

1979-MiniSymp**Interaction Of The Parkin UBL Domain With SH3-containing Proteins Involved In Synaptic Vesicle Endocytosis: Structure And Role In Protein Ubiquitination**

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Mutations in the ubiquitin ligase Parkin have been associated with the development of autosomal recessive juvenile Parkinsonism (ARJP). Mutations have been found in the RING domains, but also in the N-terminal ubiquitin-like (UBL) domain. In an effort to identify protein substrates implicated in ARJP, we identified Endophilin-A as a ligand of the rat Parkin-UBL domain. Endophilin-A is a BAR-domain containing protein that induces membrane curvature and is implicated in synaptic vesicle endocytosis and recycling. Parkin-UBL interacts directly with the Endophilin-A C-terminal SH3 domain with an affinity of 10–15 μ M. The interaction is specific as ubiquitin, Nedd8 and the Plc1-UBL domain do not interact with the SH3 domain. The Parkin-UBL domain only interacts with a subset of SH3 domains. We determined the crystal structure of rat Endophilin-A1 SH3 domain at 1.4 Angstrom resolution. Using NMR spectroscopy, we mapped the protein-protein interaction surfaces on both the rat UBL and SH3 domains. Using our SH3 structure and the crystal structure of murine Parkin-UBL, we calculated a docking model of the UBL:SH3 complex using NMR chemical shift perturbations and residual dipolar couplings. The UBL surface consists of the hydrophobic patch located around Ile44 as well as the basic C-terminal tail of the UBL domain, which is major specificity determinant. Using single-site mutagenesis, we identified key specificity and affinity determinants in the SH3 domain that explains that specificity of Parkin-UBL towards different SH3 domains. The SH3 surface involved in binding the UBL domain is centered around an invariant proline previously shown to be involved in proline-rich domain (PRD) binding. Parkin-UBL effectively competes with synaptojanin PRD for binding Endophilin-A1 SH3 domain. Finally, we show that the UBL:SH3 interaction is required for Endophilin-A ubiquitination by Parkin.

1980-MiniSymp**Mechanisms Underlying the Binding Diversity of Dynein Light Chain**

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Dynein light chain LC8 was first discovered as an essential component of the microtubule-based molecular motor dynein where it associates directly with dynein intermediate chain IC. A large fraction of LC8, however, is not associated with dynein, suggesting alternate functions for LC8 independent of its function in dynein. LC8 interacts with non-dynein proteins in diverse systems, including neuronal nitric oxide synthase (nNOS), the proapoptotic Bcl2 family protein Bim, Swallow (Swa), and Nup159 of the nuclear-pore complex. Based on the diversity of these interactions, and the fact that all known LC8 partners share a common binding groove on LC8 with dynein IC, we have proposed that the wide array of binding partners reflects the role of LC8 as an essential hub protein. In this capacity, LC8 functions not simply as a dynein cargo adaptor, as widely viewed, but as a promoter of dimerization of its monomeric, partially disordered binding partners. Here we address the mechanisms underlying LC8 binding diversity using crystallographic, solution NMR and thermodynamic characterization of several LC8/peptide complexes. Peptide binding is associated with a shift in quaternary structure that expands the hydrophobic binding surface available to the ligand, in addition to shifts in tertiary structure and ordering of LC8 around the binding groove. NMR peptide titrations show evidence of allosteric interaction between the two binding sites. Backbone ¹⁵N relaxation shows significant conformational heterogeneity in the LC8 dimer which is retained in LC8/IC but is lost in LC8/Swa, suggesting that the degree of ordering is ligand dependent. The reduced complexity of motion in LC8/Swa correlates with the less favorable entropy of binding of LC8 to Swa relative to IC. We propose that these properties observed for LC8 may be general to other dimeric hub proteins.

1981-MiniSymp**Structural Basis Of RIG-I Auto-inhibition And RNA-induced Activation**

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RIG-I (retinoic-acid-inducible-gene I) plays a critical role in regulating innate immune response against RNA viruses. RIG-I binds to 5' triphosphate RNA of viral origin and activates downstream signaling pathways resulting in the induction of type I interferons. RIG-I contains two caspase-recruitment activation domains (CARD) in its N-terminus, a DEXD/H helicase domain and a C-terminal repressor domain (RD). The function of RIG-I is regulated by auto-inhibition. While CARD domains activate downstream signaling, RD inhibits the activity of CARD domains in the absence of RNA. To investigate the RNA dependent structural transitions that activate RIG-I, we determined the structures of free and 5' triphosphate RNA bound RIG-I using negative stain

electron microscopy (EM) and single particle reconstruction. Preliminary biochemical studies show that free RIG-I, which is a monomer, undergoes dimerization upon binding to RNA. Analysis of the EM structure of RIG-I:RNA complex shows that dimerization is mediated by bivalent and parallel interactions between the two ends of adjacent RIG-I monomers. Structural modeling suggests that the dimer interfaces involve inter-molecular, homotypic, CARD-CARD and RD-RD interactions. In contrast to RNA bound RIG-I, which adopts an extended 'open' conformation, free RIG-I adopts a compact 'closed' conformation mediated by extensive intra-molecular interactions. Structural modeling indicates that the auto-inhibition of RIG-I is potentially mediated by a direct intra-molecular interaction between CARD and RD. Indeed, biochemical analysis demonstrates that isolated RD and CARD domain regions of RIG-I interact in the absence of RNA. We propose that the binding of 5' triphosphate RNA to RIG-I disrupts the intra-molecular interactions that mediate auto-inhibition, and promote inter-molecular interactions that result in dimerization and subsequent activation. These results provide novel structural insights into the mechanism of RIG-I auto-inhibition and RNA-dependent activation.

1982-MiniSymp**Cross-Correlation of Fluorescence-Quenching and Infrared Absorption in the Study of Protein Ligand Binding Sites**

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We have developed a generalized multidimensional spectroscopic approach to investigate long range conformational coupling in proteins. It employs the integration of fluorescence emission and infrared absorption data recorded simultaneously from the same protein sample that undergoes conformational transitions in response to an external perturbation. Long range coupling in the signal transfer through rhodopsin has recently been identified by Fluorescence-IR-cross-correlation. Using 2D-cross-correlation techniques, the kinetic asynchronicity of the emission from natural or artificial site-specific fluorophores relative to the secondary structure-sensitive IR-absorption bands can be determined. Thereby, IR absorptions can be identified in a model-free and unbiased way that can be assigned to secondary-structural elements that become specifically stabilized by ligand interactions. Here, we demonstrate in a cytoskeletal protein the correlation of the loss of ligand-dependent static quenching of intrinsic tryptophan emission during thermal unfolding with the loss of structure monitored by FTIR spectroscopy. The high signal to noise ratio in 2D-correlation and the "synchronicity tagging" of the IR bands through their correlation with an independent monitor of ligand dissociation allows detecting ligand protein interactions with an accuracy that is not achieved by FTIR-spectroscopy alone. In addition, topological information can be obtained from the emission wavelength of the tryptophans that become gradually unquenched during temperature-induced ligand dissociation. Fluorescence-IR-cross-correlation spectroscopy thus extends the IR-based conformational analysis by the inclusion of site-specific information on local physical parameters (polarity, electrostatics, etc.) specifically affecting the emission of fluorophores. We show how this approach provides structural information on flavonoid binding to actin, a cytoskeletal and nuclear protein that has recently been shown to respond to the binding of these natural compounds by flavonoid-specific conformational changes.

Platform AM: Emerging Single Molecule Techniques I**1983-Plat****Single-Molecule Fluorescence Imaging with Sub-nanometer Resolution**

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Objective: The emerging field of fluorescence nano-scopy promises to revolutionize biomedical research, and remarkable progress has been made in the measurement of nano-meter distances. Although diffraction of visible light limits the far-field optical resolution to ~200nm, the center position of spatially resolved fluorescent molecules or nano-particles can be located to much higher precision. Notably, in high resolution microwave and optical spectroscopy there are numerous examples where the line-center is determined with a precision of less than 0.000001 of the line-width. In contrast, the brightest single fluorescent emitters can be detected with a Signal-to-Noise-Ratio of ~100, limiting the localization precision to 0.01 (~1.5nm) of the microscope Point-Spread-Function (PSF) width. Moreover the error in co-localizing two or more single emitters is notably worse, remaining greater than 0.03-0.05 (5-10nm) of the PSF width. Results: We achieve two-color single-molecule imaging with 0.5nm absolute localization and registration accuracy as well as demonstrate 0.7nm absolute

accuracy in distance measurements between two different color dye-molecules attached at known positions along a surface tethered bio-molecule. The statistical uncertainty in the mean for an ensemble of $N \sim 10$ identical single molecule samples is limited only by the total number of collected photons to $\sim 0.3\text{nm}$, or ~ 0.002 of the width of the optical PSF. We further show how our method can be used to improve the resolution of many sub-wavelength, far-field imaging methods such as those based on co-localization of stochastically excited fluorescent molecules.

Conclusion: We demonstrate sub-nanometer resolution in measurements of molecular-scale distances using far-field fluorescence imaging optics, at room temperature and in physiological buffer conditions. The improved resolution will allow deciphering in real-time, at the single molecule level the structure and dynamics of large, multi-subunit biological complexes.

1984-Plat

Visualizing Single-proteins On A Single DNA Molecule With Super-resolution

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DNA-proteins interactions can be studied in great detail when precise and dynamic control of the mechanical state of a single DNA molecule can be achieved together with direct visualization of proteins interacting with the DNA. Our custom-built experimental setup combines optical trapping and wide-field epifluorescence microscopy. In this setup we can visualize single DNA-bound proteins (see figure) with an accuracy of tens of nanometers while at the same time we can control the tension applied on the DNA at a sub-PicoNewton level.

Here we report the limit of localization accuracy in combined DNA-trapping/fluorescence experiments by fitting a point-spread function to the fluorescence image of single DNA-bound proteins.

We investigate the impact of DNA dynamics on the maximum attainable accuracy for the localization of these DNA-bound fluorescent proteins (in this case a Ca^{2+} inactivated restriction enzyme EcoRV). In particular, we study the effect of tension on the DNA and identify the force regime in which single-proteins can be localized with super-resolution.



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Visualizing the Receptor Assembly Into Clathrin-coated Pits with Super-resolution Two-color PALM and sptPALM

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The dynamic assembly of receptors into endocytic structures such as clathrin-coated pits underlies cellular response to many external signals. However, the biophysical mechanisms for the regulated uptake of select receptors are unresolved. While commonly used bulk measurements access only ensemble-averaged behaviors, single molecule measurements have largely been limited to looking at only a few molecules in a single cell, lacking true ensemble information. Thus, until recently, the study of receptor capture by clathrin-coated pits has been limited by a scarcity of experimental methods capable of accessing information on ensembles of individual molecules within an individual cell. We addressed this by combining two recent technological advances to image receptors and clathrin-coated pits in living cells: two-color imaging with the photoswitchable PA-mCherry fluorescent label, and single particle tracking photoactivated localization microscopy (sptPALM).

As compared to other monomeric red photoactivatable proteins, PAmCherry1 has higher pH stability, faster maturation and photoactivation kinetics, better photostability, and similar number of photons in single-molecule imaging. Furthermore, lack of background green fluorescence makes PAmCherry1 an advanced probe for two-color diffraction-limited microscopy and super-resolution techniques such as PALM. Two-color PALM imaging of PAmCherry1 tagged to the transferrin receptor (TfR) and PAGFP fused with the clathrin light-chain (CLC) were performed. Pair correlation analysis suggests clusters of less than 200 nm in size with both distinct and overlapping distributions of the TfR and CLC chimeras at sub-diffraction 25 nm resolutions. In addition, sptPALM was used to create spatially resolved maps of the trajectories of single receptor motions in conjunction with SPT of diffraction-limited clathrin-coated pits.

1986-Plat

A Single-Molecule Study of Gene Regulation in Real Time

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Gene expression is inherently stochastic, yet must be tightly regulated to properly carry out essential cellular processes. However, many of the transcription factors responsible for this precise regulation are expressed at extremely low copy numbers in cells. To understand how noise in gene expression is controlled to overcome this challenge, it is important to directly monitor gene regulation in real time at the single-molecule level.

In this work, we use the genetic switch of lambda phage as a model system to demonstrate continuous monitoring of autoregulation of lambda repressor CI in living *E. coli* cells. CI exhibits both positive and negative feedback on its own expression at low and high intracellular concentrations, respectively. We generated a single-molecule, gene-expression reporter that enables counting of the exact number of CI molecules expressed in real time without compromising CI's regulatory activity. Over several cell generations, we observed highly clustered expression of CI separated by relatively long periods of low expression, which we attribute to the alternating positive and negative autoregulation of CI. Our results suggest that the noise in CI expression is controlled by coupled positive and negative feedback, a mechanism also implicated in regulation of the eukaryotic cell cycle and circadian rhythm. This method should be applicable to similar studies probing gene regulation in other systems.

1987-Plat

Pitfalls In Single Particle Tracking In Living Cells

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An increasing body of evidence for subdiffusion of biopolymers under typical *in vivo* conditions has been reported recently. The physical foundation of this subdiffusion remains unidentified although it is commonly ascribed to molecular crowding. Single particle tracking provides crucial information on the mechanisms behind the subdiffusion. In several such experiments the measured mean squared displacement shows a characteristic scatter (e.g., [1,2]).

Using the widely accepted continuous time random walk framework we demonstrate that pronounced scatter in time averaged quantities such as the mean squared displacement is no artefact but arises naturally from the nonexistence of a characteristic time scale separating microscopic and macroscopic events [3,4]. An expression for the broad distribution of diffusion coefficients in such measurements is derived and confirmed by simulations. The most crucial finding from our theory is that the subdiffusive nature of the particles will be masked in the time averages: What looks like normal diffusion in an experiment may in reality be subdiffusion in an ageing system. Interpretations of the reported data in [1,2] will be discussed. We provide general guidelines to properly interpret single molecule tracking data.

We also argue that ageing properties in biopolymer diffusion in living cells may be advantageous for the accuracy of genetic regulation at minimal concentrations of transcription factors. The physical picture emerging from our theory provides additional support for a more local picture of gene regulation and confirms the importance of colocalisation in the genome.

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1988-Plat

New Single Molecular Detection System from Three-Dimensional Tracking of Single Nanocrystals using Scanning Electron Microscope

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Recent technological progress in dynamical observations of individual functional single protein molecules in living cell has been achieved with several single molecular techniques and systems. In order to improve monitoring precisions and stability of the signal intensity from single molecular units under physiological conditions, we have proposed that single molecular techniques using shorter wavelength, for example, X-rays, electrons, neutron, and other accelerated ion probes. In this work, we demonstrate three-dimensional tracking of single nanocrystals using Scanning Electron Microscope. We called Diffracted Electron Tracking (DET).

Diffracted X-Ray tracking (DXT) [1] has been developed for obtaining the information about the dynamics of single molecules. This method can observe the