Energy- and temperature-dependent transport of integral proteins to the inner nuclear membrane via the nuclear pore

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Resident integral proteins of the inner nuclear membrane (INM) are synthesized as membrane-integrated proteins on the peripheral endoplasmic reticulum (ER) and are transported to the INM throughout interphase using an unknown trafficking mechanism. To study this transport, we developed a live cell assay that measures the movement of transmembrane reporters from the ER to the INM by rapamycin-mediated trapping at the nuclear lamina. Reporter constructs with small (<30 kD) cytosolic and lumenal domains rapidly accumulated at the INM. However, increasing the size of either domain by 47 kD strongly inhibited movement. Reduced temperature and ATP depletion also inhibited movement, which is characteristic of membrane fusion mechanisms, but pharmacological inhibition of vesicular trafficking had no effect. Because reporter accumulation at the INM was inhibited by antibodies to the nuclear pore membrane protein gp210, our results support a model wherein transport of integral proteins to the INM involves lateral diffusion in the lipid bilayer around the nuclear pore membrane, coupled with active restructuring of the nuclear pore complex.

Introduction

The nuclear envelope (NE) forms the boundary of the nucleus in eukaryotes (for reviews see Burke and Stewart, 2002; Holaska et al., 2002; Gruenbaum et al., 2003). It consists of inner nuclear membrane (INM) and outer nuclear membrane (ONM), which are joined at the "nuclear pore membrane." The latter occurs adjacent to nuclear pore complexes (NPCs), large proteinaceous assemblies that mediate directional trafficking of soluble macromolecules between the nucleus and the cytoplasm (for reviews see Suntharalingam and Wente, 2003; Fahrenkrog et al., 2004). In vertebrates, the NPC has a mass of ~125 MD and contains over 30 different proteins (nucleoporins), including two integral proteins (gp210 and POM121) that in part protrude into the lumenal space adjacent to the NPC and are thought to help tether the NPC to the pore membrane (for review see Vasu and Forbes, 2001). The ONM is morphologically continuous with the more peripheral ER and has many ER-like properties, including the presence of bound ribosomes engaged in protein synthesis. By contrast, the INM is lined by the nuclear lamina, a filamentous meshwork consisting of a polymer of nuclear lamins associated with more minor components (for reviews see Holaska et al., 2002; Gruenbaum et al., 2003). These minor lamina components include "resident" integral membrane proteins of the INM that are undetectable or present only at a low concentration in the peripheral ER (for review see Holmer and Worman, 2001). Approximately a dozen resident transmembrane proteins of the INM have been described, and a recent proteomics analysis suggests that there may be over 50 additional transmembrane proteins specific to the NE (Schirmer et al., 2003).

The NE increases in surface area throughout interphase, accompanied by synthesis and assembly of its constituent proteins (for review see Gerace and Burke, 1988). Thus, resident integral proteins are continuously transported to the INM during interphase after synthesis on the rough ER. Well-characterized integral proteins of the INM bind nuclear lamins and some of them interact with chromatin as well, providing a potential explanation for how these proteins are retained at the INM (for review see Holmer and Worman, 2001). However, the mechanism for transport of these proteins from the rough ER to the INM is not clear.

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Abbreviations used in this paper: BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl) ester; CHL, chicken hepatic lectin; FKBP, FK506-binding protein; FRB, FKBP-rapamycin binding domain; INM, inner nuclear membrane; LAP2β, lamina-associated polypeptide 2β; NE, nuclear envelope; NEM, *N*-ethylmaleimide; NPC, nuclear pore complex; ONM, outer nuclear membrane; PK, pyruvate kinase; VSV, vesicular stomatitis virus.

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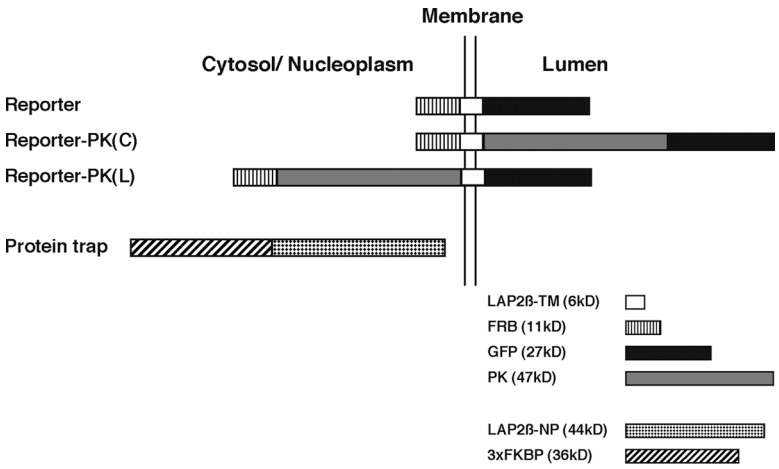

Figure 1. **Schematic diagram of protein constructs.** The standard reporter plasmid encoded residues 401–452 of LAP2β (LAP2β-TM, which comprises a short cytosolic region and its transmembrane/lumenal domains) fused to GFP and FRB as shown. In some cases, PK was fused to the lumenal (Reporter-PK (L)) or the cytoplasmic (Reporter-PK(C)) side of the reporter. The "protein trap" plasmid encoded the nucleoplasmic domain of LAP2β (residues 1–401; LAP2β-NP) and three tandem copies of FKBP. The molecular masses of the various protein segments are indicated (bottom).

Several mechanisms can explain transit of integral proteins to the INM. In one model, this is accomplished by passive diffusion of membrane proteins in the plane of the lipid bilayer between the ONM and the INM via the nuclear pore membrane (Smith and Blobel, 1993; Soullam and Worman, 1993, 1995; Holmer and Worman, 2001). In a second mechanism, the INM and ONM undergo periodic fusion to provide transient connections (Mattaj, 2004). This would allow diffusional exchange of integral proteins between the two membranes and also could be important for insertion of new NPCs in the NE. In a third mechanism, membrane vesicles bud from the ONM into the perinuclear lumen, and subsequently fuse with the INM. An analogous mechanism is known to occur in the reverse direction (i.e., from the INM to the ONM) during herpesvirus egress from the nucleus (for review see Mettenleiter, 2002). Potentially relevant to the transport mechanism are the findings that individual nucleoplasmic and lumenal domains of all well-characterized resident integral proteins of the INM are smaller than ~50 kD, and all resident INM proteins examined can move rapidly between the INM and the peripheral ER (Powell and Burke, 1990; Ellenberg et al., 1997; Östlund et al., 1999; Wu et al., 2002).

To directly analyze the mechanisms for movement of integral proteins from the ONM to the INM, we developed a live cell fluorescence imaging assay using the rapamycin-based FKBP-FRB dimerization system (Chen et al., 1995; Klemm et al., 1997). The assay involves a nucleus-restricted "trap" protein fused to the 12-kD FK506-binding protein (FKBP) domain, and a variety of ER-integrated, GFP-tagged reporter proteins that contain a FKBP-rapamycin binding domain (FRB). The reporters initially are present throughout the ER; however, addition of rapamycin to cells results in trapping and accumulation of the reporters at the INM. This allows the measurement of integral protein movement from the peripheral ER to the INM in real time. We found that movement to the INM occurs rapidly for reporters with small cytosolic or lumenal domains, but is strongly inhibited if the total size of the cytosolic or lumenal domains are increased to ~60–75 kD. The rapid movement of the small reporters that we observed at 37°C is strongly inhibited when cells are cooled to 20°C or when ATP is depleted. However, this energy-dependent movement is insensitive to inhibitors of vesicular trafficking. Because movement to the INM is strongly inhibited by antibodies to the transmembrane nucleoporin gp210, our results support a model in which movement of integral proteins to the inner membrane occurs by lateral diffusion through the nuclear pore membrane, involving an energy-dependent process that constitutively restructures the NPC.

Results

An assay for transport of integral proteins to the INM in living cells

We have developed an assay to analyze the movement of integral membrane proteins from the peripheral ER to the INM in real time. The assay uses the ability of the cell-permeable small molecule rapamycin to mediate high affinity dimerization between FKBP and FRB (Chen et al., 1995; Klemm et al., 1997). One component of this assay is a reporter protein consisting of (from the NH_2 to the COOH terminus) the 11-kD FRB (Chen et al., 1995), amino acid residues 401–452 of lamina-associated polypeptide 2β (LAP2β) containing its membrane-insertion domain (LAP2β-TM; Furukawa et al., 1995), and GFP (Fig. 1). Because LAP2β is a type II integral protein of the INM (Furukawa et al., 1995), this construct gives rise to a transmembrane protein with FRB located in the cytosol and GFP situated in the ER lumen. Additional versions of the reporter construct contained a 47-kD domain from chicken muscle pyruvate kinase (PK; Soullam and Worman, 1995) added to the lumenal or cytosolic side of the transmembrane segment. A second component of the assay was a nucleus-localized protein "trap" that lacked membrane-spanning sequences. This consisted of three tandem repeats of the 12-kD FKBP domain (Klemm et al., 1997) fused to amino acids 1–396 of LAP2β (LAP2β-NP), which contains the LAP2β lamin-binding domain and which is strongly localized to the nucleus of transfected cells (Furukawa et al., 1995).

The reporter and trap proteins were coexpressed in HeLa cells by transient transfection, and cells were examined by fluorescence microscopy (Fig. 2). Consistent with previous work showing that the short segment of LAP2β present in the reporter lacks NE targeting/retention sequences (Furukawa et al., 1998), the reporter was distributed throughout both the pe-

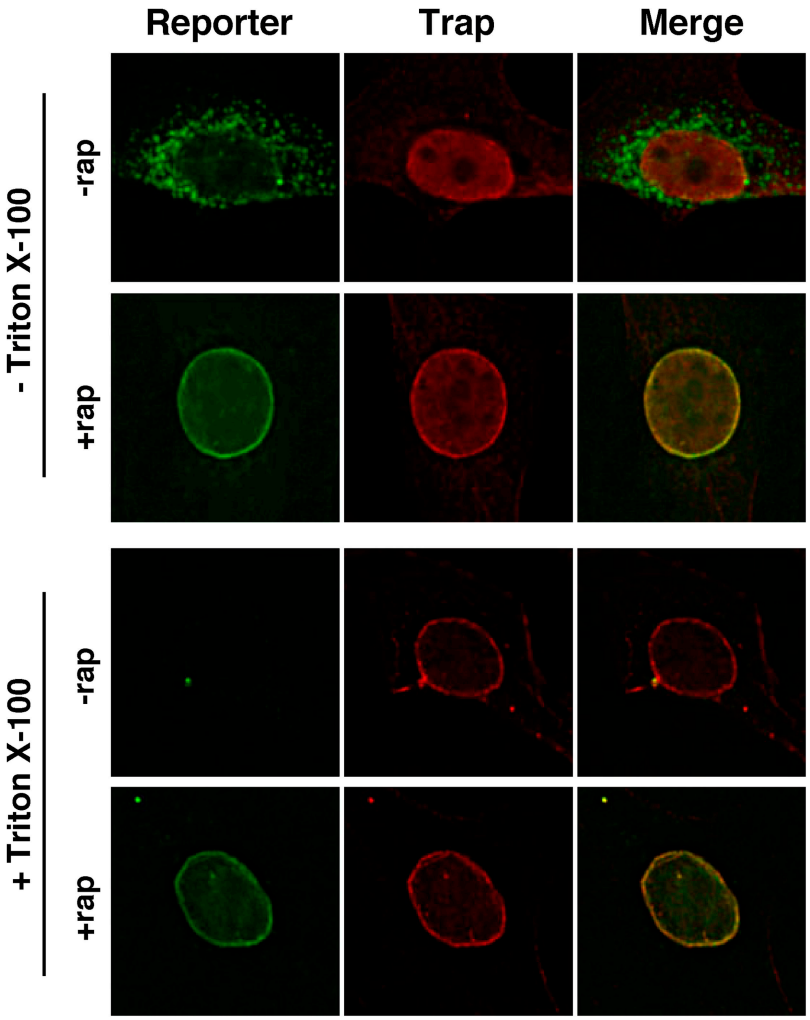

Figure 2. **Rapamycin-induced accumulation of reporter protein at the NE.** HeLa cells were cotransfected with the reporter and trap plasmids, and after 20 h were incubated with or without 150 ng/ml rapamycin (rap) at 37°C for 2 h. Cells were washed in buffer lacking or containing 1% Triton X-100 and fixed. The reporter protein was visualized by GFP fluorescence (green), whereas the trap protein was visualized by immunofluorescent staining with anti-FLAG mAb (red). Similar results were obtained upon treatment of cells with rapamycin for 30 min instead of 2 h (not depicted).

ripheral ER and the NE in the absence of rapamycin. This yielded a vesicular labeling pattern in fixed cells (Fig. 2, −Triton X-100, −rap). In contrast, the trap protein was highly concentrated in the nucleus, with elevated localization at the NE, and was essentially absent from the cytoplasm. When nonrapamycin-treated cells were washed with Triton X-100 before fixation, the reporter protein was almost completely extracted from cells. The extracted protein included the reporter initially present at the nuclear rim (Fig. 2, +Triton X-100, −rap). This behavior contrasts with that of most lamina-associated proteins, which remain bound to the lamina after Triton X-100 extraction (Furukawa et al., 1998).

When cells were treated with rapamycin, there was substantial accumulation of the reporter at the NE. The percentage of the total fluorescent reporter at the NE with rapamycin varied from cell to cell (see Fig. 3 A). This variety was apparently due to differences in the levels of reporter and/or trap protein expression, as the percentage of NE-associated reporter was always high in cells that expressed low levels of the reporter (Fig. 2, −Triton X-100, +rap; see also Fig. 3 A). When rapamycin-treated cells were extracted with Triton X-100 before fixation, a significant amount of reporter remained at the NE (Fig. 2, +Triton X-100, +rap), in contrast to nonrapamycin treated cells. This finding suggests that rapamycin-induced dimerization of the reporter with the trap protein led to immobilization of the reporter at the INM.

We used this assay to monitor the kinetics of accumulation of the reporter at the NE after rapamycin addition by real-time imaging (Fig. 3). The initial fluorescence level at the NE was normalized to a value of 1. Based on the data presented in Figs. 3 and 4, this initial NE pool apparently represents protein that is in equilibrium between the INM, ONM, and peripheral ER. With rapamycin addition the NE staining was observed to increase rapidly over the first ~10 min, concomitant with a decrease in the level of peripheral ER staining (Fig. 3 A). Quantification revealed that reporter accumulation at the NE reached a plateau at ~10–20 min, at an average intensity value ~1.4-fold higher than the initial NE labeling (Fig. 3 C).

Analysis of reporter proteins with an enlarged cytosolic or lumenal domain

Previous work showed that fusing PK to the cytosolic domain of an INM-targeting LBR fragment to increase its size to ~70 kD resulted in loss of its accumulation at the NE in transfected cells (Soullam and Worman, 1995). We examined if attaching PK to either the cytosolic or the lumenal domains of the fluorescent reporter affected NE accumulation in our assay. Consistent with the previous work, addition of PK to the cytosolic/

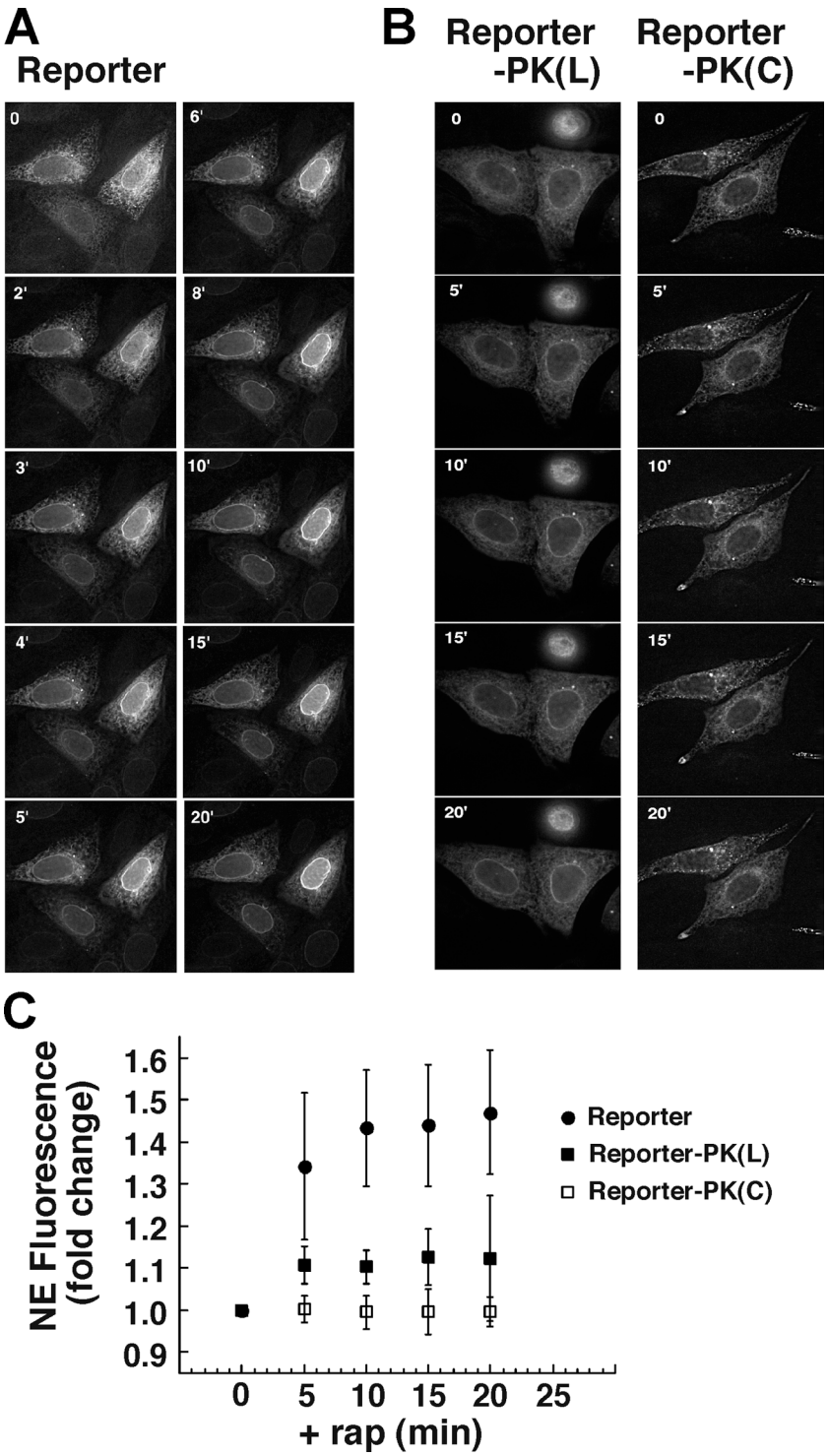

Figure 3. **Time course of accumulation of reporters at the NE after rapamycin treatment.** HeLa cells were cotransfected with reporter and trap plasmids and examined after 20 h. (A) Localization of the reporter protein was followed for up to 20 min after rapamycin treatment (as indicated) at 37°C by monitoring the GFP fluorescence using real-time imaging. (B) Localization of reporter with PK fused to the lumenal domain (reporter-PK(L)) or to the cytosolic domain (reporter-PK(C)) was also followed as in A. (C) NE fluorescence intensities of the reporter (closed circles), reporter-PK(L) (closed squares), or reporter-PK(C) (open squares) were plotted as a function of time after rapamycin addition (+rap). For each time course, the fluorescence intensities of the NE were quantified using NIH image, with the 0 min time point set at 1, and with the following time points normalized as the relative increase. The fluorescence mean and SD are given, based on the measurement of seven or more separate cells for each condition.

nucleoplasmic side of the LAP2β-based reporter completely blocked NE accumulation over the course of 20 min (Fig. 3, B and C). The loss of accumulation was not due to the change in the spacing between FRB and the membrane insertion domain of LAP2β presented by the standard reporter because a separate reporter construct in which PK was fused to the NH_2 terminus of the FRB-LAP2β-TM fusion yielded similar results (unpublished data; see Materials and methods). Accumulation of the reporter at the NE also was substantially decreased by adding PK to the lumenal domain of the reporter (~1.1-fold concentration of the reporter at the NE after 20 min, as compared with ~1.4-fold seen for the standard reporter; Fig. 3, B and C).

We performed similar studies with a reporter construct in which the membrane insertion domain of another type II integral membrane protein, chicken hepatic lectin (CHL; Chiacchia and Drickamer, 1984), was substituted for that of LAP2β. Unlike LAP2β, CHL by itself is not targeted to the NE (Soullam and Worman, 1995). Similar to the standard reporter, the CHL-based reporter became rapidly concentrated at the NE over the course of 10 min after addition of rapamycin and reached a pla-

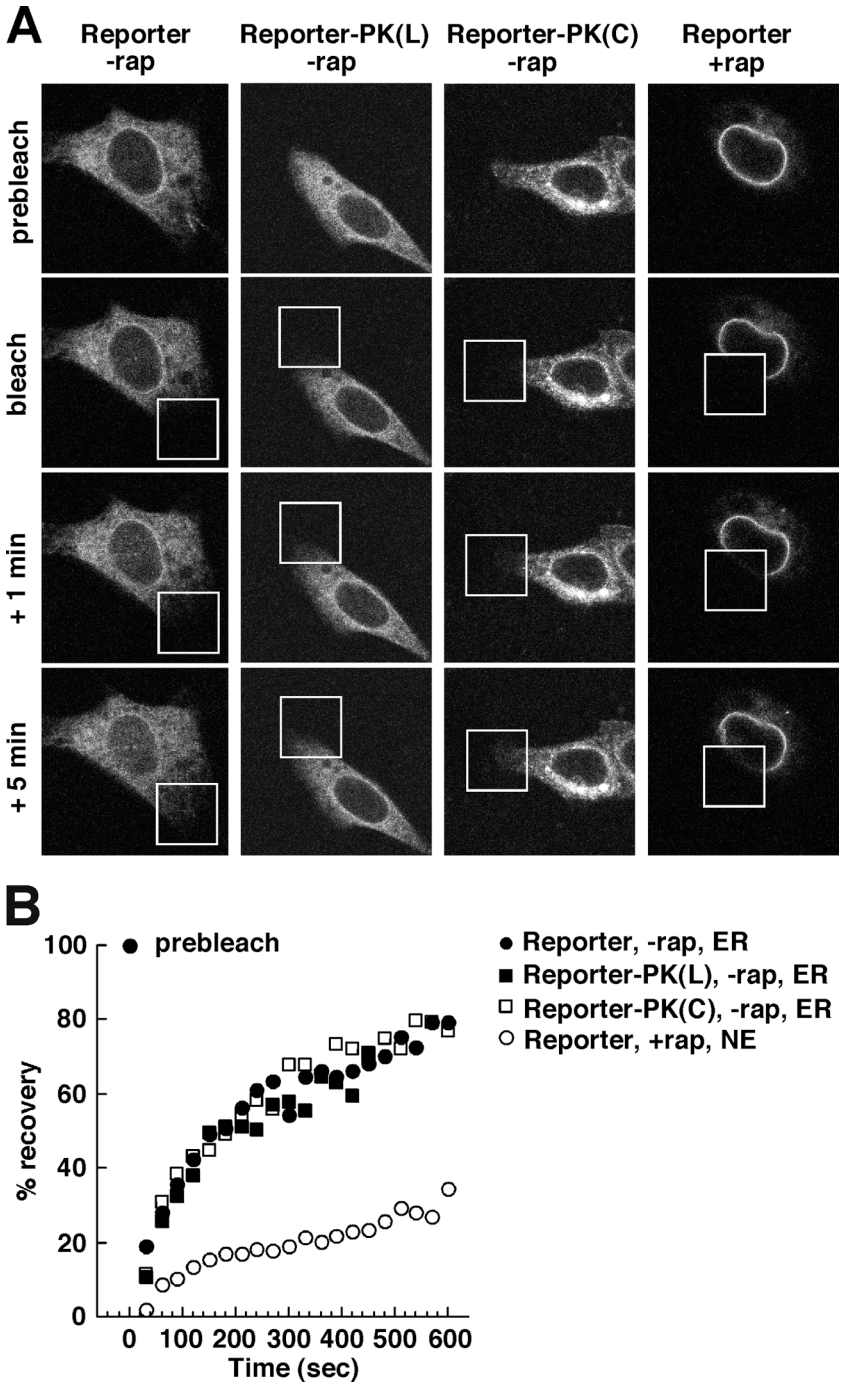

Figure 4. **Mobility of reporter constructs in the peripheral ER and at the NE.** HeLa cells were cotransfected with reporter and trap plasmids and examined after 20 h. (A) FRAP was used to measure the mobility of reporter constructs in the peripheral ER and NE at 37°C. We analyzed mobility in the peripheral ER in the absence of rapamycin (−rap) of the standard reporter (left column), reporter with PK fused to the lumenal domain (second column), and reporter with PK fused to the cytosolic domain (third column). We also analyzed mobility of the fluorescent reporter in the NE at 30 min after addition of rapamycin (+rap, right column). Fluorescence photobleaching was performed in the designated boxed areas, and the fluorescence recovery was measured at various times thereafter. Shown are cells immediately before and after photobleaching, and at 1 and 5 min thereafter. (B) Time course of recovery of fluorescence intensities in the photobleached cellular areas shown in A. Fluorescence intensities are normalized to the prebleach intensity.

teau concentration at ~1.3-fold higher than the initial level (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200409149/DC1). CHL-based reporters with PK fused to the cytosolic or lumenal domains, like the larger LAP2β-based reporters, did not significantly accumulate at the NE in the presence of rapamycin (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200409149/DC1).

To ensure that the impaired movement of the PK-fused reporters to the INM was not the result of reporter aggregation or multimerization, we examined the mobility of the reporters in the peripheral ER by FRAP analysis. For this experiment, an ~10-μm square zone over the cytoplasm was photobleached, and the fluorescence recovery was monitored thereafter (Fig. 4 and Table I). The average fluorescence intensity of the bleached zone reached ~80% of the initial prebleach level at 10 min (Fig. 4 B). The diffusion constant for the standard reporter measured by this method (D = ~0.33 $\mu m^2/s$; Table I) was very similar to values previously reported for integral proteins that are freely mobile in the ER (Cole et al., 1996; Ellenberg et al., 1997; Lippincott-Schwartz et al., 2000). Moreover, the kinetics of fluorescence recovery in the bleached cytoplasmic area for reporters containing PK were very similar to that of the standard reporter lacking PK, and statistically indistinguishable diffusion constants were

calculated (Fig. 4 and Table I). This indicates that the diminished accumulation of the PK-fused reporters at the NE in the presence of rapamycin is not the consequence of aggregation of these proteins in the ER because they retain the same lateral mobility. Considered together, our results indicate that the size of either the lumenal or cytosolic domains of integral membrane proteins restricts their movement from the ER to the INM, and that increasing the size of these domains to ~50–75 kD strongly diminishes movement.

We also used FRAP to examine the diffusional mobility of the reporter that had accumulated at the NE after addition of rapamycin. Because of the inability to clearly resolve fluorescence in the juxtanuclear peripheral ER from NE fluorescence, we only measured cells where most of the reporter protein pool was targeted to the NE. In contrast to the rapid recovery of fluorescence in photobleached cytoplasmic areas of cells, the NE fluorescence of cells treated with rapamycin recovered much more slowly (Fig. 4). We calculated a diffusion constant of ~0.10 $\mu m^2/s$ for this protein population (Table I). This value is very similar to those measured for some other INM proteins immobilized at the nuclear lamina (Östlund et al., 1999; Wu et al., 2002), and supports the results of the Triton X-100 extraction experiments (Fig. 2), suggesting that rapamycin treatment tethers the NE-associated reporter to the lamina.

Table I. **Diffusion constants (*D*) of reporter proteins**

	D	n
	$\mu m^2/s$	
Reporter, ER	0.33 ± 0.02	7
Reporter + PK (L), ER	0.30 ± 0.05	4
Reporter + PK (C), ER	0.32 ± 0.08	4
Reporter + rapamycin, NE	0.10 + 0.03	4

ER, measured in peripheral ER; NE, measured at nuclear rim; n, independent determinations.

Biochemical requirements for integral protein transport to the INM

To investigate the molecular mechanism for movement of integral proteins from the peripheral ER to the INM, we examined the effects of decreased temperature or ATP depletion on this process (Fig. 5). Either depleting ATP or incubating cells at 16–22°C strongly inhibits vesicular trafficking in cultured cells, although passive diffusion of cargo proteins in membranes of the secretory pathway is not significantly affected

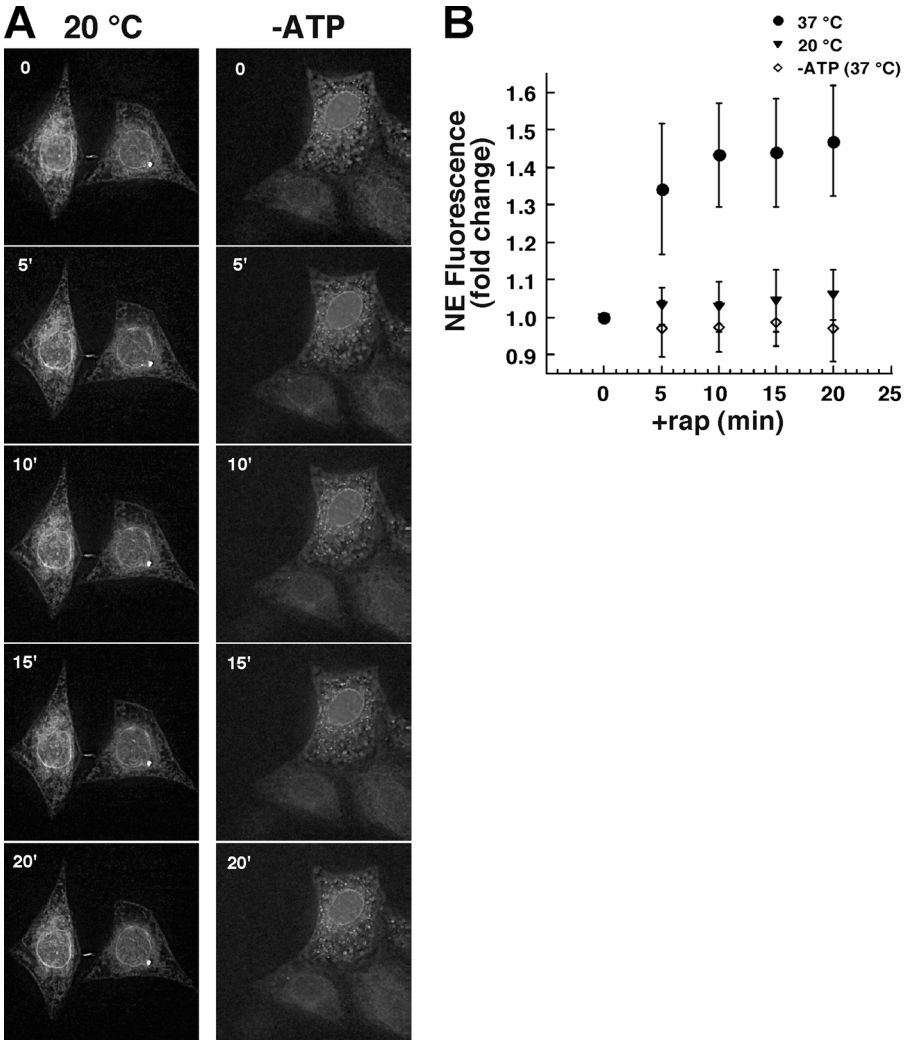

Figure 5. **Temperature and ATP dependence of reporter movement to the INM.** (A) HeLa cells cotransfected with reporter and trap plasmids were preincubated at 20°C for 10 min (left) or in glucose-free medium containing sodium azide at 37°C (−ATP) for 15 min (right). Subsequently, rapamycin was added and the localization of reporter proteins was followed by monitoring the GFP fluorescence at the times indicated. (B) Fluorescence intensities of the reporter at the NE were quantified at various times after rapamycin addition in cells maintained at 37°C in normal medium (closed circles), at 20°C in normal medium (closed triangles), or at 37°C in glucose-free medium containing sodium azide (−ATP, open diamonds). Shown are the average intensities and SD measured for 10 or more cells for each condition.

Table II. **Diffusion constants (*D*) of standard reporter at different temperatures**

	D	n
	$\mu m^2/s$	
20°C	0.33 + 0.06	5
37°C	0.33 + 0.02	4

n, independent determinations.

(Lippincott-Schwartz et al., 2000). We found that movement of the standard reporter to the INM was completely inhibited by ATP depletion, and also was strongly inhibited by cooling cells to 20°C (Fig. 5, A and B). However, neither depletion of ATP nor decrease in temperature to 20°C detectably affected the mobility of the reporter protein in the peripheral ER, as determined by FRAP (Fig. 6). The diffusion constants of the reporter protein under these conditions (Table II) were not significantly different from those measured in untreated cells at 37°C (Table I). These results indicate that the reporter proteins retained their long-range mobility in the peripheral ER under the ATP-depletion and reduced temperature conditions where transit to the INM was strongly inhibited. These findings clearly indicate that the ATP-depletion and reduced temperature do not limit access of the reporter to the ONM, and therefore, that lack of accumulation at the INM reflects a more fundamental aspect of the transport mechanism.

These results were consistent with a membrane fusion–based mechanism for movement of transmembrane proteins to the INM (Introduction). To further explore this possibility, reporter protein movement to the INM was measured after pretreatment of cells with membrane-permeable inhibitors of vesicular trafficking that block fusion between cytosolic membrane surfaces, *N*-ethylmaleimide (NEM; Beckers et al., 1989; Macaulay and Forbes, 1996), and 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl) ester (BAPTA-AM; Chen et al., 2002; Fig. 7). NEM inactivates vesicular trafficking machinery by modifying sulfhydryls of proteins such as p97/NSF (Dalal et al., 2004), whereas BAPTA-AM chelates calcium. Pretreatment of cells with 10 mM NEM or with 50 μM BAPTA-AM caused no significant inhibition of reporter movement to the INM (Fig. 7). However, these conditions efficiently inhibited the movement of the vesicular stomatitis virus (VSV) G protein from the ER to the Golgi in transfected HeLa cells, in which we performed a temperature shift with the temperature-sensitive ts045 G protein mutant (de Silva et al., 1990) to synchronize ER egress

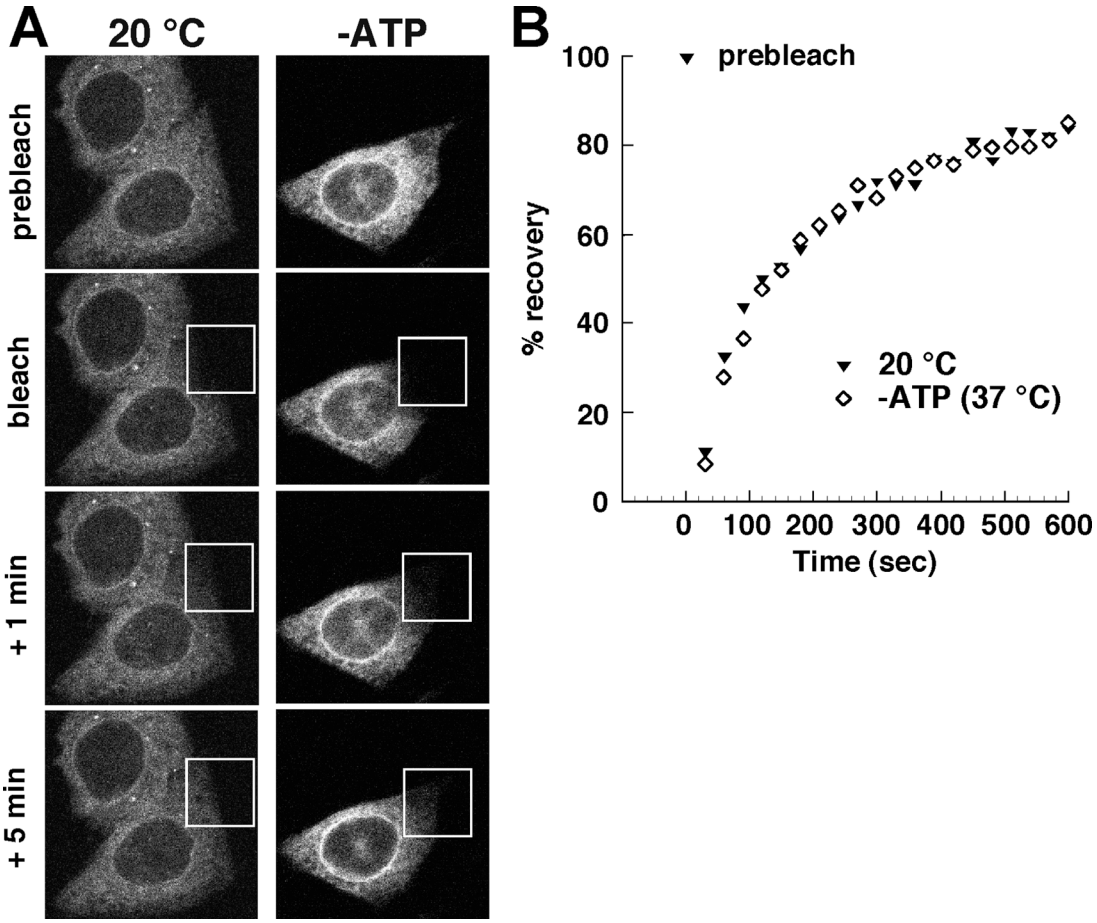

Figure 6. **Effects of reduced temperature and energy depletion on the ER mobility of reporter protein.** (A) HeLa cells cotransfected with reporter and trap plasmids were preincubated at 20°C for 10 min (left) or in glucose-free medium containing sodium azide for 15 min at 37°C (−ATP, right). The indicated boxed areas were photobleached, and the recovery of the fluorescence was measured at various times thereafter. Shown are images of cells immediately before and after photobleaching, and at 1 and 5 min thereafter. (B) Time course of recovery of fluorescence intensities in the photobleached areas shown in A. Fluorescence intensities were normalized to the prebleach intensities.

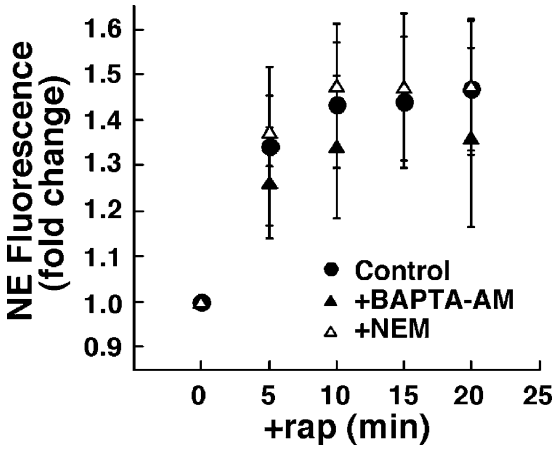

Figure 7. **Effects of membrane trafficking inhibitors on accumulation of reporter protein at the NE.** HeLa cells cotransfected with reporter and trap plasmids were preincubated with 50 μM BAPTA-AM for 30 min (closed triangles), with 10 mM NEM for 20 min (open triangles), or were maintained in normal growth medium (closed circles). Cells were then treated with rapamycin, and accumulation of fluorescent reporter at the NE at 37°C was followed as a function of time. Shown are the average intensities and SD measured for seven or more cells for each condition.

(Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200409149/DC1). Thus, membrane fusion involving cytosolic membrane surfaces is unlikely to be involved in integral protein trafficking to the INM, despite the fact that this process requires ATP and is inhibited at 20°C. These results prompted us to investigate whether or not protein trafficking to the INM might involve movement around the nuclear pore membrane in the plane of the lipid bilayer.

Role of the nuclear pore membrane in trafficking of integral proteins to the INM

For this analysis, we microinjected cells with antibodies or a lectin that bind to the NPC and could potentially impose a steric block to reporter movement. We monitored NE accumulation of the standard reporter. Because it was not technically feasible to carry out live cell imaging in these experiments, we examined cells that were fixed 30 min after rapamycin addition (Fig. 8 A). Our quantification method in these experiments involved comparing the fluorescence intensity at the NE to the intensity over cytoplasmic regions located in an area between 4–6 μm from the NE (see Materials and methods). By this method, in uninjected cells the NE to cytoplasmic fluorescence intensity ratio had an average value of approximately three before rapamycin treatment and increased to a value of approximately eight after rapamycin treatment (Fig. 8 A).

Our first experiment targeted gp210, a major transmembrane glycoprotein of the NPC containing a cytosolic tail of ~58 amino acid residues (Greber et al., 1990). We prepared affinity-purified antibodies to a peptide epitope in its cytosolic tail, which strongly and specifically labeled the NE in immunofluorescence staining (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200409149/DC1). Cells were injected with anti-gp210 and were analyzed 3 h later for reporter accumulation at the NE (Fig. 8, B and D). The injected antibodies did not detectably alter nuclear structure after 3 h (Fig. 8 D and not depicted). Moreover, there was no detectable perturbation of NPC organization in the microinjected cells, as immunofluorescence staining with the RL1 monoclonal antibody specific for a group of FG-repeat nucleoporins (Snow et al., 1987) showed no changes in the intensity or distribution of NE labeling (unpublished data). We found that accumulation of the reporter at the NE induced by rapamycin was almost completely inhibited by the microinjected anti-gp210 (Fig. 8, B and D). In this case, the average NE/cytoplasmic ratio was approximately four, as compared with the NE/cytoplasmic ratio of approximately three measured in the −rapamycin control, in which there was no rapamycin-induced trapping at the NE (Fig. 8 A). Injection of the anti-gp210 antibodies together with the cognate peptide used for immunization completely reversed the inhibitory effect, indicating that the antibody effect is specific (Fig. 8, B and D).

In a second experiment, we targeted the NPC with the lectin WGA, which binds to the cytosolically exposed *O*-glycosylated regions of FG repeat nucleoporins (Finlay et al., 1987; Snow et al., 1987; Fig. 8 C). These proteins include POM121, a type II integral membrane protein of the nuclear pore membrane (Snow et al., 1987; Hallberg et al., 1993). In this case, we obtained only slight inhibition of accumulation at the NE by injection of 0.3 mg/ml WGA (Fig. 8 C). Injection of 1 mg/ml WGA yielded ~50% inhibition of NE accumulation, as the average NE/cytoplasmic fluorescence diminished to a value of approximately five (Fig. 8 C). When nuclear import was assessed in these cells by coinjection of a fluorescent import cargo (NLS-BSA), we observed ~33 and ~66% inhibition of signal-mediated nuclear import by injection with 0.3 mg/ml and 1.0 mg/ml WGA, respectively (unpublished data). Injection of a higher concentration of WGA (5 mg/ml) caused cell rounding and detachment from the coverglass (unpublished data), precluding analysis with this condition. Considered together, our results showing inhibition of reporter movement to the INM by reagents that bind to the NPC argue that transport of transmembrane proteins to the INM occurs via the nuclear pore membrane in interphase HeLa cells. However, the strong dependence of this process on ATP and temperature argue that the transport mechanism is more complex than unrestricted diffusion in the lipid bilayer around the nuclear pore membrane (see Discussion).

Discussion

We have implemented a live cell imaging strategy to analyze the movement of integral membrane proteins from their site of synthesis and membrane integration in the peripheral ER to the INM in interphase cultured cells. Our assay is based on rapamycin-mediated trapping of GFP-fused reporter proteins at the nuclear lamina. This methodology allowed us for the first time to analyze the biochemical requirements for movement of transmembrane proteins to the INM. We found that this process is strongly inhibited at 20°C and under conditions of ATP depletion, although it is insensitive to inhibitors of membrane fusion and vesicular trafficking in the secretory pathway. Moreover, we found

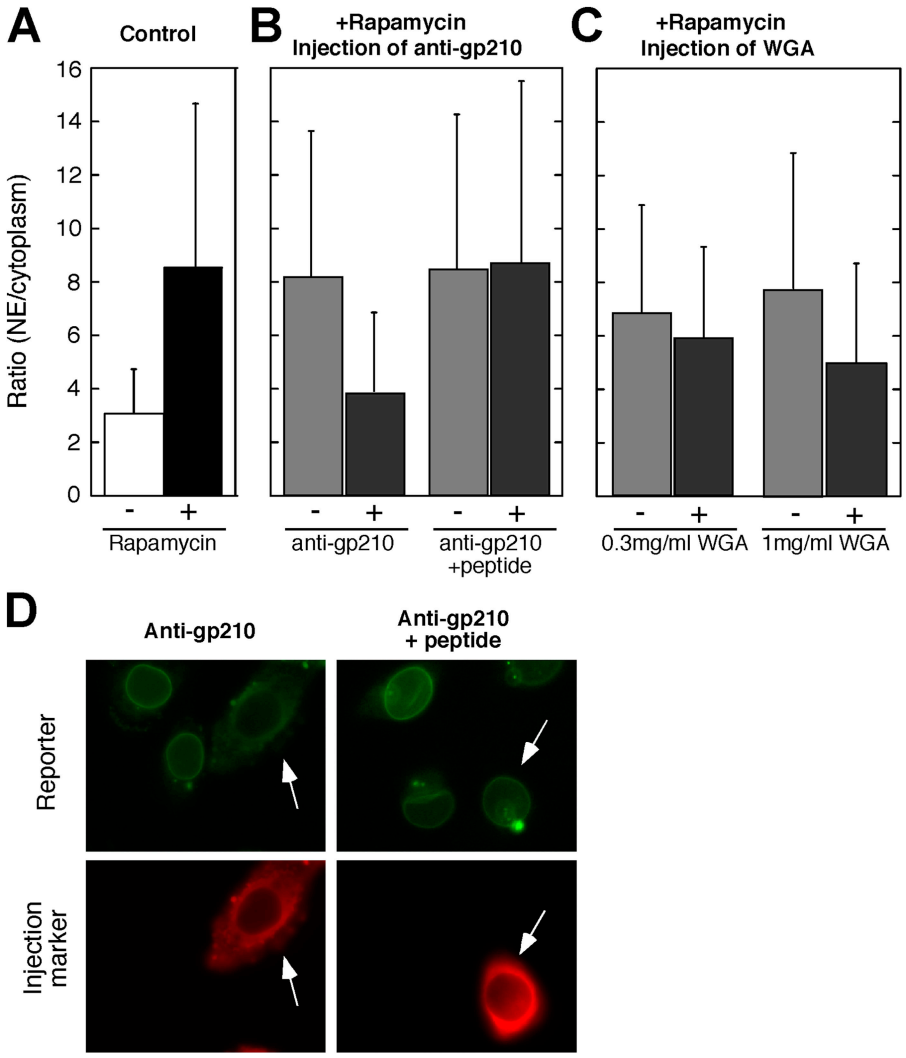

Figure 8. **Effects of microinjected anti-gp210 and WGA on reporter accumulation at the NE.** HeLa cells were cotransfected with reporter and trap plasmids and grown for 20 h. Control cultures, or cells that had been injected in the cytoplasm with anti-gp210 antibodies or WGA, were incubated for another 3 h, treated with or without rapamycin for 30 min, and fixed. The NE accumulation of reporter is represented by a ratio of the NE to cytoplasmic fluorescence intensity (see Materials and methods). Shown are the average values and SD measured for 12 or more separate cells for each experiment. (A) Uninjected cells, examined without (−) or with (+) rapamycin treatment. (B) Rapamycin-treated cells: either uninjected (−) or injected in the cytoplasm (+) with ~1 mg/ml of affinity-purified anti-gp210 or with anti-gp210 mixed with the antigen peptide. (C) Rapamycin-treated cells: either uninjected (−) or injected in the cytoplasm (+) with either 0.3 mg/ml or 1 mg/ml WGA. (D) Micrographs showing representative cells from the experiment in B, depicting the reporter localization in cells injected with anti-gp210 or with anti-gp210 + peptide (arrows). Microinjected cells were detected by immunofluorescent localization of the injected rabbit IgG. Uninjected cells in the same fields depict control levels of rapamycin-induced accumulation at the NE.

that it is potently blocked by antibodies to the nuclear pore membrane protein gp210. Considered together, our data indicate that movement of integral proteins to the INM involves energy-dependent movement around the nuclear pore membrane.

Transfection of cells with fusion proteins containing regions of various INM residents has suggested that binding to nuclear lamins and/or chromatin can specify accumulation at the INM (Introduction). Moreover, studies involving live cell imaging of GFP-tagged reporters derived from INM proteins have shown that the diffusional mobility of the reporters is substantially reduced when the proteins are assembled in the NE, as compared with their unrestricted mobility when they are dispersed throughout the ER of mitotic cells or in the peripheral ER during interphase (Introduction). This finding underscores the notion that at least many INM proteins are tethered at the nuclear lamina. A widely discussed model incorporating these findings proposes that transport of integral proteins from the ONM to the INM occurs by lateral diffusion of the proteins around the nuclear pore membrane coupled with their binding at the INM (for review see Holmer and Worman, 2001).

The proposed involvement of the nuclear pore membrane in transport to the INM has been based primarily on thin section EM, which indicates that the pore membrane contains a trilaminar lipid bilayer that connects the inner and outer membranes (for review see Gerace and Burke, 1988). In principle, this continuity could allow movement of integral protein between the two membranes. Moreover, in analysis of integral protein targeting to the INM in transfected cells, it has been observed that increasing the size of the cytosolic domain to ~60–75 kD results in loss of accumulation at the NE (Soullam and Worman, 1995; Wu et al., 2002), which could reflect steric inhibition by the NPC.

In our experimental system, we have shown that increasing the size of the lumenal as well as the cytosolic domain of integral proteins to ~60–75 kD strongly restricts movement to the INM. Moreover, we have shown by FRAP that the reporters with enlarged cytosolic or lumenal domains are as diffusionally mobile in the peripheral ER as are the smaller reporters that can efficiently accumulate at the INM. This result indicates that lack of movement of the larger reporters to the INM is not the consequence of their aggregation in the ER. Thus, our results support and extend previous data indicating the existence of a size-selective "filter" for movement of integral proteins to the INM. It remains possible that specific signals (presently hypothetical) could modify this filter to allow proteins with large extramembrane domains to reach the INM. However, such signals do not exist in the segment of LAP2β

encompassing its transmembrane and lumenal domains. This is because reporters based on the membrane-insertion domain of LAP2β are as strongly size-restricted in movement to the INM as are reporters based on CHL, a protein that normally is not targeted to the INM. Additionally, other work has indicated that the nucleoplasmic domains of the INM proteins LBR (Soullam and Worman, 1995) and MAN1 (Wu et al., 2002) do not contain signals that can modify the filter. The movement of integral proteins to the INM, which is strongly restricted by size, contrasts with signal-mediated transport of soluble macromolecular complexes through the NPC, which accommodates cargoes as large as ribosomal subunits.

Up to now there has been little direct support for the model that the nuclear pore membrane is the conduit for movement of integral membrane proteins from the ONM to the INM. This model now is robustly supported by our findings that the process is inhibited by anti-gp210 antibodies and to a significant, albeit lesser, degree by WGA. We consider it most likely that the antibodies and lectin act by sterically occluding passageways for movement to the INM. However, it also is possible that the binding of anti-gp210 or WGA induces conformational changes in the NPC, which could indirectly affect protein movement channels that allow integral proteins to pass around the pore membrane. Whatever the precise molecular mechanism of inhibition, our results clearly implicate the nuclear pore membrane in this process in cultured cells during interphase.

Our results indicate that interphase growth of the NE in cultured cells involves different mechanisms from those involved in NE reassembly at the end of mitosis (Ellenberg et al., 1997; Yang et al., 1997). NE reassembly has been biochemically analyzed in *Xenopus laevis* egg extracts and related systems and involves a complex set of reactions including membrane fusion and growth, NPC assembly, and targeting of integral proteins to the INM (for reviews see Vasu and Forbes, 2001; Holaska et al., 2002; Mattaj, 2004). Studies on NE assembly have implicated several factors in this process, including RanGTP, importin β, the AAA-ATPase p97, and nuclear lamins. Whereas NE/NPC reassembly is sensitive to BAPTA-AM (Sullivan et al., 1993; Macaulay and Forbes, 1996) and involves NEM-sensitive factors such as p97 (Hetzer et al., 2001), we found that movement of integral proteins to the INM in interphase HeLa cells is insensitive to BAPTA and NEM. This finding argues that bulk movement of integral proteins to the INM during interphase does not involve vesicular trafficking or fusion between cytosolic membrane surfaces. Nonetheless, it remains possible that these mechanisms could play a role in movement of proteins between peripheral ER and INM in some types of animal cells and/or have a quantitatively minor role in our cell system.

NEM and BAPTA are known only to block fusion at cytosolic membrane surfaces, and it is unclear if these inhibitors block fusion between lumenal membrane surfaces. Thus the lack of inhibition by these reagents does not rule out the model that integral protein movement to the INM occurs as a consequence of transient fusion between the lumenal surfaces of inner and outer membranes (Introduction). However, the strong size restriction we have observed for transmembrane protein movement to the INM is clearly inconsistent with the latter model.

In view of our strong evidence that movement of integral proteins from the ONM to the INM occurs via the nuclear pore membrane, why is this process inhibited at 20°C and under conditions of ATP depletion? Simple diffusion around the lipid bilayer of the pore membrane is not predicted to show these requirements (Zhang et al., 1993). EM averaging and tomographic analysis of the NPC embedded in the NE of *X. laevis* oocyte provides no evidence for channels adjacent to the pore membrane that would allow unrestricted passage of the cytosolic domains of integral proteins (Akey and Radermacher, 1993; Stoffler et al., 2003). Based on these findings, we propose that the NPC normally undergoes continuous, energy-dependent restructuring involving dissociation and/or conformational changes of nucleoporins. This would create transient channels through the NPC at the nuclear pore membrane, thereby permitting lateral diffusional movement of integral proteins in the lipid bilayer between the INM and ONM. In light of our finding that movement to the INM is restricted by the size of the lumenal as well as cytosolic domains of transmembrane reporters, hypothetical restructuring of the NPC also might involve its lumenal components, which in part include the large lumenal domain of gp210 (Greber et al., 1990; Akey and Radermacher, 1993; Stoffler et al., 2003).

The hypothesized ATP- and temperature-dependent restructuring of the NPC could reflect the involvement of ATPases of the heat shock protein family (for review see Hendrick and Hartl, 1993) acting on nucleoporins at the cytosolic and/or lumenal sides of the pore membrane. Because ATP is required to dissociate many heat shock proteins from their substrates, ATP-depletion could lead to stable binding of heat shock proteins to nucleoporins. Thereby, this might create a steric impediment to movement similar to that speculated for anti-gp210 antibodies. Alternatively, there could be other enzymes involved in dynamic restructuring of the NPC, such as kinases and phosphatases. However, we have not observed any reduction in the movement of our standard reporter to the INM when cells are pretreated with 5 μM of okadaic acid (unpublished data), which inhibits major cellular phosphatases (Gehringer, 2004).

Temperature dependence has been observed for many vesicular trafficking pathways, wherein transport is strongly inhibited in the 16–22°C range in cultured cells (Lippincott-Schwartz et al., 2000). The biochemical basis for this temperature restriction is unknown, but may reflect the involvement of multiple enzymes in this process, effectively yielding a much greater reduction in transport rate with decreasing temperature than typically obtained with a single enzyme. Alternatively, and not mutually exclusively, the temperature dependence might reflect specialized lipid microdomains (Brown and London, 1998), which form liquid ordered structures at reduced temperature that could limit the diffusional movement of transmembrane proteins. Both multi-enzyme systems and specialized lipid microdomains in the nuclear pore membrane might be relevant to the effects we have observed on transport of integral proteins to the INM. The methodology we have developed should now allow a more refined biochemical and biophysical analysis of these issues.

Materials and methods

Plasmids

Reporter proteins were cloned into the mammalian expression vector pEGFP-N3 (CLONTECH Laboratories, Inc.), for expression of the proteins as GFP fusions. For construction of the reporter protein, a BamHI–PstI-start codon-BglII linker (GGATCCCTGCAGACTATGCATAGAAGGAGATCCAGATCT) first was inserted into the BamHI–BglII sites of pBluescript, to create "pBluescript+linker." The FKBP12-rapamycin binding domain (FRB; obtained from J. Crabtree, Stanford University, Stanford, CA) was amplified by PCR as BamHI–BglII fragment and cloned into the BglII site of the pBluescript+linker (Stratagene). A fragment encoding residues 401–452 of LAP2β (ΔN5LAP2; Furukawa et al., 1995) was amplified by PCR with BamHI and BglII sites and cloned into the BglII site of pBluescript+linker-FRB. The FRB-ΔN5LAP2 fragment was excised from this construct using the vector's PstI site and the 3′ BglII site and cloned into the PstI–BamHI sites of pEGFP-N3, to encode a fusion protein consisting sequentially of FRB, residues 401–452 of LAP2β, and GFP. The type II membrane insertion segment of LAP2β in this construct contains a cytosolic region of nine residues, a transmembrane segment (residues 410–433), and a lumenal domain of 19 residues (Furukawa et al., 1995). To construct the reporter containing PK added to the COOH terminus of the lumenal domain, a BamHI–BglII fragment encoding chicken muscle PK, obtained from a construct provided by W. Greene (University of California, San Francisco, San Francisco, CA), was cloned into the BglII site of pBluescript+linker-FRB-ΔN5LAP2, and the latter construct was cloned into the BamHI site of pEGFP-N3.

To construct the standard reporter containing PK added to the NH_2 terminus of the cytosolic domain, the PK fragment was cloned into the BglII site of pBluescript+linker-FRB, and subsequently, the ΔN5LAP2 fragment was cloned into the BglII site of pBluescript+linker-FRB-PK. The FRB-PK-ΔN5LAP2 fragment was excised as the BamHI–BglII sites and cloned into the BamHI site of pEGFP-N3. An alternative reporter with PK cloned at the NH_2 terminus of the FRB-ΔN5LAP2 fusion was constructed as follows: The ΔN5LAP2 fragment was cloned into the BamHI–BglII site of pBluescript II-TKS (Ichihara and Kurosawa, 1993), and the FRB fragment was cloned into the BamHI site of pBluescript+linker-ΔN5LAP2. Next, the PK fragment was cloned into the BamHI site of pBluescript+linker-FRB-ΔN5LAP2, and the PK-FRB-ΔN5LAP2 fragment was excised as a BamHI–BglII fragment and cloned into the BglII site of pBluescript+linker. The PK-FRB-ΔN5LAP2 fragment from the latter then was cloned into the BglII site of pEGFP-N3.

To construct the trap plasmid, three tandem copies of FKBP 12 were cloned into pBJ5 (Schmitt and Gerace, 2001). A segment comprising residues 1–398 of LAP2β (ΔC1LAP2; Furukawa et al., 1995) was amplified by PCR as a SalI–BamHI fragment and cloned into the SalI–BamHI site of pBJ5-3xFKBP. The 3xFKBP-ΔC1LAP2 fragment was excised and cloned into the HindIII–BamHI site of pFLAG-CMV2 (Sigma-Aldrich).

We also constructed reporters containing the membrane insertion domain of another type II protein, CHL, using a cDNA for CHL provided by K. Drickamer (University of Oxford, Oxford, UK). The CHL membrane insertion segment, consisting of a cytosolic domain of 23 amino acids, a transmembrane segment of 25 amino acids, and COOH-terminal lumenal domain of 64 amino acids (Soullam and Worman, 1995), was cloned into the reporter plasmid by the same strategies as used for ΔN5LAP2 (detailed above in this section).

Transfection and rapamycin-induced in vivo dimerization

HeLa cells were grown in DME supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells that were 70–80% confluent in 3.5-cm wells were cotransfected with a mixture of 0.5 μg of reporter and 0.5 μg of trap plasmids by Lipofectamine 2000 (Invitrogen). 20 h after transfection, cells were analyzed for reporter accumulation at the NE by treatment with 150 ng/ml rapamycin for various times, in some cases after pretreatment with inhibitors (detailed below in this section). When fixed cells were examined, the cells were incubated for either 30 min or for 2 h at 37°C in the presence of rapamycin, washed with PBS containing an additional 0.1 M NaCl with or without 1% Triton X-100, fixed with 4% formaldehyde, and treated with 0.2% Triton X-100 in PBS for 2 min at RT. Immunofluorescent staining to detect the trap protein was performed by incubation of samples with an anti-FLAG mAb (Sigma-Aldrich) for 30 min at RT, followed by washing in PBS and incubation for 30 min with Alexa 594–labeled anti–mouse IgG antibody (Molecular Probes). Stained cells were mounted in fluoromount G (EM Sciences). A stack of 10 images at 2-μm intervals in the Z dimension were obtained with a microscope (model IX-70; Olympus) equipped with the liquid cooled CCD camera (model CH350L; Photometrics), using a Planapo 60× oil immersion objective (Olympus; NA 1.4). The images were deconvolved with DeltaVision software softWoRx version 2.5, and images reflecting a midplane through the nucleus were cropped using Adobe Photoshop 7.0. To test the effects of calcium or free sulfhydryls on reporter movement to the INM, cells were incubated in DME with 50 μM BAPTA-AM (Sigma-Aldrich) for 30 min or with 10 mM NEM (for 20 min at 37°C before rapamycin treatment and fixation). We validated that our conditions of BAPTA and NEM treatment strongly inhibited vesicular trafficking from the ER to the Golgi in our cell system using HeLa cells transiently transfected with the ts045 mutant of the VSV G protein (de Silva et al., 1990).

Time course of localization of the reporter protein

HeLa cells were grown on coverslips (1.5 thick × 40 mm diameter; Bioptechs) in 6-cm wells. At 70–80% confluency, cells were cotransfected with a mixture of 1.5 μg of reporter and 1.5 μg of trap plasmids and were incubated for 20 h at 37°C. Coverslips were mounted in a perfusion chamber (http://www.bioptechs.com/Products/FCS2/fcs2.html) maintained at the specified temperatures, the medium was replaced with growth medium containing 150 ng/ml rapamycin, and the localization of the reporter was monitored by visualizing GFP fluorescence with a microscope equipped with the liquid cooled CCD camera, using a Planapo 60× oil immersion objective (NA 1.4). A stack of 10 images at 2-μm intervals in the Z dimension were captured at each time point. Images were deconvolved with DeltaVision software softWoRx version 2.5. To quantify the mean NE fluorescence intensity of the reporter, a section representing a midsection of the nucleus was chosen, images were cropped using Adobe Photoshop 7.0, and scanned using NIH image at eight independent points of the nuclear rim per cell, taken from a linescan across the nucleus. To test the energy requirements, cellular ATP was depleted by treatment of cultures with 6 mM deoxyglucose and 10 mM sodium azide in glucose-free DME containing 0.5% dialyzed serum for 15 min before rapamycin treatment.

FRAP analysis

FRAP of reporter-expressing cells was performed with an inverted microscope (model Axiovert S100TV; Carl Zeiss MicroImaging, Inc.) using a 63× Planapo oil immersion objective (NA 1.4; Carl Zeiss MicroImaging, Inc.), interfaced with a confocal system (model 1024; Bio-Rad Laboratories) with a high zoom 488-nm laser scan using a 60-mW Ar/Kr laser. The outlined box (Figs. 4 and 6) was photobleached at full laser power and the recovery of fluorescence was measured by scanning the whole cell using low power at 30-s intervals. Images were cropped using Adobe Photoshop and mean fluorescence intensities were measured by NIH image, as described in the previous section. The diffusion constant *D* was determined as described by Ellenberg et al. (1997).

Microinjection experiments

All injected reagents were in PBS. Polyclonal rabbit anti-gp210 antibodies recognizing the cytosolic side of gp210 (Rb-68; Greber et al., 1990) were affinity purified on a matrix of the immunizing peptide (residues 1868–1886) coupled to CNBr-activated Sepharose 4B (Amersham Biosciences). HeLa cells were cotransfected with reporter and trap plasmids and were incubated for 20 h at 37°C. Subsequently, anti-gp210 (1 mg/ml) or a mixture of anti-gp210 + immunizing peptide (1 mg/ml antibody + 1 mg/ml peptide) was injected into the cytoplasm of cells. Alternatively, WGA (Calbiochem) at either 0.3 or 1.0 mg/ml was cytoplasmically injected together with a detection marker (1 mg/ml rabbit IgG; Sigma-Aldrich). After microinjection, cells were incubated for 3 h at 37°C, treated with rapamycin for 30 min, fixed with 4% formaldehyde, treated with 0.2% Triton X-100 in PBS for 2 min at RT, and labeled with Alexa 488 anti–rabbit IgG (Molecular Probes) to identify injected cells. After mounting in Vectorshield H-1000 (Vector Laboratories), the specimens were examined using a microscope (model Axiophot; Carl Zeiss MicroImaging, Inc.) equipped with a 40× Apochromat objective (NA 1.0; Carl Zeiss MicroImaging, Inc.) and a spot enhanced camera (Diagnostic Instruments). Images were cropped using Adobe Photoshop, and NIH image was used to quantify the NE and cytoplasmic fluorescence using two separate linescans across the nucleus and cytoplasm for each cell. The cytoplasmic fluorescence was defined as the average intensity over ~1-μm-long lines centered at ~5 μm from the nuclear rim. To measure nuclear import, GST fused with the NLS of the SV40 T antigen (GST-NLS) was injected into cytoplasm of cells with or without WGA (at either 0.3 or 3.0 mg/ml). After 30 min incubation at 37°C, cells were fixed and labeled with rabbit anti-GST polyclonal antibodies (Santa Cruz Biotechnology, Inc.) followed by Alexa 488 anti–rabbit IgG (Molecular Probes).

Online supplemental material

Figs. S1 and S2 compare the rapamycin-induced NE accumulation of reporters constructed with the transmembrane domain of either CHL or LAP2β or of reporters containing the transmembrane domain of CHL with PK fused to either the lumenal or cytosolic domain. Fig. S3 shows the localization of the ts045 mutant of the VSV G protein after shift to the permissive temperature of 32°C, without inhibitor treatment or after pretreatment with NEM or BAPTA-AM. Fig. S4 shows immunofluorescent staining of HeLa cells with the anti-gp210 antibodies used for microinjection in Fig. 8. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200409149/DC1.

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