Nucleocytoplasmic Transport

1598-Pos

Choreography of Importin α/CAS Complex Assembly and Disassembly at the Nuclear Pore Complex

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Nuclear pore complexes (NPCs) mediate the exchange of proteins and RNAs between the cytoplasm and the nucleoplasm of eukaryotic cells. Signal-dependent transport cargos must assemble with soluble nuclear transport receptors to form transport complexes, which must then be disassembled after transport. The assembly and disassembly of transport complexes is promoted by proteins near the cytoplasmic and nucleoplasmic exit sides of the NPC. Here we report the use of single molecule fluorescence resonance energy transfer (smFRET) and particle tracking to investigate the choreography of importin alpha/CAS complex assembly and disassembly in permeabilized cells. Importin alpha acts with importin beta to transport cargos into the nucleus. We directly show that importin alpha/CAS complexes form in the nuclear basket region of the NPC, at the termination of the nuclear import process. These newly formed importin alpha/CAS complexes are preferentially released into the nucleus rather than immediately exported to the cytoplasm. The rapid dissociation of some importin alpha/CAS complexes at the NPC is consistent with the formation of a transient (2-4 ms lifetime) cargo/importin alpha/CAS complex, which disassembles either by losing a CAS or a cargo molecule. These data indicate that assembly and disassembly reactions occur concomitantly and stochastically. Importin alpha mutants confirm that the nucleoporin Nup50 promotes importin alpha/CAS complex assembly and that the smFRET signals observed at the NPC result from specific interactions rather than chance encounters. The disassembly of importin alpha/CAS complexes occurs after export in the cytoplasmic filament region of the NPC. This disassembly reaction is promoted by RanGTPase activating factors, indicating that GTP hydrolysis catalyzes the dissociation process. Thus, we clearly demonstrate the power of single particle tracking and smFRET for monitoring molecular interactions with high time resolution (2 ms) in a complex system.

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New Insights Into Intranuclear mRNP Dynamics

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Messenger ribonucleoprotein particles (mRNPs) move randomly within the nucleoplasm on their way to the nuclear pore [1]. In interphase the decondensed chromatin of mammalian cells largely governs the structure of the intranuclear environment and therefore has a strong impact on mRNP mobility. The salivary gland cell nuclei of *Chironomus tentans* harbor giant polytene chromosomes, where chromatin is compact and surrounded by extended regions of nucleoplasm void of chromatin. This allows studying mRNP mobility under conditions, which presumably correspond to that of interchromatin channels in mammalian cell nuclei. In this domain we examined the intranuclear movement of a specific endogenous mRNP, the BR2 mRNP, by high-speed single molecule fluorescence microscopy [2]. mRNP dynamics could be characterized by four diffusion coefficients, 0.015$\mu m^2/s$, 0.23$\mu m^2/s$, 0.7$\mu m^2/s$ and 3.7$\mu m^2/s$, respectively.

We now used 2'-O-Me-RNA oligonucleotides and molecular beacons to label the mRNPs *in vivo,* and studied the mobility of the BR2 mRNPs by complementary state-of-the-art fluorescence microscopy techniques: single molecule tracking, FCS, line scanning FCS and raster image correlation spectroscopy. These quantitative methods revealed that mobility components $>3\mu m^2/s$ were likely due to unbound oligonucleotides. The remaining components were shown to be typical for BR2 mRNP movement with all techniques and labelling approaches used.

The BR2 mRNPs moved in a discontinuous manner in the chromatin-free nucleoplasm. That reflects transient interactions between diffusing BR2 mRNPs and submicroscopic intranuclear structures not containing chromatin. To reveal the nature of the transient mRNP immobilization we examined the impact of the nuclear actin-binding protein hrp65-2, as well as hrp65-1 on mRNP mobility. Altogether, our experiments provided a comprehensive view on the intranuclear trafficking of native mRNPs.

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1600-Pos

Observation of Single Nuclear Export Receptors at the Nuclear Pore Complex

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Nucleocytoplasmic transport across the nuclear pore complex (NPC) permanently occurs in mammalian cells via a multitude of pathways. The NPC is the only entrance to and exit from the nucleus for macromolecules. Therefore detailed study of the transport process is of great importance for both an understanding of the mechanism as well as development of specific manipulation techniques relevant e.g. for drug delivery.

Single molecule fluorescence microscopy allows observing particles and molecules during the transit across the nuclear envelope revealing dwell times, which reflect the time needed for translocation. We were able to determine dwell times of several import receptors in vitro [1] and in live cells [2].

Here, we focus on nuclear export, which is difficult to explore, because the nuclear interior and its functional factors are experimentally less accessible than the cytoplasm. We examined the NPC translocation of soluble export receptors: CRM1, which among others exports NES bearing cargos, CAS, which exports Importin alpha, Tap and NXT1, which function as mRNA export factors. Dwell times are observed both in an in vitro system based on digitonin-permeabilized cells and in vivo microinjecting receptors into the nucleus. To maintain their functionality the mentioned proteins can only be labelled with a low dye to protein ratio and are therefore prone to photobleaching and rather dim. Hence HILO illumination [3] has been applied to perform experiments. Selective illumination of only a thin sheet within the cells next to the coverslip allows working with relatively low laser power and yields a signal-to-noise ratio superior to that obtained with epi-illumination.

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1601-Pos

Oligomerization and Nucleocytoplasmic Transport of NTF2 Revealed by Brightness Analysis and Two-Photon Photoactivation

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Small molecules - less than 40 kD - diffuse freely through nuclear pores between the cytoplasm and nucleus. Large proteins and macromolecular complexes require active transport across the nuclear envelope via soluble transport factors or carrier molecules. Some carrier molecules themselves form complexes as they cycle between the nucleus and cytoplasm. One such transport factor is NTF2, which is known to play a primary role in the maintenance of the Ran gradient, but the oligomeric state of NTF2 and its function in transport are not well established. We observe that NTF2 exists as a monomer-dimer equilibrium in the cell, and we characterize several NTF2 mutants to investigate the influence of NTF2 dimerization on nucleocytoplasmic transport. We apply two-photon activation in vivo to examine the transport of photoactivable GFP-tagged carrier proteins. We also use fluorescence fluctuation spectroscopy and brightness analysis in the cell to investigate the oligomerization of the NTF2 mutants. We establish the efficacy of brightness analysis in a cell free expression system and use it to check oligomerization of NTF2 and its mutants in vitro. This work is supported by NIH grant R01GM064589.

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A Functional Map of the Nuclear Pore Complex Via High Precision Tracking of Single Molecules

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All materials entering or exiting the eukaryotic cell nucleus pass through Nuclear Pore Complexes (NPCs), large transport channels embedded in the nuclear envelope. NPCs allow passive diffusion of small molecules, while larger cargos require transport receptors to facilitate passage. How NPCs achieve this exquisite selectivity remains unclear. We have developed a single molecule imaging assay, based on small (18nm diameter) custom protein-coupled Quantum Dots (QDs), to study the motion of cargos as they approach, translocate and exit the NPC. Optical tracking of the QD cargos with a mean spatial precision of 6nm and a temporal resolution of 25ms, allowed us to characterize individual steps involved in the import reaction and characterize NPC selectivity. Single cargo trajectories reveal a size-selective cargo barrier positioned in the cytoplasmic moiety of the central channel and the majority of the QDs are rejected early rather than spending long periods of time partitioned in the channel.