A Pathway Separate from the Central Channel through the Nuclear Pore Complex for Inorganic Ions and Small Macromolecules

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Nuclear pore complexes (NPCs) provide the sole gateway for transport between the nucleoplasm and the cytosol. NPCs are supramolecular structures with an approximate molecular mass of 125 MDa and comprise 30–50 different proteins, with multiple copies of each. NPCs act as a selective barrier that controls the diffusion of macromolecules. The size of the meshwork has been estimated at ~45–50 nm in diameter (3–5). This is contradicted by the size exclusion limit for the passive diffusion of macromolecules being ~9 nm (6). In fact, electron microscopy and the results of electron microscope data indicate the presence of a large central channel ~45–50 nm in diameter (3–5). This is visible in the cross-sections of NPCs. The central plug must be somewhat flexible, allowing the transport of ions and small molecules through the central channel, the route for ions and small macromolecules through the pore periphery.

This study provides direct evidence for a pathway for ions and small molecules separate from the central channel. This pathway is not blocked by transport factors that appear to be the most hydrophobic soluble proteins, such as importin-β (12). The size of the mesh has been estimated at ~3 nm (18).

The first computer model of the NPC by Hinshaw et al. in 1992 (4) led to speculation that ions and small macromolecules use small channels with a radius of ~10 nm, located in the periphery of NPCs. Subsequent data provided further evidence for the existence of these peripheral channels (3, 21), although their functionality remained unclear.

The above observations show that the central channel is not an open gap, but rather is occupied by a meshwork formed by hydrophobic FG repeats that can only be opened by specific hydrophobic keys (transport factors). This gives new reason to suspect an additional permeability in the periphery of the pore because macromolecules up to 9 nm in diameter are able to diffuse freely through the NPC (6). So far, only a few studies have favored the hypothesis of a separate route for ions and small molecules (4, 22–24). Shahin et al. (24) revealed that calcium depletion of the perinuclear space, which leads to inhibition of signal-promoted protein transport (25, 26), has a contrary promotional effect on ion conductivity.

This study provides direct evidence for a pathway for ions and small solutes that is separate from the central channel. To achieve this, we combined three methods: (i) the nuclear hourglass technique (NHT) (22), which allows measurement of electrical resistance of the nuclear envelope; (ii) atomic force microscopy (AFM), which allows topographic measurements of NPCs; and (iii) permeability assays with fluorescently labeled dextran molecules, which provide information regarding the function of the nuclear envelope barrier.

**EXPERIMENTAL PROCEDURES**

Preparation of Cell Nuclei—Xenopus laevis females were anesthetized with 0.1% ethyl m-aminobenzoate methanesulfonate (Serva, Heidelberg, Germany), and their ovaries were
removed. Oocytes were dissected from ovary clusters and stored in modified Ringer’s solution (87 mM NaCl, 6.3 mM KCl, 1 mM MgCl₂, 1.5 mM CaCl₂, 10 mM HEPES, 100 units/100 μg penicillin/streptomycin, pH 7.4) until used. For isolation of the cell nuclei, the oocytes were transferred into nuclear isolation medium (NIM) composed of 90 mM KCl, 26 mM NaCl, 5.6 mM MgCl₂ (corresponding to a free Mg²⁺ concentration of 2 mM), 1.1 mM EGTA, and 10 mM HEPES and titrated to pH 7.4. We added 1.5% polyvinylpyrrolidone (Mₚ = 40,000; Sigma) to compensate for the lack of macromolecules in NIM, mimicking the intact cytosol. The presence of polyvinylpyrrolidone is crucial to prevent the swelling (>100% in the absence of polyvinylpyrrolidone) of total nuclear volume that occurs instantaneously after isolation in pure electrolyte solution. We used X. laevis oocytes because they provide the unique possibility to manually prepare the nucleoplasmic and cytoplasmic faces of a nuclear envelope for AFM investigations.

**NHT**—The technical aspects of this method and its application to isolated cell nuclei have been described previously in detail (22). In brief, this method uses a tapered glass tube that narrows in the middle to two-thirds the diameter of the nucleus. A current of up to 1 mA is injected via two Ag/AgCl electrodes through either end of the glass tube. The voltage drops across the cell nucleus are measured with two conventional microelectrodes, the tips of which are placed near the narrow part of the capillary opposite each other so as to measure the electrical resistance of the fluid column between them. Because current and voltage are measured simultaneously, the resistance can be calculated on-line and monitored during the measurements. The nucleus is sucked into the narrow part of the capillary by gentle fluid movement (see Fig. 2A and supplemental Movie 1). Thus, the current flows through the nucleus. The resulting increase in total electrical resistance indicates the specific electrical resistance of the cell nucleus. In this study, a direct current of 15 μA was used to induce electrophoretic movement of FITC-labeled bovine serum albumin (BSA) molecules toward the nuclear envelope. FITC-BSA was either present from the beginning of the measurement or added subsequently using a stretched pipette tip. In both cases, the nucleus was kept for 5 min in the narrowed part of the capillary in the presence of 15 mg/ml FITC-BSA. At the same time, nuclear resistance was recorded as described above. The nuclear resistance after incubation with importin-β-(45–462) was measured using alternating square-wave current pulses of 15 μA.

**Preparation of Nuclear Envelopes**—After the oocytes were placed in NIM, nuclei were isolated manually by piercing the oocyte with two pincers. Individual intact nuclei were picked up with a Pasteur pipette and transferred to a glass coverslip placed under a stereomicroscope. The chromatin was then carefully removed using sharp needles, and the nuclear envelope was spread on BD Biosciences Cell-Tak™-coated glass, with the nucleoplasmic side facing downwards or upwards. Finally, the specimens were washed with deionized water and dried.

**Fluorescence Detection of FITC-BSA at the Nuclear Envelope**—The fluorescence of whole nuclei was measured in NIM in small Petri dishes using a Zeiss Axiovert 100 inverted microscope equipped with a cooled CCD camera and the imaging software MetaMorph (VisiTron Systems GmbH, Puchheim, Germany).

![FIGURE 1. Application of an electric field (direct current) in the presence of FITC-labeled albumin.](image)

**FIGURE 1. Application of an electric field (direct current) in the presence of FITC-labeled albumin.** A, schematic of the working hypothesis. An NPC is viewed from the cytoplasm. A putative ring permeability is indicated by peripheral channel openings. A direct current is applied in the presence of negatively charged small solutes (ovals). In the left panel, it is assumed that the central channel is the main pathway for ions. The electric field (dashed lines) guides ions (not depicted) and small solutes toward the central channel, where they are detained because of their size. In the right panel, it is assumed that the main electrical conductivity is found in the peripheral channels. In this case, the central channel remains untouched (free), and the material is accumulated at the cytoplasmic ring. B, representative recording of the nuclear electrical resistance with a clamped direct current of 15 μA before and after local addition of 3 μl of FITC-BSA (15 mg/ml). Black arrows (from left to right) depict the insertion of the nucleus into the hourglass, the local addition of FITC-BSA after ~2 min, and the removal of the nucleus from the hourglass. C, electrical resistance of nuclei before (control) and 5 min after the addition of FITC-BSA. Data are the mean ± S.E. (n = number of nuclei). ***, p < 0.007. Ω, ohms; n.s., not significant.

Fluorescence measurements of membrane preparations were performed with the same setup by placing the coverslips onto a microscope slide that was mounted on a holder.

**AFM**—The application of AFM to nuclear envelopes has been described in detail (27). We used a MultiMode™ atomic
force microscope (with a NanoScope IIIa controller; Digital Instruments, Santa Barbara, CA) equipped with an optical microscope, a video camera, and a monitor to visualize the nuclear envelope and the AFM tip on its stage. We used standard V-shaped 200-μm-long silicon nitride cantilevers with a spring constant of 0.06 newtons/m and pyramidal tips with an estimated tip diameter of 10 nm (Digital Instruments). The images were recorded with 512 lines per screen at constant force (height mode) in contact mode with a scan rate of 3–10 Hz. The forces applied during the scanning procedure were minimized by retracting the AFM tip until it lost contact with the sample surface and re-engaging the tip at a set point (i.e., force value) as close as possible to the lift-off value. The scanning forces were usually below 3 nanonewtons with this approach. Although the AFM tip physically interacts with the nuclear envelope, the repeatability of results from multiple scans indicated that the preparation was not damaged. Moreover, scanning at low forces (3 nanonewtons or less) left no visible marks in the preparation.

**Macromolecule Permeability Assays**—X. laevis oocyte nuclei were isolated, incubated for the indicated times in NIM (controls) or in NIM containing 0.5 M importin-β-(45–462) at room temperature, and washed for 5 min with NIM. Nuclei were then mounted onto a chamber on the stage of an Olympus CLSM FluoView confocal laser-scanning microscope and superfused with NIM containing 19.5-kDa FITC-dextran (Sigma). The light source of the confocal microscope was an argon/krypton ion laser (Omnichrome) with an excitation wavelength of 488 nm. Comparison of diffusion rates under different experimental conditions allows conclusions to be drawn about the passive NPC permeability (28–30). During the measurement procedure, the nucleus was placed into a superfusion chamber (volume of 10 μl), which, in combination with a perfusor, allowed permanent superfusion and fast changes in perfusion solutions. Cell-Tak™ was used to immobilize the cell nucleus. The diffusion of 19.5-kDa FITC-dextran across the nuclear envelope was measured and analyzed by confocal fluorescence microscopy as described previously (29, 30).
Expression of Importin-α (45–462)—The expression plasmid for importin-α (45–462) was a kind gift from Prof. Dirk Görlich (Zentrum für Molekulare Biologie Heidelberg). Importin-α (45–462) was expressed and purified as described previously (31). Briefly, the expression plasmid pQE60 (Qiagen Inc., Hilden, Germany), containing the coding sequence of human importin-α (45–462) and an N-terminal His tag, was used to transform *Escherichia coli* BLR/Rep4. Expression was at 18 °C for 3 h. After centrifugation, the bacterial pellet was resuspended in 50 mM Tris-HCl, pH 7.5, 20 mM imidazole, 200 mM NaCl, and 5 mM mercaptoethanol, and lysis was performed by sonication. The lysate was cleared by centrifugation at 14,000 g for 30 min and loaded onto a column containing nickel-agarose. The column was washed with the same buffer and eluded with 300 mM imidazole. The buffer was exchanged for 50 mM Tris-HCl and 250 mM sucrose.

RESULTS

Electrophoretic Decoration of Ion-conductive Sites—We employed two approaches to test the hypothesis that two separate pathways exist. In the first approach, we attempted to visualize the entry site for ions through NPCs by combining the NHT with AFM. Fig. 1A illustrates this approach, in which an electric field applied across the nucleus drives charged impermeable macromolecules (albumin) toward the cytoplasmic face of NPCs. Albumin is too big to enter the nuclear interior through the ion-conductive sites of the nuclear envelope and thus gets stuck at the surface. The position of the albumin molecules at the pore surface indicates the position of the ion-conductive entry gates. We decided to use albumin for several reasons. First, albumin has a certain (yet unspecific) affinity for various surfaces and therefore would remain in place after the current was turned off. Second, the albumin stickiness is not strong enough to “glue” the nucleus to the NHT capillary, a fact that would make electrical measurements with the NHT impossible. Third, because there is no specific affinity of albumin for nuclear pore proteins, we expect that the position of the albumin molecules on the nuclear pore surface is determined exclusively by the electrical current flow. We used the NHT to expose *X. laevis* oocyte nuclei to an electric field in the presence of FITC-BSA. By applying a direct current, we detected the electrophoretic movement of FITC-BSA toward the nuclear envelope surface facing the cathode. The accompanying electrical measurements revealed a significant increase in nuclear resistance (Fig. 1, B and C). Fig. 1B shows a recording in which FITC-BSA was added locally after the nucleus had been inserted into the hourglass. This method allowed us to avoid possible interaction between FITC-BSA and the electrodes. To ensure that the observed increase in resistance was due only to the directed movement of FITC-BSA and not to some unspecific binding of FITC-BSA to the nuclear envelope, we repeated the experiment with an alternating current (Fig. 1C, right bar). The data indicate that the increase in electrical resistance is due to the direct current-driven albumin-pore interaction and not to the simple presence of albumin molecules that randomly stick to the nuclear envelope surface.

Fluorescence Detection of FITC-BSA at the Nuclear Membrane—After the nucleus was removed from the electrical setup, a bright fluorescent spot was still detectable on the apex of the nucleus that had been facing the cathode during the electrical measurement (Fig. 2B). In the next step, nuclear envelopes were spread on glass with the cytoplasmic side facing up for AFM measurements. Supplemental Movies 2 and 3 and supplemental Fig. 1 show how we routinely prepared the membranes of both cytoplasmic and nucleoplasmic sides. We chose...
to work with dried preparations because FITC-BSA molecules should remain attached to the envelope surface and thus should be detectable by AFM. Fluorescence microscopy of the nuclear envelope spread on glass allowed us to distinguish between stained and unstained membrane areas (Fig. 2C).

AFM Measurements of “Electrophoretically” Decorated Nuclear Pores—AFM was applied to fluorescent and nonfluorescent areas of the nuclear envelope (Fig. 2, D and E). We would like to emphasize that the membrane areas in Fig. 2, each 1 µm², are representative of far larger areas (several thousands of µm²) investigated. We assumed that a “central channel-borne conductivity” should lead to an obstructed central channel opening or, in other words, reduction in the depth of the channel. In contrast, a “ring-borne peripheral conductivity” should lead to decoration of the cytoplasmic ring with albumin molecules. This should result in an increased height difference between the deepest measurable point of the central channel and the NPC ring surface.

Fig. 2 (D and E) indicates that the central channels of pores were deeper at fluorescent than at nonfluorescent sites. To investigate the observed changes, we analyzed sections as well as rim profiles of single pores (Fig. 2, F and G), with nuclear pores located in nonfluorescent areas serving as controls. The histogram in Fig. 3A shows that the ring height (measured from the deepest point of the central channel) was significantly increased in pores of fluorescent areas relative to nonfluorescent areas. We assumed that binding of albumin molecules to the pore rim should lead to an increased roughness. To quantify this, we analyzed rim profiles and tested three different roughness parameters (Fig. 3A–C). The first parameter, the average roughness, describes the arithmetic mean of the absolute distances of the surface points from the mean line. The second parameter, the peak-peak distance, is the distance between the highest and lowest peaks of a rim profile. The third parameter, the 10-point height, results from the average distances of the five highest peaks from the mean line plus the average distances of the five lowest peaks. All three parameters show an increased rim roughness of nuclear pores that were located at fluorescent sites of the nuclear envelope. This indicates that albumin molecules accumulated at the pore rims.

Plugging of the NPC Central Channel Using Importin-β-(45–462)—In a second approach, the “separate pathway” hypothesis was tested by addressing whether the central channel is required for the passive diffusion of inorganic ions and small macromolecules. To this end, we blocked the central channel with the mutant importin-β-(45–462), a truncated version of the well studied nuclear transport factor (31). Importin-β-(45–462) lacks the binding site for importin-α and for GTP-bound Ran, which is required for the dissociation of importin-β from the pore after its arrival in the nucleoplasm (32–35). It is known that this results in the accumulation of the mutant in the central channel (36), which blocks the active transport of proteins (31). In this study, we analyzed the impact of this effective blocking protein on ion conductivity as well as on small solute permeability. After incubation of whole X. laevis oocyte nuclei with purified importin-β-(45–462), we prepared nuclear envelopes for AFM measurements.

AFM Detection of Importin-β-(45–462) at the Nuclear Membrane—Compared with preparations of untreated control nuclei, virtually all the pores appeared to be plugged (Fig. 4, A–D), as quantified by profile measurements of individual pores.
pores (E and F). Whereas the data in Fig. 4 were obtained from the cytoplasmic side of the nuclear envelope, the data in Fig. 5 (A and B) were collected from the nucleoplasmic side. Again, all central channels appeared to be plugged, in contrast to controls. The results indicate the transport of importin-β-(45–462) through the central channel, consistent with the observations of Görlich et al. (33), who found that an importin-β mutant stuck to the nuclear basket that was likewise deficient in the Ran-binding site.

As the plugs mentioned above are visible from both sides of the NPC, it becomes conceivable that accumulation of importin-β molecules has taken place in the interior of the NPC central channel. Again, we would like to emphasize that the membrane areas, each 4 μm², displayed in Figs. 4 and 5 are representative of far larger areas (several thousands of μm²) investigated.

We tested different incubation times and found the plugging to be most efficient after 60 min (Fig. 5C). This incubation time was then used for the following experiments.

**Electrical Measurements and Macromolecule Permeability Assays—** After we found that the importin mutants consistently stuck in the NPC central channels, we performed electrical and fluorescence measurements under identical conditions. To our great surprise, neither the electrical conductivity of the nuclear envelope, which corresponds to passive ion transport across the nuclear envelope (24), nor the nuclear envelope permeability to 19.5-kDa FITC-dextran showed any significant impairment (Fig. 5D). Thus, plugging of the central channel with importin-β-(45–462), as visualized by AFM, did not affect the permeability of the nuclear envelope for ions and small solutes.

**DISCUSSION**

In summary, we conclude that there exists in NPCs a route for inorganic ions and small solutes that is separate from the central channel. Electric field-driven albumin cannot enter the central channel and plugs this pathway. Rather, albumin molecules reside at the cytoplasmic ring, as indicated by the apparent swelling of the NPC ring structure and the decrease in the ion permeability of the nuclear envelope. The data also indicate that the ion permeability of the central channel must be lower than that of the NPC rim. This “nonparticipation” of the central channel in the ion permeability of the nuclear envelope is further supported by mechanical blocking of the central channel with importin-β-(45–462) changing neither the ion permeability nor the small solute permeability. These findings are in apparent contradiction to those of Bustamante et al. (37), who found a decrease in ion conductivity after blocking the central channel. However, the antibody used in their study to block the central channel binds to four different pore proteins, two of which are indeed located on the cytoplasmic ring (38). Recent support for peripheral routes of the NPC have been provided by Ohba et al. (39), who investigated the transport route of integral.
membrane proteins toward the inner nuclear membrane. They showed that integral membrane proteins move between the outer and inner nuclear membranes by lateral diffusion at the periphery of the NPC.

Strong support in favor of our findings comes from a more recent study in which the interconnection between passive and active transport was investigated. Naim et al. (40) found that active transport and passive transport of macromolecules are largely uncoupled and concluded that both pathways are spatially separated.

Our findings suggest that the meshwork comprising the FG repeat domains inside the central channel (12) could be denser than assumed previously. The hydrophobic interactions upon which the meshwork appears to be based are known to operate across long distances (41). Therefore, a repulsive effect for ions and charged molecules is likely to occur. The proposed alternative “peripheral” route for ions and small solutes is therefore presumably hydrophilic in nature. Some further support is provided by the observation that the diameter of the peripheral channels, 10 nm (4), matches the molecule size exclusion limit of ~9 nm for passive diffusion (6).

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REFERENCES

1. Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas, K., Zhao, Y., and Chait, B. T. (2000) J. Cell Biol. 148, 635–651
2. Ryan, K. J., and Wente, S. R. (2000) Curr. Opin. Cell Biol. 12, 361–371
3. Akey, C. W., and Radermacher, M. (1993) J. Cell Biol. 122, 1–19
4. Hinshaw, J. E., Carragher, B. O., and Milligan, R. A. (1992) J. Mol. Biol. 239, 251–254
5. Paine, P. L., Moore, L. C., and Horowitz, S. B. (1975) J. Cell Biol. 69, 1133–1141
6. Stoffler, D., Feja, B., Fahrenkrog, B., and Aebi, U. (2003) J. Cell Biol. 152, 109–114
7. Paine, P. L., Moore, L. C., and Horowitz, S. B. (1975) Nature 254, 109–114
8. Reichelt, R., Holzenburg, A., Buhle, E. L., Jr., Jarnik, M., Engel, A., and Aebi, U. (1990) J. Cell Biol. 110, 883–894
9. Wang, H., and Clopham, D. E. (1999) Biophys. J. 77, 241–247
10. Pante, N., and Kann, M. (2002) Mol. Biol. Cell 13, 425–434
11. Lim, R. Y., Huang, N. P., Koser, J., Deng, J., Lau, K. H., Schwarz-Heron, K., Fahrenkrog, B., and Aebi, U. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 9512–9517
12. Denning, D. P., Patel, S. S., Uversky, V., Fink, A. L., and Rexach, M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2450–2455
13. Bednienko, J., Cingolani, G., and Gerace, L. (2003) Traffic 4, 127–135
14. Fahrenkrog, B., Koser, J., and Aebi, U. (2004) Trends Biochem. Sci. 29, 175–182
15. Ribbeck, K., and Görlich, D. (2001) EMBO J. 20, 1320–1330
16. Santella, L., and Carafoli, E. (1997) J. Cell Biol. 138, 1091–1109
17. Danter, T., Mazzantii, M., Tonini, R., Rakowska, A., and Oberleithner, H. (1997) Cell Biol. Int. 21, 747–757
18. Shahin, V., Danner, K., Schlune, A., Buchholz, I., and Oberleithner, H. (2003) Cell. Physiol. Biochem. 17, 181–192
19. Shahin, V., Ludwig, Y., Schafer, C., and Oberleithner, H. (2005) J. Membr. Biol. 196, 147–155
20. Ludwig, Y., Schafer, C., Altermann, L., Oberleithner, H., and Shahin, V. (2006) Cell. Physiol. Biochem. 17, 181–192
21. Cutay, U., Bischoff, F. R., Kostka, S., Kraft, R., and Görlich, D. (1997) Cell 90, 1061–1071
22. Chook, Y. M., and Blobel, G. (2001) Curr. Opin. Struct. Biol. 11, 703–715
23. Görlich, D., Seewald, M. J., and Ribbeck, K. (2003) EMBO J. 22, 1088–1100
24. Mossamaparast, N., and Pemberton, L. F. (2004) Trends Cell Biol. 14, 547–556
25. Weis, K. (2003) Cell 112, 441–451
26. Jaggi, R. D., Franco-Obregon, A., and Ennslin, K. (2003) Biophys. J. 85, 4093–4098
27. Bustamante, J. O., and Liepins, A. (1995) J. Membr. Biol. 146, 239–251
28. Saitoh, H., Cooke, C. A., Burgess, W. H., Earnshaw, W. C., and Dasso, M. (1996) Mol. Biol. Cell 7, 1319–1334
29. Ohba, T., Schirmer, E. C., Nishimoto, T., and Gerace, L. (2004) J. Cell Biol. 167, 1051–1062
30. Naim, B., Brumfeld, V., Kapon, R., Kiss, V., Nevo, R., and Reich, Z. (2007) J. Biol. Chem. 282, 3881–3888
31. Singh, S., Houston, J., van Swol, F., and Brinker, C. J. (2006) Nature 442, 526