

Sequence and Characterization of Cytoplasmic Nuclear Protein Import Factor p97

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Abstract. Nuclear location sequence-mediated binding of karyophilic proteins to the nuclear pore complexes is one of the earliest steps in nuclear protein import. We previously identified two cytosolic proteins that reconstitute this step in a permeabilized cell assay: the 54/56-kD NLS receptor and p97. A monoclonal antibody to p97 localizes the protein to the cytoplasm and the nuclear envelope. p97 is extracted from nuclear envelopes under the same conditions as the O-glycosylated nucleoporins indicating a tight association with

the pore complex. The antibody inhibits import in a permeabilized cell assay but does not affect binding of karyophiles to the nuclear pore complex. Immunodepletion of p97 renders the cytosol inactive for import and identifies at least three other cytosolic proteins that interact with p97. cDNA cloning of p97 shows that it is a unique protein containing 23 cysteine residues. Recombinant p97 binds zinc and a bound metal ion is required for the nuclear envelope binding activity of the protein.

THE movement of proteins and ribonucleoproteins between the cytoplasm and nucleus is an important control mechanism in cellular regulation. This transfer of molecules is mediated by the nuclear pore complexes (NPC)¹ which form channels for diffusion and active transport across the double membrane bilayer of the nuclear envelope (NE) (Forbes, 1992; Gerace, 1992). The pore complex is a large 125 MD proteinaceous assembly spanning both membranes of the NE. The eightfold symmetrical NPC consists of two annular subunits, a central transporter and filamentous components extending into both the cytoplasm and the nucleus. Analysis of NPC functions is complicated by its size and number of distinct constituent proteins (Reichelt et al., 1990). Nevertheless, genetic and biochemical analyses have demonstrated the role of NPC proteins in both NE structure and transport (Rout and Wenthe, 1994).

Much of what is known about nuclear protein import has come from systems that reconstitute aspects of the process in vitro (Newmeyer et al., 1986; Adam et al., 1990). The most productive assay uses digitonin permeabilized cultured cells that require exogenous cytosolic factors and energy sources to accumulate karyophilic pro-

teins (Adam et al., 1990). The nuclear accumulation of proteins is mediated by short sequences of basic amino acids called nuclear localization sequences or NLSs (Dingwall and Laskey, 1991). These sequences are necessary and sufficient to direct a protein or inert carrier to the nuclear interior. Early experiments suggested that protein transport is a receptor-mediated process (Goldfarb et al., 1986), prompting the search for proteins that recognize the NLS. A number of groups independently identified possible candidates for such a protein (Yamasaki and Lanford, 1992), but only a 54/56-kD pair of NLS-binding proteins purified from bovine erythrocytes have been shown to be good candidates for an NLS-receptor in a functional assay (Adam and Gerace, 1991).

The import process can be separated into two distinct steps: NLS-dependent binding to the NPC followed by translocation into the nuclear interior (Newmeyer and Forbes, 1988; Moore and Blobel, 1992). Both steps require multiple cytosolic factors, and thus far five have been identified. The binding step involves at least two cytoplasmic factors, the 54/56-kD NLS receptor and a 97-kD protein (Adam and Adam, 1994). Translocation has been reconstituted in permeabilized cells with a crude preparation of binding factors and two purified proteins, the GTP-binding protein Ran/TC4 (Moore and Blobel, 1993; Melchior et al., 1993) and a small Ran-associated protein originally identified as a 15-kD placental protein of unknown function (Moore and Blobel, 1994). Recently, a 60-kD protein was isolated from *Xenopus* oocytes that, with Ran/TC4, reconstitutes import in permeabilized cells (Gorlich et al., 1994). The heat shock protein hsc70/hsp70, restores import activity to cytosol extracts depleted with ATP agarose

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1. *Abbreviations used in this paper:* APC-NLS, allophycocyanin conjugated to synthetic peptides containing the SV40 large T antigen; EST, expressed sequence tag; NE, nuclear envelope; NLS, nuclear localization sequence; NPC, nuclear pore complexes.

(Shi and Thomas, 1992) and antibodies to hsp70 inhibit import when microinjected into cells (Imamoto et al., 1992). Hsp70 may not be required for the nuclear import of all proteins and the exact step affected in import has not been identified (Yang and DeFranco, 1994).

In the current study, we describe the cDNA cloning of the mRNA encoding p97 and determine the intracellular localization of the protein. A specific monoclonal antibody localized p97 to both the cytoplasm and the nuclear envelope. The solubility characteristics of p97 are investigated, addressing potential functions for this protein. The monoclonal antibody inhibits import and can be used to deplete p97, along with several other cytosolic proteins, from soluble import extracts. This antibody will be a potentially valuable tool to investigate the protein import pathway.

Materials and Methods

Cell Culture

Madin-Darby bovine kidney cells (MDBK) were grown in high glucose DMEM containing 10% neonate bovine serum (Biocell Laboratories, Rancho Dominguez, CA), nonessential amino acids and penicillin/streptomycin (GIBCO BRL, Gaithersburg, MD). Cultures were maintained in a humidified incubator at 37°C with 5% CO₂ atmosphere. Cells were removed from plastic dishes by trypsinization and replated on 18 × 18 mm glass coverslips 18–24 h before use. For binding or import experiments, the culture medium was replaced with fresh medium 2–4 h before use.

In Vitro Nuclear Import and Binding

Digitonin extraction of cells and binding/import reactions were carried out as described (Adam and Gerace, 1991; Adam and Adam, 1994). Bovine p97 and 54/56 kD NLS receptor were purified as described previously (Adam and Adam, 1994). The fluorescent transport substrate was allophycocyanin conjugated to synthetic peptides containing the SV40 large T antigen NLS (APC-NLS) as described (Adam et al., 1990). In all experiments, 50- μ l import mixes contained 50–60% reticulocyte lysate, 1 μ g APC-NLS, 0.5 mM ATP, 10 mM creatine phosphate, and 20 μ g/ml creatine phosphokinase. The lysate was prepared from reticulocyte rich rabbit blood (Pel-Freez Biologicals, Rogers, AR) as described (Adam et al., 1991). For quantitation of binding or import, the coverslips were observed by epifluorescence illumination on a Zeiss Axioskop microscope equipped with a 63 × 1.25 NA objective. Images were captured onto a personal computer with a CCD camera (Electrim Corp., Princeton, NJ). The images were analyzed by a computer program written by Dr. Guenter Albrecht-Buehler (Northwestern University). Intensity values were averaged over the entire rim of the nucleus for binding experiments or over the entire nucleus for import experiments.

Preparation of Monoclonal Antibodies

20–50 μ g of purified p97 emulsified in RIBI adjuvant (RIBI Immunochem Research Inc., Hamilton, MT) was injected subcutaneously and intraperitoneally into a NZB mouse (Harlan Sprague-Dawley, Inc., Indianapolis, IN) at 4-wk intervals. After four injections, ~5 μ g of soluble p97 in PBS was injected into a tail vein and the mouse was sacrificed 3 d later. Splenic lymphocytes were fused to Sp2/0-AG14 myeloma cells with 35% polyethylene glycol and distributed into 96-well culture dishes. Hybridomas were screened by immunoblotting using a multi-channel apparatus (Idea Scientific Co., Minneapolis, MN). Positive hybridomas were expanded and cloned by limiting dilution. Antibodies were collected from culture supernatants of hybridoma 3E9 grown in suspension. A crude IgG fraction was obtained by precipitation with 60% ammonium sulfate and stored at 4°C in a 100-fold concentrated form. Mouse IgG was separated from bovine immunoglobulins by affinity chromatography on Sepharose-linked goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO). The purified antibodies were concentrated by dialysis in PBS/50% glycerol and stored at 1–2 mg/ml at –20°C.

cDNA Cloning and DNA Sequence Analysis

p97 was purified from bovine erythrocytes as described (Adam and

Adam, 1994). 100 μ g of protein was resolved by 10% SDS-PAGE and electrotransferred to PVDF membrane. The region containing p97 was visualized by staining with Ponceau S and excised from the blot. Internal peptides were generated by trypsin digestion and the resulting peptides were separated by reverse phase chromatography at the Harvard Microchemistry Facility (Cambridge, MA). Three separate peptide sequences were obtained and used to design degenerate oligonucleotides to the first and third peptides shown in Fig. 5. A 500-bp DNA fragment was obtained by PCR amplification of single-stranded cDNA generated by reverse transcription of oligo(dT) primed MDBK polyadenylated RNA. The fragment was subcloned into pBluescriptII SK– (Stratagene, Inc., La Jolla, CA) for sequencing. Sequencing of double-stranded plasmids was performed in two directions with Sequenase version 2.0 (United States Biochemical, Cleveland, OH). The sequence of the 500-bp fragment was used to search GenBank identifying a 208-bp single stranded human mRNA sequence (T07554 EST) that was 99% identical to the fragment (Adams et al., 1993). A 2.4-kbp clone of T07554 was obtained from the Amer. Type Culture Collection (Rockville, MD). For sequencing, restriction fragments of T07554 were subcloned into pBluescriptII SK–. The 3' end of the coding sequence was obtained by 3' rapid amplification of cDNA ends (3' RACE) of a HeLa λ gt11 library (Clontech Laboratories, Inc., Palo Alto, CA). The full-length sequence was concatenated and subcloned into pBluescriptII SK–. Sequences were checked against known gene sequences in GenBank using the blastx, fasta and the wordsearch programs (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, WI 53711).

In Vitro Transcription and Translation

The full-length cDNA clone containing 5' and 3' untranslated sequences was subcloned into pBluescriptII SK–. The vector was linearized 3' to the inserted gene with XhoI and transcribed in vitro with T7 RNA polymerase (Promega Biotech, Madison, WI). 4 μ g of uncapped mRNA was translated in a rabbit reticulocyte lysate in the presence of 40 μ Ci [³⁵S]methionine. The translation mixture was diluted in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris-HCl, pH 8.0), and 1.4 μ g mAb3E9 bound to 10 μ l protein G agarose was added and mixed for 2 h. After washing in RIPA buffer, the bound proteins were eluted in 1× SDS-PAGE sample buffer.

Immunofluorescence Staining of Cells

MDBK cells grown on glass coverslips were washed in PBS and fixed in 2% formaldehyde/PBS for 20 min at room temperature. For digitonin-extracted cells, cells on coverslips were rinsed in import buffer, permeabilized for 5 min at room temperature with import buffer containing 50 μ g/ml digitonin, and then fixed in 2% formaldehyde/import buffer for 20 min at room temperature. Both sets of cells were finally extracted with 0.1% Triton X-100 in PBS for 2 min at room temperature and stored in PBS until used. Primary antibodies were diluted in 0.2% BSA/PBS at 1 μ g/ml for mAb3E9 and 1:10,000 mAb414 ascites (Berkeley Antibody Co., Berkeley, CA) and incubated on the cells at room temperature for 1 h. After washing the cells extensively, rhodamine-labeled goat anti-mouse (Cappel Research Products, Durham, NC) was diluted in the same buffer at 10 μ g/ml and incubated on the cells for 30 min. After washing in PBS, the coverslips were mounted in 80% glycerol, 50 mM Tris-HCl, pH 8.0, and 0.1% *p*-phenylenediamine.

Antibody Inhibition and Depletion

For antibody inhibition experiments, MDBK cells on coverslips were permeabilized with 50 μ g/ml digitonin in import buffer for 5 min at room temperature. mAb3E9 or mouse IgG was added to rabbit reticulocyte cytosol, binding extract, or import buffer to a final concentration of 50 μ g/ml. Digitonin permeabilized cells were incubated for 15 min on ice in import buffer containing 50 μ g/ml mAb3E9 or mouse IgG. The coverslips were washed in import buffer and inverted on a 50- μ l drop of import or binding mix containing 50 μ g/ml mouse IgG, mAb3E9, or no antibody (for antibody preincubated coverslips). Import and binding incubations were carried out at 30°C and on ice, respectively, for 30 min. After rinsing in import buffer, the cells were fixed in 2% formaldehyde/import buffer for 10 min. The coverslips were mounted in import buffer containing 0.1% *p*-phenylenediamine.

For antibody depletion experiments, mAb3E9 or mouse IgG was bound to protein G agarose in 0.1 M sodium acetate, pH 5.0, at 1 mg Ab/ml agarose. After extensive washing in import buffer, 100 μ l of 3E9-agar-

ose or mouse IgG-agarose was incubated with 500 μ l rabbit reticulocyte lysate for 2 h at 4°C with mixing. The agarose beads were pelleted by centrifugation at 1,000 g in a microcentrifuge and the supernatant was transferred to a fresh tube. The beads were washed 10 \times with import buffer and resuspended in 2 vol of 1 \times SDS-PAGE sample buffer for analysis. Standard import assays were assembled using the depleted lysates at 60% of the final reaction volume (Adam and Gerace, 1991). Reconstitution of import with purified factors was carried out by the addition of 250 ng purified recombinant p97, 200 ng purified native p97 or 5 μ l of crude binding extract. Crude binding extract used in the inhibition and depletion experiments was prepared from bovine erythrocyte lysate as follows. PBS washed bovine erythrocytes were lysed in 2 mM magnesium acetate, 2 mM DTT, and 0.1 mM PMSF on ice for 20 min. The red cell ghosts were pelleted at 100,000 g for 30 min at 4°C, and the supernatant was collected taking care to avoid the loose pellet. The supernatant was bound to a phenyl sepharose column equilibrated in the same buffer and washed extensively. The column was eluted with 20 mM Hepes, pH 7.4, 2 mM magnesium acetate, 80% ethylene glycol and 2 mM DTT. The eluant was extensively dialyzed against import buffer containing 2 mM DTT. This crude fraction is the starting material for purification of the NLS receptor and p97 from erythrocytes (Adam and Gerace, 1991; Adam and Adam, 1994). When the binding mix is incubated on permeabilized cells with the APC-NLS, binding to the nuclear pore complex is observed with very little translocation to the nuclear interior (Adam and Adam, 1994).

Preparation and Fractionation of Nuclear Envelopes

Nuclear envelopes were prepared and extracted under various conditions as described previously for the characterization of NPC protein p62 (Davis and Blobel, 1986).

Bacterial Expression of Recombinant Protein

The full-length p97 sequence was subcloned into pGEX-4T-1 (Pharmacia Biotech, Piscataway, NJ) and transfected into *E. coli* PR745 (New England Biolabs, Beverly, MA). Cells were grown in 500 mL 2XYT-G (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 2% glucose) media containing 100 μ g/ml ampicillin. The cultures were grown to an $A_{600} = 1.0$ at 37°C. Fusion protein expression was induced by the addition of 0.1 mM isopropyl thio- β -D-galactoside (IPTG) for 2 h at 30°C. The cells were collected by centrifugation at 8,000 g for 30 min and lysed in import buffer containing 2 mM DTT and protease inhibitors by freeze thaw in liquid nitrogen. Soluble rp97 was further purified by gel filtration on a Superose 12 column (Pharmacia) in import buffer containing 2 mM DTT.

Zinc-binding Assay

p97-GST fusion protein (rp97) was grown as described with the exception that the 2XYT-G was supplemented with 1 mM ZnCl₂. Column purified rp97 was dialyzed at 4°C against 3 L of 25 mM Hepes, pH 8.0, 10% glycerol, 50 mM KCl, 1 mM EDTA, 0.1 mM PMSF, and 2 mM DTT for 12 h with three buffer changes to remove loosely bound metal ions. The sample was then dialyzed against four changes of 25 mM Hepes, pH 8.0, 10% glycerol, 50 mM KCl, and 0.1 mM PMSF and concentrated fourfold by vacuum dialysis (Schleicher and Schuell, Keene, NH). Protein concentrations were determined by BCA protein assay (Pierce Chemical Co., Rockford, IL). Bound zinc was determined by treating 250 μ g of rp97 in 1 ml with saturating amounts of *p*-hydroxymercuriphenyl sulfonic acid (PMPS) to displace the metal. The free zinc was quantified by the addition 20 μ L 5 mM 4-(2-pyridylazo)resorcinol (PAR) at pH 8.8, and measuring the change in absorbance at 500 nm in an LKB Ultrospec II (Pharmacia) (Erdman and Burtis, 1993).

Other Methods

Proteins were analyzed by SDS-PAGE as described (Dreyfuss et al., 1984). Silver staining of gels was by the method of Rabilloud et al. (1988). Proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes with a Royal Genie Blotter (Idea Scientific, Minneapolis, MN). Membranes were blocked in 5% nonfat dry milk in TBST (25 mM Tris-HCl, pH 8.0, 140 mM NaCl and 0.1% Tween 20). Antibodies were diluted to 0.2 μ g/ml for mAb3E9 and 1:10,000 for mAb414 ascites. Detection on immunoblots was with luminol based chemiluminescence (Schneppenheim et al., 1991) and Kodak XAR5 film. For quantitation of lumigraphs, films were scanned with an LKB laser scanning densitometer (LKB Instruments, Uppsala, Sweden).

For preparation of figures, images on X-ray film or dried gels were scanned directly with a Hewlett Packard ScanJet IIC flatbed scanner using DeskScan II software. After lettering, using Microsoft Powerpoint, the completed figures were printed on a Tektronix Phaser IIDX printer.

Results

Subcellular Localization of p97

Cytoplasmic factors are generally accepted to be required for nuclear protein import in digitonin permeabilized cells. Although these soluble factors are required, knowledge of their localization is necessary to understand the mechanisms in which they take part. We identified p97 originally as a soluble protein from bovine erythrocytes, that in conjunction with the 54/56-kD NLS receptor, reconstituted the NPC-binding step of protein import in digitonin-permeabilized cells (Adam and Adam, 1994). To determine the intracellular distribution of p97 in a nucleated cell, a monoclonal antibody to p97, mAb3E9, was produced. Prior characterization of the *in vitro* import system showed that low concentrations of the glycoside digitonin permeabilized the plasma membrane and released between 20 and 40% of the total cellular protein as soluble protein. However, membrane-bound organelles remained intact, in particular the nuclear envelope (Adam et al., 1990).

Under these conditions, at least 80% of p97 was removed from MDBK cells as determined by immunoblotting with mAb3E9 (Fig. 1). mAb414, a specific antibody for O-glycosylated nucleoporins, was used to compare the solubility of p97 to known NPC proteins (Davis and Blobel, 1986). Under the same extraction conditions, only a

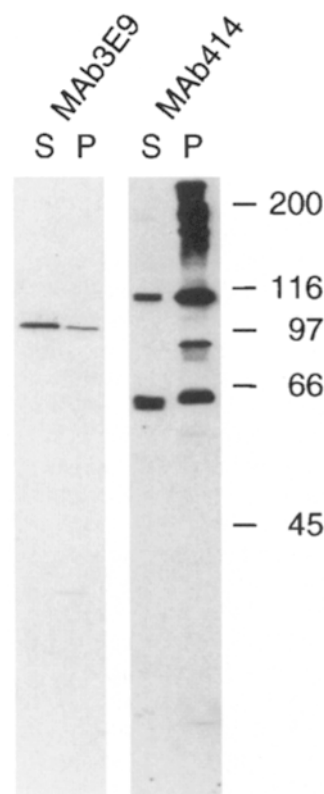


Figure 1. Digitonin solubility of p97 in MDBK cells. Cells grown on plastic dishes were permeabilized with 50 μ g/ml digitonin in import buffer for 5 min on ice. The extraction buffer was removed and centrifuged at 10,000 g for 5 min to remove dislodged cells. The supernatant was precipitated by the addition of trichloroacetic acid to 10% and resuspended in 1 \times SDS-PAGE sample buffer. The unextracted cell material remaining on the dish was rinsed once with import buffer and solubilized in 1 \times SDS-PAGE sample buffer. An equal number of cell equivalents was loaded on each lane. Immunoblotting with mAb3E9 and mAb414 was as described in Materials and Methods. Quantitation of the immunoblots was achieved by scanning lumigraphs exposed within the linear range of the film with an LKB scanning laser densitometer. Molecular masses of standard proteins are indicated on the right.

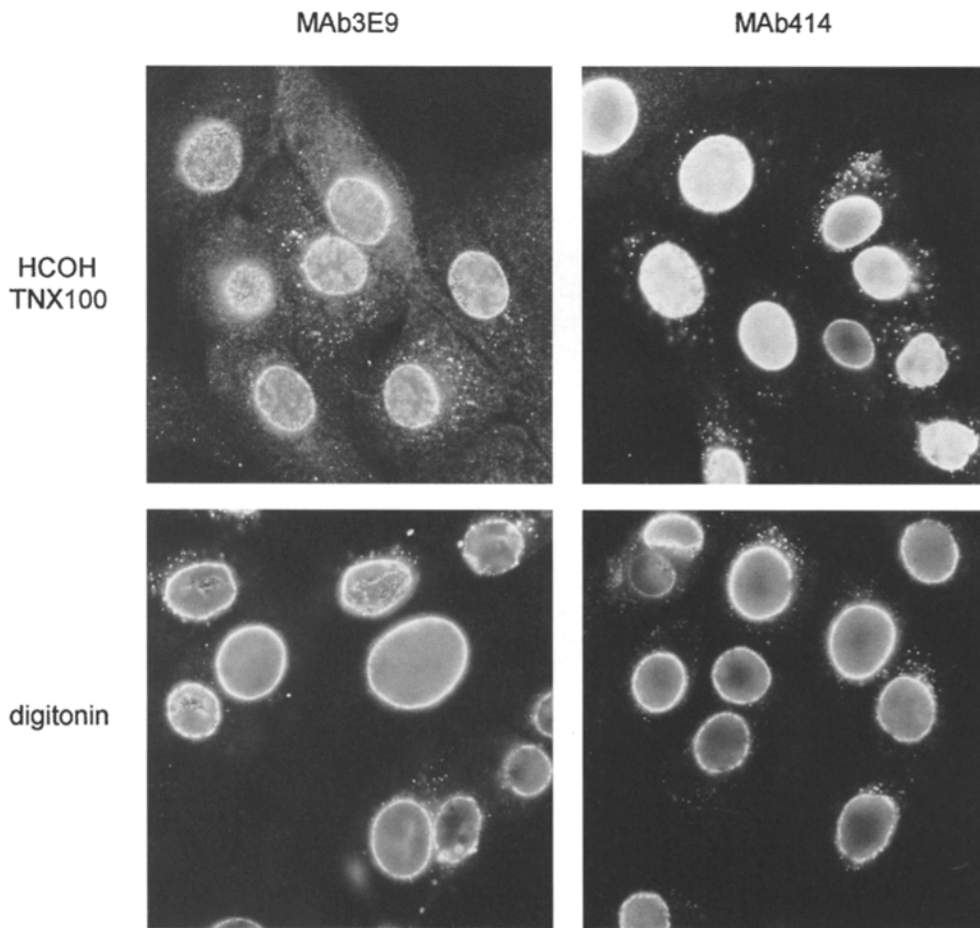


Figure 2. Localization of p97 in MDBK cells by immunofluorescence microscopy. MDBK cells were formaldehyde fixed with (*digitonin*) or without (*HCOH/TNX100*) preextraction with *digitonin*. After solubilization of membranes with Triton X-100, the cells were stained with mAb3E9, a monoclonal antibody to p97, or mAb414, a monoclonal antibody to O-glycosylated nuclear pore complex proteins. The focal plane in each image is near the nuclear surface to highlight the punctate nature of the p97 distribution. The weaker diffuse staining within the area bordered by the nuclear envelope is due to out of focus surface staining in these very flat nuclei. The lack of intranuclear staining is evident by the absence of any defined structure or exclusion of staining from nucleoli.

small amount of the O-glycosylated nucleoporins were released, with less than 20% of p62 solubilized. This soluble p62 likely represents newly synthesized protein that had not yet been assembled into the pore complex (Davis and Blobel, 1986). The low solubility of pore complex proteins is consistent with the retention of nuclear protein import function in permeabilized cells. The high solubility of p97 confirms our earlier results that this cytosolic factor functions in nuclear protein import as a soluble component (Adam and Adam, 1994).

Consistent with the immunoblotting results, indirect immunofluorescence staining of MDBK cells revealed both a cytoplasmic and an NE localization of p97 (Fig. 2). The NE staining with mAb3E9 was punctate and discontinuous along the rim; similar in appearance to staining with mAb414 and wheat germ agglutinin (data not shown). Both antibodies detected multiple large punctate regions in the cytoplasm that likely represent annulate lamellae or precursors to NPC components associated with cytoplasmic structures (Davis and Blobel, 1986). p97 also exhibited a diffuse cytoplasmic localization that is not observed with the NPC glycoproteins. This cytoplasmic p97 was lost from the cells upon permeabilization of the plasma membrane with *digitonin* under the same conditions used to prepare cells for the import assay and for immunoblotting (Fig. 1). The NE localization of p97 or NPC proteins was not altered by the *digitonin* permeabilization nor were the large cytoplasmic spots extracted. Like the NPC proteins, p97 appeared to be largely excluded from the nuclear interior,

although we could not exclude the possibility that some of the diffuse signal contained within the boundaries of the NE was due to internal staining and not out of focus signal from the nuclear surface. Masking of the epitope for mAb3E9 within the nuclear interior also cannot be ruled out.

Because the NE localization patterns were similar with both mAb3E9 and mAb414, rat liver NEs were extracted to determine if p97 fractionated from the NE similarly to the pore glycoproteins. The data in Fig. 3 illustrates that the extractability of p97 from the NE was virtually identical to that reported for p62 (Davis and Blobel, 1986) and other NPC glycoproteins (Snow et al., 1987). p97 was resistant to extraction from the NE with 2% Triton X-100 or monovalent salt concentrations less than 0.5 M. However, the combination of detergent and high salt almost completely extracted p97. These results indicated that NE-

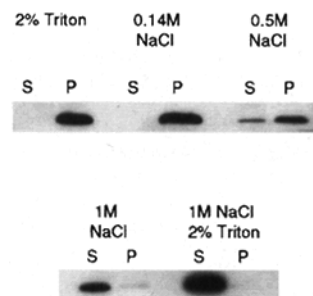


Figure 3. Extraction of p97 from rat liver nuclear envelopes. Rat liver nuclear envelopes were prepared and extracted as described (Davis and Blobel, 1986). The equivalent of 3×10^7 nuclear envelopes was loaded on each lane. Immunoblotting with mAb3E9 and mAb414 was as described in Materials and Methods.

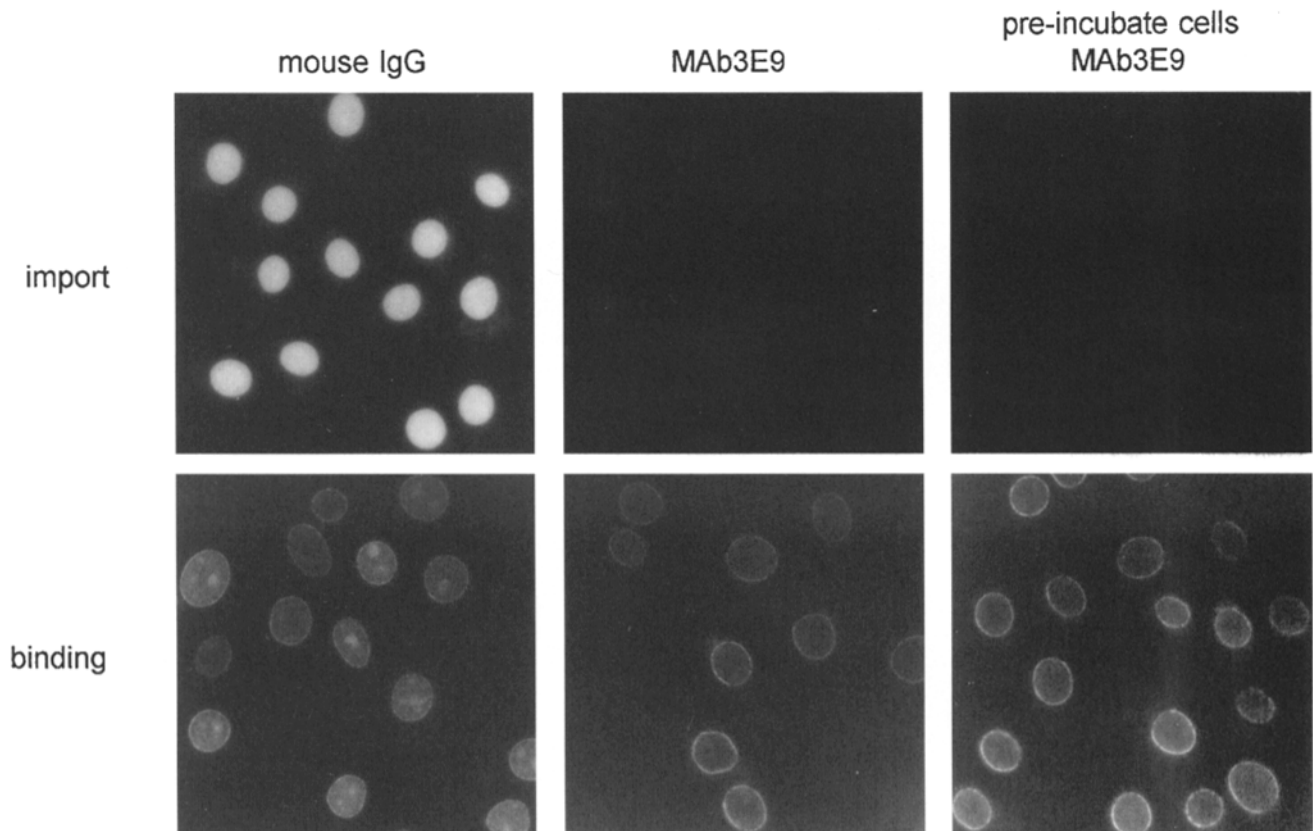


Figure 4. Inhibition of import and pore complex binding with mAb3E9. mAb3E9 or polyclonal mouse IgG was added to 50 $\mu\text{g}/\text{ml}$ to import mixture (*transport*) or to a crude-binding fraction (*binding*) and incubated on ice for 15 min. For preincubation, digitonin permeabilized cells were incubated in import buffer containing 50 $\mu\text{g}/\text{ml}$ mAb3E9 for 15 min on ice. Cells not preexposed to antibody were left in ice cold import buffer for 15 min. After the preincubation, the coverslips were rinsed in five changes of import buffer over a 5-min period. The import and binding reactions were then carried out without added antibody.

localized p97 was not membrane associated and was as tightly associated with the NPC as the pore glycoproteins.

Inhibition of Import with mAb3E9

Further experiments were carried out with mAb3E9 to characterize the role of p97 in the binding of NLS-bearing proteins to the NPC and translocation into the nucleus. The addition of mAb3E9 to cytosol in the import incubation abolished the accumulation of APC-NLS within the nucleus (Fig. 4). To determine the effect of mAb3E9 binding to NPC-associated p97, permeabilized cells were preincubated with the antibody before the import reaction was carried out. Under these conditions, import was completely inhibited also. Whether import was blocked by addition of the antibody to the import reaction or by preincubation of the antibody with the permeabilized cells, no accumulation of the NLS-APC at the pore was observed. We have observed that reticulocyte and red cell lysates contain an activity that prevents binding to the pore when translocation is inhibited (Adam et al., 1990; Adam and Adam, 1994). This inhibitory activity can be physically separated from binding activity by phenyl sepharose chromatography (Adam and Adam, 1994).

To determine the effect of mAb3E9 on NLS-mediated binding to the pore, the antibody was added to a partially purified cytosol extract containing components required for binding to the NPC (see Materials and Methods) or

preincubated on the permeabilized cells as described above. Purified NLS receptor and p97 were not used in these experiments because results of experiments presented below suggested that additional factors might participate in binding. APC-NLS binding to the pore was not decreased in the presence of antibody and, in fact, binding was reproducibly increased when antibody was preincubated with the cells. This may be due to cross-linking of binding complexes at the pore by the bivalent antibody or alternatively, additional binding sites may become exposed on the NE in the presence of the antibody by displacement of bound import complexes. It is interesting to note that the small amount of import seen in the presence of binding components was completely inhibited by mAb3E9. Together, these results suggest that the antibody inhibition of import was due not to immunoprecipitation of p97 from the cytosol, but to a direct interaction of mAb3E9 with p97. The point of inhibition must be at a step subsequent to binding at the NPC since binding was unaffected.

cDNA Cloning of p97

Protein sequences of p97 from internal peptide fragments generated by trypsin digestion of purified bovine p97 were used to isolate a full-length cDNA clone for the protein. Three peptide sequences were obtained and used to design degenerate oligonucleotide primers for PCR amplification of bovine cDNA. A specific DNA product was iso-

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-45  cgggccgtgctcttaggaggatgcgcccgcggccactcgcgcatgagctgatcaccattctcgaagaccgtgctcccgcgg
      M E L I T I L E K T V S P D R
45  ctgagctggaagccgcccgaagtctcggagcgtgcggcgtaggagaccctgccaccctctcttggaactgccagctgccagctgctgca
      L E L E A A Q K F L E R A A V E N L P T F L V E L S R V L A
135  aatcaggaacacgctcaggtccgagctgagctgctcacaatcaagaactcttgacatcaagctcagatcagatcagagccaca
      W P G S H S A R V D A E G S I P S D P A I S P R P D
225  taccagcagggctgctcttagctatgctcaatgctcagcagaagctcaagaactctgcttgacacacatggatcagcaaacctaccg
      Y Q O R W L A I D A N A R E V K N Y V L H T L G T E T Y R
105  cctagttctcctcagctgctgctgctgcttctgctgagagctccagcagtaaacacagctgcccagaactctctcagctggtggcc
      P S S A S O C V A G I A C A E I P V N Q W P E L I P Q L V A
135  aatgctacaacccccacagcagcagcagcagctgagggagctgcaatggagaccatcgttatttgcacagatagaccagccagccag
      W V T W P N Y I S T E R H K E S T L E A I G Y I C G D I D P P E Q
405  ctcaagaataaacctcaatgagattctgctgcctcaatccagggagtaggaaagagagccttagtaataatgtagagctgctgca
      L O D K S N E I L T A I I Q G M R K E E P S N N V K L A A T
195  aatgactcctcgaactctggagctccacaagaacacttgatnaagagctgaaagacccttatatgacagctgctggaagc
      N A L L R S L E E T R Y H F D K E S E R H F I W G V V C E A
675  acagctgctcagatcagggatcagggctgctctcagagctgctgtagagctccttccttcctcagctcagatggagcactat
      T O C P D T R V R V A A L Q N L V K I M S L Y Y O Y M E T Y
225  atggtctgctctcttgcactcaatcagaagcaagaactgatgaggtgctctcagaagcagatagactctgctcact
      M G P A L F A I T I E A M K S D I D E V A L Q G I E F W S N
285  gctctgctggaagatggatttgcaccatcgaagctcagagggcagcagaagagggcccccagcaccagcagctttatgctg
      V C D E E M D L A I E R S E A A E Q E R P P P E H T S K F Y A
855  aagggagcactcagctcgttccactctcaccagcagcactcaactcaacagggagcaaatgatgagcagctgagcagcctcctc
      K G A L Q Y L V P I L T Q T L T K G D E N D D D D W N P C
345  aagagcaggggtgctcctcagctcgtctggaagctgctgcaagctgcaactgctccctcctcaatcaagaacactc
      K A A G V C L M L L A T C C E E D D I V P H V L P F I K E H I
375  aagaccagctgctgagctcagagctcagagctgagcttctgctgcttctggaagaccagagccctcagctcaaacaccactc
      K N P D G S Y R D A E G S I P S D P A I S P R P D
405  gttatcagagctatgcccaccctcaatgagatgattgaagaccagctgctgctgctcagagctcagctgctgagctcagagc
      V I Q A M P T L I E L M K C C C C S V T V V I R D T A A W T V G R I
435  tggagctctcctcgaagctgctcagctgctcagctcctcctgctcagctgctcagctcagctcagctcagctcagctcagc
      C E L L P L C L P L E G L A E P R R
465  gggctctcaatgctgctgctgctcctcagctcctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctg
      V A S N V C W A F S S L A E A Y E A A D V A D D D E E P A
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      T Y C L S S F E L I V Q K L L E T T D R P D G G H Q N N L R
525  agtttgcattgactctgtaggaatctgaaacagctgccaagagctgctcctcctcctcagagcagctcagctcagctcagct
      S A A Y E S L M E I V E N S A K D C Y P A V V Q K T L V I N
555  gaagcagctcagagctcctcagagctcagagctcagagctcagagctcagagctcagagctcagagctcagagctcagagct
      E R L Q Q V L Q M E S H I G C S T D R I Q F N D L Q S L L C
585  gcaactcctcagaagtctcctcaggaagctgcaacatcaagctctgcaagctcctgctgctgctgctgctgctgctgctgct
      K T L Q N V L R K V Q H Q D A L Q I S D V V M A S L L R M F
615  caaacccagctgctgctgagggatcagagggatgctcctcctcctcagagctgctcagagctgctgagagctgctgagagct
      Q S T A G S S G V Q G E D A L M A V S T L V E V L G G E F L K
645  tacaagggccttaaacctcctcagagctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctg
      Y M E A F K P F L L G I G L K N Y A E Y Q V C L A A V G L V G
675  gacttgctgctgctgctgctcctcagagctcagagctcagagctcagagctcagagctcagagctcagagctcagagctc
      D L C R A L D S W I T P F C D E V H Q L L E M L G M E N V
705  cacagctgctgagagctcagagctcagagctcagagctcagagctcagagctcagagctcagagctcagagctcagagctc
      L N T L Q O A S Q G D M V D K S D Y D M V D Y L N E L R E S C
735  tggagcctcagctgagagctcagagctcagagctcagagctcagagctcagagctcagagctcagagctcagagctcagag
      L E A Y T G I V Q G L K G D G E N V H P D V M L V Q P R V E
765  tctatctgctcctcctcagagctcagagctcagagctcagagctcagagctcagagctcagagctcagagctcagagctc
      F I L S L D N I A G D E H D V L V C A A L L A L
795  ttacagcatttgggaagagctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctg
      C T A F G K D V L K L V E A R P M I H E L L T E G R S K T
825  aacaaagcacaacacctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctg
      N K A K T L A R W A T K E L R K L K N Q A *
846  ctgagggcccccaggagatccctcctcttggaa

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Figure 5. Sequence of p97 cDNA. The entire open reading frame encoding p97 is shown with full 5' and partial 3' flanking sequences. The 3' end extends ~300 nucleotides to the poly(A) tail. Nucleotides are numbered on the left with the A of the start codon numbered 1. Amino acid residues are numbered on the right with the first methionine numbered 1. Single underlines indicate the sequences of the peptides obtained from protein sequencing. The first and the third peptide were used to design degenerate oligonucleotides for PCR amplification. The double underlines indicate a repeated sequence in the carboxyl-terminal portion of the protein. These sequence data are available from GenBank/EMBL/DBJ under accession number L39793.

lated and sequenced and the sequence was used to search the nucleotide sequence databases for related sequences. The search revealed complete homology for the DNA fragment to a sequence in the expressed sequence tag (EST) database, clone T07554 (Adams et al., 1993). A 2.4-kbp clone was obtained from American Type Culture Collection and sequenced (Fig. 5). The 5' end of the EST clone contained a putative start site for translation (Kozak, 1987), but no termination codon was found in frame in the remainder of the sequence. The 3' end of the sequence was obtained by 3' RACE from a λ gt11 HeLa cDNA library and sequencing of multiple products. The open reading frame of the sequence shown in Fig. 5 encoded a 97,233-kD protein with a predicted isoelectric point of 4.54. 31% of the amino acids contain hydrophobic side chains in agreement with the hydrophobic nature of the protein observed during purification of p97 (Adam and Adam, 1994). A search of the sequence databases with the entire coding sequence did not yield any sequences with significant homology. The protein contained 23 cysteine residues, which correlated well with the observation that the pore complex-binding of p97 was sensitive to inactivation by sulphhydryl alkylating reagents (Adam and Adam,

1994). The cysteines were well distributed throughout the sequence with no apparent cysteine-rich domains. An additional sequence of 12 amino acids was present twice in the carboxyl-terminal portion of the protein with a third apparently degenerate sequence between the two. The function of this sequence is not known, although the conserved cysteine residues in the sequence should be noted. After the submission of this manuscript, the sequence of a rat 97-kD protein implicated in the binding step of import was reported by Radu and coworkers (Radu et al., 1995). The sequence of rat p97 is 99% identical to the sequence of human p97 reported here.

The entire cDNA coding sequence was subcloned into a vector encoding a glutathione-S-transferase fusion protein and expressed in bacteria (rp97). mAb3E9 was unable to detect the bacterially expressed rp97 by immunoblotting or immunoadsorption suggesting that the epitope recognized by this antibody is a eukaryotic posttranslational modification (data not shown). Quantitative immunoadsorption of p97 from 32 P-labeled HeLa cells indicated that p97 was not phosphorylated (data not shown) although other potential modifications are currently being investigated. In vitro translation of the mRNA transcribed from the cDNA clone by T7 RNA polymerase produced a 97-kD protein that was immunoadsorbed by mAb3E9 (Fig. 6). However, the addition of rp97 to a rabbit reticulocyte lysate failed to convert the protein to a form recognized by the antibody. Thus, either the potential modification is added cotranslationally, or the reticulocyte lysate is incapable of efficiently performing the modification.

Depletion of Import Activity with mAb3E9

mAb3E9 bound to protein G-agarose beads was also used

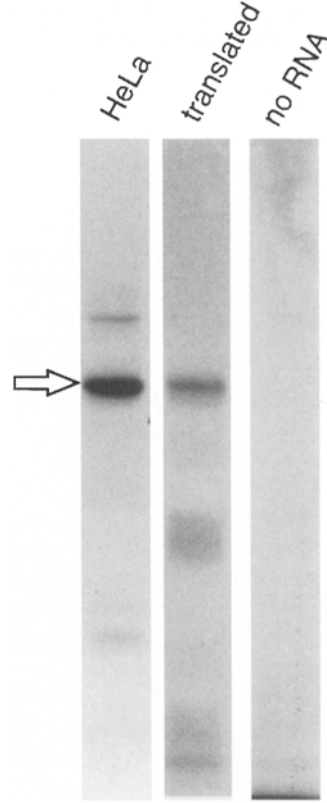


Figure 6. In vitro translation of p97 mRNA. p97 mRNA was transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate. The translation products from the recombinant mRNA and a reaction with no RNA added were immunoadsorbed with mAb3E9 and resolved by SDS-PAGE. For comparison, HeLa cells were labeled with [35 S]methionine, solubilized in RIPA buffer, and p97 immunoadsorbed with mAb3E9. The arrow indicates p97.

A

	percent of control
control	100
mouse IgG	110
MAb3E9	17
+ recombinant	38
+ native p97	39
+ binding mix	82

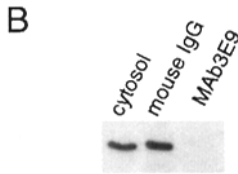


Figure 7. Immunodepletion of import activity with mAb3E9. Rabbit reticulocyte lysate was immunodepleted with mAb3E9 or mouse IgG bound to protein G-agarose beads. (A) Equivalent amounts of cytosol were assayed for import activity. Quantitation of import was as described in Materials and Methods. rp97, purified native p97 or a crude binding mix was used to complement the depleted activity. (B) Immunoblot analysis of the depleted lysates.

to deplete p97 from cytosol before the import assay. Removal of p97 from the cytosol decreased the accumulation of import substrate by ~80% when compared to cytosol incubated with protein G-agarose beads containing mouse IgG (Fig. 7 A). Immunoblot analysis of the depleted cytosol demonstrated that greater than 95% of p97 was removed by the mAb3E9 beads, consistent with the degree of import inhibition (Fig. 7 B). Import was not completely inhibited, probably due to the residual p97 and other cytoplasmic factors left in the permeabilized cell (Figs. 1 and 2) combined with cytosolic factors that were not depleted.

Only a fraction of the import capacity of the cytosol could be restored by the addition of either rp97 (fraction A, Fig. 9 B) or native p97. However, addition of a crude fraction containing pore complex binding activity restored most of the import activity to the depleted cytosol. These data suggested that additional cytoplasmic factors were depleted along with p97 by mAb3E9. To test this possibility, the proteins bound to the mAb3E9 beads used for the depletion were eluted in SDS-PAGE sample buffer and analyzed (Fig. 8). In addition to p97, a protein of 116 kD was adsorbed specifically in approximately equivalent amounts to p97. Two other proteins with relative molecular weights greater than 200 kD were adsorbed specifically also, but in much lower amounts. It is likely that the 54/56-kD NLS receptor was adsorbed to the beads, although it is not possible to tell from this analysis because the receptor comigrates with the IgG heavy chain. Proteins of similar molecular weights are also coadsorbed with p97 from [³⁵S]methionine-labeled MDBK cells (manuscript in preparation). We were unable to elute these proteins from the beads in a way that preserved their ability to restore import to the depleted cytosol (data not shown).

Zinc Ions Are Essential for Activity of p97

The number of cysteines and the *N*-ethylmaleimide (NEM) sensitivity of p97 suggested that the protein might bind one or more metal ions (Adam and Adam, 1994). However, the amino acid sequence of p97 did not fit any of the known consensus sequences for metal-binding proteins. To determine if p97 was dependent upon zinc for its activity, bacteria producing the recombinant protein were grown in the presence of zinc and rp97 was purified by gel filtration chromatography. Our initial characterization of

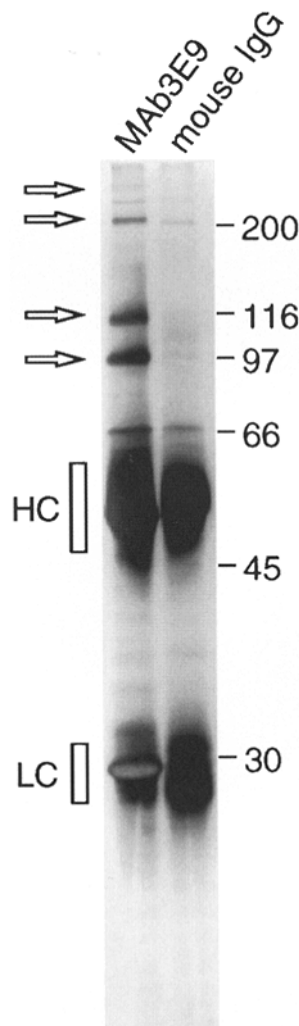


Figure 8. SDS-PAGE analysis of proteins immunoadsorbed from reticulocyte lysate by mAb3E9. The proteins bound to the protein-G agarose in the immunodepletion were eluted in SDS sample buffer and resolved by SDS-PAGE. The bound proteins were detected by silver staining. The immunoglobulin heavy and light chains are indicated by open boxes. The four proteins specifically depleted with mAb3E9 are indicated by arrows.

p97 failed to detect the protein in high molecular mass complex as had been suggested for the binding activity from *Xenopus* oocytes (Newmeyer and Forbes, 1988; Moore and Blobel, 1994). Sizing of the recombinant protein indicated that rp97, a GST fusion protein, migrated as if it were a 200-kD protein by gel filtration (data not shown). We have not yet determined whether the apparent size of p97 was due to addition of the 30-kD GST fusion protein or if rp97 dimerized under these conditions.

Two consecutive fractions from the gel filtration column containing rp97 were assayed for their ability to reconstitute NPC binding in the presence of native purified NLS receptor. Only one of the two fractions, designated A in Fig. 9, A and B, was able to reconstitute binding of the APC-NLS to the NPC. Neither NLS receptor nor rp97 alone had binding or translocation activity. In agreement with our previous results, *N*-ethylmaleimide treatment of rp97 abolished the binding activity seen with purified NLS receptor (data not shown; Adam and Adam, 1994).

Each of these fractions was then assayed for the presence of zinc using a spectrophotometric detection system to identify sulfhydryl coordinated heavy metal ions (Erdman and Burtis, 1993). Only the fraction that was able to reconstitute binding, A, contained detectable zinc in an approximately 1:1 molar ratio with rp97 (Table I). The res-

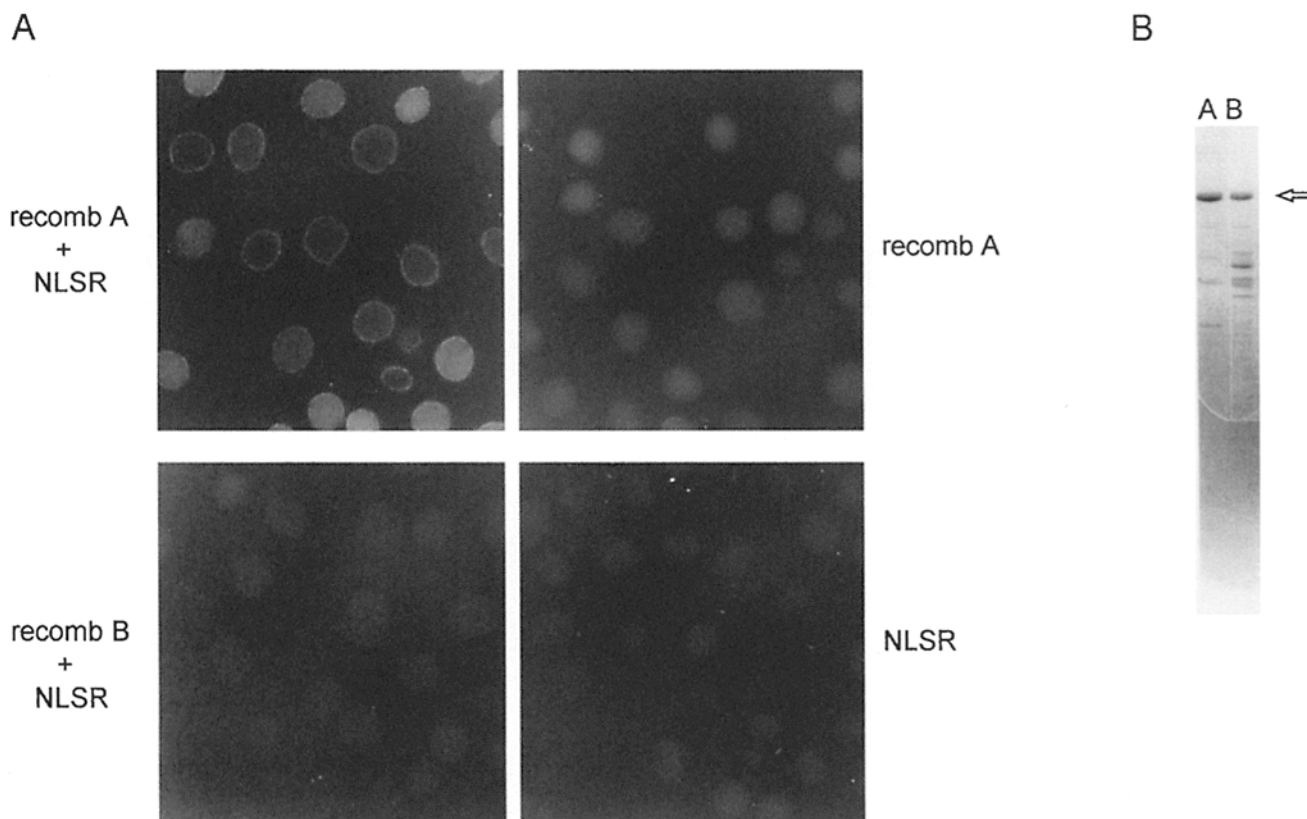


Figure 9. Binding activity of recombinant p97. Recombinant p97 grown in the presence of zinc was partially purified by gel filtration chromatography. (A) Two fractions, A and B, containing rp97 were assayed for reconstitution of binding with purified NLS receptor. Recombinant A and NLS receptor alone are shown. (B) SDS-PAGE of recombinant A and B stained with Coomassie blue. The arrow indicates the rp97.

elution of the gel filtration column was such that typically 90% of an individual protein species will elute in a 1-ml fraction. Therefore, fractions A and B probably represent two distinct populations of rp97, and the small amount of zinc associated with fraction B may be from the trailing edge of the peak of fraction A. The amount of zinc bound should be considered a preliminary estimate of the true molar ratio of protein to metal due to the presence of other minor protein species or rp97 degradation products in each fraction. We also cannot say with certainty that zinc is the active metal ion present in p97 until atomic adsorption analysis of the native protein is completed.

Discussion

A number of proteins have been identified as putative components of the nuclear protein import machinery. These proteins include the 54/56-kD NLS receptor/importin, Ran/TC4, B2/pp15, p97, and hsc70/hsp70 (for review

see Powers and Forbes, 1994). While the general biochemical activities of some of these are known (GTP hydrolysis, NLS binding), the role of others remains unknown. Other than general nuclear or cytoplasmic localization, none of these factors have been localized in sufficient detail to aid in determining their function in the import process. Such inadequate localization and characterization make any attempts at constructing models to explain import speculative at best. The localization and biochemical characterization of one of these factors, p97, are important steps in understanding the role of cytoplasmic factors in protein import.

NLS-mediated Targeting to the Pore Complex

Cytosolic activities required for binding of karyophiles to the pore complex have been identified in both *Xenopus* oocytes and bovine erythrocytes (Newmeyer and Forbes, 1990; Moore and Blobel, 1992; Adam and Adam, 1994; Gorlich et al., 1994). These cytoplasmic factors likely include molecules for the recognition of an NLS and delivery to the pore complex. Binding activity was identified in *Xenopus* oocyte extracts and partially purified into crude fractions called NIF-1 (Newmeyer and Forbes, 1990) and fraction A (Moore and Blobel, 1992). Fraction A has recently been analyzed in more detail and found to contain a 97-kD protein and two 55-kD protein components (Radu et al., 1995). A 60-kD *Xenopus* oocyte protein that alone reconstitutes NLS-mediated binding to the NPC in perme-

Table 1. Zinc Binding by Recombinant p97

Fraction	Mass of protein	Moles of protein	Moles zinc	Zn/protein
A	.257 mg	2.65 nmol	2.50 nmol	0.95
B	.225 mg	2.32 nmol	0.30 nmol	0.13

Purified recombinant p97 shown in Fig. 9 was quantitated by BCA assay and bound zinc determined by displacement of the metal from the protein and detection of free metal with 4-(pyridylazo)resorcinol (see Materials and Methods).

abilized cells has also been purified and sequenced (Gorlich et al., 1994). Interestingly, this protein is 44% identical to a yeast protein originally identified as a suppressor of temperature sensitive mutants of RNA polymerase I, Srp1p (Yano et al., 1992). Several forms of Srp1p have been identified in human cells; human SRP1 and Rch1, both which interact with the recombination activating gene product RAG-1 in yeast two hybrid assays (Cuomo et al., 1994; Cortes et al., 1994) and NPI-1 which interacts with the influenza virus nucleoprotein (O'Neill and Palese, 1995).

Our previous results on the identification of erythrocyte cytosol factors involved in the binding step of import in permeabilized cells indicated that at least two factors were involved: the 54/56-kD NLS receptor and a 97-kD protein (Adam and Adam, 1994). Protein and cDNA sequence analysis of the 54/56-kD bovine NLS receptor identify them as homologs of Srp1p, also (manuscript in preparation). It has not been shown that the *Xenopus* 60-kD protein specifically binds an NLS, but given the sequence similarity to the bovine NLS receptor, it seems likely that it will.

An Import Complex for Recognition of NLS-bearing Proteins

Although the NLS receptor and p97 are sufficient to target a NLS-bearing protein to the pore complex, the coadsorption of other previously unidentified proteins with antibodies to p97 suggests that multiple proteins may be involved in both the binding and translocation steps in the form of an import complex. Some of these proteins may not be essential, but may act as accessory factors to increase the efficiency of binding or translocation. Alternatively, they may represent factors that are not required for import in permeabilized cells, but are required in intact cells. It should be noted that digitonin-permeabilized cells may be partially or wholly uncoupled from regulatory mechanisms that influence transport, pore complex-mediated processes, or other NE functions (Moll et al., 1991; Rihs et al., 1991; Greber and Gerace, 1995).

The stable association of both the NLS receptor and p97 with the nuclear envelope suggests a strong association of an import complex with the pore (Adam et al., 1989). This may be due to multiple interactions of the import complex with nucleoporins or a single strong interaction through one component. In yeast, Srp1p has been localized primarily to the nucleus and nuclear envelope (Yano et al., 1992; Belanger et al., 1994). Belanger and coworkers identified Srp1p as a component of the nuclear pore complex interacting with the nucleoporins Nup1p and Nup2p in a two hybrid assay, and by adsorption to immobilized Nup1p (Belanger et al., 1994). A GST-Nup1p fusion protein specifically adsorbs both Srp1p and a 95-kD protein from yeast lysates. In addition, the carboxyl terminus of Nup1p binds a 100-kD protein. It is possible that either the 95- or 100-kD protein is the yeast homolog of human p97. The inhibition of NLS-mediated binding to the pore complex by WGA also points to a role for the O-glycosylated nucleoporins in recognition of an import complex (Moore and Blobel, 1992; Adam and Adam, 1994; Radu et al., 1995).

What are the possible roles of the NLS receptor and p97

in binding to the pore complex and translocation through the pore channel? We suggest that, since both NLS receptor and p97 have a strong interaction with the pore complex and are required simultaneously for binding karyophiles to the pore (Adam and Adam, 1994), the two proteins together form a structure that is recognized by multiple elements of the pore complex. While it is possible that the initial interaction with the pore is through cytoplasmic filaments extending from the cytoplasmic annulus (Richardson et al., 1988), analysis of pore complex proteins interacting with the import complex and immunoelectron microscopy will be required to resolve this issue. Although in yeast Srp1p interacts directly and independently with Nup1p and Nup2p (Belanger et al., 1994), it is possible that this represents an intermediate step in translocation after an initial binding event mediated by p97. Biochemical fractionation of rat liver cells suggests that the NLS receptor is at least partially localized to the nuclear interior (Adam et al., 1989). Indirect immunofluorescence results presented here indicate that p97 probably does not enter the nucleus, although either rapid recycling or masking of the epitope cannot be excluded. At some point in translocation, the import complex must be dissociated, with only part of the complex entering the nucleus.

Zinc Binding by p97

The predominant functions of zinc in proteins are to stabilize the tertiary folding structure of a protein forming a DNA or protein interaction domain, or as a catalytic component of an enzyme. The role of zinc in p97 function is unknown. The sequence of p97 does not contain any of the known consensus sequences for metal-binding proteins. The strongest sequence similarity is to the TFIIIA type zinc finger (Berg, 1990), although the spacing between cysteines and histidines is different and the orientation of the residues is reversed. If other amino acid residues thought to be involved in metal-binding domains, such as aspartate and glutamate are considered, additional possibilities for zinc-binding regions arise. Clarification of the metal-binding properties of p97 will require a much more detailed spectroscopic analysis to identify the potential amino acid residues involved. A pore complex protein, Nup153, contains four zinc fingers and binds DNA in vitro (Sukegawa and Blobel, 1993). We have not yet determined if p97 is capable of binding DNA, but given its predominantly cytoplasmic localization, it would seem unlikely that DNA binding would be the role of a zinc-binding domain.

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