Nucleocytoplasmic Transport

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Probing Nucleocytoplasmic Transport with Fluorescence Fluctuation Spectroscopy and Two-photon Activation of Photoactivable GFP

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Large proteins and macromolecular complexes have to enter and leave the nucleus in an efficient and selective manner. Macromolecules that are greater than 40 kD are transported actively across the nuclear envelope through nuclear pore complexes using soluble transport factors or carrier molecules that cycle between the cytoplasm and nucleus. The carrier proteins themselves interact with each other in order to transport cargo proteins across the nuclear pore complexes. In this work, we apply dual-color time-integrated fluorescence cumulant analysis (TIFCA), a fluorescence fluctuation spectroscopy technique, to investigate the protein interactions of the carrier proteins directly in cells. In addition, we apply two-photon activation to directly examine the nucleocytoplasmic transport of photoactivable GFP tagged carrier proteins. With these two approaches, we are able to probe the nucleocytoplasmic transport process of NTF2 directly inside cells and under equilibrium conditions. We investigate the oligomerization of NTF2 in cells and its transport properties when crossing the nuclear pore complexes.

The experiments identify the presence of NTF2 dimers in the cytoplasm and nucleus of cells, while the measured transport properties across the nuclear pore complex are not consistent with dimeric NTF2. We will discuss the implications of these results for functions of nuclear envelope and models of nucleocytoplasmic transport. This work is supported by the National Science Foundation (PHY-0346782) and NIH grant R01GM064589.

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Discontinous Movement of mRNPs in Nucleoplasmic Regions Devoid of Chromatin

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Messenger RNP particles (mRNPs) move randomly within nucleoplasm before they exit from the nucleus through nuclear pore complexes. To further understand mRNP trafficking, we have studied the movement of a specific mRNP, the BR2 mRNP, in the nuclei of salivary gland cells in Chironomus tentans larvae. These polytene nuclei harbor giant chromosomes separated by vast regions of nucleoplasm, which allows to study mRNP mobility without interference of chromatin. The particles were fluorescently labeled by hybridization with microinjected RNA or DNA oligonucleotides, and their movement was examined by single particle tracking. To rule out the possibility that the hybridization-based labeling interferes with mRNA trafficking, we also labeled mRNPs by incorporation of recombinant fluorescent hrp36, an hnRNP A1-like protein in C. tentans, which is incorporated into mRNPs during transcription and contained therein until translation. The BR mRNPs moved randomly, but unexpectedly in a very discontinuous manner. When mobile, they diffused with a diffusion coefficient of 3 to 4 μm2/s. This value corresponded to the theoretical expectation according to the Stokes-Einstein law considering their diameter of 50 nm and an intranuclear viscosity of 3 cP. Between mobile phases the mRNPs were slowed down 10 to 250-fold but were never completely immobile. Earlier electron microscopy work has indicated that BR particles can attach to nuclear fibrogranular clusters. We propose that the observed discontinuous movement reflects transient interactions between the BR particles and these fibrogranular clusters. Our results support the view that it is important to consider the length scale of intranuclear mobility measurements: short length scale movements as observed by FCS will most accurately reflect the movement of the particles, while long scale movements are hampered by various interactions and restrictions, the effect of chromatin being most dominant.

Ryanodine Receptors Type I

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Structural Characterization of FKBP Interactions with RyR Channels Using Site-Directed Fluorescent Labeling and FRET

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The small FK506-binding proteins (FKBP12/12.6) tightly bind to ryanodine receptor (RyR) channels and may stabilize channels in a closed conformation. To investigate the structural basis of FKBP12.6 binding to the RyR1 and RyR2 isoforms, we used cysteine mutagenesis, fluorescent labeling, direct-binding measurements, and FRET. Single cysteines were introduced at five positions distributed over the surface of FKBP12.6 (T14C, N32C, D41C, R49C, and T85C). Results showed that each of the five FKBP12.6 mutants retained high-affinity binding to the RyR1. Furthermore, high-affinity binding was retained following the covalent attachment of a 720 Da fluorescent donor at four of the five positions (14, 32, 49, and 85), suggesting that these positions are removed from the major RyR1 binding interface. By comparison, attachment of the fluorescent donor at one position (41) resulted in a marked decrease in FKBP12.6 binding. FRET from the different donor-labeled FKBPs to an acceptor attached within the RyR CaM subunit was examined to determine the orientation of FKBP12.6 bound to the RyR1 and RyR2. Results showed that FRET was dependent on the donor's position on FKBP12.6, and that the rank order of FRET efficiency from the different positions was the same for the two RyR isoforms (position $49 > 85 \geq 14 > 32$). These results indicate that in binding either RyR1 or RyR2, FKBP12.6 is oriented such that position 49 is nearest and 32 furthest from the RyR CaM binding site. Together, our results are consistent with the model of FKBP binding proposed by Samso and coworkers (2006), and point to loop 39–46 as a key component of the RyR binding interface.

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Mapping the Ryanodine Receptors Pore Region Using the Substituted Cysteine Accessibility Method

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Skeletal and cardiac muscle contraction require the activation of the type 1 and type 2 ryanodine receptor/calcium release channel (RyR1 and RyR2) respectively. RyRs are comprised of four identical subunits, each of ~ 565 kDa forming a single pore.

We are using the Substituted Cysteine Accessibility Method (SCAM) to identify the resides that line the pore region of the channel, to size the channel pore and to determine differences in the structure of the pore in different functional states. To map pore-lining cysteines in both RyR1 and RyR2 channels, we plan to use methyl thiosulfonate (MTS) compounds of different diameters and charges to probe accessible amino acid side chain in the pore of RyR channels after cysteine-substitution mutagenesis.

Earlier work (K. E. Quinn, B. E. Ehrlich, 1997. *J Gen Physiol* **109**, 255) showed that cysteines in the predicted membrane-domain of RyR are accessible to methanethiosulfonate ethylammonium (MTSEA) when the channel is open. It was inferred that the cysteines were exposed in the conduction pathway.

We have applied 5 mM MTSEA to wild-type RyR channels in planar lipid bilayers membrane and find that it decreases the current amplitude, the open probability and the mean-open time. Also, the RyR activator calcium increases the susceptibility of RyR to MTSEA. In order to identify the cysteines responsible for the MTSEA modification effects, single point mutations of cysteines (C4876A and C4882A) located in the predicted pore-forming region of RyR1 are being generated.

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A FRET-based Assay To Measure Molecular Distances Within The Ryanodine Receptor Type 1 (RyR1)

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The type 1 ryanodine receptor (RyR1) is an intracellular Ca2+-release channel that mediates excitation contraction coupling in skeletal muscle. This enormous homotetrameric protein has a subunit molecular weight of 565 kDa and is associated with numerous regulatory proteins that modulate its function in vivo. Understanding the structure and conformational dynamics of this immense macromolecular complex is a key topic in skeletal muscle biology. In this report, a novel structural assay has been devised that relies upon Förster resonance energy transfer (FRET) to measure distances between defined primary sequence elements of RyR1. These FRET measurements require site-specific incorporation of a fluorescence donor and a fluorescence acceptor into the primary sequence of RyR1. In this system, green fluorescent protein (GFP) fused to the N-terminus of RyR1 acts as the fluorescence donor. The FRET acceptor is an affinity reagent that site-specifically binds to poly-histidine segments (i.e. His tags) engineered into RyR1 where it can accept fluorescence energy from the N-terminal GFP. This report describes the characterization of the FRET acceptor as well as the recombinant His-tagged GFP-RyR1 fusion proteins used for these measurements. In addition, experiments measuring FRET from the N-terminal GFP to the FRET acceptor targeted to His tags introduced into 3 primary sequence elements poorly conserved among the 3 RyR isoforms (i.e. divergent regions) are described. (Supported by NIH grants K01ARO52120 and R21ARO56406).