

Depletion of Calcium from the Lumen of Endoplasmic Reticulum Reversibly Inhibits Passive Diffusion and Signal-mediated Transport into the Nucleus

Urs F. Greber* and Larry Gerace‡

*Yale University School of Medicine, Department of Cell Biology, New Haven, Connecticut 06520; and ‡Departments of Cell and Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

Abstract. Nuclear pore complexes provide channels for molecular transport across the nuclear envelope. Translocation of most proteins and RNAs through the pore complex is mediated by signal- and ATP-dependent mechanisms, while transport of small molecules is accomplished by passive diffusion. We report here that depletion of calcium from the lumen of the endoplasmic reticulum and nuclear envelope with ionophores or the calcium pump inhibitor thapsigargin rapidly and potently inhibits signal mediated transport of proteins into the nucleus. Luminal calcium depletion also inhibits passive diffusion through the pore complex. Signal-mediated protein import and passive

diffusion are rapidly restored when the drugs depleting luminal calcium are removed and cells are incubated at 37°C in calcium-containing medium. These results indicate that loss of calcium from the lumen of the endoplasmic reticulum and nuclear envelope reversibly affects properties of pore complex components located on the nuclear/cytoplasmic membrane surfaces, and they provide direct functional evidence for conformational flexibility of the pore complex. These methods will be useful for achieving reversible inhibition of nucleocytoplasmic trafficking for *in vivo* functional studies, and for studying the structure of the passive diffusion channel(s) of the pore complex.

NUCLEAR functions are segregated from the cytoplasm by the nuclear envelope (NE),¹ a subcompartment of the ER having both ER-like and specialized properties. The outer nuclear membrane, which functions as rough and smooth ER, is morphologically continuous with more peripheral ER, leading to direct luminal continuity between the NE and remainder of the ER system. Specialized functions of the NE are associated with the nuclear lamina, a karyoskeletal element localized on the nucleoplasmic face of the inner nuclear membrane, and nuclear pore complexes (NPCs).

The NPC is a supramolecular assembly with a mass of $\sim 125 \times 10^6$ D that spans the nuclear envelope and mediates molecular transport between the nucleus and cytoplasm (Forbes, 1992; Gerace, 1992; Panté and Aebi, 1993). It contains aqueous channels with functional diameters of ~ 9 nm, which allow low molecular mass solutes and macromolecules smaller than ~ 20 – 40 kD to passively diffuse be-

tween nucleus and cytoplasm (Peters, 1986). Most proteins and nucleic acids cannot efficiently cross the NPC by passive diffusion. Instead, they are transported through the NPC by ATP- and signal-dependent mechanisms involving a gated transport channel that can expand to a diameter of ≤ 26 nm (Gerace, 1992). Signals that specify the nuclear import of proteins (nuclear localization sequences [NLSs]) are typically short stretches of amino acids highly enriched in basic residues (Dingwall and Laskey, 1992). A qualitatively distinct signal appears to be involved in the nuclear import of small nuclear RNAs, based on kinetic competition studies (Michaud and Goldfarb, 1992).

The framework of the NPC consists of eight central "spokes" connected to peripheral "rings" facing the nucleoplasm and cytoplasm (Panté and Aebi, 1993). The spokes surround a central structure that contains the gated channel involved in signal dependent transport of macromolecules (Panté and Aebi, 1993). Eight aqueous channels with a diameter of ~ 10 nm occur between adjacent spokes, and they probably represent passive diffusion sites (Hinshaw et al., 1992; Akey and Radermacher, 1993). Extending outward from cytoplasmic and nuclear ring structures are short fibrils with lengths of 30–50 and 50–100 nm, respectively (Jarnik and Aebi, 1991), which may have a role in initial ligand interaction with the NPC.

The NPC is thought to contain a large number of polypeptides, but relatively few of its components have been

Address correspondence to Larry Gerace, The Scripps Research Institute, Department of Cell Biology, 10666 North Torrey Pines Road, La Jolla, CA 92037. Tel.: (619) 554-8514. Fax: (619) 554-6253.

1. *Abbreviations used in this paper:* AM, acetoxymethyl; BAPTA, 1,2-bis(2-aminophenoxyethane)*N,N,N',N'*-tetraacetic acid; IP₃, inositol triphosphate; NE, nuclear envelope; NLS, nuclear localization sequence; NPC, nuclear pore complex; NRK, normal rat kidney.

identified up to now (Gerace, 1992; Panté and Aebi, 1993). In vertebrates, these include a group of at least eight proteins modified with O-linked *N*-acetylglucosamine (GlcNAc) (Gerace, 1992). At least some of the O-linked glycoproteins are involved in signal-mediated transport (Gerace, 1992), although the detailed functions of these components are unclear. While most characterized NPC components are peripheral membrane proteins, two integral membrane proteins of the NPC have been identified in vertebrates. These are the N-linked glycoprotein gp210 (Gerace et al., 1982; Wozniak et al., 1989; Greber et al., 1990) and the O-linked glycoprotein POM121 (Hallberg et al., 1993). Both gp210 and POM121 appear to contain single membrane-spanning domains. Topological mapping has shown that a large (~200 kD) domain of gp210 is situated in the NE lumenal domain, and only a small tail is exposed on the extraluminal membrane surface (Greber et al., 1990). By contrast, most of the mass of POM121 is proposed to occur on the extraluminal surface of nuclear membranes (Hallberg et al., 1993). These integral membrane proteins could play a role in anchoring the NPC to nuclear membranes and/or in mediating NPC assembly. Binding of a monoclonal antibody to the lumenal domain of gp210 inhibits both signal mediated nuclear import and passive diffusion across the NPC *in vivo*, presumably caused by transmembrane effects on NPC structure (Greber and Gerace, 1992). These data indicate that gp210 is directly or indirectly connected to key functional components of the NPC.

NLS-mediated nuclear import can be experimentally dissected into two steps. A step involving ligand association with the cytoplasmic surface of the NPC can happen at low temperature and under conditions of ATP depletion, while subsequent ligand translocation through the gated channel requires ATP and physiological temperature (Newmeyer and Forbes, 1988; Richardson et al., 1988). NLS-mediated import of proteins depends on multiple cytosolic factors (Adam et al., 1990; Moore and Blobel, 1992; Newmeyer and Forbes, 1990; Sterne-Marr et al., 1992), including an NLS receptor (Adam and Gerace, 1991) and the small GTPase Ran/TC4 (Melchior et al., 1993; Moore and Blobel, 1993). Recent studies have indicated that hsc70, which has a putative role in many aspects of intracellular protein assembly and disassembly, also is involved in nuclear protein import (Shi and Thomas, 1992; Imamoto et al., 1992).

Transport of many macromolecules across the NE appears to occur constitutively, but nuclear import of certain proteins such as transcription factors and kinases can be regulated by masking/unmasking of NLSs or reversible anchoring of these proteins to cytoplasmic structures (Gerace, 1992; Whiteside and Goodbourn, 1993). Nuclear import also can be regulated by changes in the properties of the transport machinery itself, since addition of growth factors to the medium of serum-starved cells significantly enhances the functional pore diameter for mediated protein import (Feldherr and Akin, 1993). Moreover, the functional diameter of the diffusional channel may change in different cell states (Jiang and Schindler, 1990; Feldherr and Akin, 1990). Mechanisms for regulation of the nuclear transport apparatus are presently unknown.

The ER is the major calcium storage compartment in the cell and is thought to contain calcium concentrations in the millimolar range (Gill et al., 1992; Koch, 1990). Lumenal

ER calcium is important for maintenance of the structure of the ER, for protein folding and assembly, and for intracellular signalling (Sambrook, 1990; Berridge, 1993). In this study, we investigated whether depletion of lumenal ER/NE calcium stores would affect nuclear import of microinjected proteins in mammalian cells. For calcium depletion, we used both calcium-selective ionophores (Pressman and Fahim, 1982) and thapsigargin, an inhibitor of the ER/NE-resident calcium pump (Thastrup et al., 1990; Inesi and Sagara, 1992; Lanini et al., 1992). All conditions that depleted lumenal ER/NE calcium resulted in rapid and reversible inhibition of both nuclear protein import and passive diffusion across the NPC. These results indicate that the functional properties of the NPC can be regulated by components and/or conditions in the NE lumen, and they provide direct evidence that the NPC can be conformationally flexible. Our findings will be useful for achieving reversible inhibition of nuclear transport *in vivo* and thereby for studying mechanisms and functions of nucleocytoplasmic trafficking. They also will be helpful for studying the structure of the passive diffusion channel of the NPC.

Materials and Methods

Cells and Microinjections

Normal rat kidney (NRK) and HeLa cells were obtained from American Type Culture Collection (Rockville, MD). Monolayers of cells were grown in DME containing 10% FBS, 1 mM L-glutamine, 1% penicillin/streptomycin, and 1% nonessential amino acids in a humidified incubator with a 5% CO₂ atmosphere at 37°C. All cell culture reagents were purchased from GIBCO BRL (Gaithersburg, MD).

For microinjection experiments, cells were seeded on indexed glass coverslips (Bellco Glass Inc., Vineland, NJ) that had been pretreated with Cell-Tak (Collaborative Research Inc., Lexington, MA). Initially, cells were incubated for 10 min at 37°C with thapsigargin (0.5 μg/ml, GIBCO BRL) or ionophores (20 μg/ml A23187 or 5 μg/ml ionomycin; Sigma Immunochemicals, St. Louis, MO) in calcium-free Joklik's medium (GIBCO BRL) supplemented with 5% dialyzed fetal bovine serum, 1% nonessential amino acids, 15 mM HEPES/NaOH, pH 7.4, and 1.1 mM EGTA, 0.1 mM CaCl₂. Control cells were treated without inhibitors in Joklik's medium containing EGTA or 1 mM CaCl₂. Subsequently, petri dishes were chilled at 0°C for 10 min, and cells were given cytoplasmic injections of nucleoplasm-Texas red (Robbins et al., 1991; Titus et al., 1982), fixable dextran-Texas red (1 mg/ml; Molecular Probes, Inc., Eugene, OR), or HRP (5 mg/ml, 175 U/mg; Sigma), all dialyzed against microinjection buffer (Greber and Gerace, 1992). Other cells were injected with nucleoplasm-Texas red containing 10 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA)-tetra potassium salt (Molecular Probes, Inc.). The injected volume was between 1 and 10% of the cell volume. Injections were conducted at room temperature and some warming of the medium occurred. Afterwards, cells were incubated for 30 min at 37°C (after aspiration of cold medium and replacement with warm medium) for analysis of mediated nuclear import, or they were incubated for 60 min at 0°C for analysis of passive diffusion before fixation and analysis.

Quantitation of Nuclear Transport

Microinjected cells were fixed for 10 min with 3% paraformaldehyde, mounted in PBS/0.05% sodium azide/2% 1,4-diazobicyclo-[2,2,2]-octane (Harlow and Lane, 1988), and analyzed on a confocal imaging system (MRC-600; Bio Rad Laboratories, Hercules, CA) equipped with a microscope (Axiovert 10; Carl Zeiss, Inc., Thornwood, NY) and a 40× Planapochromat lens. Confocal settings were 0.3 mW laser power, Kalman filters, 1 s per frame, eight frames per image. The photomultiplier gain was set so that none of the pixels reached the saturation levels of 256 units. The confocal aperture was adjusted to 5, yielding optical slices of ~1 μm thickness. Images of an area of 200 × 280 μm² and a raster size of 512 × 756 pixels were taken through the centers of the cells, collected through the rhodamine or fluorescein channels. The average pixel values of the nuclear

and cytoplasmic areas were determined using the Bio Rad area software. Photographs from injected cells were taken on TMAX 400 film (Eastman Kodak Co., Rochester, NY) using an axiophot microscope (Carl Zeiss, Inc.) equipped with a 40× Planapochromat lens. The intracellular localization of microinjected HRP was qualitatively determined by cytochemistry using diaminobenzidine as a substrate (Doxsey et al., 1985).

Calcium and ATP Determinations

NRK or HeLa cells were loaded with the calcium dye fluo-3 acetoxymethyl (AM) ester (Molecular Probes, Inc.) by incubation for 40 min at 37°C in a solution containing 4 µg/ml fluo-3, 10 mM Hepes/NaOH, pH 7.4, 25 mM glucose, 137 mM NaCl, 5.3 mM KCl, 3 mM CaCl₂, 1 mM MgCl₂, and pluronic acid as a wetting agent (van den Pol et al., 1992). Cells were washed twice in this solution lacking fluo-3 and twice in cold Joklik's medium containing 1.1 mM EGTA/0.1 mM CaCl₂/5% fetal bovine serum. The coverslip was mounted onto a perfusion chamber on top of an inverted confocal laser scanning microscope with an argon laser and a fluorescein filter set (Bio Rad). Cells were stimulated with warm Joklik's medium containing 0.5 µg/ml thapsigargin, and real time video images were recorded as described (van den Pol et al., 1992). About 10–20 s after the addition of thapsigargin, ~30% of the cells showed an intense fluorescein signal for a short period (1–2 s). About 30 s after stimulation, ~20% of the cells were transiently fluorescent, and after 1 min, ~5% of the cells were stained. Once stimulated, the cells quickly returned to a nonfluorescent state. This was in agreement with calcium measurements using fura-2 as a calcium indicator in thapsigargin-stimulated smooth muscle cells (Gill et al., 1992).

ATP levels in whole cells treated with calcium inhibitors were determined with a bioluminescence assay based on luciferin and luciferase (Boehringer Mannheim Biochemicals, Indianapolis, IN). NRK cells were grown in 60-mm dishes to ~60% confluency, treated with thapsigargin (1 µg/ml), A23187 (20 µg/ml), or ionomycin (5 µg/ml) in Joklik's EGTA/calcium/fetal bovine serum medium for 30 min at 37°C. Control cells were kept in Joklik's/EGTA medium or in Joklik's/1 mM calcium medium. Cells were washed in cold PBS and detached from the dish by incubation with 0.25% trypsin in PBS/EDTA (GIBCO BRL) for 20 min on ice. Trypsin was neutralized with cold growth medium, and cells were collected by centrifugation and washed in PBS. ATP was extracted from the cell pellet with 20 µl 10% trichloroacetic acid for 45 min on ice. The sample was neutralized with 15 µl of 1 M Tris/HCl, pH 9.5, clarified by centrifugation at 15,000 rpm for 15 min in a table-top centrifuge, and analyzed for ATP in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA). 2×10^5 cell equivalents yielded between 3,450 and 3,700 photons per second, corresponding to 1.19–1.33 pmol of ATP.

Results

Nuclear Protein Import Is Rapidly Inhibited by Depletion of Intracellular Stores of Calcium

We investigated whether altering calcium levels within internal cellular stores affected NLS-mediated nuclear protein import in vivo in NRK cells. In most experiments, we measured nuclear import using the *Xenopus* oocyte nuclear protein nucleoplasmin, which contains a well-defined bipartite NLS (Robbins et al., 1991). Cells were preincubated in the presence of reagents affecting calcium stores for 10 min at 37°C and chilled to 0°C. Subsequently, nucleoplasmin labeled with the chromophore Texas red was microinjected into the cytoplasm, and cells were shifted to warmed preincubation medium and incubated further at 37°C for 30 min. After this incubation, the intracellular distribution of fluorescent nucleoplasmin was quantitatively determined by confocal laser microscopy in sections across the cell centers. The average pixel values across the nuclear area divided by the average pixel intensity present over the cytoplasm (N/C) was taken as a measure for nuclear import.

In control experiments, we measured nuclear import in cells that were incubated in "low calcium medium" containing a calcium/EGTA buffer that yields ~100 nM free cal-

cium. This value is ~10,000 times lower than the normal extracellular calcium in growth medium and approximately corresponds to the calcium level in the cytoplasm of resting cells (Minta et al., 1989). Low calcium in the extracellular medium had essentially no effect on the nuclear transport of nucleoplasmin (Figs. 1 and 2). After 30 min of incubation, the nuclear/cytoplasmic concentration ratio of the injected protein was ~4.3 (Fig. 2). In the presence of normal calcium-containing medium, a similar accumulation of nucleoplasmin was obtained, yielding a transport index of 4.7. The amount of transport did not increase when the cells were incubated for another 30 min at 37°C with or without extracellular calcium (data not shown), suggesting that most import-competent nucleoplasmin had accumulated in nuclei at this point. Similar results were obtained with HeLa cells (data not shown) and with nucleoplasmin conjugated to colloidal gold (Greber and Gerace, 1992). When the cells were kept on ice after the microinjection, the nuclear/cytoplasmic ratio was 0.22, indicating that low temperature inhibits nuclear import of the nucleoplasmin-Texas red preparation, similar to previous results on NLS-mediated nuclear import (Richardson et al., 1988). The low level of transport in the "0°C" sample is probably caused by a modest warming of the medium during the injection procedure.

To examine effects of depletion of intracellular calcium stores on nuclear protein import, cells were treated with the calcium ionophores A23187 or ionomycin in low calcium medium. A23187 and ionomycin are mobile carriers that shuttle calcium across biological and synthetic membranes and equilibrate existing calcium gradients (Liu and Herman, 1978; Pressman and Fahin, 1982). Ionomycin is more specific for divalent ions than A23187, but both antibiotics are highly selective for calcium and much less selective for other divalent cations present in cells (Kauffman et al., 1980). Treatment with A23187 or ionomycin substantially inhibited nuclear import of the microinjected nucleoplasmin, resulting in nuclear/cytoplasmic ratios of 0.28 and 0.17, respectively, at 30 min (Figs. 1 and 2). Similar results were obtained with HeLa cells (not shown). We confirmed that the luminal stores were largely depleted of releasable calcium upon incubation of cells with A23187 in low calcium medium, using calcium dye experiments with NRK cells loaded with fluo-3 (van den Pol et al., 1992). After treatment with A23187 and subsequent challenge with thapsigargin (see below), these cells did not display any calcium increase in the cytosol reflected by fluo-3 fluorescence (data not shown; see Materials and Methods).

It was possible that nuclear import was inhibited because of side effects from the ionophores, such as a low affinity for magnesium or a change of intracellular pH (Asem et al., 1992). To control for these possibilities, we used a second method to deplete calcium from the ER lumen, involving treatment of cells with thapsigargin in the presence of low extracellular calcium. Thapsigargin is an inhibitor of an ATP-dependent calcium pump in the endoplasmic reticulum (Thastrup et al., 1990) and NE (Lanini et al., 1992). Thapsigargin-treated cells become rapidly depleted of calcium in the ER lumen because of constitutive release of calcium from these stores combined with a block in reuptake into the ER (Thastrup et al., 1990). Under these conditions, free cytosolic calcium levels are maintained at low levels because calcium is ejected by plasma membrane pumps into

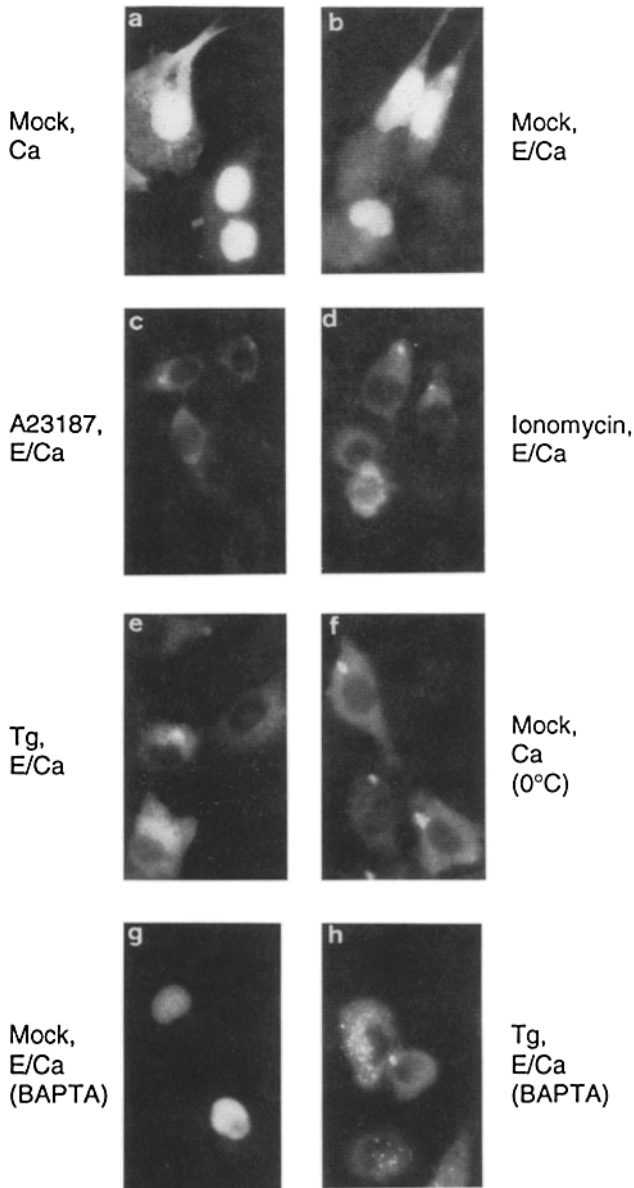


Figure 1. Inhibition of nuclear import of nucleoplasmin by intracellular calcium depletion. Nucleoplasmin-Texas red was injected into the cytoplasm of NRK cells that had been preincubated in various experimental media for 10 min at 37°C. Cells were subsequently incubated for 30 min at 37°C in the various media, and the nuclear and cytoplasmic fluorescence was examined by fluorescence microscopy after cells were fixed with formaldehyde (see Materials and Methods). Control cells were incubated in the absence of drugs (*Mock*) in normal calcium-containing medium (*a*, *Ca*) or in EGTA-buffered low calcium medium (*b*, *E/Ca*). Other samples were incubated in EGTA-buffered low calcium medium in the presence of A23187 (*c*), ionomycin (*d*), or thapsigargin (*e*, *Tg*). Some mock-treated cells were also kept on ice for 30 min after the injection of nucleoplasmin (*f*). To buffer cytoplasmic calcium, some samples of mock-treated or thapsigargin-treated cells were injected with 10 mM BAPTA together with the nucleoplasmin-Texas red (*g* and *h*, respectively).

the low calcium medium. In the presence of thapsigargin, nuclear import was also inhibited, and the nuclear/cytoplasmic ratio of injected nucleoplasmin was ~ 0.3 (Figs. 1 and 2).

To test if the inhibition of nucleoplasmin import by thap-

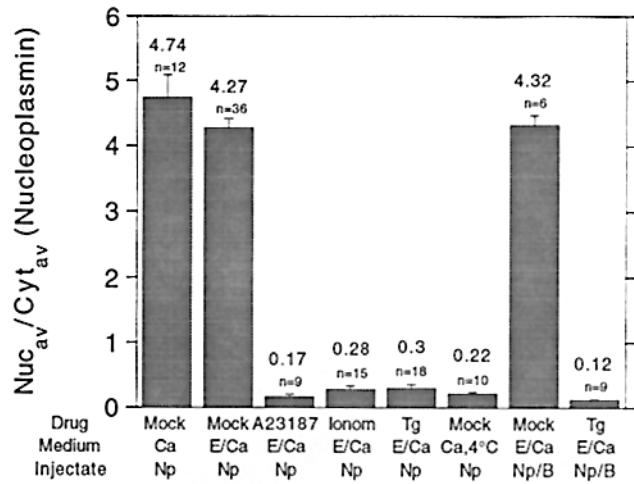


Figure 2. Quantitation of nuclear import inhibition induced by intracellular calcium depletion. Cells were preincubated with calcium ionophores A23187, ionomycin (*ionom*), or with the ATPase inhibitor thapsigargin (*Tg*) in low calcium medium buffered with EGTA (*E/Ca*), or they were treated without inhibitors (*Mock*) in either normal calcium containing medium (*Ca*) or in low calcium medium (*E/Ca*). Nucleoplasmin-Texas red was then injected into the cytoplasm, in some cases with 10 mM calcium buffer BAPTA (*Np/B*), and subsequently incubated at 37°C for 30 min or at 4°C for 30 min as described in Fig. 1. Nuclear transport of nucleoplasmin was quantitated by confocal microscopy, and the results expressed as the mean ratio of nuclear/cytoplasmic average pixel intensity for each condition based on the indicated number of cells (*n*), including the standard deviation (see Materials and Methods).

sigargin could be caused by long-term calcium fluxes in the cytoplasm, the membrane-impermeable calcium chelator BAPTA was microinjected into the cytoplasm together with the transport substrate after a brief thapsigargin pretreatment. BAPTA acts as a calcium buffer that maintains free calcium concentrations near the physiological resting level of 100 nM (Minta et al., 1989). The intracellular concentration of BAPTA achieved in this experiment (0.1–1 mM; see Materials and Methods) is sufficient to block ionophore-induced increase of cytosolic calcium in *Xenopus* oocytes maintained in calcium-free medium (Capco and Bement, 1991). No inhibition of nuclear import was observed when control (non-drug-treated) cells were injected with BAPTA and nucleoplasmin (Figs. 1 and 2). After 30 min at 37°C, the nuclear/cytoplasmic ratio was 4.3, similar to control injections in the absence of drugs (see Figs. 1 and 2). However, when thapsigargin-pretreated cells were injected with BAPTA and nucleoplasmin, nucleoplasmin import was effectively blocked (transport index of 0.12; Figs. 1 and 2). This suggests that transport inhibition by thapsigargin cannot be explained by long-term cytoplasmic calcium fluxes. Because of the difficulty of carrying out two sequential injections of cultured cells in a short time interval, we did not analyze nucleoplasmin import in cells preinjected with BAPTA.

Different findings were obtained when cells were preincubated in medium containing the membrane-permeable reagent BAPTA-AM at a concentration of 10 μ M. BAPTA-AM is cleaved to the membrane-impermeable BAPTA after it enters intracellular compartments and the cytosol (Minta et al., 1989), and consequently strongly reduces the free cal-

cium concentration in the ER and other intracellular stores (Preston and Berlin, 1992). When BAPTA-AM-loaded cells were analyzed for ability to support nuclear import, transport of nucleoplasmin was inhibited to the same degree as cells treated with ionophores or thapsigargin (data not shown). Collectively, these data indicated that the inhibition of nucleoplasmin import by the calcium ionophores and thapsigargin was likely to be caused by depletion of intracellular calcium stores, rather than by something else. Because of the specificity of thapsigargin for the ER calcium pump, the calcium stores involved in this effect are likely to be located in the nuclear envelope and the endoplasmic reticulum.

We examined whether inhibition of nuclear import by ionophores or thapsigargin was specific for nucleoplasmin or was seen for other NLS-containing nuclear import ligands as well. A second ligand we analyzed was fluorescein-conjugated bovine serum albumin coupled with synthetic peptides comprising the NLS of the SV-40 T antigen. This synthetic transport ligand, which has been well-characterized in previous studies (e.g., Michaud and Goldfarb, 1992), was efficiently transported into the nuclei of control NRK cells (Fig. 3).

However, when the NLS-BSA was injected into thapsigargin-treated cells, almost complete inhibition of nuclear import was obtained (Fig. 3). Interestingly, the fluorescent transport ligand accumulated at the NE in a rim-like pattern. This pattern is reminiscent of ligand binding to the NPC achieved with highly multivalent NLS-containing ligands (Newmeyer and Forbes, 1988; Richardson et al., 1988), but further analysis is required to determine whether this reflects accumulation at an intermediate stage of transport. A similar inhibition of nuclear import upon treatment of cells with ionophores or thapsigargin was obtained when we analyzed microinjected protein A (data not shown), a bacterial protein that fortuitously contains a functional NLS (Li and Thomas, 1989). Thus, we conclude that inhibition of mediated nu-

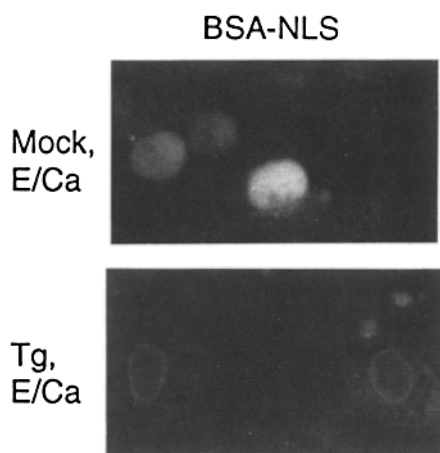


Figure 3. Inhibition of nuclear import of BSA-NLS in thapsigargin-treated cells. NRK cells were preincubated with thapsigargin (Tg) in EGTA-buffered low calcium medium (E/Ca) or without drug (Mock) and injected with fluorescein-labeled bovine serum albumin conjugated to the SV40 large T nuclear localization sequence peptide (BSA-NLS). Cells were then incubated for 30 min at 37°C, fixed in formaldehyde and analyzed by fluorescence microscopy.

clear import by treatment of cells with ionophores or thapsigargin is seen for diverse NLS-containing ligands, and is not restricted to nucleoplasmin.

The Calcium Depletion-induced Import Block is Readily Reversible

It was conceivable that inhibition of nuclear import by calcium ionophores or thapsigargin was caused by the degradation or precipitation of injected transport ligand. To address this possibility, we examined the reversibility of transport inhibition after drug treatment. Nucleoplasmin was injected into the cytoplasm of cells treated with A23187 or thapsigargin, and cells were incubated for 20 min at 37°C in the presence of the drugs. The drugs were then removed and the cells were further incubated with calcium-containing medium for another 20 min at 37°C. As demonstrated in Fig. 4, nuclear transport was effectively blocked at the end of the first incubation in the presence of the inhibitors (nuclear/cytoplasmic ratio of 0.24 and 0.22, respectively), but was almost completely restored when the inhibitors were washed out and calcium was resupplied. Transport in the A23187- and thapsigargin-treated cells achieved nuclear/cytoplasmic ratios of 4.2 and 2.4, respectively (Fig. 4). Similar results as with A23187 were obtained in ionomycin washout experiments (data not shown). These results indicated that the ionophore-blocked nucleoplasmin remained fully transport-competent after the drug treatment, and was neither degraded nor precipitated. The thapsigargin arrest was only partially reversed in the time window we have examined, presumably because of tight or irreversible drug binding to the calcium ATPase (Sagara et al., 1992).

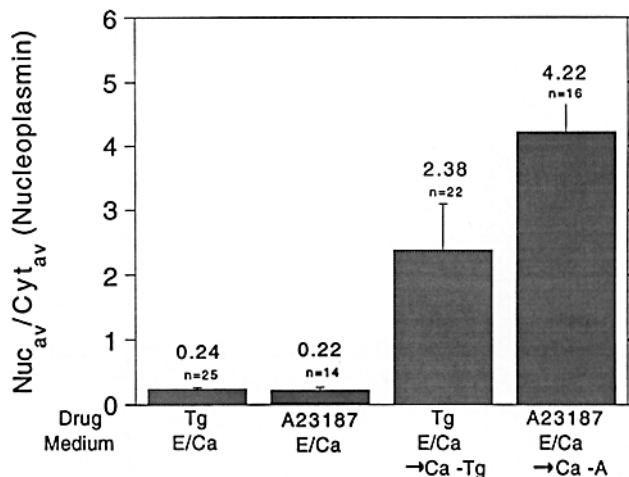


Figure 4. Reversibility of the block in mediated nuclear import caused by intracellular calcium depletion. NRK cells pretreated with thapsigargin (Tg) or A23187 in EGTA-buffered low calcium medium (E/Ca) were microinjected in the cytoplasm with nucleoplasmin-Texas red and incubated at 37°C for 20 min. In one set of dishes, the inhibitors were washed out and the cells were incubated for another 20 min at 37°C in inhibitor containing medium (Tg, E/Ca and A23187, E/Ca) or in inhibitor-free medium (-Ca -Tg and -Ca -A), and nuclear transport of nucleoplasmin was analyzed as described in Fig. 2. The average transport index with the standard deviation is indicated for each condition based on the number of cells analyzed (n).

It was possible that inhibition of nucleoplasmin accumulation by calcium ionophores and thapsigargin was caused by hypothetical side effects of the drugs resulting in disruption of the NE. To evaluate this possibility, we injected nucleoplasmin directly into the nucleus of A23187- or thapsigargin-treated cells. The injected protein remained nuclear for ≥ 30 min after injection (data not shown), suggesting that the NE remains intact in the presence of these drugs. The integrity of the NE is further demonstrated by microinjection of dextran into the cytoplasm of drug-treated cells (see below).

To investigate whether depletion of intracellular calcium resulted in loss of NPCs from the NE or in large-scale altera-

tion in NPC structure, we analyzed the ionophore- or thapsigargin-treated cells by light and electron microscopy (Fig. 5). Immunofluorescence staining with the antibody RL1, which recognizes O-linked glycoproteins of the NPC with a role in nuclear import (Snow et al., 1987; Sterne-Marr et al., 1992), indicated that RL1 antigens were not detectably released from the nuclear envelope by these inhibitors or by treatment of cells with low calcium medium (Fig. 5). Furthermore, thin section electron microscopy of fixed cells showed that the pore complexes were present at roughly normal levels after thapsigargin treatment, and they were not detectably disrupted at this level of structural resolution (Fig. 5).

Since signal-mediated nuclear import is dependent on ATP *in vivo* and *in vitro* (Newmeyer and Forbes, 1988; Breuwer and Goldfarb, 1990), it was possible that ionophores and thapsigargin inhibited this process by altering ATP levels. Therefore, we examined the overall cellular ATP levels in cells treated with calcium ionophores or thapsigargin. After 30 min of incubation with the inhibitors at 37°C in low calcium medium, the cellular ATP levels were indistinguish-

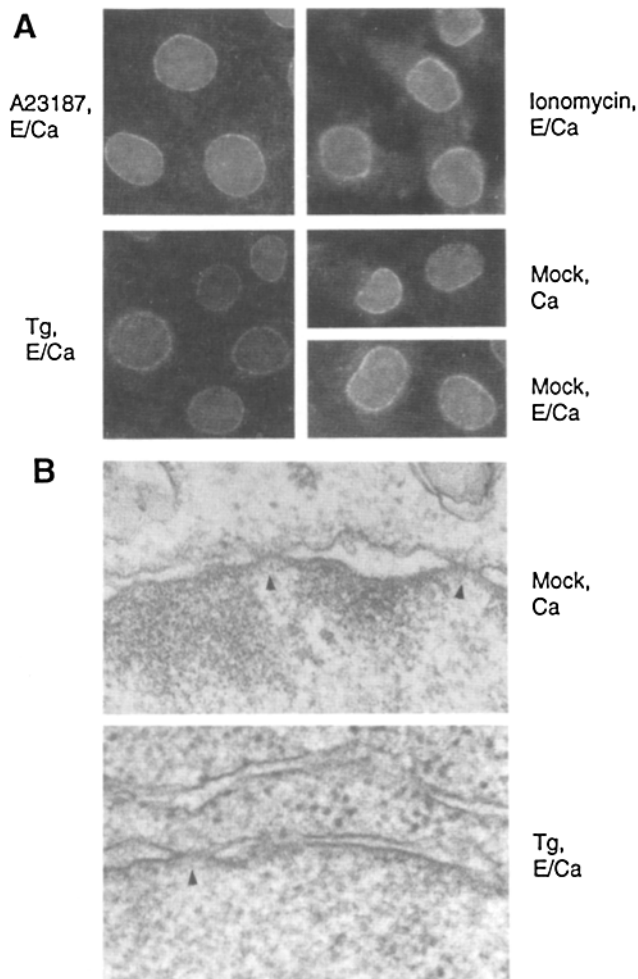


Figure 5. Indirect immunofluorescence labeling of nuclear pore complex proteins in calcium-depleted cells. (A) NRK cells were pretreated with ionophores (A23187 or ionomycin) or with the calcium pump inhibitor thapsigargin (*Tg*) in EGTA-buffered low calcium medium (*E/Ca*) as described in Fig. 1. Cells treated without inhibitors (*Mock*) were incubated in calcium containing medium (*Ca*) or in EGTA-buffered low calcium medium (*E/Ca*). Cells were stained for O-linked glycoproteins of the nuclear pore complex with the monoclonal antibody RL1 (Snow et al., 1987). (B) HeLa cells were incubated without inhibitor (*upper panel*, *Mock*) or with thapsigargin (*lower panel*, *Tg*) in low calcium medium for 60 min, fixed in glutaraldehyde, and processed for thin-section EM by conventional procedures (Griffiths, 1993). Shown are transverse views of the NE. In the *Tg* panel, the NE is the lower of the two membrane cisternae, and an NPC is indicated by an arrowhead.

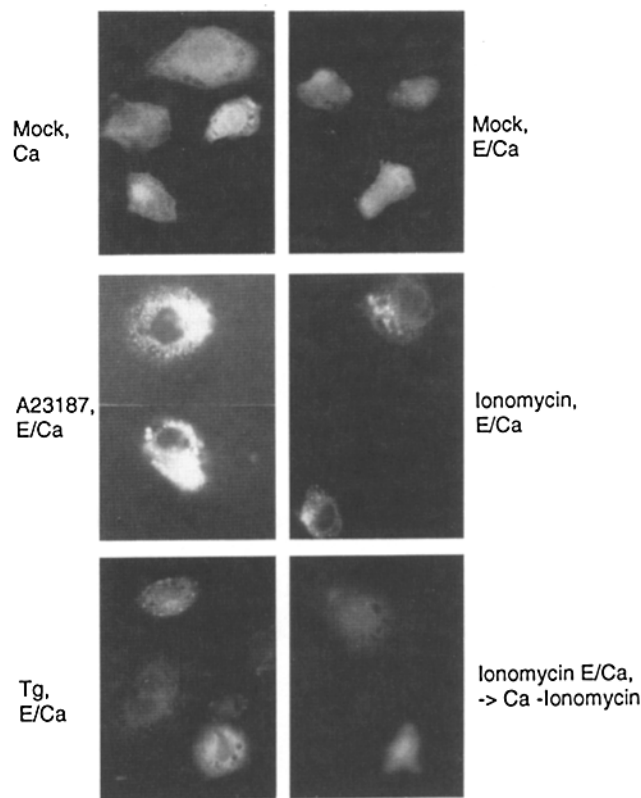


Figure 6. Inhibition of passive diffusion of 10-kD dextran into the nucleus of calcium-depleted cells. Texas red-labeled 10-kD dextran was injected into the cytoplasm of NRK cells that had been preincubated in EGTA-buffered low calcium medium (*E/Ca*) or calcium-containing medium (*Ca*), without (*Mock*) or with thapsigargin (*Tg*), ionomycin, or A23187. Cells were further incubated for 60 min at 0°C, fixed, and analyzed by fluorescence microscopy. The bottom panel in the right column shows ionomycin-treated cells that were injected with dextran, incubated for 30 min at 0°C, returned to drug-free calcium-containing medium for 10 min at 37°C, and finally incubated for an additional 30 min on ice before analysis.

able from the levels in control cells kept in calcium-containing medium without inhibitors (see Materials and Methods). The results indicated that the ionophores and thapsigargin had no general deleterious effects on the overall cellular energy levels under our experimental conditions.

Passive Diffusion into the Nucleus Is Also Reversibly Inhibited by Depletion of Intracellular Calcium Stores

We next tested whether the depletion of intracellular calcium stores affected passive diffusion across the NE in addition to signal-mediated nuclear import. For this experiment, we microinjected a small fluorescent dextran (10 kD) into the cytoplasm of ionophore- or thapsigargin-treated NRK cells to monitor diffusional permeability of the pore complex. After 60 min incubation at 0°C, cells were fixed and qualitatively examined for dextran localization by light microscopy (Fig. 6) or quantitatively analyzed by confocal microscopy (Fig. 7). Under conditions of calcium depletion by thapsigargin, dextran was largely excluded from the nucleus and confined to the cytoplasm, while in untreated cells, it readily entered the nucleus (Fig. 6). The nuclear/cytoplasmic ratio of the injected dextran in cells treated with A23187, ionomycin, or thapsigargin was 0.31, 0.33, and 0.36, respectively (Fig. 7). By contrast, the nuclear/cytoplasmic ratio of dextran in control cells was 2.3. The apparent accumulation of dextrans in the nuclei of untreated cells was presumably caused by cytoplasmic exclusion, i.e., a lower available water space in the cytoplasm compared to the nucleoplasm (Paine, 1992). Similar results were obtained with another diffusional probe, the NLS-lacking 40-KD protein HRP

(Greber and Gerace, 1992), which also was excluded from nuclei of drug-treated cells (data not shown).

When microinjected drug-treated cells were incubated for 10 min at 37°C in inhibitor-free, calcium-containing medium followed by a 30-min incubation at 0°C (Fig. 7), the inhibition of dextran diffusion by A23187 and ionomycin was almost completely reversed (nuclear/cytoplasmic ratios of 2.16 and 2.2, respectively), while inhibition of dextran diffusion by thapsigargin was partially reversed (with a nuclear/cytoplasmic index of 1.35). However, the blocks remained effective when the inhibitors were washed out and cells were maintained at 0°C (data not shown). This suggested that reversal of the defect induced by calcium depletion required re-acquisition of luminal ER calcium and/or some other metabolic process(es).

Discussion

Our data show that treatment of cultured cells with calcium-selective ionophores or the calcium pump inhibitor thapsigargin results in rapid inhibition of both NLS-mediated nuclear protein import and passive diffusion across the NE. Mediated nuclear import in thapsigargin-treated cells is likely to be inhibited by depletion of intracellular calcium stores rather than by long-term cytoplasmic calcium fluxes, since injection of the membrane impermeant BAPTA into cells to buffer long-term changes in cytoplasmic calcium did not protect cells from transport inhibition. The calcium stores associated with inhibition of mediated transport and diffusion probably occur in the lumen of the ER and NE, in view of the selectivity of thapsigargin for an ER/NE calcium pump (Inesi and Sagara, 1992; Lanini et al., 1992). Calcium depletion from the ER lumen by ionomycin or thapsigargin rapidly inhibits protein translation (Preston and Berlin, 1992; Price et al., 1992). However, the inhibition of nuclear import observed in our studies is not due to an effect on translation, since inhibition of translation with cycloheximide in cultured cells has no influence on NLS-mediated nuclear import (Martin and Helenius, 1991; Greber, U., unpublished observations).

Inhibition of NLS-mediated nuclear import was reversed completely (for ionophores) or partially (for thapsigargin) by incubation of pretreated cells in drug-free, calcium-containing medium at 37°C. Since it was possible to measure passive diffusion at 0°C, we could assess the temperature dependence for reversal of this block. We found that recovery of the capacity for passive diffusion required incubation of cells at 37°C in drug-free medium, and did not occur if cells were maintained at 0°C. This is compatible with the notion that reversal of transport inhibition (at least for passive diffusion) requires replenishment of ER luminal calcium stores.

During certain types of signal transduction, calcium is transiently released from the ER via the inositol trisphosphate (IP₃) receptor channel (Irvine, 1992; Berridge, 1993). Whether depletion of the IP₃-sensitive calcium stores would affect transport across the NE is unclear, since the IP₃-sensitive pool is only a subset of the thapsigargin-released pool (Gill et al., 1992). Even if depletion of the IP₃-sensitive calcium stores were to influence nuclear transport, it is likely that these effects would be transient, since IP₃-released stores are rapidly replenished by extracellular calcium (Randriamampita and Tsien, 1993).

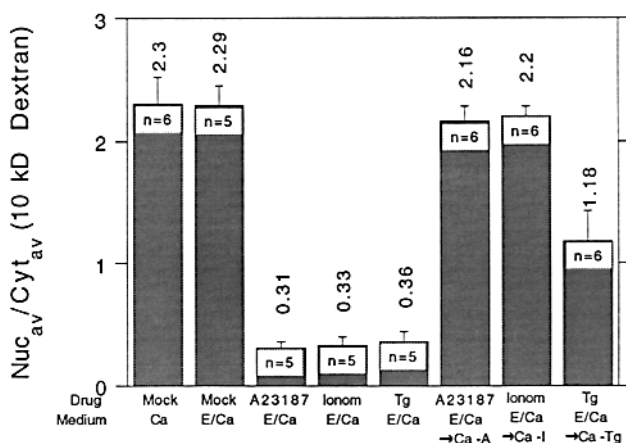


Figure 7. Quantitation of diffusion of 10-kD dextran into the nucleus of calcium-depleted cells. Texas red-labeled 10 kD dextran was injected into the cytoplasm of NRK cells treated with calcium ionophores A23187 or ionomycin (*ionom*) or with the calcium pump inhibitor thapsigargin (*Tg*) in EGTA-buffered low calcium medium (*E/Ca*) or calcium-containing medium (*Ca*). Cells were incubated for 60 min on ice, fixed with paraformaldehyde, and analyzed for nuclear and cytoplasmic dextran by quantitative confocal microscopy. Alternatively, after dextran injection and a 30-min incubation on ice, drug-containing medium was replaced with drug-free calcium-containing medium, and cells were incubated for 10 min at 37°C followed by 30 min on ice before analysis by microscopy. Results are expressed as the mean ratio of nuclear/cytoplasmic average pixel intensities for each condition based on the indicated number of cells (*n*), including the standard deviation.

We have been unsuccessful in obtaining inhibition of NLS-mediated nuclear import *in vitro* by adding calcium ionophores or thapsigargin (Paschal, B., and L. Gerace, unpublished observations) to permeabilized cell systems in which the plasma membrane is perforated by digitonin treatment (Adam et al., 1990) or mechanical disruption (Smythe et al., 1992). The reason for the ineffectiveness of the drugs in inhibiting *in vitro* nuclear import is unclear, but could reflect changes in the ionic composition of the ER lumen generated by cell permeabilization, or disruption of a more subtle regulatory feature of cells. However, this disparity cannot be ascribed solely to a reduction or loss of ongoing protein synthesis in the *in vitro* systems, since pretreatment of cells with cycloheximide to inhibit protein synthesis did not protect cells from inhibition of nuclear import by thapsigargin or calcium ionophores *in vivo* (Greber, U., unpublished observations).

What components of the nuclear transport machinery are affected by depletion of luminal ER calcium *in vivo*? Changes in the structure of NP diffusional channels are most likely responsible for inhibition of passive diffusion. The dramatic reversible effects obtained by calcium depletion suggest that the diffusional channels of the NPC are "flexible" structures whose diameter may be modulated in different physiological states, compatible with previous studies (Jiang and Schindler, 1990; Feldherr and Akin, 1990). Structural studies on isolated amphibian NEs also support the possibility that NPCs are conformationally flexible (Akey and Radermacher, 1993). Alterations in either the structure of the NPC or the activity of cytoplasmic transport components could be responsible for inhibition of NLS-mediated nuclear import. However, nuclear transport inhibition cannot be ascribed to wholesale loss of NPCs from the NE or decreases in total cellular ATP pools, since we could detect no changes in these parameters after drug treatment.

A number of possible mechanisms could explain how the nuclear transport apparatus, located on the nuclear/cytoplasmic surfaces of nuclear membranes, could be affected by calcium depletion in the ER lumen. One mechanism proposes that the luminal domain(s) of certain transmembrane proteins of the NPC such as gp210 directly bind calcium, and that loss of luminal ER calcium causes a change in their structure. A conformational change could be transmitted from the luminal to the extraluminal domain of the NPC by membrane-spanning segments of NPC proteins. A precedent for transmembrane alteration of NPC structure was established in previous work, showing that binding of a monoclonal antibody to the luminal domain of gp210 inhibits both mediated nuclear import and passive diffusion (Greber and Gerace, 1992). Our inspection of the published sequence of gp210 (Wozniak et al., 1989) reveals five amino acid stretches containing six appropriately spaced calcium-coordinating amino acids resembling the calcium-coordinating loop region of the EF hand calcium-binding motif (Kretsinger, 1976; Strynadka and James, 1989). These are located at residues 160-171, 424-435, 580-591, 1413-1424, and 1610-1621 of gp210. However, binding of calcium to gp210 has not yet been tested.

A second mechanism proposes that loss of luminal ER calcium disrupts interactions of the luminal domain of the NPC with other ER luminal proteins that are required for NPC function. These hypothetical luminal factors could in-

clude molecular chaperones such as BiP. The function of BiP (and possibly other ER luminal proteins) may require calcium, as suggested by studies showing that BiP binding to unassembled T cell receptor subunits is lost upon calcium depletion (Suzuki et al., 1991).

A third mechanism proposes that downregulation of nuclear transport upon calcium depletion is part of the stress response caused by the accumulation of unfolded proteins in the ER lumen that is known to be caused by ER calcium depletion (Wileman et al., 1991; Lodish et al., 1992). This stress response appears to activate a transmembrane signaling pathway that in part involves a membrane-spanning ER protein with a cytoplasmically disposed kinase domain (Cox et al., 1993; Mori et al., 1993). The signaling pathway leads to upregulation of the synthesis of certain luminal ER chaperones such as GRP94 and BiP (Watowich and Morimoto, 1988; Price et al., 1992), and also could affect cytoplasmic components of the nuclear transport machinery.

Consistent with the possibility that a stress response caused by aberrant conditions in the ER lumen could lead to inhibition of nuclear transport, we have found that treatment of cells for 4 h with tunicamycin results in substantial inhibition of nucleoplasmic import into the nucleus (Greber, U., and L. Gerace, unpublished observation). Tunicamycin is an inhibitor of N-glycosylation of luminal ER proteins that leads to accumulation of unfolded proteins in the ER (Doms et al., 1993). However, we cannot rule out that this effect derives from deficient folding of newly synthesized integral membrane proteins of the NPC rather than a stress response.

It is a plausible notion that nucleocytoplasmic trafficking could be downregulated in response to ER stress conditions and possibly to more general cellular stress conditions (e.g., heat shock). It could be advantageous for stressed cells to diminish rates of nucleocytoplasmic trafficking to spatially restrict damage and facilitate the recovery process. Nuclear import of cytoplasmic stress proteins and nuclear export of stress-related mRNAs clearly occurs after induction of cellular stress responses such as the heat shock response (Morimoto, 1993), but it is not evident whether this transport involves conventional kinetics, signals, and pathways. To our knowledge, systematic analysis of NLS-mediated nuclear import in stressed cells has not been carried out.

In conclusion, the methods described in this report will provide useful tools to achieve reversible inhibition of nucleocytoplasmic trafficking *in vivo*. This will facilitate further analysis of mechanisms of nuclear transport and its role in cell function. Moreover, our work suggests that the passive diffusion channel(s) of the NPC has the potential for being a conformationally dynamic entity, and it provides a basis for studying its different structural states.

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References

- Adam, S. A., and L. Gerace. 1991. Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. *Cell*. 66:837-847.
- Adam, S. A., R. Sterne Marr, and L. Gerace. 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* 111:807-816.
- Akey, C. W., and M. Radermacher. 1993. Architecture of the *Xenopus* nuclear pore complex revealed by three-dimensional cryoelectron microscopy. *J. Cell Biol.* 122:1-19.
- Asem, E. K., M. Li, and B. K. Tsang. 1992. Calcium ionophores increase intracellular pH in chicken granulosa cells. *J. Mol. Endocrinol.* 9:1-6.
- Berridge, M. J. 1993. Inositol trisphosphate and calcium signalling. *Nature (Lond.)*. 361:315-325.
- Breeuwer, M., and D. S. Goldfarb. 1990. Facilitated nuclear transport of histone H1 and other small nucleophilic proteins. *Cell*. 60:999-1008.
- Capco, D. G., and W. M. Bement. 1991. Analysis of cellular signaling events, the cytoskeleton, and spatial organization of macromolecules during early *Xenopus* development. *Methods Cell Biol.* 36:249-70.
- Cox, J. S., C. E. Shamu, and P. Walter. 1993. Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. *Cell*. 73:1197-1206.
- Dingwall, C., and R. Laskey. 1992. The nuclear membrane. *Science (Wash. DC)*. 258:942-947.
- Dingwall, C., S. V. Sharnick, and R. A. Laskey. 1982. A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. *Cell*. 30:449-458.
- Doms, R. W., R. A. Lamb, J. K. Rose, and A. Helenius. 1993. Folding and assembly of viral membrane proteins. *Virology*. 193:545-562.
- Doxsey, S. J., J. Sambrook, A. Helenius, and J. White. 1985. An efficient method for introducing macromolecules into living cells. *J. Cell Biol.* 101:19-27.
- Feldherr, C. M., and D. Akin. 1990. The permeability of the nuclear envelope in dividing and nondividing cell cultures. *J. Cell Biol.* 111:1-8.
- Feldherr, C. M., and D. Akin. 1993. Regulation of nuclear transport in proliferating and quiescent cells. *Exp. Cell Res.* 205:179-186.
- Forbes, D. J. 1992. Structure and function of the nuclear pore complex. *Annu. Rev. Cell Biol.* 8:495-527.
- Gerace, L., Y. Ottaviano, and K. C. Kondor. 1982. Identification of a major polypeptide of the nuclear pore complex. *J. Cell Biol.* 95:826-837.
- Gerace, L. 1992. Molecular trafficking across the nuclear pore complex. *Curr. Opin. Cell Biol.* 4:637-645.
- Gill, D. L., T. K. Ghosh, J. Bian, A. D. Short, R. T. Waldron, and S. L. Rybak. 1992. Function and organization of the inositol 1,4,5-trisphosphate-sensitive calcium pool. *Adv. Second Messenger Phosphoprotein Res.* 26:265-308.
- Greber, U. F., A. Senior, and L. Gerace. 1990. A major glycoprotein of the nuclear pore complex is a membrane-spanning polypeptide with a large luminal domain and a small cytoplasmic tail. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1495-1502.
- Greber, U. F., and L. Gerace. 1992. Nuclear protein import is inhibited by an antibody to a luminal epitope of a nuclear pore complex glycoprotein. *J. Cell Biol.* 116:15-30.
- Griffiths, G. 1993. Fine Structure Immunocytochemistry. Springer Verlag, Heidelberg, Germany.
- Hallberg, E., R. W. Wozniak, and G. Blobel. 1993. An integral membrane protein of the pore membrane domain of the nuclear envelope contains a nucleoporin-like region. *J. Cell Biol.* 122:513-521.
- Harlow, E., and D. Lane. 1988. Antibodies, A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Hinshaw, J. E., B. O. Carragher, and R. A. Milligan. 1992. Architecture and design of the nuclear pore complex. *Cell*. 69:1133-1141.
- Imamoto, N., Y. Matsuoka, T. Kuribara, K. Kohno, M. Miyagi, F. Sakiyama, Y. Okada, S. Tsunasawa, and Y. Yoneda. 1992. Antibodies against 70-kD heat shock cognate protein inhibit mediated nuclear import of karyophilic proteins. *J. Cell Biol.* 119:1047-1061.
- Inesi, G., and Y. Sagara. 1992. Thapsigargin, a high affinity and global inhibitor of intracellular Ca²⁺ transport ATPases. *Arch. Biochem. Biophys.* 298:313-317.
- Irvine, R. F. 1992. Inositol lipids in cell signalling. *Curr. Opin. Cell Biol.* 4:212-219.
- Jarnik, M., and U. Aebi. 1991. Toward a more complete 3-D structure of the nuclear pore complex. *J. Struct. Biol.* 107:291-308.
- Jiang, L. W., and M. Schindler. 1990. Nucleocytoplasmic transport is enhanced concomitant with nuclear accumulation of EGF binding activity in both 3T3-1 and E6F receptor reconstituted NR-6 fibroblasts. *J. Cell Biol.* 110:559-568.
- Kauffman, R. F., R. W. Taylor, and D. R. Pfeiffer. 1980. Cation transport and specificity of ionomycin. Comparison with ionophore A23187 in rat liver mitochondria. *J. Biol. Chem.* 255:2735-2739.
- Koch, G. L. 1990. The endoplasmic reticulum and calcium storage. *Bioessays*. 12:527-531.
- Kretsinger, R. H. 1976. Calcium-binding proteins. *Annu. Rev. Biochem.* 45:239-266.
- Lanini, L., O. Bachs, and E. Carafoli. 1992. The calcium pump of the liver nuclear membrane is identical to that of endoplasmic reticulum. *J. Biol. Chem.* 267:11548-11552.
- Li, R. H., and J. O. Thomas. 1989. Identification of a human protein that interacts with nuclear localization signals. *J. Cell Biol.* 109:2623-2632.
- Liu, C., and T. E. Hermann. 1978. Characterization of ionomycin as a calcium ionophore. *J. Biol. Chem.* 253:5892-5894.
- Lodish, H. F., N. Kong, and L. Wikstrom. 1992. Calcium is required for folding of newly made subunits of the asialoglycoprotein receptor within the endoplasmic reticulum. *J. Biol. Chem.* 267:12753-12760.
- Martin, K., and A. Helenius. 1991. Transport of incoming influenza virus nucleocapsids into the nucleus. *J. Virol.* 65:232-244.
- Melchior, F., B. Paschal, J. Evans, and L. Gerace. 1993. Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J. Cell Biol.* 123:1649-1659.
- Michaud, N., and D. Goldfarb. 1992. Microinjected U snRNAs are imported to oocyte nuclei via the nuclear pore complex by three distinguishable targeting pathways. *J. Cell Biol.* 116:851-861.
- Minta, A., J. P. Y. Kao, and R. Y. Tsien. 1989. Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J. Biol. Chem.* 264:8171-8178.
- Moore, M. S., and G. Blobel. 1992. The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. *Cell*. 69:939-950.
- Moore, M. S., and G. Blobel. 1993. The GTP binding protein Ran/TC4 is required for protein import into the nucleus. *Nature (Lond.)*. 365:661-663.
- Mori, K., W. Ma, M.-J. Gething, and J. Sambrook. 1993. A transmembrane protein with a cdc2⁺/CDC28-related kinase activity is required for signaling from the ER to the nucleus. *Cell*. 74:743-756.
- Morimoto, R. I. 1993. Cells in stress: transcriptional activation of heat shock genes. *Science (Wash. DC)*. 259:1409-1410.
- Newmeyer, D. D., and D. J. Forbes. 1988. Nuclear import can be separated into distinct steps in vitro: nuclear pore binding and translocation. *Cell*. 52:641-653.
- Newmeyer, D. D., and D. J. Forbes. 1990. An *N*-ethylmaleimide-sensitive cytosolic factor necessary for nuclear protein import: requirement in signal-mediated binding to the nuclear pore. *J. Cell Biol.* 110:547-557.
- Paine, P. L. 1992. Diffusion between nucleus and cytoplasm. In Nuclear Trafficking. C. M. Feldherr, editor. Academic Press, San Diego, CA. pp. 3-14.
- Panté, N., and U. Aebi. 1993. The nuclear pore complex. *J. Cell Biol.* 122:977-985.
- Peters, R. 1986. Fluorescence microphotolysis to measure nucleocytoplasmic transport and intracellular mobility. *Biochim. Biophys. Acta*. 864:305-359.
- Pressman, B. C., and M. Fahim. 1982. Pharmacology and toxicology of the monovalent carboxylic ionophores. *Annu. Rev. Pharmacol. Toxicol.* 22:465-490.
- Preston, S. F., and R. D. Berlin. 1992. An intracellular calcium store regulates protein synthesis in HeLa cells, but it is not the hormone-sensitive store. *Cell Calcium*. 13:303-312.
- Price, B. D., R. L. Mannheim, and S. K. Calderwood. 1992. Brefeldin A, thapsigargin, and AIF4 stimulate the accumulation of GRP78 mRNA in a cycloheximide dependent manner, whilst induction by hypoxia is independent of protein synthesis. *J. Cell. Physiol.* 152:545-552.
- Randriamampita, C., and R. Y. Tsien. 1993. Emptying of intracellular Ca²⁺ stores releases a novel small messenger that stimulates Ca²⁺ influx. *Nature (Lond.)*. 364:809-814.
- Richardson, W. D., A. D. Mills, S. M. Dilworth, R. A. Laskey, and C. Dingwall. 1988. Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. *Cell*. 52:655-664.
- Robbins, J., S. M. Dilworth, R. A. Laskey, and C. Dingwall. 1991. Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell*. 64:615-623.
- Sagara, Y., F. Fernandez-Belda, L. de Meis, and G. Inesi. 1992. Characterization of the inhibition of intracellular Ca²⁺ transport ATPases by thapsigargin. *J. Biol. Chem.* 267:12606-12613.
- Sambrook, J. F. 1990. The involvement of calcium in the transport of secretory proteins from the endoplasmic reticulum. *Cell*. 61:197-199.
- Shi, Y., and J. O. Thomas. 1992. The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. *Mol. Cell Biol.* 12:2186-2192.
- Smythe, E., T. Redelmeier, and S. Schmid. 1992. Receptor mediated endocytosis of semi-intact cells. *Methods Enzymol.* 119:223-234.
- Snow, C. M., A. Senior, and L. Gerace. 1987. Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. *J. Cell Biol.* 104:1143-1156.
- Sterne-Marr, R., J. M. Blevitt, and L. Gerace. 1992. O-linked glycoproteins of the nuclear pore complex interact with a cytosolic factor required for nuclear protein import. *J. Cell Biol.* 116:271-280.
- Strynadka, N., and M. James. 1989. Crystal structures of the helix-loop-helix calcium-binding proteins. *Annu. Rev. Biochem.* 58:951-998.
- Suzuki, C. K., J. S. Bonifacio, A. Y. Lin, M. M. Davis, and R. D. Klausner. 1991. Regulating the retention of T-cell receptor alpha chain variants within the endoplasmic reticulum: Ca²⁺-dependent association with BiP. *J. Cell*

- Biol.* 114:189-205.
- Thastrup, O., P. J. Cullen, B. K. Drobak, M. R. Hanley, and A. P. Dawson. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+} -ATPase. *Proc. Natl. Acad. Sci. USA.* 87:2466-2470.
- Titus, J. A., R. Haugland, S. O. Sharrow, and D. M. Segal. 1982. Texas red, a hydrophilic, red-emitting fluorophore for use with fluorescein in dual parameter flow microfluorometric and fluorescence microscopic studies. *J. Immunol. Methods.* 50:193-204.
- van den Pol, A. N., S. M. Finkbeiner, and A. H. Cornell-Bell. 1992. Calcium excitability and oscillations in suprachiasmatic nucleus neurons and glia in vitro. *J. Neurosci.* 12:2648-2664.
- Watowich, S. S., and R. I. Morimoto. 1988. Complex regulation of heat shock- and glucose-responsive genes in human cells. *Mol. Cell. Biol.* 8:393-405.
- Whiteside, S. T., and S. Goodbourn. 1993. Signal transduction and nuclear targeting: regulation of transcription factor activity by subcellular localisation. *J. Cell Sci.* 104:949-955.
- Wikstrom, L., and H. F. Lodish. 1993. Unfolded h2b asialoglycoprotein receptor subunit polypeptides are selectively degraded within the endoplasmic reticulum. *J. Biol. Chem.* 268:14412-14416.
- Wileman, T., L. P. Kane, G. R. Carson, and C. Terhorst. 1991. Depletion of cellular calcium accelerates protein degradation in the endoplasmic reticulum. *J. Biol. Chem.* 266:4500-4507.
- Wozniak, R. W., E. Bartnik, and G. Blobel. 1989. Primary structure analysis of an integral membrane glycoprotein of the nuclear pore. *J. Cell Biol.* 108:2083-2092.