Cryo-electron Tomography Provides Novel Insights into Nuclear Pore Architecture: Implications for Nucleocytoplasmic Transport

Daniel Stoffler1†, Bernhard Feja1†, Birthe Fahrenkrog1, Jochen Walz2†, Dieter Typke2 and Ueli Aebi1*

1Biozentrum, M.E. Müller Institute for Structural Biology University of Basel CH-4056 Basel, Switzerland
2Max-Planck Institute for Biochemistry, D-82152 Martinsried, Germany

To go beyond the current structural consensus model of the nuclear pore complex (NPC), we performed cryo-electron tomography of fully native NPCs from Xenopus oocyte nuclear envelopes (NEs). The cytoplasmic face of the NPC revealed distinct anchoring sites for the cytoplasmic filaments, whereas the nuclear face was topped with a massive distal ring positioned above the central pore with indications of the anchoring sites for the nuclear basket filaments and putative intranuclear filaments. The rather “spongy” central framework of the NPC was perforated by an elaborate channel and void system, and at the membrane pore interface it exhibited distinct “handles” protruding into the lumen of the NE. The most variable structural moiety of the NPC was a rather tenuous central plug partially obstructing the central pore. Its mobile character was documented by time-lapse atomic force microscopy. Taken together, the new insights we gained into NPC structure support the notion that the NPC acts as a constrained diffusion pore for molecules and particles without retention signal and as an affinity gate for signal-bearing cargoes.

Keywords: cryo-electron microscopy; electron tomography; nuclear pore complex; nucleocytoplasmic transport; 3-D reconstruction

Introduction

Nuclear pore complexes (NPCs) are large supramolecular assemblies embedded in the double-membrane nuclear envelope (NE) that allow passive diffusion of ions and small molecules, and mediate signal-dependent transport of proteins, RNAs and RNP s in and out of the nucleus.1 The 3-D architecture of the NPC has been unveiled by extensive electron microscopy (EM) studies including 3-D reconstructions of amphibian NEs, according to which the vertebrate NPC exhibits a tripartite architecture with an 8-fold rotational symmetry, a maximum diameter of ~120 nm in the plane of the NE, an overall dimension of ~180 nm perpendicular to the plane of the NE, and a total mass of ~125 MDa.2–8 The ~55 MDa central framework of the NPC, i.e. the NPC moiety residing in the double-membrane of the NE, consists of eight spokes, sandwiched between a ~32 MDa cytoplasmic ring moiety and a ~21 MDa nuclear ring moiety.2 From the cytoplasmic ring eight ~50 nm long, kinky filaments emanate, whereas the nuclear ring is capped with a cage-like structure termed nuclear basket, which is assembled from eight tenuous, ~75 nm long filaments joined distally by a massive ~55 nm-diameter distal ring. The ring-like central framework harbors a large central pore, which is implicated in signal-dependent bi-directional nucleocytoplasmic transport. The central pore often appears plugged with a “particle” of variable size and appearance, termed central plug or transporter.5,9 The functional role of the plug remains to be established, although it is conceivable that this polymorphic structure may in fact be

†These authors have equally contributed to this work.

‡Present addresses: D. Stoffler, The Scripps Research Institute, La Jolla, CA 92037, USA; B. Feja, Tietz Video and Image Processing Systems GmbH, D-82131 Gauting, Germany; J. Walz, Siemens AG, D-80312 Munich, Germany.

Abbreviations used: NPC, nuclear pore complex; NE, nuclear envelope; AFM, atomic force microscopy; EFTEM, energy-filtering transmission electron microscopy.

E-mail address of the corresponding author: ueli.aebi@unibas.ch

© 2003 Elsevier Science Ltd. All rights reserved

0022-2836/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved
mobile, for example, represent cargo caught in transit, rather than representing a stationary component of the NPC. A 3-D reconstruction of detergent-released (dform), negatively stained Xenopus NPCs further revealed that the central framework of the NPC exhibited a high degree of 822 symmetry, i.e. 8-fold symmetric relative to its central axis, and 2-fold symmetric relative to its central plane. The 822 symmetrized 3-D map exhibited eight distinct peripheral channels with an average diameter of ~9 nm, which were suggested to represent sites for passive diffusion of ions and small molecules. A 3-D reconstruction of dform Xenopus NPCs imaged in amorphous ice unveiled similar basic features for the domain architecture of the central framework. It further yielded a massive, hollow transporter being seated in the central pore and exhibiting a tripartite morphology that spanned the entire width of the central pore. Due to its high variation among distinct NPCs, this central transporter/plug was omitted in the Hinshaw et al. reconstruction. Similar to the 3-D reconstruction in negative stain, eight peripheral channels could be mapped, although the radial position of these putative diffusion channels was distinct from that of Hinshaw et al.

Compared to the dform NPCs, the 3-D reconstruction of membrane-associated (iorm) Xenopus NPCs in amorphous ice revealed significant differences in the relative location, orientation and interactions of the radial spokes comprising the central framework. Contrary to the dform NPCs the central plug was omitted in the 3-D reconstruction of these iorm NPCs. The observed structural differences between the dform and iorm 3-D reconstructions might have been caused by detergent extraction (dform), osmotic shock within the lumen of the NE and/or mechanical distortions during specimen preparation. Mechanical distortions and surface tension might also have led to a collapse of the cytoplasmic filaments and the nuclear baskets during specimen preparation so that these peripheral structural components were not visible in both the dform and iorm 3-D maps.

A 3-D map of the yeast dform NPC from frozen-hydrated specimens provided a direct comparison with the vertebrate dform NPC. Accordingly, the yeast NPC 3-D reconstruction revealed a rather flat ~822 symmetric NPC without, however, exhibiting a distinct cytoplasmic or nuclear ring moiety. Similar to the vertebrate dform, the yeast 3-D reconstruction revealed eight peripheral channels within the central framework. Consistent with its smaller linear dimensions, the mass of the yeast NPC was determined to be ~60 MDa. By comparing the overall dimensions of the yeast and vertebrate 3-D reconstructions, the yeast NPC must therefore be significantly more compact than the vertebrate NPC, since otherwise its mass would amount to only ~30 MDa. The existence of cytoplasmic filaments and a nuclear basket has also recently been documented for the yeast NPC, which again, could not be depicted in the 3-D reconstruction.

To go beyond the current consensus model of the NPC, we have employed energy-filtering transmission electron microscopy (EFTEM) and tomographic 3-D reconstruction of fully native (i.e. no detergent treatment, no chemical fixation, and no heavy metal staining) Xenopus oocyte NEs embedded in thick (i.e. 200–300 nm) amorphous ice. A first attempt has been made to incorporate the novel structural insights emerging from this 3-D reconstruction into a functional model of the NPC that describes the mechanistics of nucleocytoplasmic transport at the single pore level. This model also accounts for the temperature-dependent plugging and unplugging of the central pore, as depicted by time-lapse atomic force microscopy.

**Results**

The NPC is a highly flexible structure and therefore susceptible to surface tension and mechanical stress. Observed structural differences among NPCs may therefore predominantly represent mechanical deformations caused by specimen preparation rather than distinct functional states of the NPC. Negatively stained and frozen hydrated NPCs embedded in an amorphous ice layer of variable thickness tend to collapse during specimen preparation. To minimize artifacts caused by sample preparation and to achieve the best possible structural preservation of the NPCs, we first analyzed the effect of the ice thickness on the overall NPC architecture of native Xenopus NPCs.

**The ice thickness affects NPC conformation**

Native (i.e. no detergent treatment, no chemical fixation, and no heavy metal staining) Xenopus NEs were spread over holey carbon support films leaving most of the NPCs unsupported, and completely embedded in an amorphous ice layer. As documented in Figure 1, thus prepared samples exhibited ice thickness gradients extending over single holes so that the effect of the ice thickness on individual NPCs residing on one and the same grid could be examined directly. The ice thickness gradients were produced by slowly heating up the cryo-stage from −180 °C to −120 °C within a period of 30 minutes, followed by re-cooling to −180 °C within another 30 minutes, thereby minimizing dehydration damage. The heat was dissipated fastest near the carbon moiety of the perforated film, so that the ice layer was thickest in the center and thinnest along the borders of each hole (Figure 1(a)). No cubic ice was visible before, during or after the sublimation procedure. As documented in Figure 1(b) (lower half), NPCs embedded in thin-ice tended to collapse and/or deform due to surface tension.
Figure 1. Representative area of a chemically unfixed and unstained native *Xenopus* nuclear envelope spread over a perforated carbon support film and embedded in a thin layer of amorphous ice after partial dehydration. Towards the center of each hole, the ice thickness increases, thereby forming a smooth gradient extending radially over each hole (a). A higher magnification view of the same sample (b) revealed partially damaged NPCs residing at the border of the hole covered by a relatively thin ice layer (lower half). Filamentous connections between individual NPCs might represent collapsed cytoplasmic and/or nuclear filaments. Towards the center of each hole the ice thickness increased (upper half) and, consistently, the corresponding NPCs exhibited less damage.

Figure 2. Contrast enhancement by zero-loss filtering. Image pair of unfixed/unstained NPCs embedded in a 350 nm-thick ice layer. (a) Unfiltered and (b) zero-loss filtered image.
they lost their round shape, and their 8-fold rotational symmetry became effaced. Filamentous connections between individual NPCs were depicted, most likely representing collapsed cytoplasmic and/or intranuclear filaments. In contrast, NPCs embedded in thick ice appeared less damaged with a more regular, round shape and 8-fold rotational symmetry (Figure 1(b), upper half). Hence, thick-ice embedding yielded superior structural preservation of the NPCs, i.e. less surface tension and/or mechanical stress.

**Zero-loss EFTEM improves contrast and resolution of NPCs embedded in thick ice**

Imaging of thick-ice embedded unstained specimens comes along with a significant loss of contrast and resolution due to the increased number of inelastically scattered electrons produced (see Figure 1). To overcome this drawback, images were recorded in an EFTEM. Zero-loss energy filtering removes the inelastically scattered electrons, which in turn, are subject to chromatic aberration by the objective lens of the EM, thereby causing a significant loss of contrast and resolution. Direct comparison of an unfiltered (Figure 2(a)) with a zero-loss filtered (Figure 2(b)) image of native Xenopus NPCs embedded in ~350 nm thick amorphous ice documents the significant gain in contrast of the energy-filtered image. The logarithm of the intensity ratio for an unfiltered (Figure 2(a)) and the corresponding zero-loss filtered (Figure 2(b)) image of a given specimen area is proportional to the relative ice thickness. Hence, multiplication of this logarithm with the mean free electron path yields the absolute thickness of the ice in this specimen area. Implemented in the image acquisition software, this calculation allows direct on-line determination of the ice thickness at any given specimen site.

As documented in Figure 3(a), NPCs embedded in ~250 nm amorphous ice and imaged by zero-loss EFTEM exhibited a regular round shape with well preserved 8-fold rotational symmetry. In several instances the central plug appeared ring-like with struts emanating radially (Figure 3(a), arrows; see also Figure 6(a), arrows), most likely representing intact nuclear baskets. The ring-like plugs were located above rather than residing within the central pore, i.e. as judged from stereo pairs (data not shown), most likely representing the distal ring of the nuclear baskets (see below and Figure 4). Correlation averaging of thick-ice embedded NPCs (Figure 3(b)) yielded remarkable substructure, particularly of the radial spokes, but also in terms of preserving the 8-fold rotational symmetry.

**Tomographic 3-D reconstruction of thick-ice embedded NPCs revealed novel structural features**

For tomographic 3-D reconstruction of native NPCs, tilt series of thick-ice (i.e. 200–300 nm) embedded Xenopus oocyte NEs (see Figures 3(a) and 6(a)) were recorded automatically over tilt angles ranging from −60° to +60° in 5 degree increments on a Philips CM120 Biofilter electron microscope operated in the zero-loss imaging mode. The fully automated data collection that included tasks such as tilting the grid, recentering the specimen area, refocusing, and centering the energy-selecting slit aperture under low-dose conditions, minimized beam-induced specimen damage or deformation during image acquisition. 3-D maps of native, thick-ice embedded Xenopus NPCs were calculated from 446 individual NPCs selected from ten tilt series (see Materials and Methods).

The 3-D mass-density map of the averaged (i.e. over 446 individual NPCs; see Materials and Methods) NPC exhibited strong 8-fold rotational symmetry of the central framework (Figure 4(a)–(d)) with a rather tenuous plug residing in the central pore (Figure 4(b) and (d), marked CP). The central framework is perforated by eight...
On the cytoplasmic face, rudiments of the eight cytoplasmic filaments decorating the cytoplasmic moiety were resolved (Figure 4(b) and (c), marked CF). Due to the high flexibility of the cytoplasmic filaments it was not possible to capture them in a unique spatial conformation, so that only their anchoring sites could be enhanced.

The central framework consists of two similar halves put together back-to-back

Although not enforced in the tomographic 3-D reconstruction, the central framework of the NPC appears to consist of two similar halves that have been put together back-to-back (Figure 5, central panel). After closer inspection the nuclear ring moiety of the central framework looks slightly more massive compared to the cytoplasmic ring moiety (Figure 5, left panel: compare NR with CR). Cross-sections through the central framework in 6 nm steps confirmed this impression (Figure 5, right panel: compare slices 1, 2 and 3 with slices 11, 12). Slice 1 cuts the central framework close to its nuclear face whereas slice 12 yields a cross-section close to its cytoplasmic face. Slice 7 represents the midplane of the central framework. The 13 cross-sections also depicted the pronounced vorticity of the central framework: the nuclear half (Figure 5, right panel: slices 1–6) exhibits an anticylockwise vorticity, which turns via slice 7, having no net vorticity, into a clockwise vorticity of the cytoplasmic half (Figure 5, right panel: slices 8–13), just as described by Akey & Radermacher for their dform NPC.

Towards understanding the nature of the central plug

The NPCs often appear plugged in projection images of both negatively stained and frozen hydrated NEs, with an overall highly variable size and shape from NPC to NPC (Figures 3(a) and 6(a)). Cross-sections through the tomographic 3-D reconstruction yielded a solid, poorly structured mass in the center of the pore (Figure 5) with a high degree of variation of the overall size and shape in the “even” and “odd” subaverages computed from the 446 individual NPCs comprising the complete data set (see Materials and Methods; data not shown). In thick-ice embedded NPCs the central plug often appears as a ring-like structure (Figure 6(a), marked by arrows; and insets), which in stereo-pairs of such micrographs appear to be located above instead of residing within the central pore. This view is consistent with our 3-D reconstruction of the NPC (see Figure 4) where the dominating structural feature about the central axis is the distal ring. When viewed from the nuclear side, quick-frozen, frozen-hydrated and metal-shadowed NPCs exhibited a well-preserved nuclear basket topped with a massive distal ring (Figure 6(b), bottom; see arrows). In contrast, when viewed from the cytoplasmic side, most peripheral holes (Figure 4(a) and (c), marked PH) with a ~10 nm diameter. Attached radially to the central framework are eight distinct “handles” (Figure 4(c) and (d), marked HA) that might actually reside in the lumen of the double-membraned NE thereby anchoring the NPC within the NE.

Located above the nuclear entry of the central pore is a distinct massive ring-like structure (Figure 4(a), (c) and (d), marked DR), evidently representing the distal ring of the NPC’s nuclear basket. The distal ring revealed eight “bumps” (Figure 4(a) and (c), marked BF), most likely representing the attachment sites of the nuclear basket struts. Towards the nuclear interior eight “stumps” emanated from the distal ring (Figure 4(a) and (c), marked IF), most likely being the anchoring sites of the intranuclear filaments. In fact, the intranuclear filaments were cut off right above the distal ring during the reconstruction process (see Materials and Methods).
NPCs did not harbor any significant mass in their central pore (Figure 6(b), top). In fact, in some instances the basket was visible by looking through the empty central pore, suggesting that the distal ring might significantly add to what appears in projection images as the central plug.

To better understand the nature of the central plug and how it might be involved in or related to nucleocytoplasmic transport, we performed time-lapse atomic force microscopy (AFM) of native Xenopus NEs spread on EM grids and kept in physiological buffer (see Materials and Methods). Before spreading the NEs, the oocytes were equilibrated at 4 °C for at least one hour, a condition known to strongly attenuate nucleocytoplasmic transport. When kept at 4 °C, AFM images of the cytoplasmic face of spread NEs yielded typically 45% of the NPCs being plugged (Figure 7(a)). In contrast, after raising the sample temperature to 25 °C (i.e. being permissive for nucleocytoplasmic transport) and re-imaging the same NE area, only about 12% of the NPCs appeared plugged (Figure 7(b)). In particular, NPCs that were plugged at 4 °C became unplugged at 25 °C (Figure 7: compare the plugged NPCs marked in (a) with the corresponding unplugged NPCs marked in (b)). These AFM data suggest that what appears as a central plug judged from the cytoplasmic surface topography of NPCs, represents at least in part cargo caught or arrested in transit.

Discussion

To determine the 3-D architecture of vertebrate NPCs in more detail, a tomographic 3-D reconstruction was computed by weighted backprojection from single-axis tilt series recorded from native (i.e. no detergent treatment, no chemical fixation, and no heavy metal staining) Xenopus

Figure 5. Cross-sections through the central framework calculated from the 3-D map. Left panel: the central framework (CF) is averaged over slices 4–10, its cytoplasmic ring moiety (CR) over slices 11–13 and its nuclear ring moiety (NR) over slices 1–3. Right panel: cross-sections through 3-D reconstruction in ~6 nm steps. Slice 1 cuts the 3-D reconstruction close to the nuclear face, whereas slice 13 represents a cross-section close to the cytoplasmic face. Slice 7 roughly represents the midplane of the NPC, which yields the highest degree of 822 symmetry. The cross-sections depict the vorticity of the NPC: the nuclear half (i.e. slices 1–6) exhibits an anti-clockwise vorticity, which smoothly turns into a clockwise vorticity of the cytoplasmic half (i.e. slices 8–13). Slices 4–10 reveal a solid central plug, however, with no indication of a hollow interior.
oocyte NEs embedded in thick (200–300 nm) amorphous ice. This novel 3-D reconstruction is in good qualitative agreement with previous reconstructions\textsuperscript{4,5,11} and, additionally, resolved for the first time the anchoring sites of the cytoplasmic filaments residing on the cytoplasmic ring moiety and the massive distal ring of the nuclear basket positioned above the nuclear entry to the central pore in a 3-D reconstruction representing an average over 446 NPCs. Distinct structural features of

![Image](image_url)

**Figure 6.** A closer look at the central plug. (a) In projection NPCs often appear plugged (see arrows). Higher magnification images (see the four insets, bottom left) of native, thick-ice embedded NPCs revealed donut-shaped, hollow rings most likely representing the distal ring of the nuclear basket. Correlation averages over 100 pores (inset, bottom right) revealed a donut-shaped ring residing in the center of the pore. (b) Quick-frozen, frozen-hydrated and metal-shadowed NPCs (Jarnik & Aebi\textsuperscript{3}), when viewed from the cytoplasmic face, most did not harbor any significant mass in their central pore (top). Cytoplasmic filaments are highlighted with arrowheads. When viewed from the nuclear side, the NPCs revealed a rather well preserved nuclear basket topped with a massive distal ring (bottom; see arrows). Scale bars represent 200 nm.

![Image](image_url)

**Figure 7.** Watching temperature-dependent plugging or unplugging of individual NPCs by time-lapse AFM. The same specimen area, adsorbed with its nuclear face, was imaged by AFM in contact mode at 4°C (a) and after warming the system up to 25°C (b). Previously plugged individual NPCs (a) become unplugged upon raising the temperature (b). Four corresponding NPCs are marked by blue arrowheads for better comparison. Insets represent magnifications of two NPCs marked by blue arrowheads.
the distal ring most likely were the origins both of the nuclear basket filaments and the intranuclear filaments. Moreover, time-lapse AFM enabled us to directly correlate the plug frequently obstructing the central pore of the NPC with cargo caught or arrested in transit.

A closer look at NPC architecture

2-D back-projection of the sections spanning the intra-NE moiety of the central framework revealed three concentric rings (sections 4–10 in Figure 5, left panel marked CF). This 2-D back-projection is surprisingly similar to the averages of 2-D projection images of both negatively stained and frozen-hydrated dform NPCs, indicating that the cytoplasmic and nuclear ring moieties (see Figure 5, left panels marked CR and NR) were disordered or lacking from the NPCs of both dform preparations, probably due to the detergent treatment.

Distinct handles protruding radially from the central framework into the lumen of the NE were resolved in our 3-D reconstruction (see Figure 4(c) and (d), marked HA) but were absent from the 3-D reconstruction of negatively stained dform NPCs. It is conceivable that these might have collapsed onto the bulk of the central framework, or that the heavy metal staining filled the space between the handles and the lumenal spoke domain. 3-D reconstructions of both dform and mform NPCs embedded in amorphous ice revealed radial arms protruding from the lumenal spoke domains. The dominant structural feature at the center of every NPC, thus the dominant assembly undergoes distinct and reversible conformational changes. In contrast, depending on the NPC's mid-plane.9 Evidently, during Balbiani ring particle translocation through the central pore this 2-fold symmetric transporter assembly undergoes distinct and reversible conformational changes.9 In contrast, depending on isolation, sample handling and specimen...
preparation, earlier EM data have documented the abundance as well as the overall size and shape of the central plug to be highly variable. Our tomographic 3-D reconstruction of native NPCs embedded in thick amorphous ice yielded a rather tenuous solid particle residing in the central pore whose mass amounted to about 40% of that of the distal ring (see Figure 4). Subaverages computed from our data set comprising 446 NPCs revealed this particle to be highly variable in terms of its overall size and shape, indicating that it might predominantly represent cargo caught in transit rather than a bona fide NPC component, i.e. a transporter (see below).

Nucleocytoplasmic transport can be arrested at 4°C. Hence when intact isolated Xenopus nuclei were kept for one hour in physiological buffer at 4°C prior to being prepared for EM, over 90% of the NPCs’ central pore appeared plugged. Similarly, when intact isolated nuclei were kept for one hour in physiological buffer in the absence of ATP before being spread on EM grids, almost all of the quick-frozen, freeze-dried and metal-shadowed NPCs revealed an abundant plug in their central pore. In contrast, in the presence of 1 mM ATP, under otherwise the same conditions, the NPCs’ central pore appeared predominantly unplugged (N. Panté and U.A., unpublished results). In the present investigation the temperature-dependent appearance/disappearance of a plug in the central pore of the NPC was complemented by time-lapse AFM experiments (Figure 7), suggesting that there exists a direct correlation between the transport state of the NPCs and the abundance of material obstructing the central pore. In an optimal transport state, the central pores appear mostly unplugged, whereas in a transport inhibited state, the central pores appear obstructed by cargo arrested in transit. AFM images were recorded in contact mode, in accordance with previous AFM experiments. Under these low-force scanning conditions it is highly unlikely that, for example, the central mass depicted in Figure 7 could be displaced from the central pore by the scanning tip without visibly compromising the cytoplasmic ring moiety. As to the nature of the central plug depicted in 2-D projection images of the NPC (cf. Figures 1(b), 3(a) and 6(a)), for the larger part it represents the distal ring of the nuclear basket positioned above the central pore, and as a more variable contribution it is most likely cargo in transit through the central pore.

**Implications for nucleocytoplasmic transport**

The primary finding of the present investigation is that the major, and probably exclusive, transport channel of the NPC is a roughly cylindrical pore, ~50 nm in diameter and ~90 nm in length without any evidence for a massive, central transporter that may assume multiple functional configurations. By the fact that the NPC is able to transport macromolecules with diameters of up to 40 nm, the central pore cannot be “obstructed” with a stationary structure of even the relatively small size depicted in our 3-D reconstruction. If real, this structural moiety would have to be highly mobile or deformable in nature.

Mechanistically, a central pore of the size and shape described here is ideally suited for factor-mediated transport of signal-bearing cargoes. *A priori*, there is indeed no need for a physical gate, as long as the central pore acts as an affinity gate for the cargo, i.e. via transport factors accompanying the cargo through the pore that interact with FG-repeat nucleoporins. The question is more as to how such a large pore can act as a permeability barrier to particles that do not have an affinity for the NPC either directly or via a transport factor to which they bind. Experimentally, the flux of inert particles larger than 20–40 kDa becomes 10–100 times slower than calculated by Fick’s first law. A *priori*, depending on the exact morphology and physical properties (e.g. charge and hydrophobicity) of the central pore, in concert with molecular crowding occurring in the near field of the NPC, an attenuation of this magnitude may indeed be achieved. More explicitly, it has recently been proposed that the FG-repeat nucleoporins may form a meshwork-like assembly within the central pore that restricts the passage of inert particles to just small molecules. While consistent with a number of functional data, immuno-EM of yeast and vertebrate FG repeat nucleoporins do not readily support this model. Rather, these nucleoporins have predominantly been localized to either the cytoplasmic or nuclear periphery of the NPC (reviewed by Fahrenkrog & Aebi). Nevertheless, it is conceivable that, as described recently for Nup153, FG repeat domains more generally are highly mobile and extended so that, while being tethered to the cytoplasmic or nuclear periphery, they may reach into or even through the central pore (see below). This way they could interact with each other so as to form a “transient plug” that may obstruct the central pore and thereby attenuate passive diffusion of cargo that is not complexed with a transport factor.

The other major finding of the present investigation is the massive, ~8 MDa distal ring that is positioned above the nuclear entry of the central pore. The observation that it acts like an iris diaphragm, i.e. it can open and close in response to chemical or physical stimuli, renders this distinct NPC moiety a morphological hallmark for cargo while traversing the NPC. In fact, a similar iris-like behavior of the NPC’s central transporter during cargo translocation has been described previously. This latter observation raises the question as to whether Akey was actually observing the distal ring, rather than a central transporter, that might have been pushed into the central pore by surface tension in his projection images of thin-ice embedded spread NEs. To this end, it is interesting to note that the overall size and shape of
the distal ring are such that it fits quite snugly into the nuclear entry of the central pore (see Figure 4), thereby plugging it.

A major molecular constituent of the distal ring is the FG-repeat containing nucleoporin Nup153.35,36 Whereas its N-terminal domain maps to the nuclear ring moieties,35,37 its central Zn-finger domain resides at the distal ring, and its FG-rich C-terminal half (i.e. harboring ~35 FG-repeat motifs) is highly mobile, i.e. reaching from the distal ring via the nuclear ring all the way to the cytoplasmic opening of the central pore.35 Evidently, this 602-residue-long FG-rich C-terminal domain of Nup153 is mostly unstructured, spanning a distance of about 180 nm when fully extended.35 Acting as a terminal docking site for nuclear import cargoes,36 the FG-rich C-terminal domain of Nup153 exhibits a relatively high binding affinity for the transport factor importin-β.35 That property combined with the high mobility of the FG-rich C-terminal domain of Nup153 that is tethered to the distal ring via Nup153’s Zn-finger domain, render it well suited to bind cargo at the cytoplasmic entry of the central pore and escort it through the pore into the nuclear basket where it is discharged into the nucleus by the action of a “molecular switch”, i.e. as soon as RanGTP binds to the cargo complex via importin-β.

Materials and Methods

Preparation of native nuclear envelopes

Oocytes were removed from female Xenopus laevis as described.3 Individual stage-6 oocytes were placed in a small Petri dish filled with low salt buffer (LSB; 1 mM KCl, 10 mM Hepes, pH 7.5). Nuclei were manually isolated and transferred into a 5 μl drop of LSB residing on a 200 mesh/inch copper grid coated with a holey carbon film.40 The nucleus was opened manually as described,10 and the NE was spread over the grid. After washing the grid with LSB, a 5 μl droplet of LSB was applied and the system equilibrated for 15 minutes. All preparation procedures were performed at room temperature. After blotting, the grids were quick-frozen in liquid ethane at −180 °C using a custom-built pneumatic plunge-freezer.16

Zero-loss filtered cryo-electron microscopy

Zero-loss filtered images for 2-D image analysis were recorded on an EM912 Omega EFTEM (LEO Electron Microscopy, Oberkochen, Germany) equipped with an Oxford cryo-holder (Oxford CT3500 cryo-transfer system, Oxford Instruments, Oxon, UK). The images were recorded digitally with a 1024 × 1024 pixel, 14 bit deep slow-scan CCD camera (Proscan, Scheuring, Germany). The microscope and the camera were controlled by a VIPS-1000 (Tietz Video and Image Processing Systems, Gauting, Germany). The sample thickness was determined on-line on an absolute scale from a zero-loss-filtered/unfiltered image pair by a macro routine applying the log/ratio method16 combined with the experimentally determined partial inelastic mean free electron path. All images were recorded at 120 kV acceleration voltage. The magnification was 15,600 × on the camera, and the defocus was 1.5 μm. The electron dose ranged between 300 e−/nm² and 500 e−/nm².

Automated cryo-electron tomography

For 3-D reconstruction the data were collected by recording automated zero-loss filtered, single-axis tilt series using a CM120 Biofinner TEM (Philips, Eindhoven, The Netherlands) equipped with a post-column energy filter (Gatan imaging filter GIF100) and a Gatan cryo-holder (Gatan Inc., Pleasanton, CA). The programs used for recording fully automated tilt series were implemented in the software package Digital Micrograph (Gatan) on an Apple Macintosh computer. All images were recorded digitally with a 1024 × 1024 pixel, 14 bit deep slow-scan CCD camera (Gatan 500 kHz read-out Multiscan camera), at an acceleration voltage of 120 kV under low-dose conditions (i.e. 2000–2800 e−/nm² per tilt series), with a magnification of 14,500 × on the camera and a defocus setting of 1.5 μm. The specimens were tilted from −60° to +60° in 5° increments.

2-D Image processing

2-D image processing was performed on a DEC Alpha workstation running VMS. For alignment and correlation averaging of individual NPC projection images the Semper image processing system was used. For display, all NPC projection averages were 8-fold rotationally symmetrized, contrast inverted, and circularly masked. For better recognition of the structural features, ten equidistant contour lines were put into the upper half of the mass density maps of the averaged NPC images.

3-D Image processing

Alignment of individual projection images of a given tilt series and 3-D reconstruction by weighted back-projection were performed using the EM system running on an SGI workstation (Silicon Graphics, Mountain View, CA). Individual projections of each tilt series were first aligned relative to a common origin using cross-correlation, and the reconstructed volumes of ten tilt series were calculated with sizes up to 1024 × 1024 × 384 pixels. The x, y-positions of individual NPCs were marked manually, and 446 sub-volumes were extracted, with sizes between 96 × 96 × 192 and 96 × 96 × 384 pixels. After coarse visual determination of the z-position of each particle within its data cube the volume was reduced to 96 × 96 × 96 pixels. Re-projections in z-direction were aligned with respect to x and y-shift and in-plane rotation. A first average was calculated by summing the aligned cubes, and this average was used as a first reference for refinement of the z and phi-alignment via cross-correlation in cylindrical coordinates by five iterative cycles. Following this, an average was calculated with the alignment parameters determined up to this point. This time, the exact weighting function was calculated from the projection geometry of the tilt series, and the alignment parameters were applied to the particle projections before performing the back-projection. At this stage, the 8-fold symmetry of the particles was imposed to enhance their signal-to-noise ratio. This second average was then used as a reference for refinement of the six alignment parameters.
Atomic force microscopy
For AFM, samples were prepared as described in the cold room at 4°C. Following mounting of the sample, the Petri dish holding the grid with the spread NE was placed onto the vacuum clamp of a BioScope (Digital Instruments, Santa Barbara, CA93117, USA). Samples were viewed at 8-fold magnification through the eye-pieces of a Zeiss Axiophot inverted light microscope on which the BioScope AFM system was mounted. Images were recorded in contact mode exactly as described. Samples were first scanned at 4°C and rescanned after warming up the buffer and equilibrating it at 25°C.

Acknowledgements
This work was supported by the M. E. Müller Foundation of Switzerland, an NCCR grant awarded by the Swiss National Science Foundation (to U.A.), a Human Frontier in Science Program (HFSP) grant (to U.A.), and a grant from the Chemical Industry Foundation of Basel (to D.S.).

References
3-D Reconstruction of Native Nuclear Pore Complexes

Edited by W. Baumeister

(Received 24 October 2002; received in revised form 17 February 2003; accepted 17 February 2003)