pseudotyped adenovirus to selectively target specific cell types in the retina. By using the appropriate fiber type, we can deliver transgenes to cells, such as photoreceptors, that ordinarily are not targeted by adenovirus.

PUBLICATIONS


Nucleocytoplasmic Transport and Role of the Nuclear Lamina in Higher Level Nuclear Organization

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The nuclear envelope is a specialized domain of the endoplasmic reticulum that forms the boundary of the nucleus in eukaryotic cells. The envelope consists of inner and outer nuclear membranes, the nuclear lamina, and nuclear pore complexes (NPCs). The nuclear lamina, a protein meshwork lining the inner nuclear membrane, provides a structural framework for the nuclear envelope and an anchoring site at the nuclear periphery for interphase chromosomes. NPCs are large supramolecular assemblies that span the nuclear envelope and provide channels for molecular transport between the nucleus and the cytoplasm.

We are using biochemical, structural, and functional approaches to investigate the functions of NPCs and the lamina. A recent highlight of our studies was the identification of more than 50 novel integral proteins associated with the nuclear envelope, some with potential disease links. These results provide a new framework for dissecting the functions of the nuclear envelope.

NUCLEOCYTOPLASMIC TRANSPORT MECHANISMS

Transport of most proteins through NPCs is an energy-dependent process mediated by nucleocytoplasmic shuttling receptors of the importin β superfamily. These receptors bind to specific transport signals on cargo molecules and are translocated through NPCs by interaction of the receptors with NPC proteins (nucleoporins) that contain multiple repeats of a phenylalanine-glycine motif. A key regulator of many nuclear transport pathways is the small GTPase Ran, which promotes the binding of cargo to export receptors and dissociation of cargo from import receptors in the nucleus.

We are using in vitro assays with digitonin-permeabilized cells to analyze the events that specify transport of cargo-receptor complexes through NPCs. Our results support a model in which movement of cargo-receptor complexes occurs by facilitated diffusion though a dense meshwork formed by phenylalanine-glycine repeat nucleoporins. This process appears to be promoted by Ran-GTP–mediated dissociation of receptors from nucleoporins and by an increase in the binding affinity of receptor-cargo complexes for nucleoporins at progressively more distal sites in the transport pathway.

We defined several critical tryptophan residues involved in the binding of the adaptor importin α to importin β; the binding may trigger mutually induced conformational changes in the receptor and the adaptor upon their association. Using X-ray crystallography, we solved the structure of a fragment of importin β complexed with parathyroid hormone–related protein, which represents a novel class of cargo. Unlike importin α, which interacts with the carboxyl-terminal part of importin β, parathyroid hormone–related protein binds to the amino-terminal half of importin β. Because this fragment also contains binding sites for both Ran and nucleoporins and supports import of parathyroid hormone–related protein in vitro, it may be a prototypical nuclear transport receptor. Consistent with this prediction, we identified a nucleoporin-binding site in the carboxyl-terminal half of importin β that contributes strongly to the import function of importin β.

To define principles of the transport of viral genomes through NPCs, we are analyzing the nuclear import of adenovirus DNA. Our results suggest that this process is driven by adenoviral proteins, which interact with the nuclear import machinery to potentiate viral docking and uncoating at NPCs and subsequent translocation of viral DNA into the nucleus. Whereas the major capsid protein, hexon, appears to be involved in viral docking at NPCs, our data indicate that protein VII, the major DNA-packaging protein of adenovirus, contains multiple nuclear import signals that may drive the nuclear transport of the associated DNA.

In an analysis of the nuclear import of hexon, we found that protein VI, another viral structural protein, acts as a nucleocytoplasmic shuttling adapter for hexon import. Interestingly, the carboxyl-terminal sequence of protein VI that mediates shuttling is cleaved by proteolysis at the time of viral maturation, an example of regulation of protein function by cleavable nuclear transport signals.

NUCLEAR LAMINA AND HIGHER LEVEL NUCLEAR ORGANIZATION

The nuclear lamina of higher eukaryotes consists of 2–4 related intermediate filament proteins called lamins and several minor lamina-associated polypeptides. The importance of the lamina in cell function is underscored by recent findings that mutations in lamin A and in certain lamina-associated polypeptides cause a host of human diseases, including muscular dystrophies, developmental disorders, and premature aging.
In recent proteomic analysis, done in collaboration with J.R. Yates, Department of Cell Biology, we identified 67 novel nuclear envelope transmembrane proteins, a number of which have potential disease links. Most of these proteins appear to be lamina-associated polypeptides. We are using expression profiling to identify components of the lamina proteome that may be functionally linked. We are also screening the functions of these proteins in a muscle differentiation assay, in which the functional redundancy of lamina components appears to be limited. The goal of these studies is to understand the basic functions of the lamina: how it contributes to nuclear organization, gene expression, and linkage of the nucleus to the cytoskeleton.

PUBLICATIONS


Structure and Function of Integrins


The development and functioning of multicellular animals depends on integrins. These adhesion receptors link to the actin cytoskeleton, resulting in transmission of biochemical signals and of force during cell migration and interactions with the extracellular matrix. Control of the affinity of integrins for ligands (integrin activation) is essential for normal cell adhesion and migration and for assembly of an extracellular matrix. Integrin activation is usually mediated through the cytoplasmic tail of the integrin β subunit and can be regulated by many different biochemical signaling pathways. We found that specific binding of the cytoskeletal protein talin to the cytoplasmic tails of integrin β subunits leads to the conformational rearrangements of integrin extracellular domains that increase the affinity of the domains. Thus, regulated binding of talin to integrin β tails is a final common element of cellular signaling cascades that control integrin activation.

The principal binding site for integrin β tails in talin is a FERM domain, composed of 3 subdomains: F1, F2, and F3. Previous studies of integrin αIIbβ3 indicated that both F2 and F3 bind the β3 tail, but only F3, or the F2-F3 domain pair, induces activation. To explore integrin activation mechanisms, we examined talin-induced perturbations of β3 nuclear magnetic resonances. F3 and F2-F3, but not F2, perturbed the membrane-proximal region of the β3 tail. All domains also perturbed more distal regions of the β3 tail that appear to form the major interaction surface, because the β3(Y747A) mutation suppressed those effects. These results suggest that perturbation of the membrane-proximal region of the β3 tail is associated with talin-mediated integrin activation.

PUBLICATIONS


In one approach, we used protein domains and/or antibody fragments modified so that they generate a fluorescent signal when they find and bind to a protein with a particular conformation. In this way, we visualized localized GTP binding of each of the Rho GTPases (Rac, Rho, and Cdc42), showing that the enzymes are turned on with precise timing at different positions within a moving cell. These 3 GTPases participate in a signaling cascade to control one another’s activation. By developing dyes of different wavelengths, we can systematically examine this entire signaling pathway in the same cell to see how the proteins regulate one another during rapid, precisely orchestrated behaviors.

We identified adhesion molecules, including vinculin, that affect the activation of the MAP kinases and Rho family molecules to shift the balance between (1) apoptosis and (2) motility or proliferation. Specific interactions between vinculin and its ligands affect the dynamics of MAP kinases and Rho proteins to generate different cell behaviors. We are investigating (1) the mechanisms by which such dynamics control signals downstream of Cdc42 and ERK2 and (2) cascades that coordinate the activity of the different Rho GTPases.

Dynamics of Adhesion Signaling in Living Cells


Cytoplasm is an essentially continuous network of organized molecules, with large structures such as cytoskeletal “girders” and organelles forming a shifting scaffold for the organization of many smaller molecular assemblies. This structure is an important element in the regulatory circuitry of the cell; it controls the precise timing and location of molecular interactions that determine cell behavior. Such supramolecular organization is difficult to understand by examining isolated proteins in vitro. Therefore, we are developing and using new tools to visualize protein activities within individual, living cells.

These new techniques depend on novel dyes that “report” many aspects of protein behavior and on novel methods for site-specific attachment of these dyes to proteins and peptides. The dyes, which are optimized for use in vivo, show large changes in fluorescence that depend on their interaction with nearby amino acids or exposure to water. When attached to proteins, the dyes are affected by the binding of specific ligands, conformational changes, and phosphorylation.

We focused on building new biosensors that report the activation of MAP kinases and the Rho family of small GTPase proteins. Each of these important signaling molecules is involved in different cellular behaviors (proliferation, apoptosis, motility), participating in essentially opposite processes through tight regulation of the timing and location of their activation. These proteins also generate an array of different cytoskeletal and morphologic changes, which are tightly controlled in time and space to produce motility and other polarized cell behaviors.

Organization and Function of the Neuronal Cytoskeleton

S. Halpain, J. Braga, B. Calabrese, L. Dehmelt, S. Maiti, B. Roger

During the past year, we made significant progress in research on the development and regeneration of neurons. In 2 main projects, we focused on cytoskeletal proteins of nerve cells, key proteins that underlie the structure and morphologic flexibility required by neurons for transmitting, storing, and pro-
cessing synaptic signals. We used biochemical, molecular biological, and microscopy-based approaches to understand the function of these molecules. Fluorescence time-lapse imaging of living neurons is an important tool that we use to uncover structure-function relationships for cytoskeletal proteins and the consequences of the dysfunction of the proteins.

One project concerns microtubule-associated proteins (MAPs). These proteins are important in regulating the assembly and stability of microtubules. We found that one such protein, MAP2, also directly binds actin filaments and induces filament bundling. Surprisingly, the region used in actin binding is the same region within the molecule that mediates microtubule binding. Using fluorescence-based time-lapse imaging and high-resolution confocal microscopy, we tracked the behaviors of microtubules and actin filaments in living neuronal cells with normal and mutant forms of MAP2. Our results indicate that MAP2 promotes neurite induction not only by stabilizing microtubules but also by coordinating their interaction with actin at the cell periphery. Despite high similarity between MAP2 and tau, a closely related molecule, tau does not bind actin filaments. It also does not initiate neurites as MAP2 does. Our studies therefore not only indicated an important function for the interaction between MAP2 and actin but also revealed unexpected distinctions between MAP2 and tau precisely where the 2 proteins previously were thought to be nearly identical in structure and function. Future studies are directed at understanding how MAP2 function is regulated in cells and identifying other molecular events crucial to neurite initiation.

A second project concerns the regulation of dendritic spines, specialized cellular protrusions at glutamate-releasing synapses. Spines are dysmorphic in many types of mental retardation and psychiatric disorders and are vulnerable to injury in diseases such as stroke and epilepsy, in which excessive release of glutamate can induce neuronal injury and subsequent cell death (a condition termed excitotoxicity). Understanding how spines form, what regulates their stability, and how they recover from injury is therefore of therapeutic interest for several neurologic conditions.

Our most recent results suggest a neuroprotective role for spines, because preventing the collapse of dendritic spines attenuated neuronal cell death induced by a subsequent lethal stimulus. The spine cytoskeleton is composed mainly of actin filaments. We discovered that actin filaments in spines are broken down within minutes of an injury-inducing stimulus. However, this damage to the spine can be reversed within minutes under appropriate conditions, indicating for the first time that spines can regrow after they collapse. We are examining the physiologic consequences of spine loss and are searching for ways to prevent the loss and promote recovery. Together these projects contribute to our understanding of molecular events in normal brain development and in regeneration of neuronal structure after injury and disease.

**PUBLICATIONS**


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Genetics and Genomics of Circadian Clocks


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An vast array of cellular processes fluctuate with a 24-hour periodicity, and an endogenous circadian clock is responsible for generating these biological rhythms. Circadian rhythms are found in all kingdoms of life and control diverse events ranging from the sleep-wake cycles in mammals to the overall rate of photosynthesis in plants. Many pathologic changes in humans, such as sleep disorders, most likely are due to a defect in circadian rhythms, so understanding how the circadian clock operates within the cell will have significance for both plants and animals. To study how circadian clocks are built inside of cells, we use molecular, genetic, and genomic approaches in 3 model systems: mouse, Arabidopsis, and Drosophila.

In mammals, the circadian clock plays an integral role in timing daily rhythms of physiology, such as blood
pressure, body temperature, and liver metabolism, in anticipation to changes in light as Earth rotates around the sun. The master circadian clock resides within a region of the brain that receives light information from the eyes. However, this brain region can keep time even in the absence of light, as occurs in some visually blind persons. Mutations in the genes that encode components of the circadian clock are manifested as abnormal activity rhythms in rodents and as sleeping disorders in humans, although which photoreceptors set the clock is unclear. Thus, although significant advancements have been made in understanding how the mammalian clock itself runs, little is known about how light transduces synchronizing signals to the clock.

To address this major question, we are using genetic and genomic approaches to identify new gene functions in circadian biology. We are generating and testing a number of mouse strains with mutations in known and potential photoreceptors and are testing the mice for defects in circadian rhythm. We determined that one photoreceptor, melanopsin, is an important contributor in maintaining synchrony between the clock and environmental light conditions. With the recently completed sequencing of the human and mouse genomes, we now know the sequences of more than 30,000 genes that can be investigated for potential roles in circadian function. We developed large-scale, in vitro, cell-based assays that can be used to rapidly determine if genes control clock activity. Combining this approach with genetic analysis will enable us to further dissect the connection between environmental stimuli, in the form of light, and the behavioral and physiologic events regulated by the circadian clock.

In recent years, researchers found that intrinsic circadian clocks exist in various peripheral tissues and cell types, directly controlling local physiology and behavior. We are studying the circadian oscillators in the liver and in the vasculature. As the first step, we are investigating the heterogeneity and distinct functions of the central and peripheral oscillators. In particular, we are examining the distinct roles of the retinoid-related orphan nuclear receptors in the clock mechanism. These nuclear receptors were recently identified in our functional genomics studies.

Second, we are asking how environmental cues, mainly light-dark cycles and feeding time, entrain or synchronize peripheral oscillators. Peripheral oscillators most likely are synchronized by hormonal outputs of the suprachiasmatic nucleus or by physiologic inputs such as feeding-mediated metabolic changes. We are using transgenic mice, mice lacking certain genes, and immortalized hepatocytes and vascular smooth muscle cells in these studies along with real-time bioluminescence imaging and biochemical and genetic approaches. We are also using high-resolution bioluminescence imaging to determine whether single neurons in the suprachiasmatic nucleus and peripheral cells are autonomous circadian clocks and to characterize the precise nature of the synchronization of the molecular clockwork of individual cells.

Flowering is a major event in the life cycle of higher plants. Many plants use seasonal changes in the length of days as a signal to flower, and higher plants use their circadian clocks to perceive these changes. Recently, we defined a molecular link between the circadian clock and day length–dependent regulation of flowering. A flowering time gene known as CONSTANS was identified several years ago and is regulated by the circadian clock. We showed that clock regulation of CONSTANS expression is the key to seasonal control of flowering in Arabidopsis. We are extending these studies by comparing gene expression profiles under conditions of long days and short days to identify other components involved in perception of day length.

By combining molecular, genetic, and genomic approaches, we are beginning to define a number of molecular links between the circadian clock and rhythmic regulation of behavior and development. Analysis of circadian rhythms in multiple organisms provides a unique opportunity to define molecular controls for the behavior of whole organisms. These results will provide targets for clinical and agricultural applications to improve the quality of life.

**PUBLICATIONS**


Molecular Mechanisms That Confer Specificity to Nuclear Receptor Signaling

A. Kralli, J. Cardenas, M.B. Hock, R. Emter, J. Villena

Small lipophilic hormones play important roles in mammalian development and physiology and have widespread applications as drugs. These hormones exert their effects by binding and activating nuclear receptors that are ligand-regulated transcription factors. The responses elicited by the activated receptors are remarkably cell-type specific and dependent on the physiologic state of the organism.

We focus on receptor cofactors, such as peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and PGC-1β, that modulate signaling and confer specificity or regulation. Understanding their mechanism of action may reveal novel interventions for activating lipophilic hormones or suppressing or altering their specificity of action.

Glucocorticoids and Response to Stress

The ability of organisms to respond and adapt to stressors is fundamental for life. Response to stress involves activation of the neuroendocrine system and the secretion of adrenal glucocorticoids. Glucocorticoids act via the glucocorticoid receptor to enable mobilization of energy resources, recovery from the stress response, and preparation for future stressors. The transcriptional response mediated by receptors for glucocorticoids integrates the hormonal signal with signals indicating the type of stressor, the physiologic state of the organism, and the cellular environment.

We are studying the molecular mechanisms and regulatory networks that enable the integration of such diverse signals in the activity of glucocorticoid receptors. In particular, we address the function of coactivators, such as PGC-1α, as integrators of such responses. PGC-1α, an inducible coactivator that coordinates transcriptional programs important for energy homeostasis, is a potent, tissue-specific modulator of glucocorticoid receptors. Our findings suggest that a regulatory network consisting of PGC-1α, glucocorticoid receptors, and the orphan nuclear receptor estrogen-related receptor α (ERRα) coordinates part of the stress response.

ERRα and Mitochondrial Biogenesis

ERRα was the first orphan nuclear receptor identified (in 1988), yet we know little about its physiologic function or the mechanisms that regulate its activity. Our studies indicate that ERRα is regulated by PGC-1α at 2 levels. First, PGC-1α induces the expression of ERRα. Consistent with this induction, expression of ERRα is tissue specific and responds to metabolic signals similar to those to which PGC-1α responds (e.g., exposure to cold and fasting). Second, PGC-1α interacts physically with ERRα and enables ERRα to activate transcription.

Our findings suggest that the 2 proteins act in a common pathway to regulate transcriptional responses that control cellular energy metabolism. Indeed, we showed that ERRα is an effector of the transcriptional coactivator PGC-1α and that it regulates the expression of genes involved in oxidative phosphorylation and mitochondrial biogenesis. The genes and pathways controlled by ERRα are downregulated in humans who are insulin resistant, suggesting that changes in ERRα activity could be linked to pathologic changes in metabolic diseases.

Publications


Novel Mechanisms of Cell Adhesion and Cardiovascular Disease


Plasminogen activator inhibitor-1 (PAI-1) is a serine protease inhibitor (serpin) that regulates proteases that remove pathologic fibrin deposits from the vasculature. PAI-1 differs from most other serpins because it is the product of an immediate-early gene and it binds to the adhesive glycoprotein vitronectin. Leptin is synthesized by adipocytes and acts on hypothalamic receptors to reduce food intake and to increase energy expenditure. Juvenile-onset obesity develops in mice that lack functional leptin (ob/ob mice) or its receptor (db/db mice). Results of structure-function, cell culture, and gene-profiling studies and of studies of genetically altered mice provide insights into the mechanisms by which PAI-1, vitronectin, and leptin influence cell adhesion and migration in vitro and angiogenesis and cardiovascular disease in obesity.

The Somatomedin B Domain of Vitronectin

PAI-1 and the urokinase receptor (uPAR, CDB7) bind to the somatomedin B (SMB) domain of vitronectin, and these interactions influence the migratory behavior of cells. Biochemical studies revealed that the 8 cysteine residues of SMB are organized into 4 disulfide bonds in an unusual linear uncrossed pattern: Cys<sup>5</sup>-Cys<sup>9</sup>, Cys<sup>19</sup>-Cys<sup>21</sup>, Cys<sup>25</sup>-Cys<sup>31</sup>, and Cys<sup>32</sup>-Cys<sup>39</sup>. Nuclear magnetic resonance studies done in collaboration with J. Dyson, Department of Molecular Biology, indicated that the 4 disulfide bonds are tightly packed in the center of the domain, replacing the traditional hydrophobic core expected for a globular protein. The few noncysteine hydrophobic side chains form a cluster on the outside of the domain, providing a distinct binding surface for PAI-1 and uPAR. The hydrophobic surface consists mainly of side chains from residues in the loop formed by the Cys<sup>25</sup>-Cys<sup>31</sup> disulfide bond and is surrounded by conserved acidic and basic side chains, which may also contribute to the specificity of the intermolecular interactions of this domain. Unexpectedly, the overall fold of the molecule is compatible with several arrangements of the disulfide bonds. Conformational energy calculations and mutagenesis studies indeed confirmed that multiple disulfide-bond arrangements can satisfy the nuclear magnetic resonance restraints and retain biological activity as long as the Cys<sup>25</sup>-Cys<sup>31</sup> disulfide bond is preserved.

In separate studies, we showed that PAI-1 inhibits uPAR- and integrin-mediated cell attachment to vitronectin by binding to SMB and that it can detach cells from a variety of proteins in the extracellular matrix. The deadhesive effects of PAI-1 are vitronectin independent; they only require the binding of PAI-1 to urokinase bound to uPAR on the cell surface. This latter interaction results in the specific inactivation and internalization of those integrins that engage the extracellular matrix and in rapid cell detachment. PAI-1 can also stimulate cell migration in chemotaxis, haptotaxis, chemokinesis, and wound-healing assays. These motogenic effects of PAI-1 are mediated by the low-density lipoprotein receptor–related protein and appear to involve activation of the Jak/Stat signaling system.

Cardiovascular Disease, Obesity, and Leptin

Obesity in humans is associated with an increased risk for thrombosis and with elevated levels of leptin. In collaboration with K. Schäfer and S. Konstantinides, Georg-August-Universität Göttingen, Göttingen, Germany, we showed that administration of leptin promotes thrombosis in leptin-deficient obese (ob/ob) mice and that inhibiting leptin leads to the formation of unstable thrombi in wild-type mice. Thus, leptin plays a critical role in thrombosis in mice and possibly in obese humans who are hyperleptinemic.

The underlying cause of death in human obesity is atherosclerosis, a chronic wound-healing process that occurs in response to endothelial injury. We found that leptin also directly promotes vascular remodeling and lesion growth in wild-type and ob/ob mice, but not in mice that lack receptors for leptin (db/db mice). Thus, a direct, leptin receptor–mediated link appears to exist between the hyperleptinemia in obesity and the increased risk for atherosclerosis.

Angiogenesis in an In Vivo Model of Adipose Tissue Development

Obesity is associated with an increased risk for cardiovascular disease and cancer. Angiogenesis is a critical component of both of these pathologic processes, and expanding adipose tissue is one of the few sites of active angiogenesis in adults. Despite the potential importance of angiogenesis in obesity, little is known about underlying mechanisms. We examined the angio-
The low density lipoprotein receptor-related protein is a chemotactic receptor.

Critical role of integrin

Increased

Disulfide bonding arrangements in active forms of the somatomedin B domain

Enhanced thrombosis in atherosclerosis-prone mice is associated

_EXPRESSION PROFILING OF GENES RESPONSIVE TO INSULIN IN INSULIN-RESISTANT ADIPOCYTES

We used microarray technology and RNA from normal and insulin-resistant 3T3-L1 adipocytes to identify a new class of genes that respond to insulin. These genes continue to respond normally to insulin even though the adipocytes themselves are metabolically insulin resistant; that is, the adipocytes have a significantly decreased rate of insulin-stimulated glucose uptake. Approximately 12,000 genes or expressed sequence tags were screened. Of these, 40 were identified that became insulin-resistant as expected. However, 61 continued to respond normally to insulin. Although some of these genes were known to be regulated by insulin (e.g., the gene for PAI-1), other novel insulin-sensitive genes also were identified (e.g., the gene for monocyte chemoattractant protein 1/chemokine ligand 2). Real time reverse transcriptase–polymerase chain reaction analysis confirmed the expression patterns of several of the differentially expressed genes.

One gene that remained insulin sensitive in the insulin-resistant adipocytes was the gene for the transcription factor Egr-1. Using an antisense strategy, we showed that genes for tissue factor and macrophage colony-stimulating factor, two cardiovascular risk factors, were targets for Egr-1 in adipocytes. Thus, some signaling pathways clearly remain insulin sensitive in metabolically insulin-resistant adipocytes. These pathways may promote abnormal gene expression in hyperinsulinemic states such as obesity and type 2 diabetes and thus contribute to pathologic changes associated with these conditions.

Reacting Oxygen Species, Antioxidants, and Cell Fate

P.A. Maher, J. Vanhnsy

The tripeptide glutathione is the major endogenous antioxidant in cells and is also a cosubstrate for a variety of antioxidant and antixenobiotic enzymes. A decrease in the intracellular level of glutathione can enhance the susceptibility of cells to oxidative, toxicologic, and pathologic injuries. In the brain, loss of glutathione has been implicated in the death of nerve cells that occurs in Parkinson's disease, after brain injury, and even in the cognitive decline associated with normal aging.

During the past few years, we developed a model of oxidative stress–induced nerve cell death initiated by loss of glutathione. Oxidative stress, which can be defined as an imbalance between the production and removal of reactive oxygen species, is implicated in the pathophysiology of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases and of eye diseases such as glaucoma and macular degeneration. We used our model to identify the critical steps that lead to oxidative stress–induced nerve cell death.

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and to characterize different approaches to neuroprotection. Currently, we are focusing on the mechanisms that regulate glutathione metabolism to determine whether or not these mechanisms can be modulated to protect nerve cells from death induced by oxidative stress. To this end, we identified several small molecules that can maintain glutathione levels in nerve cells in the presence of oxidative stress and prevent cell death.

Glutathione metabolism is quite complex and can be affected by both the levels and the activity of the enzymes involved in glutathione biosynthesis, by the levels of the 3 amino acids that make up glutathione (cysteine, glutamate, and glycine), and by the co-substrates involved in its biosynthesis and regeneration. Several of the small molecules we identified that can maintain glutathione levels in the presence of oxidative stress appear to have both acute and chronic effects on glutathione levels in cells, suggesting that the molecules act at multiple steps of glutathione metabolism.

One novel mechanism whereby these small molecules increase glutathione levels uses a cellular stress response involved in the modulation of protein synthesis. Activation of this stress response correlates with an increase in the levels of critical enzyme required for glutathione biosynthesis. In addition, the small molecules activate a second stress response pathway, which appears to enhance their ability to upregulate glutathione biosynthesis.

The pathways underlying the acute effects of the small molecules on glutathione levels are still under investigation. Nevertheless, these data suggest that low levels of certain types of stress may actually be beneficial to nerve cells because among other actions, the molecules can induce an increase in glutathione levels. This increase can protect nerve cells from severe forms of stress.

Among the small molecules that are most effective at maintaining glutathione levels in the presence of oxidative stress are flavonoids and flavonoid derivatives. Flavonoids are a family of plant-derived, polyphenolic compounds that are widely distributed in fruits and vegetables and therefore regularly a part of the diet in humans. A number of physiologic benefits have been attributed to consumption of flavonoids, including protection from cardiovascular disease and cancer, but the precise mechanisms underlying these effects remain to be determined. Flavonoids are best known for their activities as potent antioxidants and scavengers of free radicals, but, as discussed, they can also increase glutathione levels by mechanisms unrelated to their antioxidant activities. This property, along with additional characteristics that we are exploring, may make flavonoids or flavonoid derivatives particularly effective for the treatment of neurodegenerative and eye diseases associated with oxidative stress.

**Publications**


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**Viral Nanoparticles: Pathogen Inhibitors and Biomolecular Sensors**

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The goal of nanotechnology in biomedical science is to design tiny nanomachines with multiple functions that can be used to detect, target, and treat human diseases in vivo, thereby eliminating the need for invasive diagnostic or therapeutic procedures. Previously, research focused primarily on chemically derived materials such as dendrimers or polymers to develop synthetic nanosized particles (<100 nm in diameter) for biological applications such as molecular therapeutics, tumor targeting, and in vivo biomedical imaging.

We use cowpea mosaic virus (CPMV) as a nanoparticle platform for antivirals, antitoxins, vaccines, and tumor-targeting agents. CPMV is an icosahedral, 31-nm particle that can be 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.
VIRAL NANOPARTICLES AS ANTIVIRALS AND ANTITOXINS

We began working on viral nanoparticles by developing a CPMV-based antiviral that can inhibit the interaction between measles virus and CD46, the cellular receptor for measles virus. Our work with antivirals based on viral nanoparticles led us to hypothesize that nanoparticle inhibitors could also function as vaccines, by inhibiting a pathogen-receptor interaction and simultaneously inducing antipathogen immunity to protect against further exposures. Our viral nanoparticles displaying T-cell epitopes can induce pathogen-specific T-lymphocyte responses in vivo that protect against challenge with the pathogen. We are also developing combination inhibitor-vaccine approaches for other viral and bacterial pathogens.

ORAL BIOAVAILABILITY AND IMMUNOGENICITY OF VIRAL NANOPARTICLES

An important aspect of using plants for the production of nanoparticles is the potential for oral delivery of the particles. CPMV particles are highly stable and can withstand high temperature and low pH, but their stability has never been studied in the harsh environment of the gastrointestinal tract. Surprisingly, we found that viral nanoparticles traffic from the gastrointestinal tract into the bloodstream and reach a variety of tissues in vivo, including spleen, liver, kidney, lymph nodes, lung, and bone marrow. Viral nanoparticles pass through the gut endothelial lining, possibly via M cells that sample particulate matter in the gastrointestinal tract. Interestingly, high concentrations of plant viruses are commonly found in food sources. Although these plant viruses are not infectious for mammalian cells, our results suggest that systemic exposure to plant pathogens via ingestion of infected leaves most likely is common and might have pathogenic or immunologic consequences in certain individuals.

VIRAL NANOPARTICLES AS NOVEL BIOMOLECULAR SENSORS FOR TARGETING CANCER

Upon discovering that viral nanoparticles were orally bioavailable, we investigated the usefulness of the particles as biomolecular sensors for tumor targeting and imaging in vivo. Our goal, in collaboration with M.G. Finn, Department of Chemistry, and J. Johnson and A. Schneemann, Department of Molecular Biology, is to develop viral nanoparticles that can be delivered in a noninvasive manner, home to a tumor in vivo, act as an image-contrast agent for detection by magnetic resonance imaging or other noninvasive imaging techniques, and deliver an antitumor therapeutic or tumor-icidal gene. We are developing targeted viral nanoparticles that are designed to detect, image, and treat tumors in vivo. One set of these viral nanoparticles displays a tumor-targeting peptide called neuropeptide Y that allows the particles to recognize neuroblastoma cells. These nanoparticles recognize the Y1 receptor upregulated on SK-N-MC neuroblastoma cells. A second type of viral nanoparticle is chemically attached to a single-chain antibody to carcinoembryonic antigen; these viral nanoparticles specifically recognize colon carcinoma cells. Viral nanoparticles can also be labeled with molecules such as gadolinium; such labeling allows them to function as image-contrast agents for tumors in magnetic resonance imaging.

PUBLICATIONS


Regulation of Translation and Expression of Human Monoclonal Antibodies in Eukaryotic Algae


Gene expression in chloroplasts is primarily controlled during the translation of plastid mRNAs into proteins. Using genetic and biochemical analysis, we identified RNA elements and their corresponding trans-acting factors required for translation of specific chloroplast mRNAs. Using proteomic and bioinformatics analyses, we identified many of the proteins that function in chloroplast translation, including the complete set of plastid ribosomal proteins and the general initiation and elongation factors. These analyses indicated that the translational apparatus of chloroplasts is related to that of bacteria, but is more complex.

Chloroplast mRNAs contain ribosome-binding sequences, but these sequences are positioned far upstream of their bacterial counterparts. Chloroplasts contain all of the general translation factors found in bacteria, and most of the ribosomal proteins are conserved between plastids and bacteria. However, chloroplasts contain a number of additional proteins and
protein domains associated with the plastid ribosome, and some ribosomal proteins are either quite divergent or missing.

Using these proteomic and genetic analysis, we identified the set of components, both proteins and RNA, required for chloroplast translation. To understand how these components interact to provide regulated mRNA translation, we are using electron cryomicroscopy and single-particle reconstruction analysis of the chloroplast ribosome and RNA-protein complexes to analyze the structure of the components. These studies should provide the structural basis for understanding the molecular and biochemical interactions between mRNA and ribosomes during translation (Fig. 1).

On the basis of our understanding of chloroplast translation, we developed a system to express a wide array of recombinant proteins, including those used as human therapeutic agents. For example, we constructed strains of *Chlamydomonas reinhardtii* that express variants of monoclonal antibodies directed against herpes simplex virus. These antibodies assemble in the cell to form fully functional antibodies that bind herpes simplex proteins. Antibody binding to the coat protein of herpes simplex virus can block viral propagation. We also showed that this alga-based system can be used to produce a number of proteins with potential human therapeutic value. These studies indicate that eukaryotic algae have tremendous potential for the expression of recombinant human therapeutic proteins, because algae can be grown economically on a very large scale.

Using the transgenic strains we generated, we are assessing the effect of various factors on the accumulation of recombinant proteins. We found that chloroplast ribosomes preferentially translate endogenous chloroplast mRNAs over heterologous mRNAs. Increasing the translatability of heterologous mRNAs is now a primary focus of our studies, one that should lead to a greater understanding of the mechanism of chloroplast translation and to higher levels of expression of therapeutic proteins.

**PUBLICATIONS**


**Molecular Basis of Cognitive Function and Dysfunction**


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 mental retardation to psychiatric disorders such as schizophrenia with onsets in late adolescence and early adulthood to diseases of aging such as Alzheimer’s disease.

We use genetic manipulation in mice to investigate the molecular events involved in learning and memory. Of the genetically accessible experimental organisms, mice are the most similar to humans in both genetic makeup and brain structure, so that insights gained in...
mice most likely will be applicable to humans. Moreover, understanding the genes involved in a process can identify molecular targets that might be amenable to therapeutic intervention.

**CALCIUM SIGNALING AND MEMORY**

We know relatively little at a molecular level about how the brain stores new information. One hypothesis, which we tried to test, is that calcium-regulated changes in the strength of synaptic connections between nerve cells can store information. We generated 2 different types of mutant mice in which calcium signaling molecules are altered either at the synapse or in the cell nucleus. The enzyme calcium/calmodulin-dependent protein kinase is abundant at synapses and when activated by calcium can alter the strength of synaptic connections. We used genetic manipulations to indiscriminately activate this kinase at all synapses in the entorhinal cortex, a part of the brain that is important for memory and is affected in the earliest stages of Alzheimer’s disease. We found not only that the formation of new memories is impaired but also that previously established memories are 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.

The formation of stable long-lasting memories requires not just the modification of preexisting proteins in neurons but also the expression of new genes in the nucleus of neurons. We generated mutant mice in which the general calcium signaling molecule calmodulin was specifically inhibited in the nucleus to test the role that this signaling plays in long-term memory storage. We found that although these mice could learn and remember new information, the memory did not last more than a few hours. This finding suggests that calcium activation of new gene expression is required for the conversion of short-term to long-term memories.

**GENETIC MODELS OF DISEASE**

The recent determination of the complete sequence of the mouse and human genomes indicates that humans are highly similar to mice at the genetic level. One approach to understanding genetic diseases in humans is to introduce the same mutations into mice to produce models of the diseases for better understanding of the molecular pathology and for testing possible treatments.

Rubinstein-Taybi syndrome is a developmental and cognitive disorder due to a mutation in the gene **CBP**.

We produced a strain of mice with a defect in **CBP** and found that the mice had impairments in several learning and memory tasks. More important, we showed that these impairments were not due to problems in development of the brain because they could be reversed by providing a normally functioning **CBP** gene to adult mice. The protein encoded by **CBP** chemically modifies histones to allow the expression of a large variety of other genes. We found that the memory deficits in the mice could be reversed by treatment with a drug that targets this histone-modifying function, suggesting a possible treatment for this and possibly other cognitive disorders.

**PUBLICATIONS**


### Regulation of Hepatitis B Virus Replication

**A.L. Anderson, C.E. Oropeza, A. McLachlan**

**H**epatitis B virus (HBV), a major human pathogen, is a 42-nm particle with an enveloped 28-nm nucleocapsid that contains the partially double-stranded 3.2-kb DNA genome. The HBV genomic DNA in the virion is synthesized in the nucleocapsid by the reverse transcription of a greater-than-genome-length pregenomic 3.5-kb RNA. This process of viral replication is unique to the hepadnaviruses and involves several partially defined steps.

Transcription of covalently closed circular HBV DNA from the nucleocapsid promoter proceeds through the polyadenylation signal sequence on the initial passage of the cellular RNA polymerase around the viral genome, generating the 3.5-kb pregenomic RNA. The 3.5-kb pregenomic RNA is translated to produce the core and polymerase polypeptides. The HBV polymerase binds to a stem-loop structure designated **ε** at the 5’ end of the pregenomic RNA. The complex of pregenomic RNA and HBV polymerase is subsequently incorporated into the viral nucleocapsid, which is composed of core polypeptide subunits.

The polymerase synthesizes the first 3 nucleotides of the HBV minus-strand DNA by using a tyrosine residue in the amino-terminal region of the polymerase molecule as a primer and the bulge region of **ε** as a template. The polymerase and the covalently attached...
3 nucleotides are translocated from ε at the 5′ end of the pregenomic RNA to a complementary sequence present in the DR1 element located at the 3′ end of the pregenomic RNA. Regulation of this translocation step is poorly understood, but it clearly requires more than the simple complementarity between the donor sequence in the ε bulge and the acceptor sequence in the DR1 element. The pregenomic RNA may assume a conformation that brings these sequences into proximity so that the translocation step can be readily achieved. Alterations in the conformation of ε and the polymerase molecule might also be associated with this process. After the polymerase translocates to the DR1 sequence at the 3′ end of the pregenomic RNA, minus-strand synthesis can proceed by reverse transcription of the pregenomic RNA.

We identified a sequence element designated ϕ that is important for efficient viral replication. This 19-nucleotide sequence is located 32 nucleotides upstream of the DR1 element and is complementary to ε. Consequently, it appears most likely that this sequence element enhances the efficiency of viral replication by juxtaposing the HBV polymerase with the covalently attached 3 nucleotides located at the bulge region of ε with the DR1 sequence present at the 3′ end of the pregenomic RNA. By generating an alternative RNA secondary structure element between ε and ϕ sequences, the ϕ sequence could enhance the efficiency of the initial translocation step required for the elongation step in minus-strand DNA synthesis.

The observation that similar secondary structures can form between ε and ϕ sequences in woodchuck hepatitis virus and duck HBV pregenomic RNA further supports the suggestion that the ε sequence is an important element involved in mediating this translocation step in the hepadnavirus replication cycle. Small molecules that could inhibit the formation of the alternative RNA secondary structure involving ϕ would be a potentially important new class of antiviral drugs against chronic HBV infection, which affects approximately 300 million persons worldwide.

Regulation of the Plasminogen Activation System

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Assembly of plasminogen and plasminogen activators on cell surfaces is a key control point for positive regulation of cell-surface proteolytic activity necessary in physiologic and pathologic processes that require cell migration. We tested the hypothesis that plasminogen binding and activation increase during the monocyte-macrophage differentiation pathway as a mechanism to arm these cells with the proteolytic activity of plasmin during inflammation. We examined the relationship between monocyte differentiation and plasminogen binding by using a monocyte progenitor cell line in which differentiation is conditionally regulated by Hoxa9 under the control of the estrogen receptor promoter. The Hoxa9-ER4 cell line is derived from conditionally immortalized murine bone marrow precursors, is dependent on granulocyte-monocyte colony-stimulating factor, and differentiates to monocytes when estrogen is removed from the medium, therein inactivating the Hoxa9 protein. The mature monocytes respond to monocyte colony-stimulating factor. Thus, changes in specific phenotypes can be identified at discrete steps in monocytoid differentiation.

Using fluorescence-activated cell sorting, we examined binding of plasminogen to viable Hoxa9-ER4 cells at distinct stages of differentiation. In the presence of estrogen, Hoxa9-ER4 cells had low cell-surface expression of monocytoid markers. After withdrawal of estrogen and treatment with monocyte colony-stimulating factor, induction of monocytoid markers occurred, consistent with differentiation into the macrophage phenotype. As the cells differentiated, specific plasminogen binding to the viable cells increased with time, reaching a 50-fold increase by 2 days.

Thus, in controlled highly synchronous differentiation to macrophages, receptors for plasminogen were markedly upregulated. Furthermore, treatment of viable human peripheral blood monocytes with monocyte colony-stimulating factor increased the number of plasminogen-binding sites 5-fold, with an increase in affinity of approximately 10-fold. Thus, increased plas-
Enolase comes muscling in on plasminogen activation. Plasminogen binds to monocytic cells is also markedly enhanced after induction of apoptosis. Previously, we identified a major plasminogen-binding protein, TIP49a, that exposes a carboxyl-terminal lysine on the surfaces of monocytes and U937 cells and increases its membrane expression after induction of apoptosis. Therefore, we tested the hypothesis that TIP49a binds plasminogen on both viable and nonviable cells.

To probe TIP49a function, we developed and characterized a monoclonal antibody that reacts with the carboxyl terminus of TIP49a. An Fab fraction of the antibody reacted with the peptide in enzyme-linked immunosorbent assays, but not with a control peptide corresponding to an internal sequence of TIP49a. Plasminogen bound to the carboxyl-terminal peptide, and the monoclonal antibody inhibited the interaction by 90%. The monoclonal antibody also inhibited cell-dependent plasminogen activation, and the extent of inhibition depended on the concentration of cells.

Using fluorescence-activated cell sorting with the monoclonal antibody and propidium iodide, we verified that TIP49a was present on the surfaces of viable U937 cells. After treatment with apoptosis inducers, TNF-α, or cycloheximide, TIP49a was also detected on the nonviable subpopulation of cells. The antibody to TIP49a reduced plasminogen binding to viable and nonviable cells by 30% and 31%, respectively. These results suggest that TIP49a promotes plasminogen activation on the surface of monocyteic cells by binding plasminogen and are consistent with the idea that more than one plasminogen-binding protein contributes to plasminogen binding and activation on both viable and nonviable cells.

**PUBLICATIONS**


**Structure and Action of Molecular Machines**


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Macromolecular assemblies may be composed of from 2 to perhaps scores of proteins and are the functional units—the molecular machines—of the cell. We use electron cryomicroscopy and image analysis to study the structure and mechanism of action of several of these machines. We combine the 3-dimensional maps calculated from electron images of the machines with biochemical data and high-resolution x-ray structures of the individual components to provide insight into the operation of the machines. During the past year, we examined the mechanisms of microtubule stabilization and destabilization by several proteins and continued our work on backward-moving kinesins and myosins, VCP/p97, bacterial toxins, and membrane proteins.

Microtubule-associated protein 2c (MAP2c) and tau, neuronal microtubule-stabilizing proteins, are unstructured in solution but become ordered upon binding to microtubules. We showed that both MAP2c and tau bind longitudinally along the outer ridges of microtubule protofilaments, overlapping the kinesin- and dynein-binding sites. These results suggest that microtubule stabilization by these proteins is accomplished by strengthening the interactions along protofilaments. Our data also suggest that evolutionarily maintained differences in the microtubule-binding repeats in the proteins may be important for specific targeting of different repeats to either α- or β-tubulin. An examination of MAP2c and tau binding to microtubules and F-actin indicated that MAP2, but not tau, binds and bundles F-actin via its microtubule-binding domain. These findings distinguish MAP2 and tau in terms of their ability to organize cytoskeletal filaments and suggest that interactions with actin could contribute to functional differences between MAP2 and tau in neurons.

The protein doublecortin is expressed in migrating and differentiating neurons. In humans, mutations in this protein disrupt brain development, causing lissen-
cephaly. Although doublecortin is associated with and stabilizes the microtubule cytoskeleton, it has no homology with other microtubule-binding proteins such as MAP2 or tau. We found that doublecortin preferentially nucleates and binds to 13-protofilament microtubules. This specificity was explained when we discovered that the protein binds in the valleys between the protofilaments of the microtubule wall. This binding site is unique and appears to be ideally located for microtubule stabilization. In this location, doublecortin most likely contributes to both the longitudinal and the lateral interactions that stabilize the microtubule wall.

Whereas KinN and KinC kinesins move along intact microtubules, members of the KinI kinesins destabilize and depolymerize microtubules and do not appear to have motile properties. We found that a KinI fragment consisting of only the conserved motor core is necessary and sufficient for ATP-dependent depolymerization. The motor core binds along microtubules in all nucleotide states, but in the presence of a nonhydrolyzable ATP analog, depolymerization also occurs. Structural characterization of the analog-induced depolymerization products provided a snapshot of the disassembly machine at the microtubule ends.

Our data indicate that whereas conventional kinesins use the energy of ATP binding to execute a power stroke that results in unidirectional motion along the microtubule surface, KinIs at the ends of microtubules use the energy to bend the underlying protofilament, thereby destabilizing the microtubule lattice and leading to microtubule depolymerization. Furthermore, when the motor core is associated with the microtubule wall, the core is stalled in a weakly bound, nucleotide-free state. Progression to the strongly bound, ATP-containing state is only possible when the KinI encounters a microtubule end, where it can catalyze deformation of protofilaments and disassembly of microtubules. The unusual mechanochemical coupling of this kinesin provides an elegant mechanistic basis for its microtubule-depolymerizing activity.

Although the mechanism of plus end–directed, progressive motion by the conventional KinN kinesins is now well understood, the mechanism by which members of the KinC kinesins (e.g., Ncd) move toward the minus ends of microtubules is not. Likewise, in the myosin superfamily, how nucleotide-mediated conformational changes in the motor domain of class VI myosins result in “backward” motility is not known. We are elucidating the molecular mechanisms of these more unusual members of the myosin and kinesin superfamilies. (Movies showing the motions of conventional myosin and kinesin can be viewed at www.scripps.edu/milligan/projects.html.)

We extended our studies on VCP/p97, a member of the AAA ATPase family of proteins. This protein is involved in a wide variety of cellular processes, including organelle assembly, homotypic membrane fusion, and protein degradation. We examined VCP/p97 in various nucleotide states by using electron cryomicroscopy and single-particle image analysis. The resulting 3-dimensional maps of the hexameric protein assembly show that it undergoes substantial conformational changes during the ATPase cycle. Nucleotide-dependent rearrangements of the subunits are accompanied by constriction of the central channel opening and changes in the interaction geometry of the N-terminal domain of the protein.

We developed a general method for helical crystallization of proteins on lipid tubules that we are using to study the virulence factor PFO from Clostridium perfringens. PFO is a cytolysin, an important class of proteins that oligomerize and embed within membranes as part of their lytic function. We obtained helical crystals of wild-type and several mutant forms of PFO on nickel-lipid tubules. Three-dimensional maps of these proteins derived from images of the helical crystals will be used to compliment our studies of PFO pore formation on lipid layers. These studies will provide a better understanding of the pathogenic function of cytolysins. Additional studies involving tubular crystallization of membrane proteins and other bacterial toxins are opening up promising new areas for future research.

PUBLICATIONS


Molecular Mechanisms of CNS Development and Mechanosensory Perception


A disproportionately large number of genes in the genomes of vertebrates encode cell recognition molecules that mediate cell-cell interactions and interactions between cells and the extracellular matrix. This finding most likely reflects an evolutionary trend toward increasingly more complex cellular interactions in higher metazoans. The highest diversity of such interactions occurs in the CNS, where thousands of different neuronal subtypes are connected into defined neuronal circuits. We use mouse genetics, genomics, cell biology, biochemistry, and imaging technology to analyze the function of cell recognition molecules in the CNS in developing and adult mice. In another project, we are elucidating the mechanisms by which cell recognition molecules contribute to mechanosensory perception.

INTEGRIN FUNCTIONS IN THE CNS

The establishment of the 3-dimensional cytoarchitecture of the nervous system depends on interactions of receptors on neuronal cells with molecules presented within the extracellular matrix and by neighboring cells. Integrins are a class of neuronal receptors that mediate interactions with glycoproteins secreted by the extracellular matrix and with membrane-anchored counterreceptors. Originally identified as adhesion-promoting molecules, integrins were anticipated to have a rather static role, providing a mechanical link between extracellular ligands and the interior of the cell. It is now clear that integrins also have active roles in signal transduction. We analyzed integrin functions in the CNS genetically in mice by using both conventional and conditional gene inactivation.

Our data indicate that integrins have important functions in the formation of the cerebral and cerebellar cortex. The cortical defects in integrin-deficient mice resemble the pathologic changes that occur in humans who have lissencephaly, Walker-Warburg syndrome, and muscle-brain-eye disease. These abnormalities are associated with peripheral neuropathy and muscular dystrophy. Our recent results in mice indicated that development of peripheral nerve and muscle is also regulated by integrins, suggesting that defects in interactions between cells and the extracellular matrix are at the core of the pathologic changes in humans. We are investigating the integrin-dependent signaling mechanisms important for CNS development and functional interactions of integrins with other receptors and secreted signaling molecules in the CNS.

CELL RECOGNITION MOLECULES, MECHANOSENSORY PERCEPTION, AND USHER SYNDROME

Mechanosensation, the transduction of mechanical force into an electrochemical signal, allows living organisms to detect touch, hear, register movement and gravity, and sense changes in cell volume and shape. In mammals, the hair cells of the inner ear are the principal mechanosensors for the detection of sound and movement. Hair cells elaborate stereocilia that contain mechanosensitive ion channels. The stereocilia of a hair cell are interconnected by extracellular bridges into a bundle and are situated next to specialized extracellular matrix assemblies. Sound waves or head movements lead to deflection of the stereocilia bundle, changes in the ion permeability of the mechanosensitive channels, and depolarization of the hair cells. The molecules that regulate development and function of hair cells are poorly defined.

Because defects in hair cells cause inherited forms of deafness, we use human and mouse genetics as a guideline to identify and study molecules that regulate the development and function of mechanosensory hair cells. Currently, about 50 genes have been identified in which mutations lead to deafness. Many of these genes encode membrane-anchored cell recognition molecules and molecules secreted by the extracellular matrix. Mutations in the putative gene for the cell adhesion molecule cadherin 23 lead to Usher syndrome, the leading cause of deaf-blindness in humans. Mice with mutations in the gene for this adhesion molecule have stereocilia defects, suggesting that cadherin 23 regulates the function of stereocilia. Our recent results indicate that cadherin 23 is a component of the tip link in hair cell stereocilia that has been proposed to gate mechanotransduction channels. We are studying...
the mechanism of action of cadherin 23 during mechano-
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Molecular Mechanisms of
Sensory Neuron Specification
and Function

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The sensory nervous system in vertebrates enables
fine detection of varied external information. The
sense of touch consists of the perception of mul-
tiple discrete types of stimuli, including temperature,
pain, position, and pressure. A great deal remains
unknown about the molecular pathways that lead to the
diversity of somatic neurons that sense “touch” and the
ability of the neurons to detect various stimuli. We are
using genomics, imaging, and transgenic technologies
to identify and characterize proteins involved in the
development and function of these sensory neurons.

A major effort is devoted to identifying molecules
that play important roles in the actual detection of
various mechanical and thermal stimuli. One molecule
involved in such detection is vanilloid receptor 1, which
is activated by noxious heat. We cloned novel transient-
receptor-potential channels related to vanilloid recep-
tor 1 that are expressed in sensory neurons. One of
these, TRPM8, is the first channel known to be acti-
vated by cold temperatures and by a cooling agent,
menthol. We are characterizing this and other transient-
receptor-potential channels relevant to the sensory
nervous system.

We are also interested in the mechanisms by which
sensory neurons become restricted in function, so that
some neurons sense only cold temperatures, whereas
others sense only mechanical stimulation. Neurotrophins
and their Trk receptors are great candidates for a role
in this process. We are using (1) transgenic mice that
express genes for neurotrophins to show that neurotro-
phins play an instructive role during sensory neuronal
development and (2) neurophin-deficient mice and
expression array methods to identify genes uniquely
expressed in distinct classes of sensory neurons in
dorsal root ganglia.

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Functional Proteins in Tumor
Metastasis and Angiogenesis

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We established a number of in vivo model sys-
tems that can recapitulate the major cellular
and tissue events that occur during tumor
metastasis and angiogenesis. The model systems
allow quantitative measurements, microscopic analysis in
real time, biochemical and immunologic probing, and
direct molecular intervention. Recently, use of short
interfering RNA molecules directed against specific
expressed genes provided insights into the contributory
role of these gene products in tumor dissemination. In
addition, use of subtractive immunization, which is
used to generate unique neutralizing monoclonal anti-
bodies, in combination with immunoproteomics enables
us to identify specific antigenic molecules that are func-
tionally active in metastasis and angiogenesis.
**METASTASIS**

Selected human tumor cells inoculated onto the chorioallantoic membrane of developing chick embryos form primary tumors on the membrane in 4–7 days. A small percentage of the cells in the primary tumor disseminate through the vasculature and within 3–4 days, arrest and proliferate in secondary organs of the embryo. Measuring a small number of early-arriving metastatic cells (<200) growing and expanding 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 chick embryo. Chicken DNA contains no Alu sequences, so any product generated by the polymerase chain reaction indicates that human tumor cells are present in the 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 measure 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 small interfering RNA constructs to delete specific genes that might contribute to metastatic dissemination. Inoculating monoclonal antibodies directly into tumor-bearing embryos and monitoring the influence of the antibodies on metastasis are also part of our screening procedures.

We are also using a more conventional method of monitoring human tumor metastasis in immunodeficient mice. However, compared with our chick embryo metastasis assay, this method is less quantitative, requires more time (3–5 weeks), and is more difficult to use for molecular intervention. We are using the mouse metastasis assay to confirm the efficacy of various effector molecules and inhibitors that initially are identified in the chick embryo metastasis assay.

Using subtractive immunization, we generated several unique antimetastatic monoclonal antibodies. One of the antibodies recognizes an antigen identified as PETA-3/CD151, a member of the tetraspanin family of proteins. Our results indicate that PETA-3 is a transmembrane cell-surface protein that contributes to the metastatic phenotype by mediating the motility of the disseminating tumor cells. Another of the antimetastatic monoclonal antibodies was used as a tool in a functional proteomics approach to identify a novel tyrosine-phosphorylated glycoprotein that contributes to the metastatic process. This 135-kD cell-surface molecule, which we termed subtractive immunization metastasis-associated antigen (SIMA), is being biochemically and mechanistically characterized. In addition, we are examining the appearance and expression levels of these 2 specific antigens in human cancer tissue in order to link them with the progression of malignant cells.

**ANGIOGENESIS**

One of the most commonly used in vivo assays for angiogenesis is the chick embryo chorioallantoic membrane assay. We developed a quantitative variation of this assay that allows detection and measurement of the 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 characterized this specific proteolytic event and found that specific collagen-cleaving metalloproteinases are implicated directly in vessel outgrowth.

We are examining a variety of defined metalloproteinase-deficient mice and mice with specific mutations in the gene for collagen to determine their capabilities for outgrowth of blood vessels. This approach should suggest and define the rate-limiting proteolytic enzymes and their substrates in angiogenic tissue remodeling.

We are also using an in vitro model system for the formation of endothelial tubes to identify key regulatory molecules in angiogenesis. We examined a group of membrane-anchored serine proteases and secreted serine proteases for their differential mRNA expression in this vascular tube–forming assay and also in samples of human tissue. A number of unexpected serine proteases became circumstantially linked to angiogenesis and are being cloned, expressed, and characterized. In addition, we are using subtractive immunization, which worked so well in the metastasis system, in the angiogenesis model system. The results indicate that the technique can be used to identify specific antigens that function in the formation of blood vessels.

**PUBLICATIONS**


Regulation of Clathrin-Mediated Endocytosis


Clathrin-mediated endocytosis is essential for the efficient uptake of nutrients and other macromolecules into cells and for the regulation of signaling by cell-surface receptors. The process occurs at clathrin-coated pits, which concentrate receptor-ligand complexes, deform the membrane, invaginate, and eventually pinch off, forming clathrin-coated vesicles (CCVs). The major components involved in the formation of CCVs are clathrin, adaptor proteins, and dynamin.

Clathrin self-assembles into a polygonal lattice and serves as a scaffold for the formation of coated pits. Adaptor protein-2 is a heterotetrameric protein that triggers clathrin assembly at the plasma membrane and interacts directly with the cytoplasmic tails of surface receptors to concentrate the receptors into the assembling coated pit. Dynamin is a multidomain GTPase that regulates endocytosis. Each of these proteins also interacts with a myriad of accessory proteins, whose function in the formation of CCVs is poorly understood. Our goals are to define the minimum molecular machinery required, determine the role of accessory proteins, define the hierarchy of the interactions and molecular events that lead to the formation of CCVs, and understand how these interactions are regulated within the cell.

Taking a biochemical approach, we developed a cell-free assay that faithfully reconstitutes the formation of CCVs. We are using cytosolic fractionation to identify the minimum machinery required. More recently, in collaboration with C. Waterman-Storer, Department of Cell Biology, we are examining the dynamics of the assembly of clathrin-coated pits and the formation of CCVs in living cells. We are using total internal reflection fluorescence microscopy to selectively image clathrin dynamics in a narrow strip (~100 nm wide) near the bottom surface of cells. These studies revealed marked heterogeneity in coated pit dynamics and allowed us to directly visualize the effects of perturbing molecular interactions on clathrin dynamics and endocytosis.

In studies on the regulation of endocytosis, we recently identified a kinase, called adaptor-associated kinase-1, that phosphorylates the adaptor protein-2 complex and regulates its interactions with cargo molecules in vitro. This past year we found that activity of the kinase is stimulated by assembled clathrin. This observation suggests a model in which clathrin does not act simply as a passive scaffold but plays a more direct role in cargo selection by coordinating the activation of interactions between adaptor protein-2 and cargo with the assembly of coated pits.

Ample evidence supports an essential role for dynamin in endocytosis; however, the exact function of dynamin remains uncertain. Dynamin is an atypical GTPase with a low affinity for GTP, a high rate of GDP dissociation, and a relatively high intrinsic rate of GTP hydrolysis, which is further stimulated by self-assembly. Although these properties have been extensively studied in vitro, the roles of self-assembly, GTP binding, and/or hydrolysis in endocytosis are not firmly established. Therefore, we continue to focus considerable effort on elucidating the cellular function of dynamin.

Previously, we identified a domain in dynamin, termed the GTPase effector domain, that mediates dynamin assembly and has activity as an assembly-dependent GTPase-activating protein. Examination of new mutations in the domain indicated that its assembly properties are located in an amphipathic α-helix near the N terminus of the domain. One mutation completely disrupts self-assembly and generates a new class of dominant-negative dynamin mutants, establishing that self-assembly is required for dynamin function in vivo.

We also generated a series of GTPase domain mutants to probe the mechanism of GTP hydrolysis and to further test the role of GTP binding and/or hydrolysis in endocytosis. These mutants had differential and graded effects on GTP binding and hydrolysis. Analysis of transferrin endocytosis rates in cells overexpressing dynamin GTPase mutants revealed a strong correlation with the both the basal and the assembly-stimulated rates of GTP hydrolysis and a more complex correlation with the calculated ratio of free dynamin to dynamin.
complexed to GTP, suggesting that GTP binding is not sufficient and that GTP hydrolysis is required for clathrin-mediated endocytosis in vivo.

Further insight into the function of dynamin in endocytosis in vivo was derived from collaborative studies with M. Ramaswami, University of Arizona, Tucson, Arizona. Point mutations in shibire, the gene for the Drosophila homolog of dynamin, cause temperature-sensitive defects in endocytosis in fruit flies. One of these mutations, the ts2 mutation, occurs in the switch 2 region of dynamin's GTPase domain, which in other GTPases is involved in interactions with GTPase-activating proteins. We generated the ts2 mutation in human dynamin and showed that the gene product, dyn1:ts2, was defective in GTP binding, resulting in reduced assembly-stimulated GTPase activity at physiologic concentrations of GTP.

Researchers in the Ramaswami laboratory screened for suppressors of shibire (termed sushi) and identified 2 second-site sushi mutations located within the GTPase effector domain that fully rescue the defects in endocytosis associated with the ts2 mutation. Surprisingly, we found that this functional rescue was accompanied by further reduction in both the basal and the assembly-stimulated rates of GTP hydrolysis. These findings establish that, as for other members of the GTPase superfamily, the function of dynamin in vivo is negatively regulated by dynamin's activity as a GTPase-activating protein, which is encoded in the GTPase effector domain.

PUBLICATIONS
Molecular Regulation of Vascular System Development

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The cardiovascular system is the first organ system to develop in mammalian embryos. Establishing a functional circulatory system is crucial for delivery of nutrients and oxygen to the embryos, and defects result in death before birth or in congenital cardiovascular anomalies. Mouse embryos are the premier model system for studies of cardiovascular development, because detailed information on mouse embryology is available and the mouse genome can be manipulated at will. However, our understanding of the molecular mechanisms that control the formation of the heart, blood, and lymphatic system is still incomplete. Previously, we used a genetic approach to screen for novel genes involved in these processes.

A gene expressed in endothelium, Vezf1, encodes a 56-kD nuclear transcription factor. We hypothesize that Vezf1 functions in a strictly dose-dependent fashion to regulate vascular development. Using molecular genetic approaches, including studies in Vezf1-deficient mice and transgenic mice, we found that Vezf1 is essential for remodeling, integrity, and proliferation of the vascular endothelium. Embryos deficient in Vezf1 or with reduced or increased levels of Vezf1 expression have vascular and lymphatic endothelial abnormalities and hemorrhaging, and they die during midgestation. Of interest, the lethal phenotype in mice lacking the gene can be at least partially rescued by endothelial overexpression of Vezf1.

In a reductionist approach, we are studying defects in mutant embryonic endothelial cells and fibroblasts to determine the molecular pathways for Vezf1. We also identified factors that bind specifically to the promoter that are known to regulate endothelial and hema-

topoietic genes. Of interest, our preliminary studies indicate that Vezf1 does not function through the canonical vascular signaling pathways via vascular endothelial cell growth factor, angiopoietin, ephrin, or Notch. Currently, we are using subtractive hybridization and cDNA microarrays to screen for target genes that are directly regulated by Vezf1.

Embryos heterozygous at the Vezf1 locus have lymphatic abnormalities reminiscent of cystic hygromas, a human congenital syndrome of unknown etiology. We are using gain- and loss-of-function approaches to dissect the function of Vezf1 in the lymphatic endothelium, and we are determining if cystic hygromas have mutations in Vezf1.

A second endothelial gene identified in our screen, Egfl7, encodes a 30-KD secreted protein with 2 internal epidermal growth factor–like domains. We hypothesize that Egfl7 function is crucial for endothelial differentiation and homeostasis. Thus, Egfl7 expression is restricted to the embryonic vascular endothelium and to endothelial progenitor cells in the yolk sac. In the quiescent vasculature in adult mice, expression of Egfl7 is low but becomes upregulated during vascular injury and endothelial regeneration, during pregnancy in the uterus, and during tumor angiogenesis.

We are using the mouse model system and gain- and loss-of-function approaches to determine the role of Egfl7 during development and during angiogenesis in adults. We are also using the chick chorioallantois and nude mouse models to study the role of Egfl7 in tumor angiogenesis. We are determining if the gene product EGFL7 acts as a growth factor or a cytokine through direct binding to receptors or through interaction with proteins in the extracellular matrix. Of interest, EGFL7 specifically interacts with Notch in a yeast 2-hybrid assay, suggesting a role for Egfl7 in Notch signaling, possibly by acting as an antagonist to the known membrane-bound ligands of Notch. In support of this hypothesis, EGFL7 stimulates migration of endothelial cells and embryo fibroblasts but not vascular smooth muscle cells.

Using an approach in which we added an autofluorescent reporter gene, the gene for enhanced green fluorescent protein, into the mouse Egfl7 gene, we marked the endogenous Egfl7 locus. This approach will enable us to isolate populations of endothelial progenitor cells and to study the potential of the cells to contribute to or regenerate to vascular tissues in embryos and adults.
Centromeres, Kinetochores, and Chromosome Dynamics in Human Cells

K. Monier, A. Visser, K. Sullivan

During each cell cycle, the genome is packaged for transport on the mitotic spindle, the replicated chromosomes are segregated, and the nucleus is reformed to resume its normal program of gene expression. We are interested in how chromosome segregation is accomplished in mitosis and in how the large-scale organization of the nucleus is set up in postmitotic cells.

Chromosome segregation during cell division is mediated by centromeres (one on each chromosome), the structures that direct formation of the kinetochore, the microtubule-binding structure, at the surface of the chromosomes in mitosis. In metazoan chromosomes, the position and molecular composition of the centromere appear to be determined largely through epigenetic mechanisms. A centromere-specific histone H3 homolog, CENP-A, replaces normal histone H3 at centromeres and may provide a mechanistic basis for epigenetic centromere specification. Understanding how CENP-A assembles at centromeres is thus a key step in understanding centromeres. Endogenous CENP-A is expressed in the G2 phase of the cell cycle, and previously we showed that CENP-A can assemble into kinetochores in the absence of DNA replication. To determine the precise timing of CENP-A assembly, we microinjected (1) CENP-A labeled with green fluorescent protein and (2) other histones into synchronized HeLa cells. Fluorescent CENP-A was detectable within 1–2 hours as a faint nucleoplasmic staining in nucleoli and/or as brighter foci that did not correspond to centromeres. However, 4 hours after injection, fluorescent CENP-A became concentrated in centromeres irrespective of the phase of the cell cycle.

The time lag between CENP-A synthesis and assembly suggests that CENP-A must be modified before it accumulates at centromeres or that the centromeres must be made accessible for CENP-A. CENP-A also assembles into the chromosome arms under these conditions, suggesting that it can use general machinery for chromatin assembly. The preferential assembly into centromeres also suggests that at least some centromere-specific assembly factors are resident at centromeres. We are identifying those factors to determine how CENP-A is incorporated uniquely at centromeres in a normal cell cycle.

Posttranslational modification of histone subunits in chromatin is a major mechanism for regulating a variety of chromosomal functions. Mitotic phosphorylation of histone H3 and of CENP-A is mediated by Aurora B kinase. Aurora B kinase activity begins in pericentric regions of the chromosomes during the G2 phase, and these sites are fully phosphorylated before Aurora B acts on general chromatin in the chromosome arms. Using in situ hybridization, we found that phosphorylation of pericentric histone H3 does not occur at random, but preferentially starts at certain chromosomes. Immunocytochemical analysis of a number of heterochromatin markers indicated that DNA methylation is the most highly correlated feature of initiating pericentric chromatin. The analysis also indicated that Aurora B can accumulate without phosphorylation of the underlying histone H3, suggesting an activation step that is required for activation of the kinase. Disruption of DNA methylation with 5-azacytidine or antisense suppression of DNA methyltransferase 1 disrupts the normal progression of pericentric histone phosphorylation. Taken together, these results suggest that one role for DNA methylation is to organize the activation of Aurora B kinase in preparation for mitosis. Pericentric heterochromatin may function as an assembly site for the active enzyme, which then acts elsewhere in the nucleus. Because both DNA methylation and Aurora B kinase are dysregulated in tumor cells, this pathway could be important in carcinogenesis.
Ion Channels and Fast Synaptic Transmission

N. Unwin, B. Sheehan

Ion channels play a central role in the rapid transmission of electrical signals throughout the nervous system. To determine how these membrane proteins work, we are using electron microscopy to analyze their structures trapped in different physiologic states. Current studies center on the nicotinic acetylcholine receptor at the nerve-muscle synapse. We wish to find out how this ion channel achieves its ion selectivity and high transport rate and how it opens and desensitizes in response to acetylcholine released into the synaptic cleft. For our studies, we use postsynaptic membranes isolated from the (muscle-derived) electric organ of a Torpedo ray, which form tubular crystals of acetylcholine receptors.

The acetylcholine receptor is a member of a superfamily of transmitter-gated ion channels, which includes the serotonin 5-HT₃, α-aminobutyric-acid, and glycine receptors. It has a cation-selective pore, delineated by a ring of 5 similar subunits, that opens upon binding of acetylcholine to distant sites in the 2 ligand-binding (α) subunits at the subunit interfaces. In earlier studies, we obtained a description of the N-terminal ligand-binding domain of the receptor by fitting the β-sheet core structure from a homologous pentameric acetylcholine-binding protein to the 3-dimensional densities determined from electron images.

More recently, we extended the structural analysis to derive an atomic model of the closed membrane-spanning pore. We showed that the pore is shaped by an inner ring of 5 α-helices, which curve radially to create a tapering path for the ions, and an outer ring of 15 α-helices, which coil around each other and shield the inner ring from the lipids. The gate, near the middle of the lipid bilayer, is a constricting hydrophobic girdle formed by weak interactions between neighboring inner helices.

The details of this structure, together with those obtained from the receptor trapped in the open-channel form, enabled us to understand in outline the mechanism by which acetylcholine opens the pore. When acetylcholine enters the ligand-binding domain, it triggers rotations of the protein chains on opposite sides of the entrance to the pore. These rotations are communicated through the inner helices and open the pore by breaking the hydrophobic girdle.

We are now examining the 4-Å structure of the whole channel. Information on the structure should allow us to explain how the localized disturbances associated with acetylcholine binding trigger the extended conformational change to open the pore and provide a more complete account of the gating mechanism. Knowledge of the precise locations of the charged amino acid residues should also allow us to explain quantitatively how the cation selectivity and high conduction rates are achieved.

The results of these structural studies on the acetylcholine receptor are providing crucial insight into the nature of a number of neuromuscular disorders, including several well-characterized congenital myasthenic syndromes. The findings 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.

PUBLICATIONS

Microscopes and Motility: Systems Integration in Cell Migration


In cancer cells, loss of regulation of cell-cell adhesion and cell motility results in deadly metastasis. The locomotion of vertebrate tissue cells is thought to require complex and dynamic interactions between the microtubule and actin cytoskeletal polymers, the endomembrane trafficking system, and focal adhesions to the substrate. We develop quantitative microscopy methods to analyze the dynamic interactions between these complex macromolecular systems in living cells to understand how the systems are spatiotemporally coordinated to drive directed cell movement. We then use these microscopic assays to analyze cells in which specific perturbations of cytoskeletal, membrane, or adhesive proteins are made to dissect the molecular mechanisms of the regulation of the proteins and their contribution to cell morphogenesis and migration.
One way in which cytoskeletal systems interact in cells is by being coregulated by the same signaling cascades. This coregulation would result in the spatiotemporally coordinated effort of actin and microtubules in cells to mediate a specific morphogenetic response. We showed that a signaling cascade downstream of Rac1 GTPase promotes the polymerization of both actin filaments and microtubules at the leading edge of migrating cells to promote protrusion of the membrane. Rac1 interacts directly with and activates the serine-threonine kinase Pak1, whose activity is required for growth of both microtubules and actin filaments. Growth of microtubules is promoted when Pak directly phosphorylates and inactivates the microtubule-destabilizing protein Op18/stathmin.

Inhibition of PKD on the trans-Golgi network inhibited cell motile functions, some of which could be restored by exogenous activation of Rac1. However, these activities were delocalized from a single leading edge, and directed cell motility was not fully recovered. These results indicate that PKD-mediated transport across the trans-Golgi network to the plasma membrane along microtubules is required for fibroblast locomotion and localized Rac1-dependent leading-edge activity.

To aid our studies of macromolecular dynamics in living cells, we pioneered fluorescent speckle microscopy, a powerful method that allows quantitative analysis of the dynamics of macromolecular assemblies in living cells. In the past year, we enhanced the sensitivity of this technique by extending the technology to multispectral total internal reflection fluorescence microscopy, allowing the first-ever analysis of the molecular dynamics of proteins within focal adhesion complexes during cell migration. Simultaneous analysis of focal adhesion proteins and actin in living, migrating cells will allow the molecular dissection of how forces generated in the cytoskeleton are transmitted to the extracellular matrix to move a cell. Via our collaboration with G. Danuser, Department of Cell Biology, we made huge strides in our ability to extract molecular kinetic measurements from in vivo fluorescent speckle microscopy images by using computer vision image analysis algorithms.

**PUBLICATIONS**


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**Systems Biology of Yeast and Protozoa**

E.A. Winzeler, K.G. Le Roch

Systems biology is a relatively new discipline in which the complete set of proteins or genes or other molecules produced by an organism is studied by using computational methods that integrate data from genome sequencing projects, global gene expression monitoring, or full genome proteomics experiments to make new observations that would not be possible if only a single cellular process or entity were studied. We use the yeast *Saccharomyces cerevisiae* as a model organism, but we are beginning to investigate organisms with important medical relevance, such as *Plasmodium falciparum*, the parasite that causes malaria. Our goal is to use a systems biology approach to find new treatments for malaria.

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We have 2 basic areas of malaria research. First, we are interested in finding new antigens for vaccine development. Discovery of antigens in malaria has traditionally relied on the biochemical purification of proteins expressed on the cell surfaces of *P falciparum* or infected erythrocytes. However, this approach has produced no effective licensed malaria vaccine, in part because of variation in the expressed antigens within and across isolates of the parasite. We are using genome-wide analysis methods to discover genes that appear to be under selective pressure from the host’s immune system. Our goal is to find the complete set of proteins that elicit the immunogenic response that provides adults from malarious regions some immunity to the disease.

In addition, in order to find new targets for drug development, we are interested in understanding how the malaria parasite functions at the molecular level. For example, only 15% of the proteins identified in the *P falciparum* genome sequencing project have direct experimental evidence supporting their functional assignment. Because *P falciparum* is an intracellular parasite and determining its gene function by using traditional genetic methods is difficult, these numbers are unlikely to change significantly soon. However, functional information about malaria genes and the genomes of other parasites whose genome sequences have been determined can be added cost-effectively by using new high-throughput technologies, such as large-scale proteomic experiments, comprehensive 2-hybrid studies, global gene expression monitoring, and sequencing of comparative species.

We examined gene expression profiles from a large number of *P falciparum* stages and found that genes expressed at the same time and at the same place often participate in the same biological processes. These experiments provided new groups of genes that most likely are involved in processes such as hemoglobin degradation and host-parasite interactions.

New Tools and Applications for Cellular Proteomics


Several advances in high-throughput technologies have created the foundation for the global proteomic analysis of complex mixtures and cellular lysates. The success and popularity of proteomics applications in studies of complex protein interactions in biology have created a demand for more powerful tools to analyze increasingly complex systems. Mass spectrometry is a key technology for realizing these goals, and we are leaders in (1) developing mass spectrometry–based methods that allow comprehensive analysis of very complex mixtures and (2) applying these emerging technologies to studies of existing biological problems.

Multidimensional protein identification technology (MudPIT) is a tool that we use extensively to analyze a wide range of samples. MudPIT involves the direct coupling of a multidimensional chromatographic separation system to a tandem mass spectrometer, providing data we term MS/MS spectra. Hundreds of thousands of MS/MS spectra can be collected on an automated MudPIT system in a 24-hour period, and sophisticated software is required to match spectra with peptide sequences in a database.

Recently, we developed a new algorithm to complement the existing Sequest algorithm for protein identification from the MS/MS data. Use of the new algorithm increases the confidence that the peptide indicated by a spectrum is correct by providing an independent measure of the quality of spectrum-peptide matches. In addition, de novo sequencing of the spectra can be used to extract partial peptide sequences so that less stringent searches against a sequence database are required, aiding in identifying posttranslationally modified peptides. As the proteomics field shifts toward methods that can provide a quantitative measure of protein expression, software tools for analyzing quantitative MS/MS data have also been developed to streamline and simplify data analysis.

We use MudPIT in various applications. We developed methods to optimize the analysis of membrane proteins, considered by many a major limitation of
proteomics technologies, and applied the methods to studies of the proteome of the Golgi apparatus. We identified 41 previously uncharacterized proteins and characterized posttranslational modifications that had not been detected before.

Continuing with the large-scale proteomic analysis of \textit{Plasmodium falciparum}, the parasite that causes malaria, we selected candidate antigens for vaccine development from proteomic data sets to observe the efficacy of the antigens in generating an immune response. We found that a much wider diversity of antigens than predicted can elicit a response. These results suggest that our understanding of antigenic immunodominance in the host response to complex pathogens is incomplete.

Finally, we used a subtractive proteomics strategy to identify integral membrane proteins of the nuclear envelope. All known components of the nuclear envelope were identified, and 67 uncharacterized open reading frames were detected. A total of 23 of the proteins identified mapped to chromosome regions that are linked to a variety of dystrophies. Approximately 300 dystrophies remain to be linked to a responsible gene, and the proportion of genes localized to disease loci was greater than what would be expected to occur randomly, suggesting that many of the 67 identified proteins are good candidates for disease links.

\section*{PUBLICATIONS}


Macromolecular Assemblies Visualized by Electron Cryomicroscopy and Image Analysis: Membrane Proteins and Viruses


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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 extra-cellular ligand binds to a membrane receptor? How do viruses attack 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 indicates 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.

Research projects under way include the structure analysis of (1) membrane proteins involved in cell-to-cell communication (gap junctions), water transport (aquaporins), ionic transport (potassium channels), transmembrane signaling (integrins), and viral recognition (rotavirus NSP4); (2) viruses responsible for significant human diseases (rotavirus, astrovirus, retroviruses); and (3) viruses used as model systems to understand mechanisms of pathogenesis (reoviruses, nodaviruses, tetraviruses, and sobemoviruses). The following sections summarize selected projects that exemplify the themes of our research program.

CARDIAC GAP JUNCTION MEMBRANE CHANNELS

Cardiac gap junctions play an important functional role in the heart by electrically coupling adjacent cells, thereby organizing the pattern of current flow to allow a coordinated contraction of the muscle. They are therefore intimately involved in both normal coordinated depolarization of heart muscle and cardiac arrhythmias causing sudden death.

We expressed a recombinant cardiac gap junction protein, termed connexin 43, and produced 2-dimensional crystals suitable for electron cryocrystallography. The 3-dimensional map (Fig. 1) shows that the dodecameric channel is about 150 Å long, with a diameter of about 65 Å within the membranes and about 55 Å in the extracellular gap. Within the membrane interior, each hexameric connexon is formed by 24 rods of density, consistent with an \(\alpha\)-helical conformation for the 4 transmembrane domains of each connexin 43 subunit. We anticipate that this basic molecular design will be a common folding motif for gap junction channels. Recently, we extended the analysis to 5.7-Å resolution, and a more detailed molecular interpretation is under way.

INTEGRINS

Cardiovascular disease is the major cause of mortality in the United States; most of these deaths are due to myocardial infarctions (heart attacks) caused by coronary atherosclerosis. Myocardial infarction almost always is due to formation of a thrombus at the site of a ruptured atherosclerotic plaque. A key event that stimulates thrombus formation is platelet aggregation, which is mediated by the prototypical integrin \(\alpha_{IIb}\beta_3\). Integrins are a large family of heterodimeric transmembrane receptor proteins that modulate cell adhesion, such as platelet aggregation, as well as other important biological processes, such as development, angiogenesis, wound healing, and neoplastic transformation.

We used electron cryomicroscopy and single-particle image reconstruction to derive a 3-dimensional structure at 20-Å resolution of the unliganded, low-affinity state of the human platelet integrin \(\alpha_{IIb}\beta_3\). The large ectodomain and small cytoplasmic domains are connected by a rod of density that we interpret as 2 parallel transmembrane \(\alpha\)-helices (Fig. 2). The structural details revealed by these studies will provide insight into the molecular basis of integrin activation that will be relevant for the rational design of drugs to modulate integrin functions. Currently, we are examining the structure of integrins in a complex with physiologic ligands such as the protein fibronectin.

NODAVIRUSES

The nodavirus flock house virus (FHV) has a genome consisting of 2 strands of RNA that are packaged in
an icosahedral capsid formed by 180 protein subunits. FHV is an exceedingly useful system for understanding mechanisms of viral assembly. We used electron cryo-microscopy and image reconstruction to determine the structure of 4 types of FHV particles that differed in RNA and protein content (Fig. 3). RNA-capsid interactions were primarily mediated via the N and C termini, which are essential for RNA recognition and particle assembly. A substantial fraction of the packaged nucleic acid, either viral or heterologous, was organized as a dodecahedral cage of duplex RNA. The similarity in RNA tertiary structure suggests that RNA folding is independent of sequence and length. Computational modeling indicated that formation of RNA duplexes involves both short- and long-range interactions. These studies suggest that the capsid protein can exploit the plasticity of the RNA secondary structures, capturing those that are compatible with the geometry of the dodecahedral cage. Further analysis of capsid protein mutants and designed RNA molecules is under way.

PUBLICATIONS


