Reconstitution of nuclear protein export in isolated nuclear envelopes

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Signal-dependent nuclear protein export was studied in perforated nuclei and isolated nuclear envelopes of *Xenopus* oocytes by optical single transporter recording. Manually isolated and purified oocyte nuclei were attached to isoporous filters and made permeable for macromolecules by perforation. Export of a recombinant protein (GG-NES) containing the nuclear export signal (NES) of the protein kinase A inhibitor through nuclear envelope patches spanning filter pores could be induced by the addition of GTP alone. Export continued against a concentration gradient, and was NES dependent and inhibited by leptomycin B and GTPγS, a nonhydrolyzable GTP analogue. Addition of recombinant RanBP3, a potential cofactor of CRM1-dependent export, did not promote GG-NES export at stoichiometric concentration but gradually inhibited export at higher concentrations. In isolated filter-attached nuclear envelopes, export of GG-NES was virtually abolished in the presence of GTP alone. However, a preformed export complex consisting of GG-NES, recombinant human CRM1, and RanGTP was rapidly exported. Unexpectedly, export was strongly reduced when the export complex contained RanGTPγS or RanG19V/Q69L-GTP, a GTPase-deficient Ran mutant. This paper shows that nuclear transport, previously studied in intact and permeabilized cells only, can be quantitatively analyzed in perforated nuclei and isolated nuclear envelopes.

Introduction

The exchange of matter between cell nucleus and cytoplasm is mediated by the nuclear pore complex (NPC),* a large transporter spanning the nuclear envelope. The NPC selectively exports proteins, ribonucleoprotein particles, and ribosomal subunits containing nuclear export signals (NESs) while importing proteins and small RNPs containing nuclear localization sequences (NLSs). Inert molecules smaller than ∼9 nm can permeate the NPC by diffusion (for reviews see Görlich and Kutay, 1999; Mattaj and Conti, 1999; Chook and Blobel, 2001; Lyman and Gerace, 2001).

Selective nuclear protein import is a multistep process starting with the formation in the cytosol of a complex consisting of an NLS protein and its cognate import receptor, a member of the karyopherin β family (also referred to as importins, exportins, or transportins). The import complex binds to the NPC and is translocated through the NPC core by a diffusional process (Rout et al., 2000; Ribbeck and Görlich, 2001). After translocation, the complex is probably trapped on the nuclear side of the NPC at high-affinity sites (Ben-Efraim and Gerace, 2001) and released into the nuclear space by interaction with the GTP form of Ran, a small GTPase of the Ras family.

Selective nuclear export is complementary to import starting with the formation on the nuclear side of an export complex consisting of an NES protein, an exportin such as CRM1, and RanGTP. After binding to the NPC, the export complex is translocated through the NPC and released on its cytoplasmic side. Cytosolic factors, such as RanGAP and RanBP1, interact with the export complex, induce the hydrolysis of Ran-bound GTP, and thus promote the dissociation of the export complex. Ran acts as a molecular switch, making selective nuclear transport vectorial and permitting transport against concentration gradients. The necessary energy is provided by hydrolysis of Ran-bound GTP. However, previous studies suggested (Richards et al., 1997; Nakielny and Dreyfuss, 1998; Englmeier et al., 1999; Ribbeck et al., 1999) that hydrolysis of Ran-bound GTP is neither required for the translocation of the export complex through the NPC nor for its release from the NPC. A gradient of free RanGTP supposedly exists between nuclear content and

cytosol, a hypothesis (Görlich et al., 1996) supported by the fact that RCC1, the protein facilitating the exchange of Ran-bound GDP for GTP, occurs only in the nucleus, whereas factors activating the GTPase activity of Ran, such as Ran-GAP and RanBP2, are restricted to the cytoplasm.

In general, nuclear export is not as well understood as import. Concerning CRM1-mediated export, for instance, evidence has been provided for the involvement of additional factors, especially RanBP3 (Englmeier et al., 2001; Lindsay et al., 2001) and RanBP1 (Askjaer et al., 1999; Kehlenbach et al., 1999), but many important details are still to be resolved. With regard to the export of RNAs, hnRNPs, and ribosomal subunits, knowledge is in an initial phase. For tRNAs, an export receptor, exportin-t or Los1, has been identified, but found not to be required for viability (Arts et al., 1998; Hellmuth et al., 1998; Kutay et al., 1998). The export of hnRNPs involves a large set of proteins (Cole, 2000), among which the mammalian TAP (Gruter et al., 1998) and the yeast Mex 67p (Segref et al., 1997) are best studied. TAP has no structural resemblance with karyopherins (Liker et al., 2000). Also, hnRNP export is independent of Ran (Clouse et al., 2001).

Nuclear transport has been studied previously in intact and digitonin-permeabilized cells. In the search for an assay providing better defined and manipulatable conditions, we have developed optical single transporter recording (OSTR; Tschodrich-Rotter and Peters, 1998): isolated *Xenopus* oocyte nuclei are attached to isoporous filters and transport across nuclear envelope patches spanning filter pores is measured by confocal microscopy (Keminer and Peters, 1999; Keminer et al., 1999). In the present study, a both simplified and enhanced version of OSTR was employed in which nuclei are kept in a mock intracellular medium throughout, strictly avoiding dehydration or exposure to the air–water interface. Furthermore, "large" membrane patches were used, containing not single but small groups of NPCs. We thus found that NES-dependent export could be reconstituted in isolated nuclear envelopes using defined solutions of recombinant human CRM1 and RanGTP. Unexpectedly, the efficient export of preformed export complexes required hydrolysis of Ran-bound GTP.

Results and discussion

OSTR is both simplified and enhanced by using a preformed sample chamber

The central element of the OSTR version used in this study is a preformed chamber consisting of a plastic culture dish with a small hole in its bottom. Across the hole a piece of an isoporous filter is affixed. With this element, the procedure indicated in Fig. 1 A and described more completely under the Materials and methods proved to be simple, fast, and reproducible. The microscopic appearance of filter-attached nuclei and nuclear envelopes is illustrated in Fig. 1 B.

In perforated nuclei, the endogenous apparatus for signal-dependent protein export is conserved

In perforated nuclei, GG-NES (2 μM) was exported upon addition of GTP (500 μM) alone (Fig. 2 A, left). Export
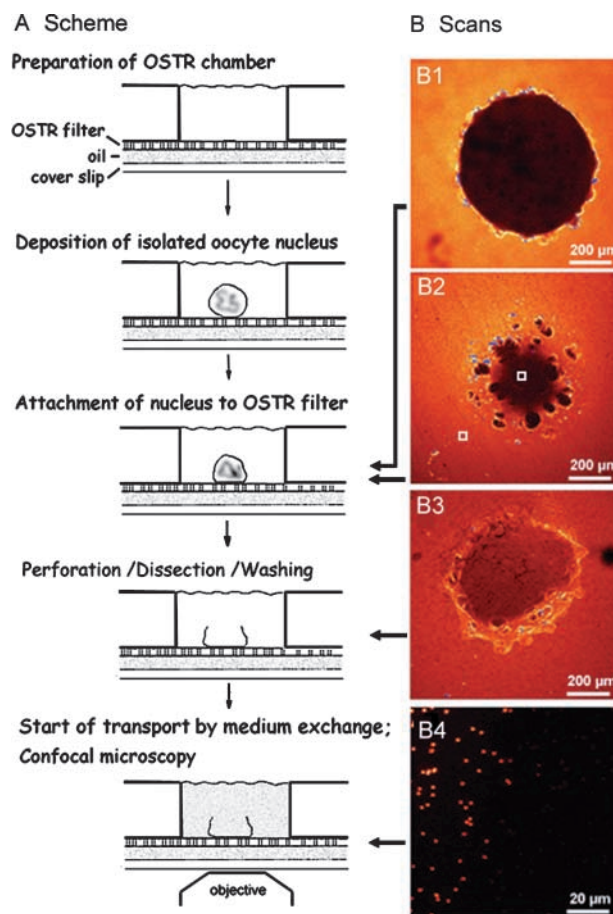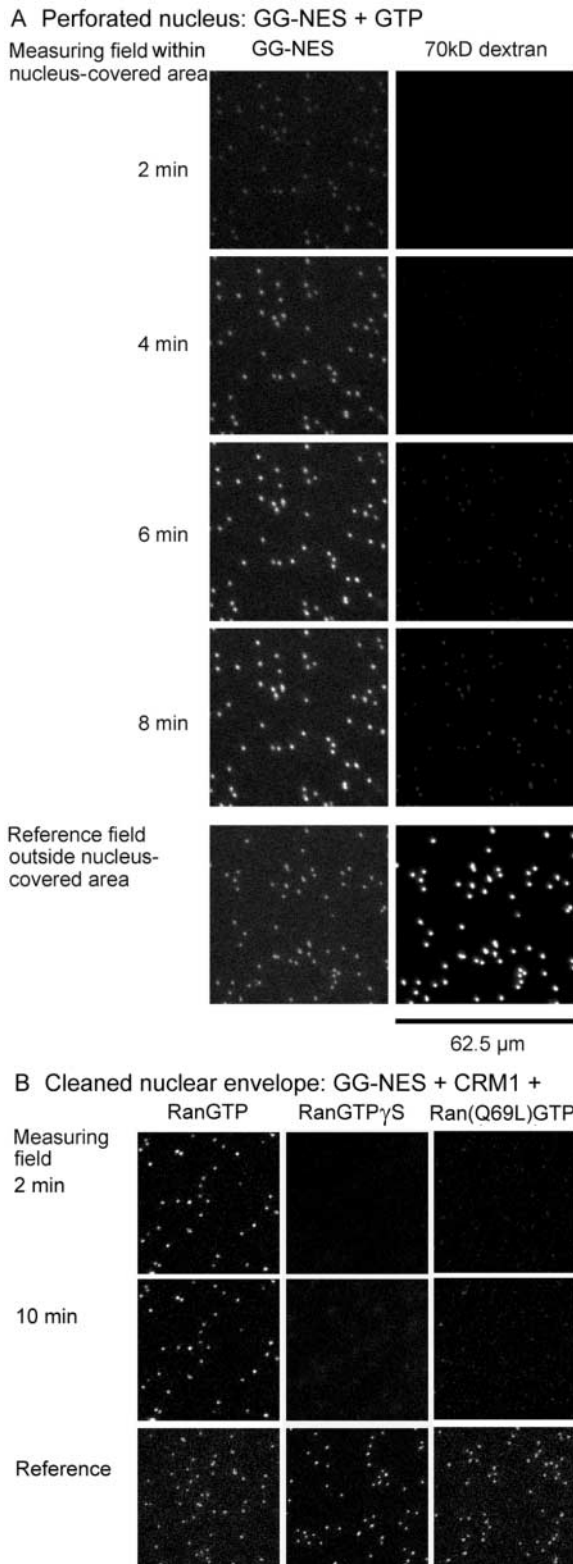

Figure 1. **The OSTR version used in this study.** (A) Sequence of events during an OSTR experiment. (B) Microscopic appearance of an OSTR specimen incubated with the NPC-impermeable molecule TRD70. (B1) Largest perimeter of a filter-attached intact nucleus. (B2) Filter-attached area of same nucleus. The typical size and position of measuring and reference fields (white squares) are indicated. (B3) Nuclear envelope after dissection and washing. (B4) Filter pores visualized at higher magnification showing the boundary of the nuclear envelope–sealed area.

continued against a concentration difference. Texas red–labeled 70-kD dextran (TRD70; Fig. 2 A, right) was largely excluded from filter pores, indicating sufficiently tight sealing and NPC integrity. Further examples of raw data pertaining to isolated nuclear envelopes are given in Fig. 2 B.

Quantitative data are demonstrated in Fig. 3. In the presence of GTP, GG-NES was rapidly exported and accumulated. Addition of an ATP-regenerating system did not further stimulate export (unpublished data). Omission of GTP reduced the initial rate of GG-NES export by ∼65%. Leptomycin B (200 nM), a specific inhibitor of CRM1 (Wolff et al., 1997), strongly inhibited export. A similarly strong inhibition was seen when GTPγS, a nonhydrolyzable GTP analogue, was used instead of GTP. Export was virtually abolished when GG, a protein similar to GG-NES but without NES, was used.

By calibrating fluorescence in terms of concentration (see the Materials and methods), we found that in transport measurements using 2.0 μM GG-NES and 500 μM GTP, the mean initial concentration increase of GG-NES

A Perforated nucleus: GG-NES + GTP

Measuring field within nucleus-covered area — GG-NES — 70kD dextran

2 min

4 min

6 min

8 min

Reference field outside nucleus-covered area

62.5 µm

B Cleaned nuclear envelope: GG-NES + CRM1 +

RanGTP — RanGTPγS — Ran(Q69L)GTP

Measuring field 2 min

10 min

Reference

Figure 2. Examples of export measurements. (A) In an OSTR chamber, a filter-attached perforated nucleus was incubated with a transport medium containing 2 µM GG-NES, 7.5 µM TRD70, and 500 µM GTP. The time after addition of the transport medium to the OSTR chamber is indicated. A region in the center of the area covered by the nucleus was selected and the filter pores imaged. The accumulation of GG-NES with time and the exclusion of TRD70 can be recognized. After completion of export, the nucleus-covered
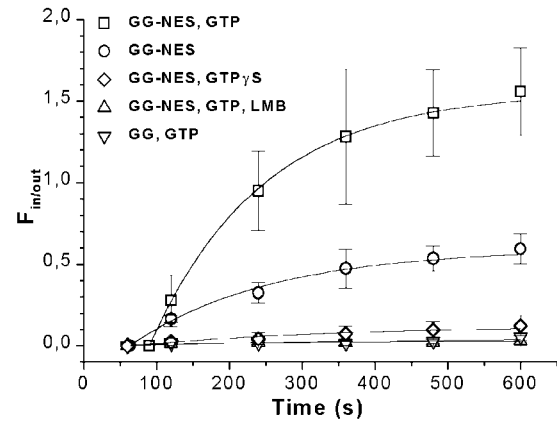

Figure 3. Export kinetics in perforated nuclei. The mean fluorescence of filter pores was plotted in normalized form versus time after the addition of transport medium. Symbols are mean ± SD of five or more measurements, each comprising ∼40 membrane patches. Lines are fits of simple exponentials to the experimental data. GG-NES was strongly exported in the presence of GTP alone (squares). Export started after a lag time of ∼50–100 s, probably needed for diffusion of export substrate to the inner side of the nuclear envelope and formation of the export complex, and proceeded beyond equilibration. If GTP was omitted (circles), export was reduced. If GTP was replaced by the nonhydrolyzable analogue GTPγS (diamonds), export was strongly inhibited. In the presence of leptomycin B (LMB) and GTP (triangles), export of GG-NES was virtually abolished. No export was seen of GG, an inert protein, in the presence of GTP (inverted triangles).

in filter pores was 20.2 nM/s. Assuming that the filter pores had a mean volume of 5 fl and that the membrane patches covering filter pores contained ∼15 free NPCs not in contact with filter walls, the transport rate was derived to be 4.0 molecules/s/NPC. For other transport media, the transport rates (molecules/s/NPC) were 1.2 (2 µM GG-NES), 0.05 (2 µM GG-NES, 500 µM GTP, 200 nM LMB), 0.2 (2 µM GG-NES, 500 µM GTPγS) and 0.02 (2 µM GG, 500 µM GTP).

Recently, RanBP3 (Müller et al., 1998) was found to be a cofactor of CRM1-dependent export (Englmeier et al., 2001; Lindsay et al., 2001). At optimal concentrations, RanBP3 stabilized the interaction between CRM1, RanGTP, and an NES protein. At higher concentrations, RanBP3 inhibited and finally completely prevented formation of that complex. We studied the effect of RanBP3 on the export of GG-NES from perforated nuclei in the presence of 500 µM GTP. Export rates of GG-NES (1 µM) were found to be 3.3, 3.6, 2.5, or 1.3 molecules/s/NPC at 0, 1, 4 or 8 µM RanBP3, respectively. Thus, only the inhibitory, not stimulatory, effect of RanBP3 was observed.

The results suggest that perforated nuclei, although permeable for macromolecules, retain a considerable amount of transport cofactors. An insoluble form of RanBP (Nup358/RanBP2) is known to be an integral

area was shifted out of the field of view and a reference field was imaged in a region of the filter where GG-NES and TRD70 had free access to the filter pores. (B) Analogue experiments with isolated filter-attached nuclear envelopes at conditions specified in Fig. 5 B.
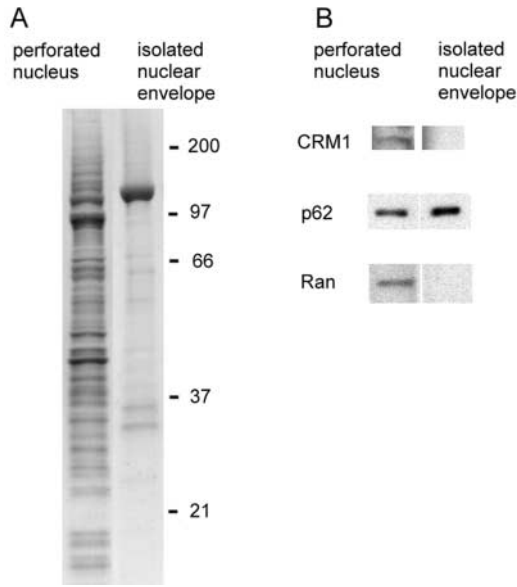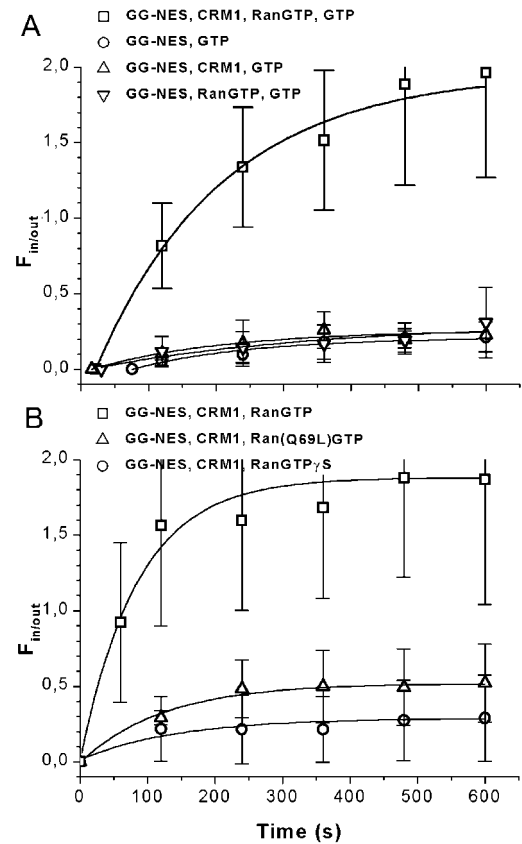
Figure 4. **Protein profiles of perforated nuclei and nuclear envelopes.** Either two perforated nuclei or four nuclear envelopes were separated by SDS gel electrophoresis and transferred to a nitrocellulose membrane. (A) Amido black staining (the prominent band at ∼120 kD is probably due to contaminating yolk proteins). (B) Western blots probed with anti-CRM1, anti-p62, and anti-Ran.

Figure 5. **Export kinetics in isolated nuclear envelopes.** The mean fluorescence of filter pores was plotted in normalized form versus time after addition of transport medium. Symbols are mean ± SD of five or more measurements, each comprising the data of ∼40 filter pores. Lines are fits of a simple exponential with off-set to the experimental data. (A) In the presence of GTP alone (circles), GTP and CRM1 (triangles), or GTP and RanGTP (inverted triangles) little export of GG-NES was observed. However, when an export complex was preformed from GG-NES, CRM1, and RanGTP and applied together with GTP (squares), export was strong, without apparent lag time, and proceeded beyond equilibration. (B) Export of export complexes in the absence of free GTP. A complex of GG-NES, CRM1, and RanGTP (squares) was very rapidly exported and accumulated in filter pores. When hydrolysis of Ran-bound GTP was prevented by using for complex formation either RanGTPγS (circles) or a GTPase-deficient Ran mutant (triangles), export was strongly inhibited.

component of the cytoplasmic filaments of the NPC. RanGAP also occurs in an insoluble, modified form covalently attached to cytoplasmic filaments of the NPC. CRM1 and Ran, present in substantial amounts (Fig. 4), may be loosely bound to nuclear constituents and gradually released after perforation. The same may hold for endogenous RanBP3, which explains why no stimulation but only inhibition of export was seen upon addition of exogenous RanBP3. Export continued against a concentration gradient, implying energy consumption. Consistently, export was inhibited when GTP was substituted by a nonhydrolyzable GTP analogue.

In isolated nuclear envelopes, endogenous CRM1 and Ran is depleted but can be substituted by recombinant heterologous proteins

Filter-attached nuclear envelopes could be effectively depleted of proteins and transport factors by washing them three times with mock 3. As shown in Fig. 4 A, the contents and diversity of proteins was largely reduced in isolated nuclear envelopes as compared with perforated nuclei. Using p62 as a loading control, Western blots (Fig. 4 B) showed that perforated nuclei contained substantial amounts of both CRM1 and Ran, whereas these substances could not be detected in isolated nuclear envelopes.

The results of export measurements using isolated nuclear envelopes are demonstrated in Fig. 5. In a first series (Fig. 5 A), all transport solutions contained 500 μM GTP. In the presence of GTP alone, export of GG-NES (2 μM) was virtually abolished (initial rate 0.4 molecules/s/NPC). Addition of either recombinant human CRM1 (2 μM) or RanGTP (2 μM) did not significantly increase export (initial rates, 0.5 and 0.4 molecules/s/NPC, respectively). However, when

an export complex was formed in advance by mixing 2 μM GG-NES with 2 μM CRM1 and 2 μm RanGTP, strong export was observed. The export rate was 4.2 molecules/s/NPC, indistinguishable from that seen in perforated nuclei with endogenous transport factors. The final level of accumulation was also very similar.

In a second series (Fig. 5 B), no free GTP was present. Nevertheless, an export complex preformed by mixing 2 μM of GG-NES with 2 μM CRM1 and 2 μM RanGTP was exported at a rate of 8.8 molecules/s/NPC. The standard deviations of all data in Fig. 5 are considerable, and it remains to be seen whether the difference in export rates in the presence and absence of free GTP (4.2 vs. 8.8 molecules/s/NPC) is significant. When hydrolysis of Ran-bound GTP was prevented by forming export complexes

from 2 μM GG-NES, 2 μM CRM1, and either 2 μM RanGTPγS or 2 μM of the GTP form of the GTPase-deficient Ran G19V/Q69L (Coutavas et al., 1993), export was strongly reduced with regard to both rate and equilibrium level. Thus, for the complex containing RanGTPγS, the rate was reduced to 0.8 molecules/s/NPC and the final $F_{in/out}$ value to 0.3. For the complex containing RanG19V/Q69LGTP, the rate was 1.7 molecules/s/NPC and the final $F_{in/out}$ value 0.5. These effects were not due to a partial inactivation of Ran and/or CRM1. When 2 μM GG-NES, 2 μM CRM1, and 5 μM RanGTPγS or 5 μM RanG19V/Q69LGTP were employed, the final $F_{in/out}$ value was 0.45, not significantly differing from equimolar conditions. Similarly, when 1 μM GG-NES, 2 μM CRM1, and 5 μM RanGTP were employed, the final $F_{in/out}$ value was 2.4 ± 0.7, similar to equimolar conditions.

Our observation that export kinetics were strongly affected by hydrolysis of Ran-bound GTP (Fig. 5 B) is somewhat unexpected. Previous studies using either nuclear injection of intact cells (Richards et al., 1997) or digitonin-permeabilized cells together with two-phase import–export protocols (Englmeier et al., 1999) had suggested that neither the translocation of an export complex through the NPC nor its release from the NPC on the cytoplasmic side would require GTP hydrolysis on Ran. One possible explanation for the difference between our studies and those performed previously could be that certain accessory export factors were not present in our assay. A candidate is RanBP1, which facilitates the release of the export complex from the NPC (Askjaer et al., 1999; Black et al., 1999; Kehlenbach et al., 1999). Another possible explanation would be that an enhancement of transport by GTP hydrolysis was not noticed in intact or permeabilized cells because in both assays the extranuclear concentration of the export complex was essentially zero throughout (even in intact cells, see Richards et al., 1997), and a persistent concentration gradient would drive export. If export should in fact be facilitated by GTP hydrolysis, as suggested by our results, the coupling between the presumably diffusional translocation through the NPC center and the release from a terminal binding site at the cytoplasmic face of the NPC would have to be quite tight.

Conclusion

In this study, we observed that in perforated nuclei, the competence for selective protein export was largely conserved. In isolated nuclear envelopes, export could be fully restored by the addition of recombinant transport factors. These findings, together with the high sensitivity and selectivity of OSTR, open new avenues for nuclear transport studies. Predictions and models based on experiments with intact or permeabilized cells may now be studied experimentally. Also, the export of large particles and subunits may be directly analyzed.

Materials and methods

Recombinant proteins and other substances
Human COOH-terminal His-tagged CRM1 was expressed in *Escherichia coli* TG1 and purified using NiNTA agarose according to Englmeier et al. (1999). Purified CRM1 was stored in mock 3 (90 mM KCl, 10 mM NaCl, 2 mM MgCl$_2$, 0.1 mM CaCl$_2$, 1.0 mM *N*-[2-hydroxyethyl]ethylene-diamine-triacetic acid [HEDTA], 10 mM Hepes, pH 7.3) plus 250 mM sucrose. Human wild-type Ran and RanG19V/Q69L (Coutavas et al., 1993) were expressed in *E. coli* BL21(DE3). Purification and loading of Ran or the Ran mutant with GTP or GTPγS were performed according to Bischoff and Ponstingl (1995). GST–RanBP3 (provided by I.J. Mattaj, EMBO Laboratory, Heidelberg, Germany) was expressed in *E. coli* BL21(DE3) and purified using GSH-agarose. GG-NES (i.e., export protein) and GG (i.e., control protein) were prepared as previously described (Keminer et al., 1999). Lysine-fixable TRD70 was obtained from Molecular Probes.

OSTR measurements
A nucleus, manually isolated and cleaned in mock 3 as previously described (Radtke et al., 2001), was placed into a preformed OSTR chamber (3.5 mm × 1.0 mm) and attached to the bottom, a filter (0.72 ± 0.8 μm pore diameter, catalog no. 7062 4708, Whatman), by gentle pressure. In some experiments, the unattached part of the nuclear envelope was perforated using a steel pin without removing the nuclear contents. In other experiments, the attached nucleus was fully opened, the nuclear content removed, and the residual filter-attached nuclear envelope was washed three times with 15 μl mock 3 before applying the transport medium. The OSTR chamber was then placed on the stage of a confocal microscope. A 40-fold objective (NA 1.0, oil) was used to image filter pores in the nuclear envelope–sealed area of the filter. Particular care was taken to avoid collapsed membrane protrusions and choose flat nuclear envelope regions. A time series of fluorescence scans was acquired recording simultaneously the entrance of GG-NES into and the exclusion of TRD70 from the filter pores. When transport was completed, a region of the chamber where the filter was not covered by the nuclear envelope was scanned for reference. Confocal scans were evaluated using ImageJ (W. Rasband, National Institute for Mental Health, Bethesda, Maryland). A special Java plug-in was created to align an image stack, place circular regions of interest on individual filter pores, determine the local background surrounding each region of interest, and compute the background-corrected fluorescence $F_{in}(t)$ for each filter pore at each time point. Then the mean fluorescence F_{out} of filter pores in reference areas was determined, $F_{in}(t)$ was normalized to F_{out}, and the resultant ratio $F_{in/out}(t)$ was plotted versus time. About 30–40 kinetic curves were obtained from a single experiment.

Fluorescence was calibrated in terms of concentration by loading OSTR chambers with transport media containing 0.5–4.0 μM GG-NES and measuring the fluorescence of filter pores at the same microscope settings employed in transport measurements. A linear relationship between filter pore fluorescence and GG-NES concentration was found in the 0.0–2.0-μM range. Above 2 μM, the slope became smaller so that at 4 μM GG-NES, the mean fluorescence of filter pores was only 1.3 times that at 2.0 μM. Hence, for $F_{in/out} \leq 1$, the concentration of GG-NES in filter pores could be calculated according to $C_{in} = C_{out} \times F_{in/out}$, where C_{out} is the substrate concentration in the transport medium.

Protein profiles of perforated nuclei and nuclear envelopes
The contents of OSTR chambers containing perforated nuclei or washed nuclear envelopes was solubilized by perfusion with SDS sample buffer. Samples containing the equivalent of either two nuclei or four washed nuclear envelopes were loaded on 4–20% Tris-glycine gradient gels (catalog no. EC60255, Invitrogen) and after electrophoresis transferred to nitrocellulose in 192 mM glycine, 25 mM Tris, 15% methanol, pH 8.3, and stained with amido black. For Western blots, the strips were first blocked in PBS-T (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 8.1 mM Na$_2$HPO$_4$, 0.1% Tween 20) plus 5% nonfat dry milk for 60 min at room temperature and exposed to the primary and secondary antibodies for 60 min each with three 5-min washes with PBS-T in between. The following antibodies were used: anti-Ran (N-20 and C-20, 1:500; Santa Cruz Biotechnology, Inc.), anti-Crm1 (H-300, 1:100; Santa Cruz Biotechnology, Inc.), mab414 against p62 (1:1,000; BAbCo), anti–rabbit or anti–mouse secondary antibodies coupled to HRP (1:15,000; Amersham Biosciences), and anti-goat HRP (1:10,000; Santa Cruz Biotechnology, Inc.). For chemiluminescence, the Westerns were developed using an ECL kit from Pierce Chemical Co. (Supersignal West Femto, catalog no. 34095).

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