Nucleocytoplasmic Transport

1598-Pos

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

Changxia Sun[1], Murray Stewart[2], **Siegfried M. Musser[1]**.
[1]Texas A&M Health Science Center, College Station, TX, USA, [2]MRC Laboratoy of Molecular Biology, Cambridge, United Kingdom.

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.

1599-Pos

New Insights Into Intranuclear mRNP Dynamics

Roman Veith[1], Johannes Anzt[1], Thomas Sorkalla[2], Eugen Baumgart[1], Hanns Häberlein[2], Jan Peter Siebrasse[1], Ulrich Kubitscheck[1].
[1]Institute of physical and theoretical Chemistry, Bonn, Germany, [2]Institute of Biochemistry and Molecular Biology, Bonn, Germany.

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

Ulrike Schmitz-Ziffels, Andreas Veenendaal, Jan Peter Siebrasse, Ulrich Kubitscheck.
University of Bonn, Bonn, Germany.

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

Patrick J. Macdonald, Yan Chen, Jinhui Li, Joachim D. Mueller.
University of Minnesota, Minneapolis, MN, USA.

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.

1602-Pos

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

Alan R. Lowe, Jake Siegel, Petr Kalab, Merek Siu, Karsten Weis, Jan T. Liphardt.
University of California, Berkeley, CA, USA.

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.

Within the channel, cargos move in a non-directional manner, consistent with anomalous subdiffusion in a crowded volume, with dimensions of ~55 nm in width and ~68 nm in length. By varying the number of import receptors on the surface of the cargo, we find that the translocation is not governed by simple receptor-NPC binding interactions and that the central channel behaves in accordance with the 'selective phase' model. Finally, in the absence of Ran, cargos still explore the entire volume of the NPC, but have a dramatically reduced probability of exit into the nucleus from the pore, suggesting that NPC entry and exit steps are not equivalent and that the pore is functionally asymmetric to importing cargos.

1603-Pos

Single Molecule Imaging of the Calcium Ion Regulation of Nuclear Pore Passive Permeability

Ashapurna Sarma, Weidong Yang.
Bowling Green State University, Bowling Green, OH, USA.

Nuclear pore complex (NPC) is the sole pathway for direct communication between the cytoplasm and the nucleoplasm of eukaryotic cell. Based on the criteria of the molecular size exclusion, the NPC allows unregulated passive diffusion of small molecules (< 40 kDa) and facilitated translocation of larger molecules (up to 50 MDa). While recent evidence suggests a third transport mode: Ca^{2+} regulated transport. In details, the nuclear pore permeability can be regulated by Ca^{2+} stored in the lumen of nuclear envelope and endoplasmic reticulum. However, the mechanism of Ca^{2+} regulated transport remains poorly understood. Here we applied a speedy single molecule fluorescence microscopy to characterize the dependence of the nuclear pore passive permeability on the Ca^{2+} store concentration by snapshots of real-time transient movements of 3 - 40 kDa dextran molecules through the NPCs. We observed novel features under real-time trafficking conditions that escape detection by ensemble measurements: decreased amount of Ca^{2+} in the store induced restricted passive diffusion of dextran molecules with longer diffusion times and lower transport efficiencies through the NPCs. Dextran molecules cannot penetrate deeply into the nuclear pore and the majority were rejected or trapped by likely barriers formed on both sides of NPCs when the stored calcium was significantly depleted. Our results suggest that a filamentous structure that occludes the NPC may be altered by the depletion of calcium in the store.

1604-Pos

Single-Molecule Snapshots of Three-Dimensional Distribution of Transient Interactions in the Nuclear Pore Complex

Jiong MA, Weidong Yang.
Bowling Green State University, Bowling Green, OH, USA.

Translocation of macromolecules through the nuclear pore complex (NPC) is hindered by the phenylalanine-glycine (FG) repeats barrier unless they are chaperoned by transport receptors in eukaryotic cells. However, challenged by measuring a series of transient interactions between the transport receptor and the FG repeats, the precise mechanism of the facilitated translocation remains unclear. We applied single-point edge-excitation sub-diffraction (SPEED) microscopy to obtain a three-dimensional density map of the transient interactions with a spatiotemporal resolution of 9 nm and 400 μs. We observed novel features under real-time trafficking conditions that escape detection by conventional electron microscopy: the actual pathway of facilitated translocation through the NPC is not completely restricted by the NPC architecture; the primary interaction sites between Importin β1 (Imp β1, a major transport receptor) and the FG repeats locate symmetrically on the cytoplasmic and nucleoplasmic sides of the nuclear pore; Imp β1 only rarely occupies a central channel of approximate 10-20 nm diameter along the NPC axis, but the Imp β1-assisted cargo molecules expand their pathways into the central channel.

1605-Pos

Squeezing through the Pore - Conformational Plasticity in Nuclear Import

Nicole Dölker, Helmut Grubmüller.
MPI for Biophysical Chemistry, Goettingen, Germany.

In eukaryotic cells, exchange of macromolecules between the cytoplasm and the nucleus is mediated by specialized transport factors. By binding to these transporters, cargo molecules which are otherwise excluded from entering the nucleus can traverse the nuclear pore efficiently. Most of the proteins mediating nuclear import and export belong to the importin beta family. The transport cycle of importin beta starts with recognition of the cargo in the cytoplasm. The importin-cargo complex then crosses the permeability barrier of the nuclear pore and enters the nucleus, where the complex dissociates upon binding of RanGTP to importin beta.

Importin beta has a superhelical structure and exhibits a great intrinsic flexibility, which is needed for recognition of a wide variety of ligands [1]. Although small angle scattering data, as well as previous molecular dynamics studies, indicate that in solution importin beta adopts a rather open conformation, most crystal structures exhibit a compact conformation of the superhelix [2, 3].

We carried out all-atom molecular dynamics simulations of importin beta in aqueous solution and in hydrophobic media and found a large influence of the hydrophobicity of the environment on conformation and dynamical properties of importin beta. Our results indicate that the structural plasticity of importin beta is not only important for cargo recognition, but that its dependence on the environment plays an important role in the transport process. We propose a mechanism by which opening and closing of importin beta during nuclear import facilitate cargo binding, transport and release.

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1606-Pos

Polymer Brushes and the Nuclear Pore Complex

Ajay Gopinathan[1], Yong Woon Kim[2], Roya Zandi[3], Michael Colvin[1], Michael Rexach[4].
[1]University of California, Merced, CA, USA, [2]Korea Institute for Advanced Study, Seoul, Republic of Korea, [3]University of California, Riverside, CA, USA, [4]University of California, Santa Cruz, CA, USA.

The nuclear pore complex (NPC) is an important macromolecular structure that gates the aqueous pores between the cytoplasm and nucleoplasm of cells and controls all nucleo-cytoplasmic transport and communication such as the import of proteins from the cytoplasm and the export of RNA from the nucleus. The NPC forms a barrier that maintains a tight seal against cytoplasmic particles larger than 4 nm while simultaneously allowing the facilitated transport of specially ''tagged'' particles up to 40 nm diameter, at speeds comparable to free diffusion! The key to the selectivity is hypothesized to be due to a large number of NPC proteins that fill the pore and potentially interact with each other and the cargo. However, despite numerous studies on the structure and properties of individual NPC proteins, the actual structure of the complex within the nuclear pore and its mechanism of operation are virtually unknown with leading models of nuclear pore transport assuming vastly different morphologies for the NPC protein complex filling the nuclear pore. Here, we use a bottom-up approach, applying the physics of polymer brushes to understand the three dimensional architecture of the complex based on experimental understanding of the properties of individual NPC proteins. Our results indicate that there exist transitions between distinct brush morphologies (open and closed states of the gate), which can be triggered by the presence of cargo with specific surface properties. This has led to development of the Discrete Gate Model - an experimental data driven theoretical model. The resulting transport mechanism, that we propose, is fundamentally different from existing models and points to a novel form of gated transport in operation within the nuclear pore complex. Our results can also be extended to designing and optimizing novel forms of biomimetic transport based on this mechanism.

1607-Pos

Formation of the Bicoid Gradient in D. Melanogaster in Unfertilized Versus Fertilized Eggs

Jeffrey A. Drocco, Eric F. Wieschaus, David W. Tank.
Princeton University, Princeton, NJ, USA.

The concentration profile of Bicoid protein in *D. melanogaster* is an example of a morphogen gradient that has been well studied by quantitative methods. Various models have been proposed which suggest that the layer of syncitial nuclei which forms in the embryo prior to gastrulation plays a significant role in the formation of this gradient. In particular, given the small cytoplasmic diffusion constant of 0.3 microns^2/s reported by Gregor *et al.*, it has been proposed that the nuclear motion coincident with mitotic divisions serves to extend the length scale of the gradient. In this work we present time series measurements of the Bicoid gradient in unfertilized eggs, which lack nuclei other than a single female pronucleus. We find that the unfertilized gradient takes a form similar to the fertilized gradient modulated by a uniform positive offset across the egg, consistent with the claim that syncitial nuclei do not extend the length of the gradient. In addition, we present results of simulation to argue that nuclear trapping combined with observed nuclear motion is insufficient to resolve the paradox of a small diffusion constant and an embryo-length gradient.

1608-Pos

Endothelin and Phenylephrine Both Trigger Nuclear IP_3 Elevation, but Differ in Ability to Activate Nuclear HDAC5 Export

Chia-Wei J. Chang[1,2], Kathryn Helmstadter[1], Gregory Mignery[2], Julie Bossuyt[1], Donald Bers[1].
[1]University of California - Davis, Davis, CA, USA, [2]Loyola University Chicago, Maywood, IL, USA.