# Identification of NTF2, a Cytosolic Factor for Nuclear Import That Interacts with Nuclear Pore Complex Protein p62

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*Abstract.* Protein import into the nucleus is a multistep process that requires the activities of several cytosolic factors. In this study we have purified a cytosolic factor that interacts with the nuclear pore complex glycoprotein p62. Isolation involved biochemical complementation of cytosol depleted of this activity by preadsorption with recombinant p62 and the use of a novel flow cytometry-based assay for quantitation of nuclear import. The purified activity (NTF2) is an apparent dimer of ~14-kD subunits and is present at ~$10^6$ copies per cell. We obtained a cDNA encoding NTF2 and showed that the recombinant protein restores transport activity to p62-pretreated cytosol. Our data suggest that NTF2 acts at a relatively late stage of nuclear protein import, subsequent to the initial docking of nuclear import ligand at the nuclear envelope. NTF2 interacts with at least one additional cytosolic transport activity, indicating that it could be part of a multicomponent system of cytosolic factors that assemble at the pore complex during nuclear import.

MOLECULAR trafficking between the cytoplasm and nucleus occurs through large, proteinaceous structures called nuclear pore complexes (NPCs)[1] (reviewed by Forbes, 1992; Gerace, 1992; Pante and Aebi, 1993; Fabre and Hurt, 1994). The NPC spans the double membrane of the nuclear envelope and accommodates two mechanistically different types of transport: passive diffusion and active transport. Ions and other small molecules passively diffuse through ~10-nm aqueous channels in the NPC. Most macromolecules are too large to pass through these diffusional channels at significant rates and are transported through a central gated channel in an ATP- and temperature-dependent manner. The information that targets a protein for nuclear import is referred to as a nuclear localization sequence (NLS; Kalderon et al., 1984; Robbins et al., 1991). NLSs are usually enriched in basic residues, though they do not adhere to a consensus sequence.

Although the molecular basis for the active transport of proteins and RNAs through the NPC is poorly understood, the emerging view of nuclear protein import is that of a multistep process involving interactions between several cytoplasmic factors and the NPC (Forbes, 1992; Gerace, 1992; Goldfarb, 1992; Fabre and Hurt, 1994). The earliest step of nuclear protein import is thought to occur in the cytoplasm, where an NLS-containing protein binds to the NLS receptor (Adam and Gerace, 1991). The second step of nuclear import occurs when the ligand–NLS receptor complex migrates to the nuclear envelope and binds to the cytoplasmic surface of the NPC (Newmeyer and Forbes, 1988; Richardson et al., 1988). This ligand–NLS receptor "docking" occurs in apparent association with 30–50-nm long filaments that emanate from the cytoplasmic surface of the NPC (Richardson et al., 1988; Jarnik and Aebi, 1991). Subsequently, the ligand–NLS receptor complex is delivered to the central channel complex (discussed in Gerace, 1992). The final steps of nuclear protein import involve gating of the central channel and translocation into the nucleus.

Insights into the biochemical requirements for signal-mediated nuclear protein import have come from the use of cell-free assays involving nuclei that are assembled or resealed with *Xenopus* egg extracts in vitro (Newmeyer et al., 1986), or digitonin-permeabilized tissue culture cells supplemented with exogenous cytosol (Adam et al., 1990). Studies with these systems have indicated that multiple cytosolic factors are required for nuclear protein import (Adam et al., 1990; Newmeyer and Forbes, 1990; Sterne-Marr et al., 1992; Moore and Blobel, 1992). One of these factors is a ~55-kD protein that specifically binds the NLS of SV40 large T antigen (Adam and Gerace, 1991). Based on its functional properties and its subcellular localization, the 55-kD protein was proposed to act as a transport carrier that shuttles between the cytoplasm and nucleus (Adam and Gerace, 1991; Gerace, 1992). Nuclear protein import also appears to

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1. *Abbreviations used in this paper*: NEM, *N*-ethylmaleimide; NLS, nuclear localization sequence; NPC, nuclear pore complex; NTF2, nuclear transport factor 2.

require Hsp70 or its cytosolic cognate, as demonstrated by depletion and reconstitution experiments using bacterially expressed Hsp70 (Shi and Thomas, 1992). More recently, the Ras-related GTPase Ran/TC4 was identified as a nuclear transport factor present in *Xenopus* (Moore and Blobel, 1993) and mammalian (Melchior et al., 1993) cytosol. Finally, a 97-kD protein has been described that appears to play a role in NLS receptor-dependent ligand binding to the nuclear envelope (Adam and Adam, 1994).

Although progress has been made in isolating soluble nuclear protein import factors, it has been technically difficult to demonstrate directly that polypeptides of the NPC participate in nucleocytoplasmic transport. The NPC, with a total mass of ~125 × $10^6$ daltons, is estimated to contain 100–200 different proteins. Only a small number of the NPC proteins have been identified (Fabre and Hurt, 1994). These include a group of eight 45–210-kD polypeptides that contain multiple copies of O-linked *N*-acetylglucosamine. These polypeptides are recognized by WGA and certain mAbs (Davis and Blobel, 1987; Snow et al., 1987). At least some of these O-glycosylated proteins contain multiple copies of degenerate pentapeptide and/or tetrapeptide repeats (Fabre and Hurt, 1994).

A transport-related function for these O-linked glycoproteins was suggested by studies showing inhibition on nuclear transport by WGA (Finlay et al., 1987; Yoneda et al., 1987; Dabauvalle et al., 1988; Bataille et al., 1990; Dargemont and Kuhn, 1992) and anti-glycoprotein mAbs (Dabauvalle et al., 1988; Featherstone et al., 1988; Bataille et al., 1990). Additional evidence that NPC glycoproteins may be involved in nuclear import was obtained in studies using a *Xenopus* nuclear reconstitution extract. NPCs assembled in the absence of most WGA-binding proteins no longer supported the active transport of NLS-ligands (Finlay and Forbes, 1990).

Using a different approach, our laboratory explored whether the family of NPC glycoproteins could interact stably with essential cytosolic transport factors (Sterne-Marr et al., 1992). Pretreatment of cytosol with immobilized NPC glycoproteins resulted in the specific depletion of at least one factor required for nuclear protein import (Sterne-Marr et al., 1992). This strongly suggested that one or more of the O-linked NPC glycoproteins plays a direct role in nuclear protein import via interactions with cytosolic transport factors.

In this study we have identified a factor for nuclear protein import that interacts with the O-linked glycoprotein p62. We modified the nuclear protein import assay developed previously in our laboratory (Adam et al., 1990) to include a rapid, quantitative analysis by flow cytometry. HeLa cell cytosol preadsorbed with immobilized recombinant p62 is substantially inhibited in its ability to support nuclear protein import when tested in the permeabilized cell assay. The activity that biochemically complements p62-preadsorbed cytosol was purified to near homogeneity from HeLa cell extract. The purified protein, which we refer to as nuclear transport factor 2 (NTF2), elutes from a gel filtration column with an apparent mass of 28 kD and has a subunit molecular mass ~14 kD on SDS-PAGE. After obtaining a cDNA for NTF2, we expressed the protein in *Escherichia coli*. Recombinant NTF2 fully restores transport activity to p62-pretreated cytosol, indicating that NTF2 is the only rate-limiting factor depleted under these conditions. We cannot detect a requirement for NTF2 in the docking of NLS ligand with the nuclear envelope, suggesting that NTF2 is involved in a subsequent transport event such as delivery to the central channel complex or translocation through the gated channel.

## Materials and Methods

### Preparation of Transport Ligand (FITC-BSA-NLS)

Nuclear protein import was measured using digitonin-permeabilized tissue culture cells (Adam et al., 1990; Melchior et al., 1993). The transport ligand was fluorescently labeled BSA conjugated with synthetic peptides containing the SV40 large T antigen NLS (Kalderon et al., 1984). The conjugate was prepared by first dissolving 10 mg of BSA (fatty acid free; Boehringer Mannheim Biochemicals, Indianapolis, IN) in 1 ml of $Na_2HCO_3$, pH 9.0. To this was added 0.1 ml of 10 mg/ml FITC isomer I (Molecular Probes, Inc., Eugene, OR) prepared in DMSO. The mixture was stirred for 60 min at room temperature, and the reaction was stopped by addition of 0.1 ml of 1.5 M hydroxylamine. The unincorporated FITC was removed by gel filtration chromatography, and 1-mg aliquots of the FITC-BSA were frozen in liquid $N_2$ and stored at −80°C.

Conjugation of NLS peptides to the FITC-BSA was performed using the heterobifunctional cross-linker sulfo-SMCC (Pierce, Rockford, IL). Each milligram of FITC-BSA was reacted with 50 μl of 20 mM sulfo-SMCC (made in DMSO) for 45 min at room temperature. The excess cross-linker was removed on a disposable Sephadex G-25 column (PD-10; Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The conjugate was mixed with 1 mg of NLS peptide (CGGGPKKKRKVED) overnight at 4°C. The unincorporated peptide was removed either by gel filtration (Sephadex G-50) or by multiple washings on a Centricon-30 filter (Amicon Corp., Danvers, MA). The FITC-BSA-NLS conjugate was frozen in liquid $N_2$ and stored at −80°C until use. This ligand, which contains an average of 20 NLS peptides per molecule of BSA, was found distributed throughout the nucleus when viewed by fluorescence microscopy. Including twofold more cross-linker and consequently more NLS peptides per molecule of BSA produces a ligand that has a strong tendency to concentrate in the nucleolus.

### Nuclear Protein Import Assay

Suspension culture HeLa cells were propagated in Joklik's modified S-MEM plus 10% FCS. To prepare cells for the transport assay, 50 ml of exponentially growing cells was harvested by centrifugation and washed once in ice-cold transport buffer (20 mM Hepes, pH 7.4, containing 110 mM potassium acetate, 2 mM magnesium acetate, and 0.5 mM EGTA). The cell pellet was resuspended to a total volume of 1 ml with transport buffer, and the cells were kept on ice. The cell concentration was determined by comparing the $A_{550}$ of a 1:50 dilution of the washed cells with values from a standard curve. The cells were then diluted to 5 × $10^6$ cells per ml using transport buffer containing 2 mM DTT and 1 μg/ml each aprotinin, leupeptin, and pepstatin. After adding digitonin (from a 10% stock in DMSO) to 0.01%, the cells were gently mixed by pipetting and held on ice for 6 min. The cells were subsequently diluted with ice-cold transport buffer (containing DTT and protease inhibitors) to 50 ml and collected by centrifugation. The cell pellet was resuspended in ~3 vol of transport buffer and stored on ice until use. The concentration of permeabilized cells in this preparation was ~5 × $10^7$/ml.

Transport reactions (total volume of 40 μl) contained HeLa cell cytosol (2–5 mg/ml), an ATP-regenerating system (1 mM ATP, 5 mM creatine phosphate, 20 U/ml creatine phosphokinase), FITC-BSA-NLS (200–800 nM), and permeabilized HeLa cells (typically 5 × $10^5$). The reactions were performed for 20 min at 30°C in 6-ml polystyrene tubes (No. 2058; Falcon Plastics, Cockeysville, MD) that fit the FACS® instruments (Becton Dickinson, Mountain View, CA). The transport reaction was stopped by transferring the samples to an ice water bath. The cells were then diluted with 100 vol (4 ml) of ice-cold transport buffer, collected by centrifugation, and finally resuspended in ~0.3 ml of transport buffer. The fluorescence of $10^4$ cells was measured for each sample using the FACScan® flow cytometer (Becton Dickinson). The mean fluorescence per cell of each sample was then determined using Lysis II software (Becton Dickinson). Assays were not usually performed in duplicate, since the standard deviation for duplicates was typically ~2%. The mean fluorescence values between different experiments are not necessarily directly comparable, owing to potential variation in the extent of cell permeabilization and the use of different

batches and amounts of NLS ligands. Note, however, that the data in Fig. 4 *A* and Fig. 5 *C*, which show that recombinant NTF2 stimulates transport to the same extent as the HeLa cell protein, are from the same experiment.

## *Preparation of HeLa Cell Cytosol*

16 liters of exponentially growing HeLa cells (~$5 \times 10^5$ cells per ml) was collected by centrifugation at 300 *g* for 10 min. The cells were washed twice with ice-cold PBS and once with transport buffer containing 2 mM DTT. The washed cell pellet (~40 ml) was resuspended in 2 vol of lysis buffer (5 mM Hepes, pH 7.4, containing 10 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, with 3 μg/ml each aprotinin, leupeptin, and pepstatin, 5 mM DTT, and 0.1 mM PMSF). After swelling for 10 min on ice, the cells were disrupted with two to three strokes in a tight fitting stainless steel homogenizer. Using this procedure, >95% of the cells were disrupted as judged by phase contrast microscopy. The homogenate was diluted with 0.1 vol of 10× transport buffer and centrifuged at 40,000 *g* for 30 min at 4°C. The supernatant was filtered through four layers of cheesecloth and centrifuged at 150,000 *g* for 60 min at 4°C. The resulting supernatant was aliquoted, frozen in liquid $N_2$, and stored at −80°C. Before use in transport reactions, cytosol was thawed, dialyzed against transport buffer containing 2 mM DTT, and stored as previously described. HeLa cell cytosol could be freeze thawed as many as three times without appreciable loss of protein import activity.

## *Preparation of p62 Affinity-depleted Cytosol*

Bacterially expressed p62 (see below) was dialyzed into 100 mM sodium bicarbonate, pH 8.3, containing 100 mM NaCl and coupled overnight at 4°C to CNBr-activated Sepharose beads (Pharmacia LKB Biotechnology Inc.) at a concentration of 3–5 mg/ml. The beads were quenched with 1 M ethanolamine for 2 h at room temperature, washed five times in transport buffer, and treated for at least 1 h with 50 mg/ml BSA in transport buffer to block nonspecific binding. Control matrices, which did not deplete cytosol of essential transport factors, were prepared in a similar manner. These included Sepharose beads coupled with BSA, total BL21(DE3)pLysS cell extract, or the putative coiled-coil domain of p62 spanning amino acids 294–525. Small scale depletions were performed by combining cytosol with an equal volume of beads and mixing end over end for 30 min at 4°C. Large scale depletions used a 10-ml disposable column containing 2 ml of p62–Sepharose. The column was treated with 4 ml of 100 mg/ml BSA followed by an 8-ml wash with transport buffer. HeLa cell cytosol (4 ml) was passed over the column three times, concentrated to 3 ml by vacuum dialysis, frozen in liquid $N_2$, and stored at −80°C.

## *Purification of NTF2*

The rationale was to assay column fractions for an activity that would biochemically complement cytosol that was pretreated with p62–Sepharose.

20 ml of HeLa cell cytosol was thawed and centrifuged at 150,000 *g* for 30 min at 4°C. The clarified supernatant was diluted 1:1 with 20 mM Hepes, pH 7.4, and loaded onto a 20-ml DEAE–Sepharose Fast Flow column (Pharmacia LKB Biotechnology, Inc.), previously equilibrated in the same buffer, at 45 ml/h. The column was washed with 60 ml of Tris-KCl buffer (20 mM Tris, pH 7.6, containing 50 mM KCl, 5 mM magnesium chloride, and 0.5 mM EDTA) and eluted with a 120-ml linear gradient of 50–300 mM KCl in Tris-KCl buffer. 100 μl of each fraction was dialyzed against transport buffer for 2 h (with one buffer change) in a microdialyzer unit (Pierce, Rockford, IL) that was previously blocked with 1% BSA. The DEAE, S100 HR, and MonoQ activity profiles were generated by assaying 10 μl of each fraction in the presence of 15 μl of p62-treated cytosol using the permeabilized cell assay.

DEAE fractions containing the NTF2 activity (eluting at ~175 mM KCl) were pooled, precipitated with solid ammonium sulfate (60% final concentration), resuspended with 1 ml of transport buffer, and clarified at 12,000 *g* for 15 min. The sample was applied to a gel filtration column (1.5 × 65 cm; S100 HR; Pharmacia LKB Biotechnology Inc.) equilibrated in transport buffer and chromatographed at 15 ml/h. NTF2 activity, eluting with an apparent molecular mass of 28,000 daltons, was pooled, concentrated using a Centricon-30 filter, frozen in liquid $N_2$, and stored at −80°C.

For the final purification step, the pooled material from the S100 column was fractionated on an FPLC MonoQ column (1 ml; Pharmacia LKB Biotechnology Inc.) using a flow rate of 0.5 ml/min and a linear gradient of transport buffer (buffer A) and transport buffer containing 1.0 M potassium acetate (buffer B). After loading the sample, the column was washed with buffer A for 5 min followed by a 10-min linear gradient of 0–100% buffer B (in buffer A). We found that NTF2 reproducibly eluted at 32–33% buffer B (400 mM potassium acetate). In an effort to minimize the trace contaminants whose elution overlapped slightly with NTF2 (see silver-stained gel of the column profile), we held the gradient at 32.8% buffer B for 3 min, during which time the NTF2 eluted completely. The samples were dialyzed and assayed as previously described. The active fractions were dialyzed against transport buffer containing 2 mM DTT, frozen in liquid $N_2$, and stored in aliquots at −80°C.

## *Protein Sequencing*

The sequences of several peptides derived from NTF2 were determined by in situ tryptic digestion and standard Edman degradation methodology (Aebersold, 1989) by Dr. John Leszyk at the Protein Chemistry Facility at the Worcester Foundation for Experimental Biology (Shrewsbury, MA). Briefly, the peak fraction of NTF2 from a MonoQ profile was concentrated by centricon filtration, electrophoresed on a 16% polyacrylamide gel, and transferred to Immobilon polyvinylidene difluoride membrane (Millipore Corp., Waters Chromatography, Milford, MA). The protein was visualized by Ponceau S staining, and the blot strip was excised and cleaved with trypsin. The resulting peptides were purified by HPLC on a $C_{18}$ reversed-phase column. The peptides were sequenced on a 477A sequencer equipped with a model 120A in-line PTH analyzer (both from Applied Biosystems, Inc., Foster City, CA).

## *Molecular Cloning and Expression of NTF2 and p62*

The sequences of the tryptic peptides indicated that NTF2 was identical to a previously cloned human protein of unknown function (Grundmann et al., 1988). Primers spanning bases 99–120 and 462–483 of the published cDNA were used to amplify the complete open reading frame by PCR from first-strand cDNA prepared from HeLa cell poly(A) RNA. The PCR product was subcloned into pET23b, which was modified to include a stop codon at the HincII site, thereby eliminating translation of the histidine tag. The PCR-amplified open reading frame was sequenced on both strands and found to correspond exactly to the published sequence.

pET23b-NTF2 was transformed into JM109 and BL21(DE3)pLysS cells, the latter of which gave better inducible expression. Fresh transformants were grown in LB containing ampicillin and chloramphenicol overnight at 37°C, collected by centrifugation, resuspended in fresh media, and diluted 1:100 in LB containing ampicillin and chloramphenicol. Upon reaching an $A_{600}$ of ~0.6, expression was induced with 0.4 mM isopropyl β-D-thiogalactopyranoside for 2 h, at which time the bacteria were collected by centrifugation at 6,000 *g* for 15 min at 4°C. The pellets were frozen in liquid $N_2$ and stored at −80°C until use.

To isolate the recombinant NTF2, the cell pellet from a 500-ml culture was thawed in 20 ml of TNE buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EDTA) containing 5 μg/ml each aprotinin, leupeptin, and pepstatin, 0.5 mM PMSF, and 5 mM DTT. After five passes in a stainless steel homogenizer, the viscous sample was centrifuged sequentially at 30,000 and 150,000 *g*. We note that though only 10–20% of the NTF2 remained soluble under these preparative conditions, our attempts to recover active NTF2 from the insoluble fraction have been unsuccessful. The supernatant from the high speed spin was stirred with solid ammonium sulfate (50% final concentration) for 30 min at 4°C. The precipitate was collected by centrifugation at 12,000 *g* for 15 min, and the pellet was resuspended in transport buffer. After centrifugation at 12,000 *g* for 30 min, the clarified sample was chromatographed on an S100 HR column (1.5 × 65 cm) preequilibrated in 2× transport buffer.

Bacterial expression of rat p62 (Starr et al., 1990) was performed essentially as described for NTF2, using a cDNA kindly provided by Dr. John Hanover (NIH, Bethesda, MD). The open reading frame was cloned into a modified pET23b that lacked the histidine tag (described above). The frozen bacterial pellet from a 500-ml culture was homogenized in 20 ml of TNE buffer containing protease inhibitors and 10 mM DTT. The homogenate was then diluted to 100 ml and centrifuged at 12,000 *g* for 15 min. The resulting pellet was washed twice in the same manner. The washed inclusion body fraction was then homogenized in 20 ml of TNE buffer containing 6 M urea, 0.1 M DTT, and 1 mM PMSF and incubated for 60 min on ice. The sample was clarified at 150,000 *g* for 60 min, and the resulting supernatant was dialyzed overnight against 100 vol of TNE buffer containing 2 M urea and 20 mM DTT. Alternatively, the 6 M urea supernatant was rapidly diluted 1:2 with TNE, without noticeable protein precipitation. The 2 M urea supernatant was dispensed in 4-ml aliquots and stored at −20°C

until use. The p62 obtained by this method was usually >70% pure, as estimated by SDS-PAGE and scanning densitometry.

### Immunological Reagents

The relative levels of Ran/TC4, RCC1, and Hsp70 in cytosol pretreated with control or p62-Sepharose were compared by immunoblotting and detection by Enhanced Chemiluminescence (Amersham Corp., Arlington Heights, IL). Polyclonal antibodies to Ran/TC4 and RCC1 were kindly provided by Drs. Peter D'Eustachio (NYU, New York) and Takeharu Nishimoto (Kyushu University, Japan), respectively. The anti-Hsp70 mAb was purchased from StressGen (Victoria, BC).

The polyclonal antibody to NTF2 was produced in mice using the amino-terminal peptide MGDKPIWEQIGS(C) coupled to KLH. Blots were probed with serum diluted 1:1,000, and detected by peroxidase-labeled secondary antibodies and ECL.

### Quantitation of Nuclear Envelope-Associated FITC-BSA-NLS (Docking Reaction)

The effect of NTF2 on the targeting/binding of NLS ligand to the nuclear envelope (docking) was analyzed by depleting ATP from the assay and measuring the envelope-associated fluorescence. It should be noted that depletion of ATP from the assay resulted in variable levels of ligand associated with the cytoplasmic compartment; therefore flow cytometry could not be used for this assay. We also noticed that docking was more readily observed using NLS ligands that were slightly overcoupled with NLS peptides. It was found that NLS conjugates of allophycocyanin and FITC-BSA that accumulated visibly in nucleoli were usually suitable for observing docking. In contrast, NLS conjugates that distributed uniformly throughout the nucleoplasm, or were excluded from nucleoli, did not seem to bind stably to the nuclear envelopes of permeabilized cells in the presence of crude cytosol. Interestingly, the "docking fraction" (described in the following section) promoted stable association of even low-coupled ligands with the nuclear envelope.

HeLa cells were grown on 18-mm coverslips, washed with transport buffer, and permeabilized with digitonin as previously described (Adam et al., 1990). The permeabilized cells were depleted of ATP by incubation with 16 U/ml hexokinase (H-5750; Sigma Chemical Co., St. Louis, MO), 5 mM glucose, and 10 mg/ml BSA in transport buffer for 10 min at 30°C in a humid chamber. The components of the docking reaction included hexokinase, glucose, and BSA (same concentrations as above), plus p62-pretreated HeLa cell cytosol (2 mg/ml), NTF2 (20 μg/ml), and FITC-BSA-NLS (200 nM). The docking reaction was performed for 5 min at room temperature. The coverslips were washed three times in transport buffer (5 min each), fixed in 4% ultrapure formaldehyde (10 min), rinsed briefly in distilled water, and mounted in 10% glycerol in PBS (SlowFade; Molecular Probes Inc.).

At least 12 fields per coverslip were photographed with a 15-s exposure using Tmax film (400 ASA; Eastman Kodak Co., Rochester, NY), and film was developed according to the manufacturer's instructions. The photographic negatives were scanned with a UC1260 instrument (UMAX Data Systems Inc., Taiwan, R.O.C.) at a resolution of 1,200 dpm, and the images were acquired and analyzed on a MacIntosh IIfx computer using the program NIH Image, version 1.55. NIH Image is public domain software written by Wayne Rasband at the NIH and is available through the Internet by anonymous ftp from zippy.nimh.nih.gov.

The nuclear envelope fluorescence was measured at five different positions per nucleus by densitometric scanning (4-pixel scan width). The mean maximum fluorescence per nucleus (minus background) was determined for at least 20 cells from two coverslips. The fluorescence density profile taken across the nuclear envelope is essentially a symmetric peak. We take this as an indication that averaging the maximum fluorescence is an appropriate measure of relative ligand accumulation.

### Purification of a HeLa Cell Docking Fraction

A modification of the procedure describing the purification of the NLS receptor and p97 from bovine cytosol (Adam and Adam, 1994) was used to prepare a fraction from HeLa cell cytosol that reconstitutes binding of NLS ligand to the nuclear envelope. HeLa cell cytosol (10 ml; prepared as previously described) was precipitated with ammonium sulfate (final concentration, 50%) for 30 min at 4°C. The precipitate was collected by centrifugation and washed once in transport buffer containing 50% ammonium sulfate. The pellet was dissolved in 15 ml of buffer A (20 mM Tris, pH 8.0, containing 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, and 2 mM DTT and dialyzed overnight. After clarification (40,000 *g* for 30 min), the sample was applied to a 10-ml DEAE-Sepharose column equilibrated in buffer A. The column was washed extensively with buffer B (same components as buffer A, except containing 200 mM NaCl) and eluted with buffer C (same components as buffer A, except containing 800 mM NaCl). The buffer C eluate was bound to a 5-ml Hydroxylapatite column (preequilibrated in 2 mM sodium phosphate, pH 7.0, containing 2 mM DTT), washed with 10 column vol of preequilibration buffer, and eluted with 200 mM sodium phosphate, pH 7.0, containing 5 mM DTT. The fractions containing docking activity (assayed using digitonin-permeabilized monolayer HeLa cells) were pooled, and BSA was added to a final concentration of 1%. The sample was concentrated twofold by dialysis in a collodion membrane against transport buffer containing 5 mM DTT. Aliquots were frozen in liquid $N_2$ and stored at −80°C.

## Results

We developed a new method for rapid quantitation of signal-mediated nuclear protein import in vitro in a transport assay involving digitonin-permeabilized tissue culture cells supplemented with HeLa cell cytosol. The import substrate consisted of fluorescent BSA coupled with synthetic peptides representing the NLS of SV40 large T antigen (FITC-BSA-NLS). We found that nuclear protein import could be accurately quantitated by measuring the total cell-associated fluorescence using flow cytometry, as shown in Fig. 1 (*left panels*).

At the end of a standard 20-min import reaction, an average intensity of 754.79 fluorescence units per cell was measured by flow cytometry (Fig. 1, *Control, left panel*). Analysis of this sample by epifluorescence and phase microscopy (*middle* and *left panels*) indicated that virtually all cell-associated FITC-BSA-NLS was within the nucleus. The signal obtained with flow cytometry was strongly diminished by conditions that inhibit import both in vivo and in vitro (reviewed by Forbes, 1992). Substantial inhibition of the accumulation was observed (Fig. 1) if the assay was performed at 0°C (86.74 fluorescence units), in the presence of WGA (88.66 fluorescence units), or if the system was depleted of ATP using hexokinase and glucose (134.58 fluorescence units). Correspondingly, fluorescence microscopy revealed the FITC-BSA-NLS was virtually absent from the nuclei of cold and WGA-blocked samples, and only a low level of ligand was observed in the nuclei of cells depleted of ATP (Fig. 1, *middle panels*). Moreover, the nuclear accumulation of NLS ligand measured by flow cytometry was inhibited 85% by including a 40-fold molar excess of NLS-containing histone H1 (Breeuwer and Goldfarb, 1990) in the transport assay (data not shown).

Together, these controls established that flow cytometry is a valid method for measuring nuclear protein import in digitonin-permeabilized cells. This assay provides a much more rapid method for quantitating protein import than previously described techniques. It should be a valuable tool for the biochemical characterization of nuclear protein import, including the identification of novel cytosolic transport factors.

Our laboratory has shown previously that preincubation of cytosol with the eight major O-linked glycoproteins of the NPC immobilized on Sepharose beads results in a loss of transport activity from the cytosol. This was apparently due to affinity depletion of an essential transport factor(s) that bound to certain of the NPC glycoproteins (Sterne-Marr et al., 1992). We found that some of this factor depletion activ-

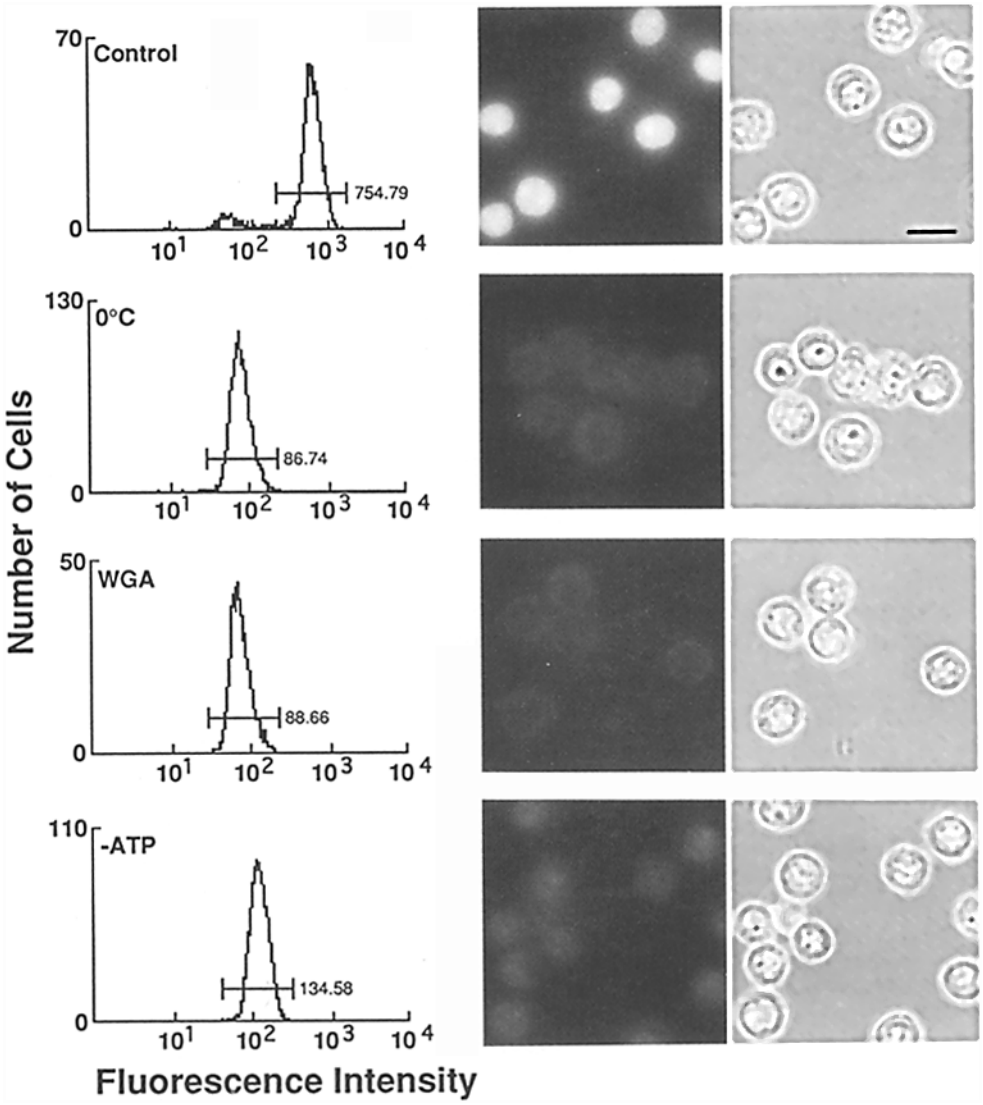


*Figure 1.* Analysis of nuclear protein import by flow cytometry and fluorescence microscopy. Nuclear transport reactions were performed under standard conditions (*control*), or under conditions that inhibit nuclear protein import in vivo and in vitro (*0°C*, *WGA*, or *−ATP*). The fluorescence output generated by the flow cytometer is plotted as a histogram, with the mean fluorescence of the bracketed region indicated (*left panels*). A small aliquot of each sample was applied to a polylysine-coated coverslip and photographed using epifluorescence (*middle panels*) and phase microscopy (*right panels*). The samples were photographed and printed at the same exposure. Bar, 20 μm.

ity was associated with the "p62 complex" (data not shown), a stable complex containing four of the major NPC O-linked glycoproteins of 45, 54, 58, and 62 kD (Finlay et al., 1991; Guan, T., and L. Gerace, unpublished data). We then tested whether recombinant p62 (Fig. 2 *A*) was itself sufficient for binding the putative transport factor(s) when coupled to Sepharose beads. Cytosol preincubated with Sepharose beads alone, or those coupled with BSA or total *E. coli* extract, specifically lost no transport activity. However, the activity of cytosol preincubated with p62–Sepharose was inhibited by 62% (Fig. 2 *B*). In five experiments the inhibition was 65.4 ± 7.3% (mean ± SD). To determine which region of the p62 polypeptide is responsible for binding the transport activity, we expressed the carboxy-terminal 232 amino acids of p62 and tested it as previously described. This domain, which contains several regions predicted to form α helical coiled coils (Starr et al., 1990), did not have detectable depletion activity (data not shown). We were unsuccessful in expressing a p62 fragment that contained residues 1–293 and therefore can only infer that the factor-binding site most likely resides in this amino-terminal region.

The transport activity depleted from cytosol using p62–Sepharose appeared to be distinct from several previously characterized factors, as illustrated by the immunoblotting experiment shown in Fig. 2 *C*. Neither Ran/TC4 (Ren et al., 1993) nor its guanine nucleotide exchange factor, RCC1 (Ohtsubo et al., 1987), was depleted from cytosol by treatment with p62–Sepharose. Three cytosolic Ran/TC4 binding proteins (relative molecular masses of 27, 85, and 110 kD) detected by blot overlay with Ran–[$\gamma^{32}$P]GTP (Coutavas et al., 1993) were also unchanged (data not shown). In addition, the level of Hsp70 was unaffected by this treatment (Fig. 2 *C*). Fractionation of HeLa cell cytosol on a gel filtration column revealed that the complementing activity chromatographed with an apparent molecular mass of 20–30 kD (see the following section). This was smaller than the NLS receptor (~50–60 kD; Adam and Gerace, 1991), and we set out to purify what appeared to be a novel transport activity.

### *Purification of a Novel Factor That Complements Cytosol Pretreated with p62–Sepharose*

Attempts to elute the transport activity from p62–Sepharose using high salt, low pH, or concentrated $MgCl_2$ were unsuccessful. Therefore, we decided to purify the factor from

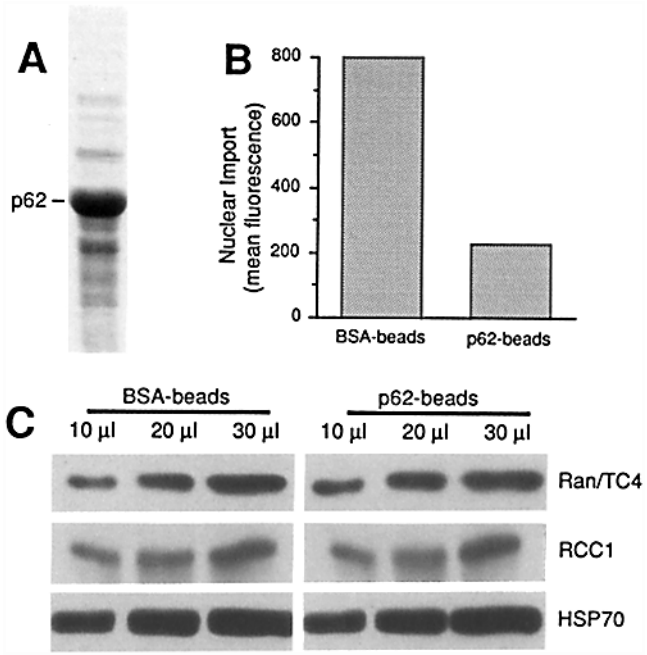


*Figure 2.* A novel cytosolic nuclear protein import factor interacts specifically with recombinant NPC protein p62. (*A*) SDS-PAGE (8% gel) of the p62 protein used in these studies. (*B*) Affinity depletion of a cytosolic transport factor using p62-Sepharose. HeLa cell cytosol was mixed with an equal volume of BSA beads or p62 beads for 30 min at 4°C, and the unbound fractions were tested in the permeabilized cell transport assay. The p62-pretreated cytosol was inhibited by 62%. (*C*) Immunoblot analysis (at three gel loadings) showing that the relative levels of cytosolic Ran/TC4, RCC1, and Hsp70 are unaffected by preincubation with p62-Sepharose.

HeLa cell cytosol by conventional column chromatography, assaying for activity using biochemical complementation of cytosol inactivated by pretreatment with p62-Sepharose. The assay involved use of a saturating concentration of cytosol (usually ~4 mg/ml) in order to avoid detecting activities that might stimulate transport simply because they were present at subsaturating levels.

HeLa cell cytosol was loaded on a DEAE-Sepharose column, washed extensively with buffer, and eluted using a linear salt gradient. A single peak of complementing activity was detected, eluting with ~175 mM KCl, in fractions 11–14 (Fig. 3 *A*, *upper panel*). The active fractions were pooled, precipitated with ammonium sulfate, and chromatographed on an S100 HR gel filtration column. The complementing activity eluted with an apparent molecular mass of 28 kD (Fig. 3 *A*, *middle panel*). Fractions 47–51 were pooled, concentrated, and applied to a MonoQ column. The maximum complementing activity that eluted from the MonoQ column was found in fraction 18 (Fig. 3 *A*, *lower panel*), exactly coincident with a low molecular mass (~14 kD) polypeptide detected by silver staining (Fig. 3 *B*). Starting with 20 ml (354 mg of protein) of HeLa cytosol, we obtained 85 µg of the factor, representing a 1,200-fold purification of the activity (Table I). Based on its apparent role in nuclear transport and the fact that it is the second novel transport factor after the NLS receptor (Adam and Gerace, 1991) purified by our

*Table I. Purification Summary of NTF2*

| Fraction | Protein | Activity* | Specific activity | Fold purification | Yield |
|---|---|---|---|---|---|
| | *mg* | *U* | *U/mg* | | % |
| Cytosol | 354 | 195,000 | 550 | – | 100 |
| DEAE-Sepharose | 13.2 | 193,534 | 14,706 | 27 | 99 |
| S100 HR | 0.63 | 117,459 | 186,442 | 13 | 61 |
| MonoQ | 0.085 | 56,514 | 664,870 | 3.6 | 36 |

* Activity is the mean nuclear fluorescence obtained using a saturating amount of p62-treated HeLa cytosol and a subsaturating amount of fractionated cytosol minus the buffer control.

laboratory, we refer to this activity as NTF2, for Nuclear Transport Factor 2.

The purified NTF2 was found to stimulate the nuclear protein import of p62-pretreated cytosol in a saturable manner (Fig. 4 *A*), indicating that it is a rate-limiting component of the latter. NTF2 is the only rate-limiting factor removed under these experimental conditions, as illustrated by complementation with recombinant NTF2 (see Fig. 5). Maximal stimulation was observed at ~3 µg/ml, corresponding to a concentration of ~200 nM. This is comparable to the concentration of purified NLS receptor required for maximum import in permeabilized cells (182 nM; Adam and Gerace, 1991). It is significantly less, however, than the concentration of Hsp70 reported to be necessary to restore full transport activity to HeLa cell cytosol that had been pretreated with reagents to deplete heat shock proteins (2.9 µM; Shi and Thomas, 1992). We note that like the NLS receptor (Adam and Gerace, 1991), NTF2 is capable of stimulating several-fold the transport activity of dilute cytosol, though NTF2 has little effect when assayed in the presence of saturating cytosol (data not shown).

Since the affinity depletion scheme involved exposing p62 beads to crude cytosol, it was formally possible that the p62-NTF2 interaction was indirect, perhaps mediated by an additional cytosolic factor. We therefore tested whether NTF2 would bind directly to p62 immobilized on Sepharose beads. Purified NTF2 was incubated with BSA-Sepharose or p62-Sepharose, and the unbound fraction of each sample was tested for its ability to complement p62-treated cytosol. We observed substantial depletion of NTF2 activity in the sample treated with p62-Sepharose as compared with control Sepharose (Fig. 4 *B*). These data indicate a direct interaction between the NPC protein p62 and the cytosolic transport factor NTF2 and provide strong evidence for a direct role of p62 in nuclear protein import. Moreover, our data indicate that NTF2 is depleted rather than simply inactivated by recombinant p62, as also illustrated by immunodetection experiments (see Fig. 7 *C*).

### *Molecular Identification and Expression of Recombinant NTF2*

To determine the primary structure of NTF2, several tryptic peptides derived from the purified ~14-kD band were analyzed by microsequencing (Table II). A search of the GenEMBL data base revealed that the sequence of each of

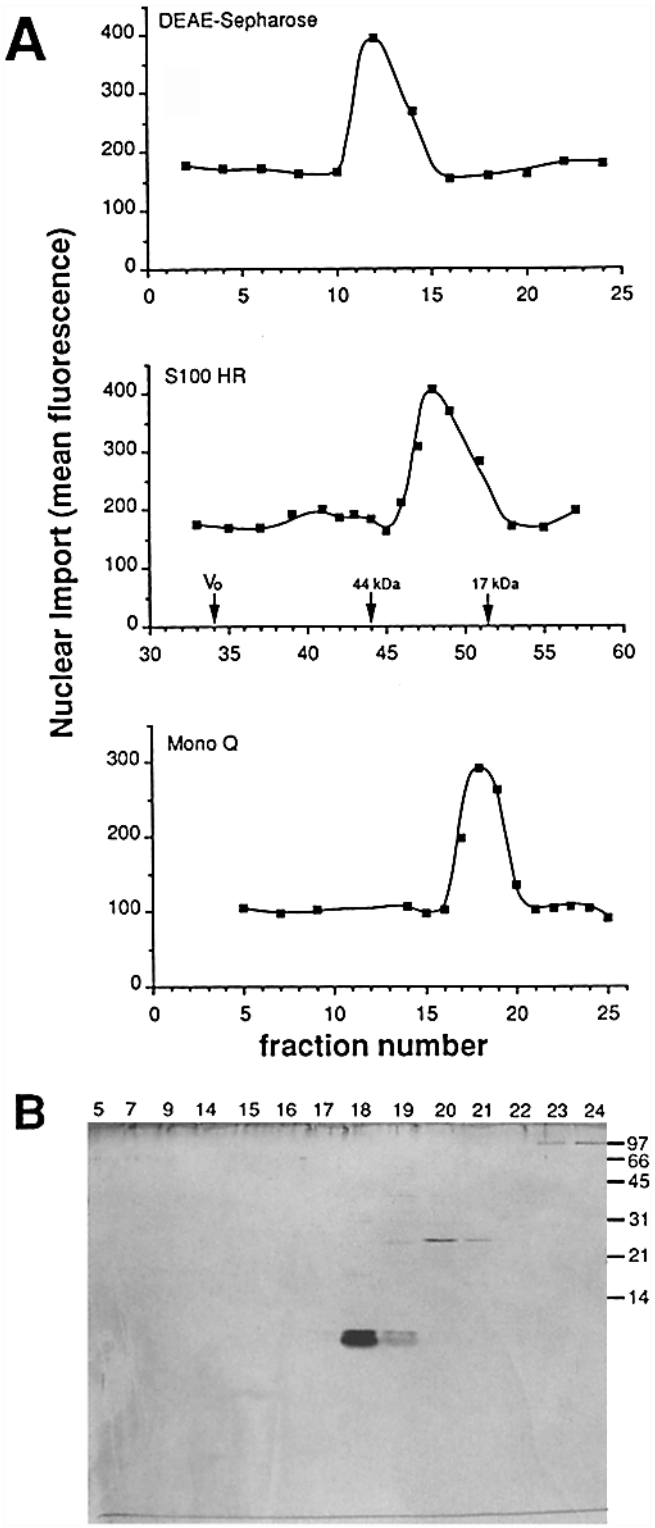


*Figure 3.* Purification of a cytosolic factor that restores nuclear protein import activity to cytosol that has been pretreated with p62–Sepharose. (*A*) Activity profiles from the sequential DEAE–Sepharose, S100 HR, and MonoQ columns used to purify NTF2. The activity profiles were generated by assaying 10 μl of each column fraction in the presence of p62-treated cytosol (see Materials and Methods). No activity was detected in the flow-through of the DEAE–Sepharose column (data not shown), indicating that NTF2 is the only complementing activity present in HeLa cytosol. (*B*) Silver-stained SDS–polyacrylamide gel (16%) of selected fractions from the MonoQ profile. The peak of protein detected by silver staining (fraction 18) coincides with the peak of protein import activity measured with the permeabilized cell transport assay.

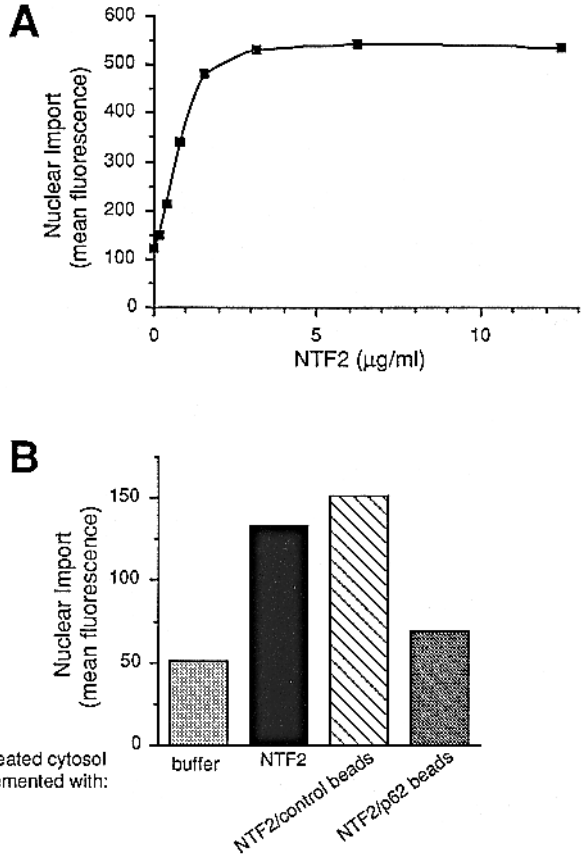


*Figure 4.* Characterization of the NTF2 protein purified from HeLa cell cytosol. (*A*) Increasing concentrations of NTF2 were added to transport reactions that contained a saturating amount of cytosol pretreated with p62–Sepharose. The extent of nuclear protein import was saturated in the presence of 200 nM exogenous NTF2. This compares favorably with the predicted final concentration of cytosolic NTF2 in transport assays using control cytosol, which based on our purification yield is ~130 nM. (*B*) Interaction of NTF2 with p62 in vitro. Purified NTF2 was diluted with 1 mg/ml BSA and incubated with BSA beads (*NTF2/control beads*) or p62 beads (*NTF2/p62 beads*) for 30 min at 4°C, and the unbound fraction was tested for its ability to complement HeLa cytosol that itself was pretreated with p62 beads.

the six tryptic peptides was contained within the open reading frame of a cDNA encoding a 127-residue human protein of unknown function (Grundmann et al., 1988). The peptide sequences matched the human protein sequence at 52 of 53 positions. The single indeterminate residue in peptide 5 is a cysteine, a residue that would not have been revealed by our analysis.

We designed PCR primers based on the published nucleotide sequence (Grundmann et al., 1988) in order to clone the open reading frame of NTF2. Using first-strand cDNA generated from HeLa cell poly(A) RNA as the template, we amplified the open reading frame and determined that the

*Table II. Amino Acid Sequences of Tryptic Peptides Derived from Purified NTF2*

| Peptide sequence | Amino acid position in human p14 |
|---|---|
| LSSLPFQK | 56–63 |
| IQHSITAQ | 64–71 |
| ADEDPIMGFHQ(M)(F)(L) | 91–104 |
| ADEDPIMGFHQMFLLK | 91–106 |
| NIN(D)AWVXTN(D)MFR | 107–120 |
| LALHNFG | 121–127 |

resulting product contained a nucleotide sequence that was identical to that of the published human sequence. NTF2 was expressed in *E. coli*, and the soluble fraction obtained after isopropyl β-D-thiogalactopyranoside induction was purified by ammonium sulfate precipitation and gel filtration chromatography (Fig. 5 *A*). The recombinant NTF2, when added to cytosol that had been pretreated with p62–Sepharose, restored transport to near control levels (Fig. 5 *B*). The maximum level of transport observed with recombinant NTF2 was comparable to that obtained with the purified human protein (Fig. 4 *A* and Fig. 5 *C*). Approximately threefold more recombinant protein was required to achieve saturation (~600 nM), which is probably attributable to the presence of inactive NTF2 in the preparation.

We tested whether NTF2, like the NLS receptor (Adam and Gerace, 1991) and p97 (Adam and Adam, 1994), is sensitive to sulfhydryl modification. Pretreatment of recombinant NTF2 with 5 mM *N*-ethylmaleimide (NEM) for 30 min at room temperature did not affect its ability to complement p62-treated cytosol (Fig. 5 *D*). This is consistent with previous data from our laboratory showing that an NEM-insensitive transport factor is depleted from cytosol by immobilized O-linked glycoproteins (Sterne-Marr et al., 1992).

Using an affinity depletion approach analogous to that used with p62, we tested whether Sepharose-immobilized NTF2 could interact with other cytosolic nuclear protein import factors. Substantial depletion of transport activity was, in fact, observed when HeLa cell cytosol was pretreated with NTF2–Sepharose (Fig. 5 *E*, *stippled bars*). Since NTF2 ap-

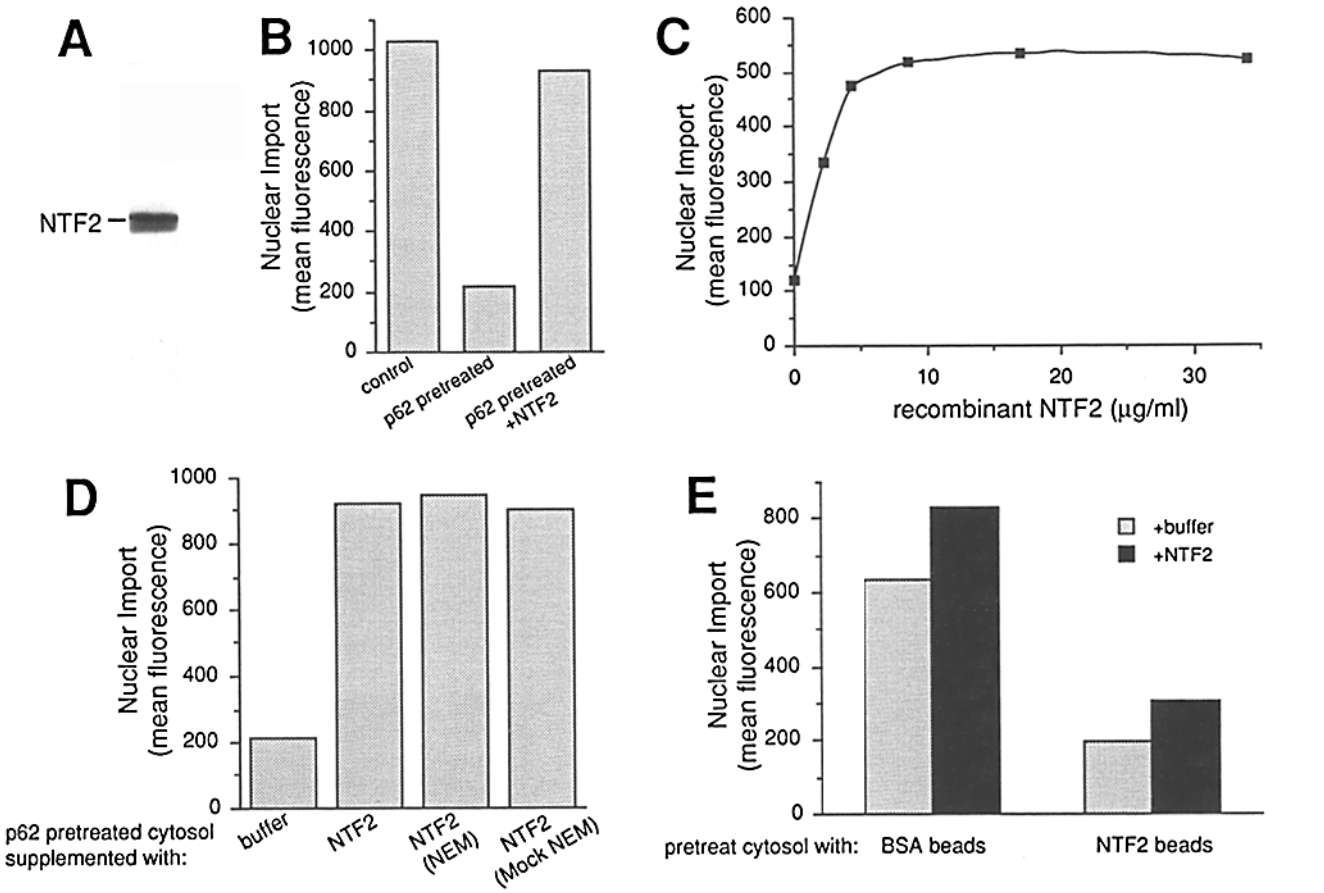


*Figure 5.* Characterization of the recombinant NTF2 protein. (*A*) Electrophoretic analysis (16% gel) of the NTF2 purified from bacterial lysate. (*B*) Addition of recombinant NTF2 to p62-pretreated cytosol restores transport to near control levels. Transport was measured in control cytosol, p62-pretreated cytosol, and p62-pretreated cytosol supplemented with 1.6 μg of NTF2. (*C*) Titration of the recombinant protein in the permeabilized cell assay. Recombinant NTF2 stimulates transport to about the same maximal level as the purified HeLa cell protein (compare with Fig. 4 *A*). (*D*) NTF2 is a NEM-insensitive transport factor. Recombinant NTF2 was treated with 5 mM NEM, quenched with excess DTT, and assayed for its ability to complement transport using p62-pretreated cytosol. (*E*) NTF2 interacts with an essential cytosolic transport factor. Recombinant NTF2 was coupled to Sepharose beads and incubated with HeLa cell cytosol, and the unbound fraction was tested in the transport assay. The substantial transport inhibition observed (~70%) indicates that NTF2 is likely to interact with an additional cytosolic factor required for nuclear protein import.

pears to dimerize (Fig. 3 *A* and Grundmann et al., 1988), we considered the possibility that the reduction of transport activity could be due to cytosolic NTF2 binding to the Sepharose-immobilized NTF2. Supplementing the control and NTF2-treated cytosols with recombinant protein resulted in only a modest increase in transport (Fig. 5 *E*, *solid bars*). Together, these data suggest that NTF2 interacts with at least one additional cytosolic transport factor that can be made rate limiting in our assay. This suggests that NTF2 could be part of a multicomponent system of cytosolic factors assembled at the NPC during nuclear protein import.

### *NTF2 Appears to Be Required for a Late Step of Nuclear Protein Import*

It has been shown previously that targeting of NLS ligands to the NPC (docking) and subsequent translocation through the nuclear pore require different cytosolic factors (Moore and Blobel, 1992). The docking reaction can be examined as a distinct transport step by depleting ATP from the transport assay and measuring the accumulation of FITC–BSA–NLS at the nuclear envelope. The level of cytoplasmic background in this assay (Fig. 6 *C*) made it necessary to measure nuclear envelope–associated fluorescence by densitometric scanning. To test whether NTF2 is involved in the initial targeting of NLS ligands to the NPC, we compared levels of transport and ligand docking in assays containing p62-pretreated cytosol without or with addition of recombinant NTF2. The level of transport, measured in an ATP-containing assay, was stimulated 3.5-fold when cytosol was complemented with recombinant NTF2 (Fig. 6 *A*). By contrast, the level of docking, measured in an ATP-depleted assay, was not significantly changed by addition of NTF2 (Fig. 6 *B*). This suggests that NTF2 functions in a step subsequent to the "ATP-independent" association of NLS ligand with the nuclear envelope.

### *Nuclear Protein Import Requires NTF2, Ran/TC4, and a Docking Fraction*

We sought to obtain additional evidence that NTF2 is required for nuclear protein import and, more specifically, to test further whether NTF2 is required for binding of NLS substrate to the nuclear envelope. To this end, we reconstituted protein import in permeabilized cells using NTF2 and the small GTPase Ran/TC4 (Melchior et al., 1993; Moore and Blobel, 1993) prepared as recombinant proteins, together with a cytosolic fraction that reconstitutes docking of NLS ligand to the nuclear envelope (Moore and Blobel, 1992; Adam and Adam, 1994). The addition of any one of these components to the permeabilized cell assay resulted in only low levels of import (Fig. 7 *A*). Addition of the docking fraction and NTF2, or docking fraction and Ran/TC4, also resulted in only a low level of transport, whereas an intermediate level of transport was obtained with NTF2 and Ran/TC4. However, the presence of all three factors resulted in a level of transport that was significantly higher than the sum of the levels supported by the individual components. Notably, adding Ran/TC4 to NTF2 and the docking fraction stimulated transport by more than fourfold. The data from this experiment indicate that NTF2 and Ran/TC4, as well as the docking fraction, perform distinct functions in nuclear protein import. Furthermore, the apparent synergy observed between these components is consistent with, but does not prove, that these factors act in a common transport pathway.

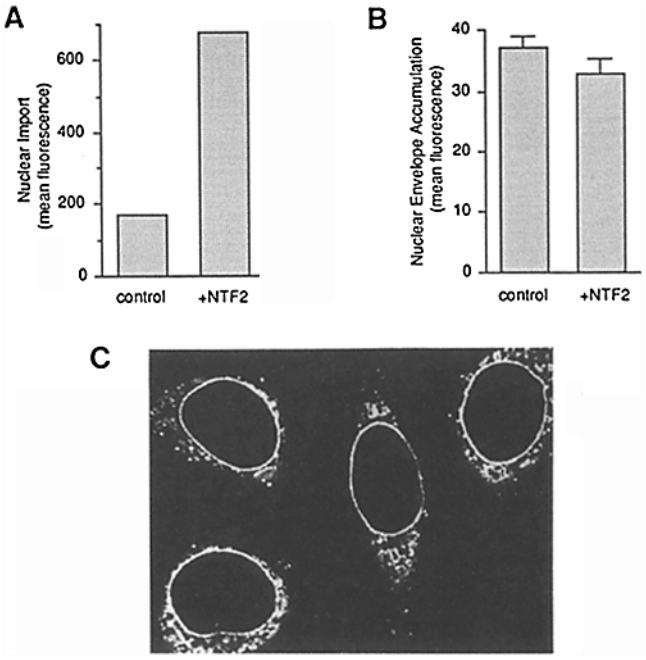


*Figure 6.* Analysis of the stage of nuclear protein import that requires NTF2. (*A*) Addition of recombinant NTF2 to p62-pretreated cytosol stimulates nuclear protein import 3.5-fold in digitonin-permeabilized cells in a 20-min assay. (*B*) NTF2 does not stimulate the association of FITC–BSA–NLS with the nuclear envelope in a 5-min docking reaction. Similar results were obtained when the docking reaction was performed for 20 min (data not shown). (*C*) Examples of docking mediated by p62-pretreated cytosol, recorded by confocal microscopy. The cytoplasmic background is due in part to the use of a highly coupled NLS ligand. The details of the docking assay are described in Materials and Methods.

The docking fraction used in these experiments was found to be sufficient for binding NLS ligand to the nuclear envelope in a punctate pattern (Fig. 7 *B*), indicating that this fraction likely contains the NLS receptor and p97 (Adam and Adam, 1994). Immunoblot analysis of the docking fraction with an antibody raised against an amino-terminal peptide of NTF2 showed that it was devoid of detectable NTF2 (Fig. 7 *C*). These results provide further evidence that cytosolic NTF2 is not required for the initial binding of NLS ligand at the nuclear envelope and suggest that it acts subsequent to the docking step.

## *Discussion*

We have developed a flow cytometry–based method for measuring nuclear protein import in permeabilized cells and used this method to isolate a novel cytosolic transport factor. This factor, which we refer to as NTF2, was purified from HeLa cell extract based on its ability to complement biochemically cytosol that was affinity depleted with NPC protein p62. Recombinant NTF2 restores transport activity to p62-pretreated cytosol to the same extent as the native HeLa cell protein, thus establishing that the complementing activity can be ascribed to NTF2 itself and not a copurifying spe-

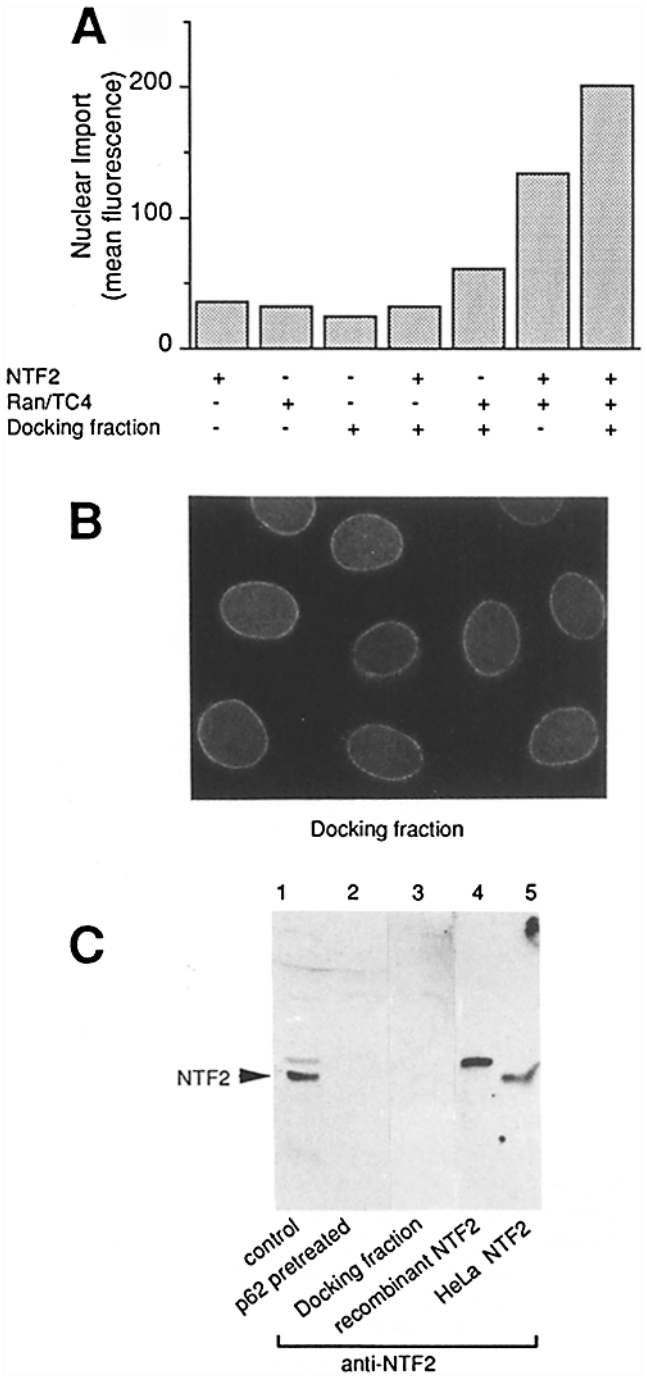


*Figure 7.* Reconstitution of nuclear protein import with recombinant proteins and a HeLa cell docking fraction. (*A*) The levels of transport supported by NTF2, Ran/TC4, and the docking fraction alone and in combination were tested. Saturating concentrations of NTF2 and Ran/TC4 were used; however, no attempt was made to use a saturating concentration of the docking fraction. Note that the level of transport supported by control cytosol was 400 units (data not shown), and the cell-associated fluorescence occurring in the absence of added factors (95 units) was subtracted from each sample. (*B*) Fluorescence micrograph of digitonin-permeabilized HeLa cells incubated with the HeLa cell docking fraction and a low-coupled NLS ligand on ice for 25 min. Docking was also observed at room temperature in the presence of 1 mM ATP, though a low level of transport was also apparent in a subset of cells (data not shown). (*C*) Immunoblot analysis of the components used in these studies using an antibody to the amino terminus of NTF2. A major reactive band was detected in HeLa cytosol (lane *1*) that comigrates with purified NTF2 (lane *5*). The minor, more slowly migrating immunoreactive species in lane *1* probably corresponds to NTF2, as we have noticed that some preparations of NTF2 resolve as a doublet (see Fig. 3 *B*). NTF2 was not detected in p62-pretreated cytosol (lane *2*) or in the docking fraction (lane *3*). The recombinant NTF2 (lane *4*) contains a 12-amino acid addition at the amino terminus, causing it to migrate more slowly than the HeLa cell protein (lane *5*).

cies. Our data suggest that NTF2 interacts directly with NPC protein p62 and exerts its effect at a relatively late step in the nuclear protein import pathway.

Interestingly, data base comparisons suggested the existence of NTF2-related proteins in phylogenetically diverse species. Human NTF2 is 46, 33, and 36% identical to partial open reading frames cloned in yeast (*Saccharomyces cerevisiae*), *Arabidopsis*, and rice, respectively (Fig. 8). Particularly striking is a highly conserved central region corresponding to residues 14–79 in the human protein in which the degree of identity to the human protein is 47–53%. The sequences of the amino- and carboxy-terminal regions of the four proteins are less well conserved. Given the significant sequence relationships that span three kingdoms, we anticipate that NTF2 will prove to be a highly conserved component of the nuclear transport apparatus.

### *Functional Involvement of O-linked Glycoproteins in Transport*

Previous studies have suggested a potential role for the group of eight O-linked NPC glycoproteins in nuclear protein import (see Introduction). Data from our laboratory indicated that at least two of these polypeptides can interact with essential cytosolic transport factors (Sterne-Marr et al., 1992). Here we have shown that NPC protein p62, prepared as a recombinant polypeptide, alone is sufficient to deplete HeLa cell cytosol of an essential transport factor. This suggests a direct role for p62 in nuclear import, and indicates that the multiple copies of *N*-acetylglucosamine found on the native protein (Holt et al., 1987) are not essential for the p62–NTF2 interaction. Whether the 45-, 54-, and 58-kD subunits of the p62 complex play a role in regulating this interaction or whether these subunits also interact directly with NTF2 will require further investigation.

Our data point to the amino-terminal domain of p62 as the site of interaction with NTF2. This region is predicted to form a $\beta$ sheet structure and contains multiple copies of a degenerate pentapeptide repeat related to the sequence GFXFG (Starr et al., 1990; Hanover, 1992). The hydrophobic nature of the repeats suggests that they might help form the core of a $\beta$ strand, with the intervening sequences constituting the loop structures. This type of repeat is found in a number of phylogenetically diverse NPC polypeptides (Fabre and Hurt, 1994), implying a potentially important role in NPC function. Surprisingly, deletion studies have failed to reveal an essential function for the repeat domains in the NSP1 (Nehrbass et al., 1990) and NUP2 (Loeb et al., 1993), proteins of *S. cerevisiae*, though this "lack of phenotype" should be interpreted with caution. Since multiple pro-

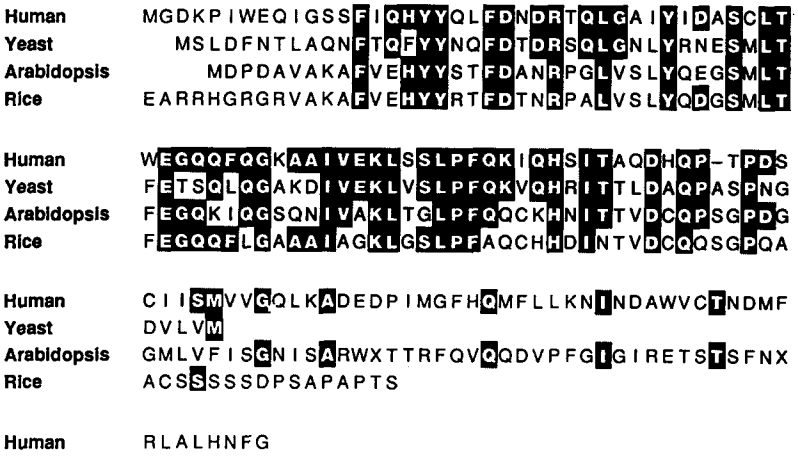


*Figure 8.* Alignment of human NTF2 with related open reading frames identified in yeast (*S. cerevisiae*), *Arabidopsis*, and rice. Amino acids that are identical to the human sequence are shown in reversed type. The percent identities (see Discussion) were determined using the GCG program BESTFIT with the default settings. The yeast sequence was translated from the antisense strand of the 5′ noncoding region of the PSL1 gene. Since there is no in-frame stop codon, the sequence is likely to extend beyond the carboxy-terminal methionine that corresponds to residue 84 in human NTF2. The GenEMBL accession numbers for these sequences are as follows: human, X07315; yeast, L22204; *Arabidopsis*, T04451; rice, D23112.

teins of each NPC contain these types of repeats (Fabre and Hurt, 1994), they may fulfill redundant functions. It will be important to determine whether NTF2 interacts directly with the conserved pentapeptide repeats of p62, or with the nonconserved sequences interspersed between these repeats. If NTF2 associates with the pentapeptide repeats themselves, it is possible that this protein interacts with other components of the NPC containing pentapeptide repeats in addition to p62, such as Nup153 (Sukegawa and Blobel, 1993) and CAN (Kraemer et al., 1994). In this regard, we note that the cytosolic transport activity bound by nup153 (referred to as p180; Sterne-Marr et al., 1992) and NTF2 are both insensitive to alkylation by NEM.

### *NTF2 Function in Nuclear Protein Import*

Our data suggest that NTF2 acts at a step distal to the initial binding of NLS ligand to the NPC. We have shown that efficient binding of NLS ligand to the nuclear envelope can be reconstituted with either p62-pretreated cytosol or a partially purified docking fraction. Both of these samples are devoid of immunologically detectable NTF2. Addition of NTF2 to either p62-pretreated cytosol or the docking fraction and Ran/TC4 stimulates transport approximately threefold but does not stimulate docking (examined in the case of p62-pretreated cytosol). The likelihood that NTF2 acts subsequent to docking is further supported by immunoelectron microscopy showing that p62 is located near the central channel complex, but not at the more peripheral cytoplasmic fibrils implicated in ligand docking (Guan, T., and L. Gerace, unpublished data).

After the original submission of this work, Moore and Blobel (1994) reported the identification of a low molecular weight transport factor in *Xenopus* cytosol that, based on peptide sequence, is likely to be the amphibian homologue of NTF2. It was shown that the *Xenopus* protein together with Ran/TC4 promoted the dissociation of NLS ligand from the nuclear envelope, supporting a role for NTF2 in a late step of transport. These observations are consistent with our quantitative analysis showing that NTF2 does not stimulate the binding of NLS ligand to the nuclear envelope. The low molecular weight *Xenopus* activity was also reported to coelute from a gel filtration column with a fraction of Ran/TC4 (Moore and Blobel, 1994), though whether these proteins directly interact remains to be established. We have found no evidence for association of Ran/TC4 with NTF2 by gel filtration of HeLa cell cytosol (Melchior et al., 1993) and have been unable to detect either GTPase activating or nucleotide exchange activity of NTF2 for Ran/TC4 (Paschal, B. M., F. Melchior, and L. Gerace, unpublished observations).

The maximum of 70% transport inhibition observed in p62-pretreated cytosol presumably reflects residual NTF2 in the permeabilized cell fraction. However, at this point we cannot formally rule out that NTF2 is a stimulatory rather than essential transport factor. This question should be resolved when transport-inhibiting anti-NTF2 antibodies become available.

Given the caveat that permeabilized cells seem to contain residual levels of cytosolic transport factors, it has been shown that reconstitution of NLS ligand binding to the nuclear envelope requires the addition of only two exogenous cytosolic factors: the 55-kD NLS receptor (Adam and Gerace, 1991) and p97 (Adam and Adam, 1994). By contrast, other data have suggested that the *Xenopus* NLS receptor (importin) alone mediates this binding step (Gorlich et al., 1994). The discrepancy is likely related to the 50-fold higher concentration of the *Xenopus* NLS receptor used in the latter study (Gorlich et al., 1994) as compared with the study using mammalian NLS receptor and p97 (Adam and Adam, 1994). Furthermore, the "complete" nuclear protein import supported by saturating levels of importin and Ran/TC4 is 30% of the transport supported by *Xenopus* cytosol (Table I; Gorlich et al., 1994). These data support the view that additional cytosolic factors, including p97 (Adam and Adam, 1994) and NTF2 (this study and Moore and Blobel, 1994), are required to reconstitute protein import fully.

Up to now, five distinct cytosolic transport factors have been identified: NLS receptor (Adam and Gerace, 1991; Gorlich et al., 1994), Hsp70 (Shi and Thomas, 1992), Ran/TC4 (Moore and Blobel, 1993; Melchior et al., 1993), p97 (Adam and Adam, 1994), and NTF2 (this study and Moore and Blobel, 1994). As a group, these factors facilitate multiple transport steps, a view supported by our observation that significant nuclear import occurs only when a cytosolic fraction that mediates NLS ligand docking at the nuclear envelope is combined with Ran/TC4 and NTF2.

Our finding that NTF2 itself can deplete/inactivate at least one additional transport activity from cytosol suggests that

it may be part of a multicomponent complex of cytosolic factors that interact with p62 in the transport cycle. A number of different functional roles can be envisaged for NTF2 in this context. In one scheme, NTF2 could serve as an adaptor molecule that mediates or regulates the hypothetical interaction between the ligand–NLS receptor complex and p62. Alternatively, it could promote structural changes in the p62 complex related to either ligand delivery to the central channel complex or gating of the central channel. The high cytosolic concentration of NTF2 and the fact that similar concentrations of NTF2 and the NLS receptor are required for maximum transport in permeabilized cells are consistent with NTF2 acting as a stoichiometric rather than regulatory component of the transport machinery.

It is interesting to note that p62 is symmetrically localized on both the cytoplasmic and nucleoplasmic sides of the NPC (Cordes et al., 1991; Guan, T., and L. Gerace, unpublished observations). This could suggest functions for putative NTF2–p62 interactions on both surfaces of the NPC during the nuclear import cycle. Alternatively, the symmetrical distribution of p62 could imply that NTF2 functions in nuclear protein export as well. One could imagine that the process of nuclear protein export is mechanistically similar to nuclear protein import, with initial binding of a transport complex to the peripheral nucleoplasmic filaments followed by interactions with the central channel complex containing p62. Although our data strongly argue that NTF2 interacts with p62 during the nuclear import process, we wish to point out that an unequivocal demonstration of this point will probably require arresting transport intermediates in vivo followed by cross-linking and immunoprecipitation.

In conclusion, we have identified NTF2 as a novel cytosolic factor for nuclear protein import that interacts with NPC protein p62. Our evidence suggests that NTF2 functions after the initial docking of NLS ligand with the NPC and that it may be part of a multicomponent system of cytosolic factors assembled at the NPC during the transport cycle. These findings support the emerging view that nuclear protein import is a cooperative effort between multiple soluble factors and the NPC.

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