Quantitative Topographical Analysis of Nuclear Pore Complex Function Using Scanning Force Microscopy

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ABSTRACT The only avenue for macromolecular communication between nucleoplasmic and cytoplasmic compartments of the cell is through the nuclear pore complexes (NPCs), which are thus situated at a key location for the control of downstream transcriptional processes. The translocation of cargo through the NPC is mediated by transport receptors, which have the difficult task of making specific, yet transitory, interactions with the NPC in order to support efficient transport. In this report we have examined two stages in the translocation process using scanning force microscopy. We show that the initial docking of importin β 45-462 is rapid and occurs at nanomolar concentrations. Later stages of transport involve translocation through the central lumen of the NPC and are thought to involve hydrophobic interactions between transport receptors and the NPC. Using calcium-depleted nuclear envelopes we argue that luminal regions of the NPC exhibit hydrophobic characteristics that are not observed for other regions of the NPC.

INTRODUCTION

Transport receptors mediate the selective translocation of macromolecular cargo across the nuclear envelope. To this end transport receptors must exhibit dual specificity. On the one hand, transport receptors interact with specific amino acid residues that line the transport pathway through the nuclear pore complex (NPC). On the other hand, transport receptors are also responsible for cargo recognition via a peptide sequence that is intrinsic to the cargo molecule. Through these associations transport receptors are then able to chaperone macromolecular cargo through the NPC in a specific and effective manner.

The importin β transport complex is the best characterized nuclear transport pathway of eukaryotes. In this transport pathway specific recognition of the NPC is mediated by importin β whereas cargo binding is relegated to an adaptor molecule known as importin α. It is thus the union of cargo-bound importin α with importin β that forms the intact transport complex, which then targets to the NPC for subsequent translocation via the interactions of importin β. In particular, importin β has affinity for nuclear pore proteins, or nucleoporins, containing Phenylalanine (F) and Glycine (G) repeating motifs (Radu et al., 1995; Stewart, 2000). The transport complex remains irreversibly bound to peripheral regions of the NPC until it becomes associated with Ran-GTP, a Ras related GTPase (Görlich and Kutay, 1999). It is the binding of Ran-GTP within the nuclear compartment that causes importin β to dissociate from peripheral components of the NPC, thus effectively terminating the transport process. By contrast, the interactions of importin β with luminal nucleoporins need to be highly transient in order for transport through the lumen of the NPC to be rapid. This mechanistic disparity might indicate that the FG-repeats of peripheral and luminal filamentous NPC proteins differ in their intrinsic importin β binding affinity. Indeed, the finding that replacing the isoleucine residue at position 178 with an aspartic acid residue greatly reduces the binding of importin β to FG-repeats of peripheral filamentous nucleoporins (Bayliss et al., 2000), while at the same time supporting efficient transport through the NPC (U. Kutay, unpublished results), may testify to such a mechanistic scenario. Interestingly, a recent report has shown that the filamentous nucleoporins of the cytoplasmic compartment may be, in fact, dispensable for translocation of importin β through the NPC (Walther et al., 2002). It thus may be that more peripheral filamentous FG-nucleoporins act to concentrate cargo at the mouth of the NPC via high-affinity interactions, whereas luminal FG-nucleoporins subserve rapid translocation of cargo via lower-affinity interactions (cf. Rout et al., 2000).

In a previous study we examined the effect of binding of mutant importin β proteins on NPC topology (Jäggi et al., 2003). We found that the extent of accumulation of bound mutant transport receptors over the luminal domain of the NPC as visualized using scanning force microscopy (SFM) agreed well with the relative binding affinity of the mutant constructs for the NPC as determined in biochemical measurements. The most dramatic case was that of the 45-462 importin β mutant, which due to the truncation of its amino terminal end is unable to interact with Ran proteins and hence binds irreversibly to the NPC (Kutay et al., 1997). Furthermore, truncation of the carboxyl terminal of this mutant impedes its interaction with importin α and hence its association with nuclear localization sequence-containing cargo. Initial biochemical studies conducted with the 45-462 mutant receptor demonstrated that it accumulated at the NPC and blocked all receptor-mediated transport through the nuclear envelope, although the exact mechanism of block remained elusive.

Transport receptor binding to the NPC is not necessarily

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synonymous with the translocation process. The translocation of cargo through the NPC involves the combination of several independent processes, namely transport receptor binding, the actual translocation of cargo through the NPC, the transport of RanGDP into the nucleus, nucleotide exchange of RanGDP to RanGTP within the nucleus, and finally the association of RanGTP with importin β. The binding of the 45-462 mutant receptor to the NPC thus only represents the unique binding kinetics of importin β to FG-repeats of the NPC. In this report, we investigate the binding characteristics of importin 45-462 as a function of incubation time and initial free transport receptor concentration.

The nuclear pore complex is a 125-MDa macromolecular complex exhibiting an octagonal, radially symmetrical structure that has been the focus of intense structural characterization on both the electron microscopic (Akey, 1995; Stoffler et al., 1999; Panté and Aebi, 1996) and scanning force microscopic levels (Stoffler et al., 1999; Nevo et al., 2000; Danker and Oberleithner, 2000). Conformational changes in the central channel region of the NPC have been previously visualized by SFM measurements under conditions of calcium store depletion (Perez-Terzic et al., 1996; Moore-Nichols et al., 2002; Jäggi et al., 2003). Specifically, depleting the perinuclear calcium stores by a variety of biochemical means causes the appearance of a molecular structure within the lumen of the NPC commonly referred to as the central translocator plug, central granule, or central channel complex. Since its initial discovery (Akey, 1990; Akey and Radermacher, 1993), the nature of the central translocator plug has been a matter of much controversy. There are those that explain its existence as cargo caught in transit (Stoffler et al., 1999). On the other hand, the fact that the central plug can be shown to extrude in real time in isolated patches lacking both cytoplasmic and nucleoplasmic constituents argues against this interpretation (Wang and Clapham, 1999) and has led to the notion that the central translocator plug is an integral part of the NPC. We have previously shown that the topological characteristics of the central translocator plug are clearly distinct from those obtained under conditions where a variety of transport receptors are bound to the NPC (Jäggi et al., 2003). In this report we further demonstrate that the surface characteristics of the central translocator plug are distinct from those of the rest of the NPC or bound transport receptors. Therefore, if the topographic characteristics of a transport receptor bound to the NPC can be equated to that of cargo undergoing transport, then the central plug is most likely not cargo caught in transit or, alternatively, represents cargo of unique structural characteristics.

MATERIALS AND METHODS

Scanning force microscopy

Imaging of nuclear envelope samples was performed in liquid with a BioScope atomic force microscope (AFM) (Digital Instruments, Veeco Metrology Group, Santa Barbara, CA) using silicon nitride cantilevers (spring constant 0.16 N/m) with oxide-sharpened tips (Olympus Optical, Tokyo, Japan) as previously described. (Jäggi et al., 2003). Tapping mode AFM was employed to minimize lateral forces exerted on the sample. Typical cantilever amplitudes were 10 nm, and setpoints used for height feedback operation were larger than 90% of the free amplitude. Scan speed was adjusted to $\sim$2 μm/s.

Data analysis

Changes in NPC topology were investigated by taking cross-sectional profiles through single NPCs as previously described (Jäggi et al., 2003). Briefly, to minimize artifacts induced by thermal drift the profile measurements were taken along the fast scan direction of the SFM. The NPC topology was characterized by measurements of upper rim diameter (d) and central pore depth (z) as indicated in the inset of Fig. 1 *B*. Measuring pore diameter at the upper rim, rather than at the outer circumference of the NPC, eliminated any uncertainties associated with limited tip asperity. Thus, diameter measurements were independent of the tip radius and aspect ratio. Measured data sets for depth and diameter were well described by single Gaussian distributions. In all cases diameter and depth data are given as mean values $\pm$ SD.

Nuclei preparation

Xenopus nuclei were prepared as previously described (Jäggi et al., 2001, 2003). Briefly, oocytes were stored (18°C) in Modified Barth's Solution consisting of (in mM); 1 KCl, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 2.4 $NaHCO_3$, 88 NaCl, and 10 HEPES, pH 7.4. Nuclear envelopes were typically prepared for scanning on the day of oocyte isolation. Nevertheless, analogous results could also be obtained on nuclear envelopes from oocytes stored in Modified Barth's Solution for up to 3 days. After dissection nuclei were immediately transferred into a low salt buffer consisting of (in mM): 1 KCl, 0.5 $MgCl_2$, and 15 Tris. Nuclei were washed several times in cold ($\sim$10°C) low salt buffer to remove any adherent cellular material and debri. After cleaning, nuclei were then transferred to Mock Intracellular Buffer consisting of (in mM); 90 KCl, 10 NaCl, 2 $MgCl_2$, 0.75 $CaCl_2$, 1.1 EGTA, and 15 Tris, pH 7.32, in which they were incubated in indicated experimental conditions. After incubation in importin β 45-462 mutant protein for the indicated times and concentrations or the incubation in 10 mM EGTA for 10 min the nuclei were fixed in 2% formaldehyde and 1% glutaraldehyde at 4°C overnight. In preparation for scanning, nuclei were placed intact onto plastic tissue culture dishes, partially dried, and rehydrated in distilled water. Since the nuclei were imaged intact it was clear that imaging was performed on the outer (cytosolic), rather than on the inner (nucleoplasmic), nuclear envelope.

Molecular biology

The generation, expression, and purification of the mutant importin β fragment, 45-462, was described previously (Kutay et al., 1997). Nuclear envelopes were incubated with mutant fragment for the indicated times and concentrations before overnight fixation.

RESULTS AND DISCUSSION

Time-dependent binding of 45-462

We previously showed that incubating nuclear envelopes with the 45-462 importin β mutant completely occluded the central channel region of the NPC after just a few minutes of

exposure (Jäggi et al., 2003). The time dependence of 45-462 binding is shown in Fig. 1. Since the binding of the 45-462 mutant is an irreversible process the accumulation of bound protein over the central channel region indicates the saturation of available binding sites for importin β. In fact, very brief exposures to the 45-462 mutant protein were necessary in order to be able to clearly resolve the central channel depression of the NPC using SFM before its occupancy with bound protein. Thus, at very short exposure times to the 45-462 mutant protein the NPCs were virtually indistinguishable from those from control samples. After only 10 s of exposure to 45-462 fragment the filling of the central channel region was apparent. Saturation of 45-462 binding was apparent shortly after only 2 min of incubation. In an earlier report Ribbeck and Görlich (2001) demonstrated that transportin import into the nuclear compartment was apparent within 5 s and reached saturation after 2 min. The mechanisms for transportin translocation and 45-462 binding, although obviously related, differ on a phenomenological level, especially at early time points when translocation through the NPC might represent a nonlinear phase of the filling of the central channel region. Nevertheless, within these boundary conditions we conclude that the temporal characteristics of transportin import into intact nuclei are strikingly similar to our data examining the irreversible binding of importin β 45-462.

Dose-dependent binding

The previous time course was conducted with a set concentration of 45-462 protein of 1 μM. We next examined the concentration dependence of 45-462 mutant receptors binding to the NPC after a fixed incubation period of 2 min. Fig. 2 shows that the accumulation of mutant protein was apparent in the nanomolar range and saturated in the micromolar range. Again, these values are similar to those of Ribbeck and Görlich (2001) for transportin import into the nuclear compartment. Due to the irreversible nature of the binding of the 45-462 mutant to the NPC, values of rate constants cannot be calculated.

Perinuclear calcium store depletion

The central translocator plug is a molecular structure that is on occasion observed after depletion of perinuclear calcium stores (Perez-Terzic et al., 1996; Stoffler et al., 1999; Moore-Nichols, et al., 2002; Jäggi et al., 2003). There is some debate as to whether it is cargo caught in transit (Stoffler et al., 1999) or represents an integral part of the structure. For a more detailed discussion of the origin of the central translocator plug see Moore-Nichols et al. (2002). In this context we would like to report on experimental findings that might shed some light on this issue. Fig. 3 shows consecutive

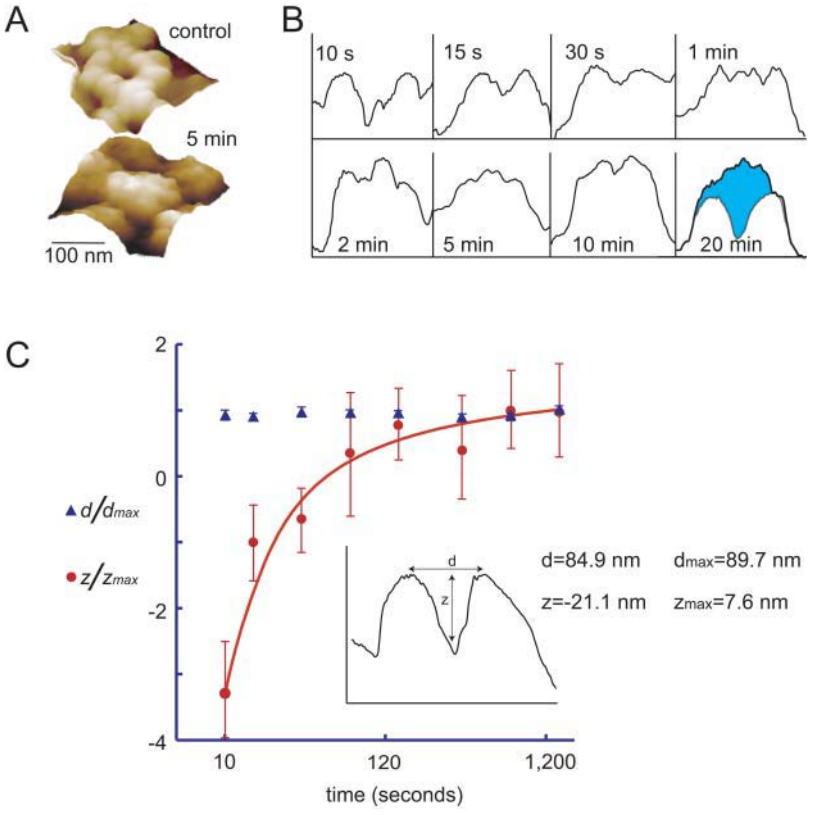

FIGURE 1 Time-dependent accumulation of 45-462 importin β over the surface of the NPC. (*A*) Scanning force microscopic images of a control NPC (*top*) and one treated with mutant transport receptor for 5 min (*bottom*). Scale bar applies to both images. (*B*) Line profiles of representative NPCs incubated with mutant protein for the indicated times. For comparison the profile of a control NPC is shown superimposed on the profile of a NPC after 20-min incubation with the 45-462 mutant receptor protein. The difference between the two profiles is shown by the shaded area. (*C*) Time response curves for normalized diameter (d/d_{max}) and vertical aspect (z/z_{max}) as a function of incubation time with 45-462 mutant protein. The data is normalized to the maximal measured mean values d_{max} and z_{max}. The line was drawn by eye and is not intended to imply a model. The inset depicts the manner in which the measurements of d and z were obtained. The number of measurements per time point are (in brackets): 10 s (50), 15 s (29), 30 s (13), 60 s (9), 120 s (14), 300 s (11), 600 s (24), 1,200 s (44). Error bars are given as standard deviation and in the diameter measurements may be smaller than the dimensions of the symbol.

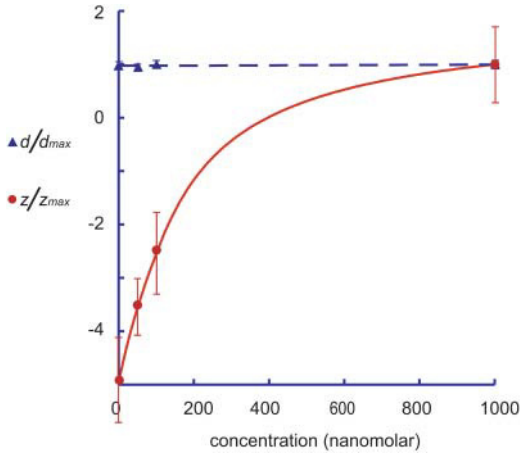

FIGURE 2 Dose dependence of 45-462 importin β binding. Filling of the central channel region becomes apparent in the nanomolar range. Incubation time at each concentration of mutant protein was 2 min. The number of measurements per concentration point are: 10 nM (46), 50 nM (86), 100 nM (33) and 1 μM (425). Error bars are given as standard deviation and in the diameter measurements may be smaller than the dimensions of the symbol. The lines through the data points were drawn by eye and imply no model.

scans performed on a single EGTA-treated NPC clearly exhibiting a central translocator plug protruding from the lumen of the NPC (Fig. 3 *A*). Interestingly, the volume of the translocator decreased with repeated scanning (3 *A*, *b* and *c*), as if portions of the translocator structure were being swept away by the scanning tip. Furthermore, the translocator surface exhibited a grainy and irregular appearance much like that often attributed to instabilities in height feedback operation. By contrast, the surrounding NPC body and the nuclear envelope surface were consistently imaged at high resolution. This is illustrated in greater detail in the profiles taken across the diameter of the same NPC (Fig. 3 *B*). The amplitude of the noise level in the height measurement increases as the cantilever scans across the surface of the central translocator (*arrow*), which is increasingly evident as more of the plug is scratched away. Furthermore, such surface characteristics were never observed in scans performed under identical AFM operation conditions examining the binding of importin β mutants on the NPC surface. On the basis of structural properties and variations in surface characteristics we are thus able to distinguish binding of transport molecules, represented by importin β 45-462, from the occurrence of the central granule.

Discussion of surface characteristics of central translocator plug

The central translocator exhibits surface characteristics differing in two principal respects from the rest of the NPC and bound importin β protein. First, large parts of the central granule can be removed with repeated scanning. Second, as more granular material is removed, pronounced cantilever instabilities become increasingly apparent. Both effects indicate increased attractive interactions between SFM tip and translocator surface. Importantly, sample material is only removed from the central granule structure, whereas the NPC rim is left intact. Moreover, importin β protein bound to the surface of the NPC was never observed to undergo such a strong interaction with the tip, even after prolonged scanning.

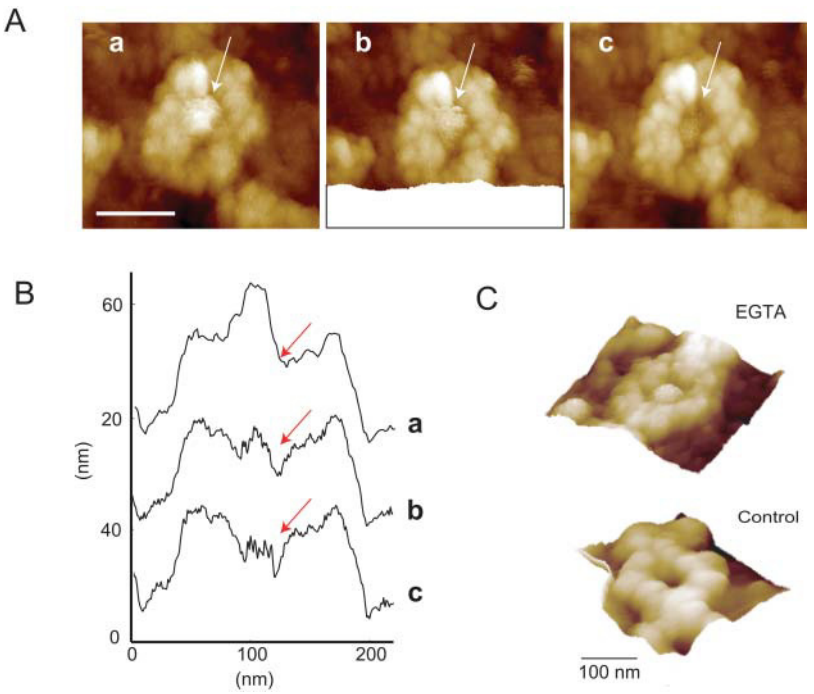

FIGURE 3 The central granule/translocator of calcium-depleted NPCs displays distinct surface characteristics and deformability from the rest of the NPC body. (*A*) Consecutive scans of the same EGTA-treated NPC. Each scan took ~8 min, and there was no delay between consecutive images. (*B*) Corresponding line profiles through the NPC shown above. The increase in noise is putatively attributed to hydrophobic interactions between the tip and the newly exposed translocator surfaces. Profile *B* (*a*) was acquired with fewer data points and thus appears smoother than *B* (*b*) and *B* (*c*). (*C*) Three-dimensional surface topography of an EGTA-treated (*top*) and control NPC (*bottom*). Perinuclear calcium store depletion was achieved by treating nuclear envelopes with 10 mM EGTA for 10 min.

Attractive forces between tip and sample can lead to cantilever instabilities and irregular height feedback if the force gradient becomes larger than the cantilever's force constant. This effect, commonly referred to as snap-in/snap-to-contact, is most frequently encountered in air due to the large capillary forces arising from the thin water film covering a sample surface. In our experiments, however, both tip and sample are completely immersed in liquids. Therefore, capillary forces are eliminated and the observed snap-in effect must therefore arise from attractive interactions between the cantilever and the central plug surface. We propose that these attractive forces are hydrophobic rather than electrostatic in nature.

In aqueous environments surface charges are strongly screened by soluble counterions, an effect attributed to the formation of a diffuse electrical double layer (Israelachvili, 1991; Müller et al., 1999). In our experiments the concentration of protein surface charges is greatly diminished through the use of formaldehyde/glutaraldehyde fixatives. Attractive electrostatic interactions are thus expected to be negligible.

Hydrophobic interactions, on the other hand, may be sufficiently strong to cause snap-in effects. Interactions between hydrophobic surfaces in aqueous liquids are always attractive (see Israelachvili, 1991, for a detailed description of hydrophobicity and hydrophobic interactions). The silicon nitride SFM tips used in our measurements were used without prior cleaning in oxygen plasma or under ultraviolet illumination, rendering them hydrophobic as determined from contact angle measurements (Knapp and Stemmer, 1999). Indeed, similar scanning instabilities are often observed between SFM tips and membranes or proteins that have been damaged, thereby exposing their hydrophobic interiors. It is thus relevant that the NPC rim and surrounding nuclear envelope was consistently imaged at higher resolution, possibly indicating that the central granule surface is more hydrophobic in nature. Importantly, the snap-to-contact effect becomes increasingly evident as the scanning tip exposes more granular material.

Relevance to previous work

The ability of transport receptors to interact with the NPC is largely determined by hydrophobic interactions (Radu et al., 1995; Stewart, 2000). These interactions are, on the one hand, mediated by the FG-repeats of nucleoporins that line the translocation pathway through the NPC and, on the other hand, by hydrophobic pockets expressed on the surface of importin β. Ribbeck and Görlich (2002) proposed that through such an arrangement transport receptor-bound cargo is able to specifically interact and thus effectively partition into the hydrophobic phase established by interconnected FG-repeats within the lumen of the NPC. While this model is still being actively discussed in the field they were able to show that small amphipathic alcohols that presumably interfere with these intraluminal hydrophobic interactions are able to disrupt the permeability barrier normally imposed by the NPC. In a previous report we showed that these small polar alcohols cause the NPC to dilate and proposed that such amphipathic alcohols, by way of disrupting the associations between luminal FG-repeats, relieve stress within the NPC structure (Jäggi et al., 2003). In further support of this interpretation Bickel and Bruinsma (2002) have recently conducted a physical analysis of the properties that would be required for cargo to partition through a confined reversible gel in a poor solvent, such as the selective hydrophobic phase proposed to fill the lumen of the NPC by Ribbeck and Görlich (2002). In the analysis put forth by Bickel and Bruinsma (2002) they proposed that intraluminal tension would intrinsically arise from the thermal fluctuations of the connections between FG-repeats comprising the reversible gel within the NPC lumen. Disrupting these connections could feasibly account for the NPC dilation observed by us (Jäggi et al., 2003) and explain the previously described breakdown in NPC specificity observed with these same small polar alcohols (Ribbeck and Görlich, 2002). Finally, the hydrophobic nature of the central translocator plug may imply that it originates from the luminal FG-repeats. The appearance of the central channel plug might thus represent the extrusion of the luminal FG-repeat matrix in response to inwardly directed pressure within the lumen of the NPC possibly (cf. Bickel and Bruinsma, 2002).

CONCLUSIONS

In this report we used scanning force microscopy (SFM) to derive quantitative measurements of transport-relevant properties of the NPC of *Xenopus* oocytes. Our values of time and concentration dependence of importin β 45-462 binding show striking similarities to the values obtained for transportin import into intact nuclei using fluorescent imaging techniques (Ribbeck and Görlich, 2001), thereby giving validity to our experimental approach. Furthermore, using this same technique we were able to show that the central translocator plug can be modified by repeated scanning with a hydrophopic tip, leaving any other domain of the NPC or nuclear envelope intact. The unique hydrophobic nature of the central translocator plug may arise from FG-repeats forming part of the NPC lumen. This result may indicate that the transport receptor binding sites (FG-repeats) expressed on the cytoplasmic rim of the NPC may be distinct in hydrophobic character and possibly binding capacity. We hence give two important examples where SFM was clearly able to provide quantitative measurements of related, although functionally distinct, transport phenomena.

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