

Molecular and Functional Characterization of the p62 Complex, an Assembly of Nuclear Pore Complex Glycoproteins

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Abstract. Macromolecular trafficking across the nuclear envelope involves interactions between cytosolic transport factors and nuclear pore complex proteins. The p62 complex, an assembly of 62, 58, 54, and 45-kD O-linked glycoproteins localized near the central gated channel of the nuclear pore complex, has been directly implicated in nuclear protein import. The cDNA cloning of rat p62 was reported previously. We have now carried out cDNA cloning of rat p58, p54, and p45. We found that p58 contains regions with FG (Phe, Gly) and PA (Pro, Ala) repeats at both its NH₂ and COOH termini separated by a predicted α -helical coiled-coil region, while p54 has an NH₂-terminal FG and PA repeat region and a COOH-terminal predicted coiled-coil region. p45 and p58 appear to be generated by alternative splicing, with p45 containing the NH₂-terminal FG repeat region and the coiled-coil region of p58. Using immunogold electron microscopy, we found that p58/p45

and p54 are localized on both sides of the nuclear pore complex, like p62. Previous studies have shown that immobilized recombinant p62 can bind the cytosolic nuclear import factor NTF2 and thereby deplete transport activity from cytosol. We have now found that immobilized recombinant p58 and p54 also can deplete nuclear transport activity from cytosol, and that p62, p58, and p54 bind directly to the cytosolic nuclear import factors p97 and NTF2. At least in the case of p58, this involves FG repeat regions. Moreover, p58 can bind to a complex containing transport ligand, the nuclear localization sequence receptor (Srp1 α) and p97. These data support a model in which the p62 complex binds to a multicomponent particle consisting of transport ligand and cytosolic factors to achieve accumulation of ligand near the central gated channel of the nuclear pore complex.

THE nuclear pore complex (NPC),¹ a large supramolecular assembly spanning the nuclear envelope (NE), mediates molecular exchanges between the nucleus and cytoplasm. The NPC contains aqueous channels that accommodate the passive diffusion of molecules smaller than ~20–40 kD, but transport of most macromolecules occurs by signal-mediated, energy-dependent mechanisms (reviewed by Fabre and Hurt, 1994; Panté and Aebi, 1994; Rout and Wentz, 1994; Melchior and Gerace, 1995; Görlich and Mattaj, 1996). Targeting signals for nuclear protein import, termed nuclear localization sequences (NLSs), are usually short stretches of amino acids enriched in basic residues that can occur as single or bipartite motifs (Kalderon et al., 1984; Robbins et al., 1991).

A combination of in vitro and in vivo studies have shown that nuclear protein import is a complex multistep process involving interactions between several cytosolic factors and NPC proteins. The pathway of nuclear import is believed to involve interaction of a transport ligand complex with the cytoplasmic periphery of the NPC (Newmeyer and Forbes, 1988; Richardson et al., 1988), movement to central regions of the NPC, and translocation to the nuclear interior (discussed by Melchior and Gerace, 1995). Understanding of NLS-mediated nuclear import has been strongly advanced by the development of in vitro assays that faithfully reconstitute this process (Newmeyer et al., 1986; Adam et al., 1990). The most frequently used assay involves digitonin-permeabilized cells supplemented with exogenous cytosol and a fluorescent NLS-containing transport ligand (Adam et al., 1990). Studies with this and similar systems have led to the identification of five different cytosolic factors involved in nuclear protein import (reviewed by Powers and Forbes, 1994; Adam, 1995; Sweet and Gerace, 1995): the NLS receptor and its homologues (karyopherin α , importin 60, Srp1 α , or Rch1) (Adam et al., 1989; Görlich et al., 1994; Moroianu et al., 1995a; Weis et al., 1995), p97 (also termed importin 90 and karyopherin β) (Adam and Adam, 1994; Chi et al., 1995;

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1. *Abbreviations used in this paper:* GST, glutathione S-transferase; FG, Phe, Gly; NE, nuclear envelope; NLS, nuclear localization sequence; NPC, nuclear pore complex; NRK, normal rat kidney; ORF, open reading frame; PA, Pro, Ala.

Görlich et al., 1995; Radu et al., 1995a), the small GTPase Ran/TC4 (Melchior et al., 1993; Moore and Blobel, 1993), NTF2 (also termed p10) (Moore and Blobel, 1994; Paschal and Gerace, 1995), and Hsp70/Hsc70 (Imamoto et al., 1992; Shi and Thomas, 1992).

The role of these cytosolic factors in nuclear import is beginning to be elucidated. The NLS receptor is believed to act as a shuttling carrier that interacts with NLS-containing ligands in the cytoplasm and carries them to the nuclear interior (Adam and Gerace, 1991; Melchoir and Gerace, 1995). The NLS receptor has been found to directly interact with p97, which may act as an adaptor for the binding of the NLS receptor-ligand complex to various sites in the NPC during the translocation process (Adam and Adam, 1994; Görlich et al., 1995; Moroianu et al., 1995a,b). Ran appears to participate in an early step of protein import at the NPC, in which GTP hydrolysis by Ran may determine commitment to transport and help specify vectoriality (Melchior et al., 1995). The cytosolic factor NTF2 appears to be involved in a transport step subsequent to initial binding of ligand to the NPC (Moore and Blobel, 1994; Paschal and Gerace, 1995), but its precise functions are unknown. The transport functions of the heat shock protein Hsp70 are undefined, and this factor appears to be required for the nuclear import of only certain NLS-containing ligands (Yang and DeFranco, 1994).

The framework of the NPC consists of peripheral cytoplasmic and nucleoplasmic rings flanking a set of central spokes (Hinshaw et al., 1992; Akey and Radermacher, 1993). The gated transport channel, which is involved in signal-mediated transport of macromolecules, is situated in the center of the spokes. It is estimated that the NPC contains >100 different proteins (nucleoporins). So far, only a few of these have been molecularly characterized (see Rout and Wentz, 1994). Vertebrate NPC proteins include a group of at least nine polypeptides ranging from 45–365 kD that are modified with O-linked *N*-acetylglucosamine (Davis and Blobel, 1987; Snow et al., 1987). The direct participation of at least some of these glycoproteins in nuclear protein import has been suggested by a large body of evidence (discussed by Melchior and Gerace, 1995).

All of the O-linked glycoproteins of the vertebrate NPC that have been characterized by cDNA cloning contain multiple (up to 20 or more) FG (Phe, Gly)-containing repeats, which are often part of larger motifs (e.g., FxFG or GLFG). Similar FG-containing motifs are found in a number of yeast nucleoporins (see Rout and Wentz, 1994), but the relationship between these proteins and specific mammalian FG repeat nucleoporins is unclear. While the functions of the FG repeat motifs are unknown, it appears that the FG-containing regions of some vertebrate nucleoporins interact with cytosolic transport factors (Paschal and Gerace, 1995; Radu et al., 1995b).

Four of the mammalian O-linked glycoproteins, having mobilities of about 62, 58, 54, and 45 kD, exist as a stable "p62 complex" that can be isolated by biochemical fractionation after chemical solubilization of NEs (Finlay et al., 1991; Kita et al., 1993). Recent studies have shown that the p62 complex forms a donut-shaped particle with a diameter of ~15 nm and have suggested that it contains one copy of each of the four subunits (Guan et al., 1995). p62

has been shown to be localized on both the nucleoplasmic and cytoplasmic surfaces of the NPC near the central gated channel (Guan et al., 1995). This is in striking contrast to all other O-linked glycoproteins that have been characterized, since these are restricted to either the nucleoplasmic or cytoplasmic side of the NPC (Panté and Aebi, 1994). The p62 complex has been directly implicated in nuclear import in two separate studies: NPCs assembled *in vitro* in the absence of this component were deficient in nuclear import (Finlay et al., 1991), and p62 was found to interact *in vitro* with the cytosolic transport factor NTF2 (Paschal and Gerace, 1995). The location of the p62 complex is consistent with a role in the collection of transport ligands near the center of the NPC and delivery to the central gated channel. Despite its apparent importance in nuclear transport, only the 62-kD subunit of the complex has been molecularly cloned so far (Starr et al., 1990; Carmo-Fonseca et al., 1991; Cordes et al., 1991).

Here we report the cDNA cloning of the 58-, 54-, and 45-kD subunits of the p62 complex. Similar to p62, both p58 and p54 contain multiple FG repeats and a region of predicted coiled-coil α -helix that could specify heterotypic interactions. A combination of data suggests that p58 and p45 are generated by alternative splicing. We found that recombinant p58 and p54 are able to deplete nuclear import activity from cytosol and further demonstrated that p62, p58, and p54 each interact directly with the cytosolic transport factors p97 and NTF2. Furthermore, at least p58 shows a strong interaction with a putative transport complex consisting of ligand, NLS receptor, and p97. Considered together, these data suggest that the p62 complex is an important binding site near the central gated channel of the NPC for a complex containing transport ligand.

Materials and Methods

cDNA Cloning of p58, p54, and p45

The p62 complex was purified from rat liver NEs as described by Guan et al. (1995), separated by SDS-PAGE, and transferred to Immobilon polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The sequences of peptides from p58, p54, and p45 (4, 6, and 2 peptides, respectively) were determined by *in situ* tryptic digestion and standard Edman degradation methodology (Aebersold, 1989) by Dr. John Leszyk at the Worcester Foundation for Biomedical Research (Shrewsbury, MA). Degenerate oligonucleotides were designed from the peptide sequences and were used to obtain partial cDNA sequences of p54 and p58 by PCR. The template for PCR reactions was single stranded cDNA synthesized from Normal rat kidney (NRK) cell polyA⁺ mRNA (kindly provided by Dr. Kazuhiro Furukawa) using oligo(dT) as primer (Sambrook et al., 1989). PCR products were cloned into the pCRII plasmid from InVitrogen Corp. (San Diego, CA) and sequenced with an automatic DNA sequencer (Applied Biosystems, Inc., Foster City, CA) at the core facility of the Scripps Research Institute. A cDNA fragment of 297 bp, corresponding to amino acids 322–420 of p58, and a cDNA fragment of 273 bp, corresponding to amino acids 226–316 of p54, were obtained by PCR. ³²P-labeled probes were prepared from these cDNAs by random priming using a Prime-it II kit (Stratagene, La Jolla, CA) and were used to screen a rat macrophage λ ZAP cDNA library (kindly provided by Dr. George Fey) as described (Sambrook et al., 1989). Positive clones were analyzed by DNA sequencing. From ~10⁶ phage plaques, we isolated five and six positive clones for p58 and p54, respectively. Each set of positive clones contained different length fragments of the same cDNAs. The longest p58 clone is 3,749-bp-long and contains 1,758 bp of p58 coding sequence, 98 bp of 5' untranslated region, and 1,893 bp of 3' untranslated region followed by polyA sequence. The longest p54 clone is 2,194-bp-long and contains 1,533 bp of p54 coding sequence, 4 bp of 5' untranslated region, and 657 bp of 3' un-

translated region followed by polyA sequence. Further 5' untranslated sequence of p54 (62 bp) was obtained by 5'-RACE with 5'-RACE-Ready cDNA (Clontech Laboratories, Inc., Palo Alto, CA) following the manufacturer's instructions. The cDNA cloning of p45 is described in text.

Northern Hybridization

Total rat liver RNA was isolated by guanidium thiocyanate extraction as described by Sambrook et al. (1989), and polyA+ RNA was further purified with the PolyATtract mRNA Isolation System (Promega Corp., Madison, WI). PolyA+ RNA from NRK cells (provided by Dr. Kazuhiro Furukawa) was isolated as described (Furukawa et al., 1995). The full-length p58 coding sequence was used as a template to generate ³²P-labeled probes with Prime-it II kit (Stratagene). RNA electrophoresis, transfer, and hybridization were performed with standard protocols (Sambrook et al., 1989).

Antibody Production

Recombinant glutathione S-transferase (GST) fusion proteins containing full-length p58 and p54 were expressed in *Escherichia coli* (see below) and separated by SDS-PAGE. Gel slices containing GST-p58 and GST-p54 were excised, and proteins were electroeluted from gel slices (Harlow and Lane, 1988) and dialyzed against PBS. Purified proteins were then emulsified with TiterMax adjuvant (Vaxcel, Inc., Norcross, GA) according to the manufacturers' instructions. 1 mg of each protein was used for the initial injection of rabbits as described (Harlow and Lane, 1988). Animals were subsequently boosted three times at 4-wk intervals using 0.3 mg of protein. Antisera were collected 10–14 d after each boost. To make an antibody against the COOH-terminal sequence of p58, a peptide containing the last 14 amino acids of p58 was synthesized, covalently linked to the carrier keyhole limpet hemocyanin, and used for injection of rabbits. ~100 mg of peptide equivalent was used for the initial injection, and ~30 mg was used for the booster injection. Injection and antisera collection schedules were the same as described above. To purify anti-p58 and anti-p54 antibodies, antisera were first adsorbed against GST coupled to CNBr-activated Sepharose beads (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), and then were incubated with GST-p58 or GST-p54 coupled to CNBr beads. Bound antibodies were then eluted with 0.1 M glycine-HCl, pH 2.2, and dialyzed against PBS. To purify p58 COOH-terminal peptide antibody, antiserum was incubated with immobilized GST-p58, and bound antibodies were eluted and dialyzed against PBS.

Immunofluorescence and Immunogold Electron Microscopy

To carry out immunofluorescence microscopy on isolated rat liver nuclei, the nuclei were mounted on poly-L-lysine-coated coverslips and fixed with 4% formaldehyde. After permeabilization with 0.2% Triton X-100, the nuclei were washed twice with 5% BSA in PBS and incubated with affinity purified anti-p58 and anti-p54 antibodies in PBS and 5% BSA for 1 h at room temperature. After washing, fluorescence-conjugated donkey α -rabbit antibody was incubated with the coverslips for 1 h at room temperature. The coverslips were washed again, mounted onto microscope slides, and examined with a Zeiss Axiophot microscope (Thornwood, NY). Images were recorded using Kodak TMAX 400 film (Rochester, NY). Immunogold EM was performed as described by Guan et al. (1995).

Expression of Recombinant Proteins

To make GST fusion proteins containing p58, p54, and p62, the full-length coding sequences of p58 and p54, and NH₂-terminal (p58N, residue 1–225), middle (p58M, residue 226–410), and COOH-terminal (p58C, residue 411–585) regions of p58 were inserted into the pGEX-2T plasmid (Pharmacia), and the full-length coding sequence of p62 was inserted into pGEX-3X (Pharmacia). Plasmids were transformed into *E. coli* strain TB1. To express GST-p58, 10 ml of overnight bacterial culture was added to 1 l of M9 medium (Sambrook et al., 1989) with 100 μ g/ml ampicillin and grown at 25°C to an OD₆₀₀ of ~0.2. Protein expression was induced by adding IPTG to 0.1 mM and incubating at 25°C for an additional 7 h. Bacteria were harvested by centrifugation and resuspended to 1/10 the original culture volume in lysis buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 0.5 M NaCl, 2 mM DTT, 1 mM PMSF, 5 μ g/ml each of leupeptin, pepstatin A, and aprotinin) plus 3 mg/ml lysozyme. After 15 min incubation at room temperature, NP-40 was added to the lysate to 0.1%. The lysate was then

sonicated one to three times with 30-s bursts until the solution was no longer viscous. Soluble proteins were then separated from insoluble material by centrifugation at 30,000 g for 20 min. The supernatant was mixed with 1.5 ml glutathione Sepharose beads (Pharmacia), and incubated together for 2 h at 4°C. The beads were then washed three times with lysis buffer, two times with PBS, and stored in 10 vol of PBS containing 2 mM Na₃N at 4°C. GST-p58N, GST-p58M, and GST-p58C were expressed in a fashion similar to GST-p58 except that bacteria were grown in LB plus ampicillin at 37°C, and protein expression was induced at an OD₆₀₀ of ~0.4 by incubating with 0.2 mM IPTG for 3 h. GST-p62 and GST-p54 were expressed the same way as different regions of p58, but they are insoluble under this condition. To solubilize them, 1.5% Sarkosyl instead of 0.1% NP-40 was added to bacterial lysates after the lysozyme digestion, and 2% Triton X-100 was added to supernatants after the 30,000-g centrifugation (Frangioni and Neel, 1993). Binding of solubilized GST-p62 and GST-p54 to glutathione Sepharose beads was essentially the same as that of GST-p58.

The GST-p97 expression plasmid for human p97 was kindly provided by Dr. Stephen Adam (Northwestern University, Chicago, IL). The protein was expressed as described by Chi et al. (1995) and purified in an identical fashion to GST-p58. To make the (His)₆-p97 fusion protein, full-length p97 coding sequence was subcloned into the pTrcHis A plasmid (Invitrogen Corp.) downstream of the coding sequence of (His)₆, and this construct was transformed into *E. coli* strain BL21. Proteins were expressed and purified similarly as described for GST-p97 except for several differences: protein expression was induced by 2 mM IPTG at OD₆₀₀~0.6; the lysis buffer (His-lysis buffer) contained 50 mM Tris-Cl, pH 7.4, 0.5 M NaCl, 2 mM β -mercaptoethanol, and protease inhibitors. Ni-NTA agarose beads (Qiagen, Chatsworth, CA) were used to purify (His)₆-p97 proteins according to manufacturers' instructions. His-lysis buffer with 1 mM imidazole was used as washing buffer. Proteins bound to the Ni-NTA beads were eluted by mixing the beads with equal volumes of 300 mM imidazole for 10 min at room temperature two sequential times. The eluates were pooled together and dialyzed against transport buffer (20 mM Hepes, pH 7.4, 110 mM KOAc, 2 mM Mg(OAc)₂, 1 mM EGTA, 2 mM DTT, and protease inhibitors) overnight. The expression plasmid for (His)₆-hSrp1 α (Weis et al., 1995) was kindly provided by Dr. Angus Lamond (EMBL, Heidelberg, Germany), and transformed into *E. coli* strain BL21. Proteins were expressed and purified as described for (His)₆-p97. The construction of the NTF2 expression plasmid and preparation of recombinant NTF2 has been described (Paschal and Gerace, 1995). Protein concentrations and purities were determined by SDS-PAGE and Coomassie blue staining. All recombinant proteins were >80% pure except for hSrp1 α , which is ~60–70% pure.

Cytosol Depletion Assay

Recombinant GST, GST-p62, GST-p58, and GST-p54 were adsorbed to glutathione Sepharose beads as described above and adjusted to the same protein concentration (~6 mg/ml) by adding glutathione Sepharose beads. 100 μ l of each immobilized protein beads were washed three times with transport buffer, preincubated with the same volume of 5% BSA in transport buffer for 30 min at 4°C, briefly washed with transport buffer, and incubated with 100 μ l HeLa cell cytosol (prepared as described by Paschal and Gerace, 1995) for 2 h at 4°C. Bead-treated cytosol samples were then separated from beads by centrifugation, and the cytosol was tested for its activity in nuclear protein import. The nuclear protein import assay was performed essentially as described by Paschal and Gerace (1995). Typically, 15 μ l bead-treated cytosol was used in each 40- μ l reaction which contains ~0.5 \times 10⁶ digitonin-permeabilized HeLa cells, fluorescently labeled BSA-NLS conjugates, and an ATP regeneration system. Reactions were incubated at 30°C for 20 min, and import activity was measured by flow cytometry (Paschal and Gerace, 1995).

To analyze the depletion of transport factors from cytosol and their binding to beads, protein beads and cytosol samples were separated after coinubation, the beads were washed three times in transport buffer, and each was analyzed by Western blotting. Normally, 2.5–5 μ l equivalents of beads or cytosol was loaded in each lane. Western blotting was performed as described by Sambrook et al. (1989). For detection of beads-bound NTF2, the washing step was omitted because it removes bound NTF2 from beads, probably due to unstable or high off rate binding of NTF2 to p62 complex subunits. Anti-NTF2 polyclonal antibody (Paschal et al., 1996), anti-Ran polyclonal antibody (Melchior et al., 1995), α -hSrp1 α polyclonal antibody (Weis et al., 1995), and α -p97 monoclonal antibody (Chi et al., 1995) were generously provided by Drs. Bryce Paschal, Frauke

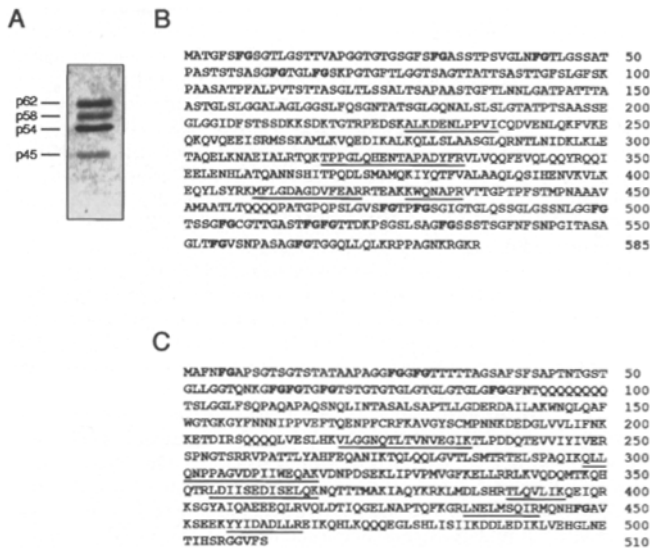


Figure 1. cDNA cloning of p58 and p54. (A) SDS-PAGE of the purified rat liver p62 complex (visualized by silver staining) used to obtain peptide sequences of the subunits. (B and C) Deduced amino acid sequences of p58 (B) and p54 (C). Underlined sequences are those determined by sequencing of peptides from the purified polypeptides. FG repeats are highlighted with bold letters. Genbank accession numbers for p58 and p54 are U63839 and U63840, respectively.

Melchior, Angus Lamond, and Stephen Adam, respectively. Signals were detected with HRP-coupled secondary antibodies and an ECL kit (Amersham Corp., Arlington Heights, IL).

Recombinant Factor Binding Assay

10 μ l of beads containing GST, GST-p62, GST-p58, GST-p54, and GST fusions of different regions of p58 were preincubated with 400 μ l 5% BSA in transport buffer for 30–60 min at 4°C and were subsequently incubated with 400 μ l transport buffer containing 5% BSA and individual recombinant cytosolic factors for 2 h at 4°C. The beads were washed three times with transport buffer (For NTF2 binding, the washing step was omitted. See above.), and 2 μ l of beads were used for Western blot analysis. In addition to 5% BSA, other blocking agents, including 5% nonfat milk, 2% gelatin, and 2% casamino acids, with or without 0.2% Tween, were also tested in binding assays. All of them showed similar binding patterns (data not shown). Concentrations of cytosolic factors in binding assay were: 0.5 μ M NTF2, and 200 nM Srp1 α and (His)₆-p97. A polyclonal rabbit anti-NTF2 antibody (see above) was used to detect NTF2, and a monoclonal antibody (anti-Xpress antibody, InVitrogen Corp.) against the linker region between His-tag and expressed proteins (i.e., Srp1 α and p97) was used to detect recombinant Srp1 α and p97 signals. The binding of Srp1 α and import ligand to immobilized GST-p97 and the binding of import ligand, Srp1 α , and (His)₆-p97 to immobilized GST-p58, GST-p58N, and GST-p58C were performed in the same way as described above. Normally, 50–100 nM biotin-labeled BSA-NLS conjugates were used in binding assays as import ligand. The ligand signal was detected by Streptavidin-HRP and ECL as above.

Results

Molecular Cloning of p58 and p54

To facilitate further characterization of the p62 complex, we carried out cDNA cloning of the 58-, 54-, and 45-kD subunits. The p62 complex was isolated from rat liver NEMs (Fig. 1 A) (Guan et al., 1995), and the sequences of several tryptic peptides of the 45–58 kD subunits were obtained. Since p45 is closely related to p58 (see below), we initially

concentrated on cDNA cloning of p58 and p54. Degenerate oligonucleotides were designed on the basis of peptide sequences obtained for these two polypeptides, and these were used to isolate cDNA fragments of p58 and p54 by PCR amplification of cDNA synthesized from NRK cell mRNA. These amplified fragments were then used to screen a rat macrophage cDNA library. From this procedure, we isolated cDNA clones of 3,749 bp and 2,194 bp that appear to encode p58 and p54, respectively (see Materials and Methods). The p58 cDNA clone contains an open reading frame (ORF) encoding a 59,261-D polypeptide of 585 amino acid residues (Fig. 1 B), while the p54 ORF encodes a 55,742-D protein of 510 amino acids (Fig. 1 C). All the peptide sequences generated from purified p58 and p54 were found in the deduced amino acid sequences of the p58 and p54 cDNAs (underlined regions in Figs. 1, B and C), validating the identities of the clones. We believe that we have obtained the full-length coding sequences for both p58 and p54. The p58 has an in-frame stop codon upstream of the putative start site. While an in-frame stop is not seen in the most proximal 66 nucleotides of the putative 5' noncoding region of the p54 cDNA, a recombinant p54 polypeptide expressed in *E. coli* comigrates with rat liver p54 on SDS-PAGE (data not shown).

The deduced amino acid sequences of both p58 and p54 contain multiple FG dipeptide motifs (bold letters in Figs. 1, B and C), similar to p62. There are 14 and 8 FG motifs in p58 and p54, respectively, compared to 6 FG repeats in p62. In all three polypeptides, the FG repeats occur in regions that have very few charged amino acids, and are enriched in glycine, serine, and threonine residues. The FG repeats of p58 are found at both its NH₂ and COOH termini (between amino acids 7–67 and 474–565), while the FG repeats of p54 are concentrated in an NH₂-terminal region (residues 5–88). Some of the FG repeats of p58 occur within degenerate versions of the sequence GFxFG, similar to p62. The FG-containing regions of p62, p58, and p54 also contain multiple repeats of the dipeptide sequence PA (see Starr et al., 1990), which is present 13, 8, and 6 times in p62, p58, and p45 respectively (usually embedded within the sequence PAS, PAT, or PAA).

Like p62, both p58 and p54 have two or more 20–40 amino acid segments predicted to have a high probability of forming α -helical coiled-coils (Fig. 2). The predicted coiled-coil region of p58 occurs between amino acids 235–415 (Fig. 2 B) and separates the two FG repeat-containing regions of this protein. The segments of p54 that are predicted to have significant coiled-coil potential occur between residues 325–415 (Fig. 2 C) and are at the COOH-terminal side of its FG repeat region. Neither p58 nor p54 showed statistically significant relatedness to other data base entries.

Molecular Characterization of p45

Whether p45 is an authentic NPC polypeptide occurring in vivo or an in vitro degradation product has been a source of controversy (discussed in Guan et al., 1995). In our hands, p45 was consistently recovered as a major band in p62 complex preparations where in vitro proteolysis was minimal, as indicated by the presence of only four major bands (e.g., Fig. 1 A), and was diminished in preparations

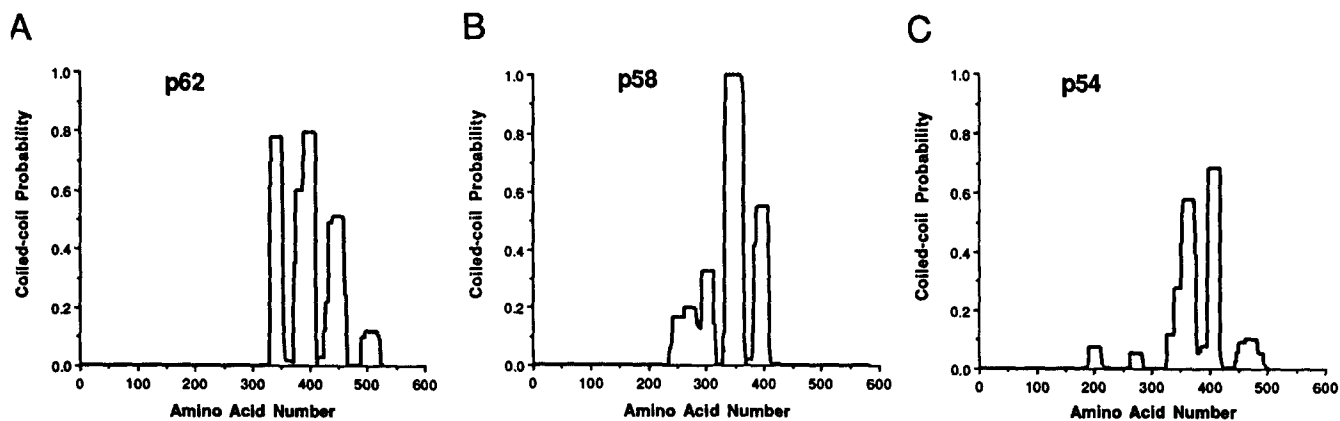


Figure 2. Prediction of α -helical coiled-coil regions in subunits of the p62 complex. The plots present the probability of α -helical coiled-coil structure in p62 (A), p58 (B), and p54 (C) as determined by the algorithm of Lupas et al. (1991).

that had undergone apparent degradation, as suggested by the presence of a complex ladder of bands (see Guan et al., 1995). Thus, p45 does not appear to be an *in vitro* degradation product. However, several lines of data indicated that p58 and p45 are very similar, as previously proposed by others (Kita et al., 1993). The HPLC profiles of tryptic peptide digests of p58 and p45 closely resembled each other but clearly differed from that of p54 (Fig. 3 A). We obtained the peptide sequences from two peaks of the HPLC gradient, resolving the p45 tryptic digests that coincided with sequenced peaks of the p58 gradient. The two p45 peptide sequences we obtained, TPPGLQHENTAPADYFR and KWQNAPR, were identical to the sequences of p58 peptides obtained at these elution positions, corresponding to residues 317–333 and 427–433 of p58, respectively. Furthermore, polyclonal antibodies raised against recombinant p58 recognized both p58 and p45 on Western blots of a p62 complex-enriched fraction of isolated rat liver NEs (Fig. 3 B) but did not react with p62 or p54. By contrast, anti-p54 polyclonal antibodies recognized p54 but not the other components of the p62 complex in Western blotting (Fig. 3 B). A very similar reaction pattern was obtained when these antibodies were used for Western blotting of NRK cells lysed directly in SDS sample buffer (Fig. 3 C). This provides further evidence that p45 is not generated by *in vitro* proteolysis. Despite the antigenic similarities between p58 and p45, antibodies against a COOH-terminal peptide of p58 recognized only p58 and not p45 in Western blotting (Fig. 3 B), indicating that the COOH terminus of p58 is not shared by p45.

Considered together, these data suggested either that p45 is generated by *in vivo* proteolysis of p58 or that p58 and p45 are generated by alternative splicing of a common precursor mRNA. To help distinguish between these possibilities, we performed Northern hybridization on mRNAs from rat liver and NRK cells, using the full length p58 coding sequence as a probe. Two major hybridizing bands of ~ 3.8 and ~ 2.5 kb were detected for both mRNAs (Fig. 4, lanes 1 and 3). These two bands were not due to nonspecific reaction with 18S and 28S rRNAs because 50 μ g of total rat liver RNA did not show any hybridization signal (Fig. 4, lane 2). These results support the possibility that p58 and p45 are related by alternative splicing.

To more conclusively analyze the nature of p45, we used PCR to clone putative p45 cDNAs from the library used for the cDNA cloning of p58 and p54. Because p58 and p45 are very similar by tryptic peptide mapping but p45 does not contain epitopes found at the COOH terminus of p58 (above), it seemed likely that the two polypeptides share the region of p58 that lies between the two common sequenced peptides (residues 317–433 of p58) and differ downstream of this sequence. We therefore used PCR to search for a unique 3' portion of a putative p45 cDNA. A p58 cDNA segment located between the two peptides shared by p58 and p45 was chosen as the 5' primer, and a sequence from the library vector at the 3' ends of the cDNA inserts was used as the 3' primer. The major PCR product amplified from the cDNA library with these primers, which was seen in four separate PCR reactions, was ~ 400 bp long (data not shown). This is distinct from the ~ 2.5 -kb cDNA fragment of p58 that was predicted to be generated by this procedure. DNA sequencing of the cloned 400-bp fragment from two separate PCR reactions showed that the 5' half of this PCR product perfectly matched the p58 cDNA sequence up to nucleotide 1492, where the two sequences diverged (Fig. 5 A). The last four identical nucleotides preceding the sequence divergence (CAGG) match the consensus C/AAGG/A marker for mRNA splicing sites (Fig. 5 A) (Sharp, 1981; Breathnach and Chambon, 1981; Cech, 1983), suggesting that this is a site of alternative splicing. This putative splice site follows the predicted coiled-coil domain and precedes the second FG repeat region of p58. The putative ORF of p45 contained in this PCR product is identical to the sequence of p58 between residues 417–465, and then is followed by three different amino acid residues before it terminates (Fig. 5 B). A poly A tract was found ~ 120 bp after the putative splice site (Fig. 5 A).

Further PCR amplifications were performed using a fragment of the 3' untranslated region of p45 as the 3' primer and a piece of 5' vector sequence as the 5' primer. The longest PCR product obtained by this approach (~ 1.4 kb long) contained ORF encoding sequences identical to p58 from residue 40 to the putative splice site plus the unique 3' segment of p45 (data not shown). We have been unable to obtain p45 cDNAs that extend further in the 5' direc-

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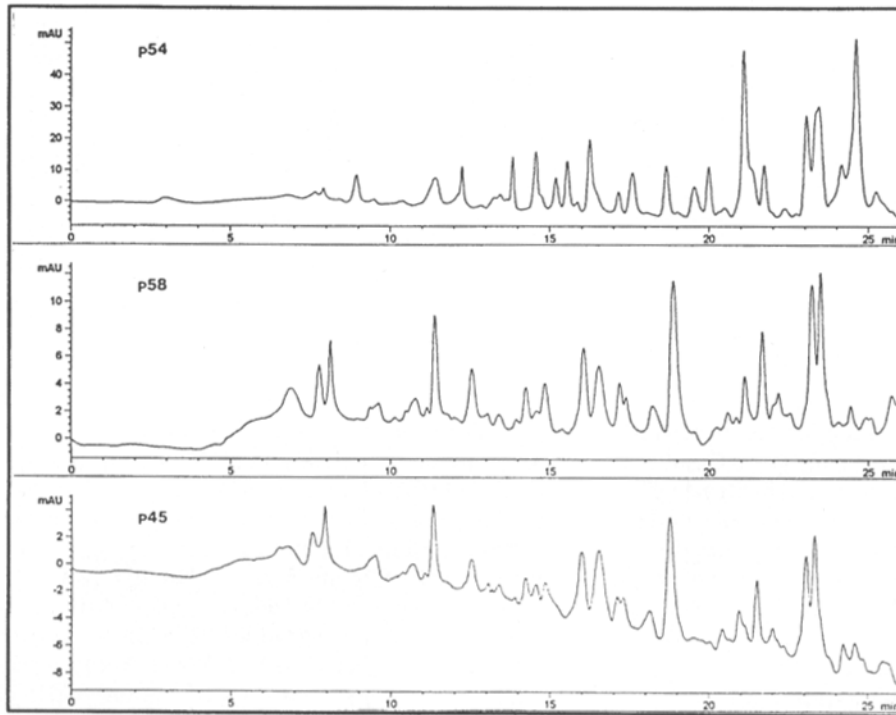
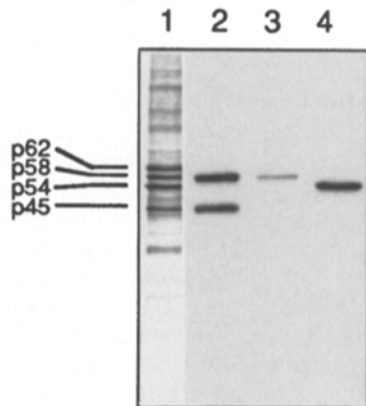
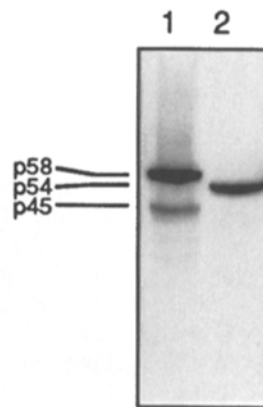


Figure 3. Analysis of relationship between p58 and p45. (A) Analysis of tryptic digests of p54 (top), p58 (middle) and p45 (bottom) on a C₁₈ reversed phase HPLC column (see Materials and Methods). X-axes represent elution time, and Y-axes represent A₂₁₀. (B) Analysis of WGA-binding proteins of rat liver NEs (Guan et al., 1995) by immunoblotting with anti-p58 and anti-p54 antibodies. The WGA-binding proteins from 5 OD₂₆₀U of NEs were loaded on each lane and separated on a 10% SDS gel. Lane 1 was visualized by silver staining, while lanes 2–4 were transferred to a nitrocellulose membrane and probed with various affinity purified antibodies: anti-p58 (lane 2), anti-p58 COOH-terminal peptide (lane 3), and anti-p54 (lane 4). (C) Western blotting of a whole NRK cell lysate with affinity-purified anti-p58 and anti-p54 antibodies. Cells were directly lysed in SDS gel sample buffer (Sambrook et al., 1989), electrophoresed on a 10% SDS gel, and probed with affinity purified anti-p58 (lane 1) or anti-p54 (lane 2) antibodies.

B



C



tion than residue 40 of p58 despite repeated attempts, and we do not know whether p58 and p45 share the same NH₂ terminus. Nevertheless, these data indicate that p45 and p58 are likely to be generated by alternative splicing.

Immunolocalization of p58/p45 and p54

We next carried out immunocytochemical localization of p58/p45 and p54 using affinity-purified polyclonal antibodies (characterized in Fig. 3, B and C) raised against recombinant fusion proteins containing the p58 and p54 ORFs. It

was not possible to analyze the localization of p58 separate from p45 in these studies, as the only monospecific antibody to p58, which was generated against the COOH terminal peptide of this protein (Fig. 3 B), did not give a positive immunofluorescence reaction. In immunofluorescent staining of isolated rat liver nuclei, distinct NE-like nuclear rim staining was obtained with both anti-p58/p45 (Fig. 6 A) and anti-p54 (Fig. 6 B) in a discontinuous fashion reminiscent of NPC localization. These results further validate the identity of our cDNA clones and are consistent with the results of immunofluorescence localization carried out with

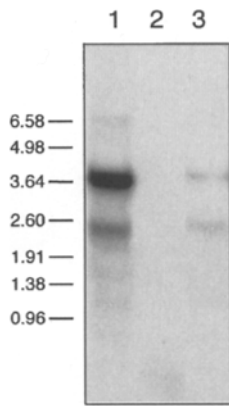


Figure 4. Northern blot analysis of mRNAs that hybridize to a p58 probe. A full-length p58 coding sequence probe was used to analyze 25 µg NRK cell polyA+ mRNA (lane 1) 50 µg total rat liver RNA (lane 2) and 10 µg rat liver polyA+ mRNA (lane 3). Mobilities of molecular weight markers in kb are indicated to the left of lane 1.

polyclonal antibodies raised against rat liver p58 and p54 (Finlay et al., 1991). When these antibodies were used to label isolated rat liver NEs by immunogold electron microscopy, both anti-p58/p45 (Fig. 7, *a-g*) and anti-p54 (Fig. 7, *h-o*) decorated the cytoplasmic and nucleoplasmic surfaces of the NPC. Gold labeling was found most frequently near central regions of the NPC and was approximately equally distributed on the cytoplasmic and nucleoplasmic surfaces based on the counting of gold particles in prints of electron micrographs (data not shown). The localization of p58/45 and p54 determined by this work agrees well with the recently described localization of p62, which is found on both the cytoplasmic and nucleoplasmic sides of the NPC near the central gated channel (Guan et al., 1995).

Interactions between p62 Complex Subunits and Cytosolic Transport Factors

Previous work in our laboratory showed that preincubating cytosol with immobilized recombinant p62 results in the loss of its ability to support nuclear import in digitonin-permeabilized cells. This is due, at least in part, to depletion of the transport factor NTF2 from cytosol (Paschal and Gerace, 1995). Thus, p62 appears to have a direct role in nuclear import through interaction with at least one cytosolic transport factor. To determine whether p58 and p54 also interact with cytosolic transport factors, we conducted more detailed cytosol depletion studies using different subunits of the p62 complex. GST fusion proteins containing

A

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1      10      20      30      40      50      60      70
TGTTGTTTGAAGCCAGGAGAACAGAGCCAGAGAGTGGCAGATGCACCCAGAGTTACTACTGGACCCACG
*****
TGTTGTTTGAAGCCAGGAGAACAGAGCCAGAGTGGCAGATGCACCCAGAGTTACTACTGGACCCACG
1346   1355   1365   1375   1385   1395   1405   1415

71      80      90      100     110     120     130     140
CTTTTCAGCACCATGCCAAACGACAGCCGTTGCCATGGCTGCAACACTTACACAGCAGCAACAGCCCTG
*****
CCTTTCAGCACCATGCCAAACGACAGCCGTTGCCATGGCTGCAACACTTACACAGCAGCAACAGCCCTG
1416   1425   1435   1445   1455   1465   1475   1485

141     150     160     170     180     190     200     210
CTACAGGTGATGGTGGTGAATGTAAGTGTAGCTGAGCTGAAGACTAGAATCAATAATCACTTTGTGAACAG
*****
CTACAGGGCCACAGCCGCTCTCTGGGAGTTAGTTTGGAAACGCCATTCGGCTCAGGATTGGCACTGGCTT
1486   1495   1505   1515   1525   1535   1545   1555

211     220     230     240     250     260     270     280
TTGGAATTGTCACTGTACTGTGTAATGGTTTGGCTTTAAGATGAACAATAAATAAATAAATAAATAA
*****
GCAATCAAGTGGCTTAGGTTCTTCAAACTGGAGGATTTGGAAGTACTGCTGGTTTCGGATCGCGTACC
1556   1565   1575   1585   1595   1605   1615   1625

281     290
AAAAAAAAAAAA
**
ACAGGGGCTTCC
1626   1635

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B

```

1      10      20      30      40      50
VFEARTEAKKQWQNAAPRVITGPTPFSTMPNAAVAVMAATLTQQQPATGDGW*
*****
VFEARTEAKKQWQNAAPRVITGPTPFSTMPNAAVAVMAATLTQQQPATGQPS
417     426     436     446     456     466

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Figure 5. Sequence analysis of a partial-length putative p45 cDNA. A cDNA containing the 3' sequence of p45 mRNA was obtained by PCR (see text). Shown are comparisons of (A) the nucleotide sequence of this partial length cDNA of p45 (*upper line*) and the corresponding segment of p58 (*lower line*), and (B) the amino acid sequence encoded by the p45 ORF and that of p58 in this region. Numbers above the p45 sequences are the relative positions of residues in the partial length sequences, while numbers below the p58 sequences indicate actual positions of residues in the full-length p58 sequence. Stars between upper and lower sequences indicate identical residues. The star at the end of the p45 amino acid sequence indicates a stop codon.

recombinant p62, p58, and p54 were prepared and immobilized on glutathione Sepharose beads. Cytosol was then preincubated with the immobilized proteins and tested for its activity in nuclear import assays involving permeabilized HeLa cells. Under our standard conditions, an ~50% loss of import activity was obtained when cytosol was pre-

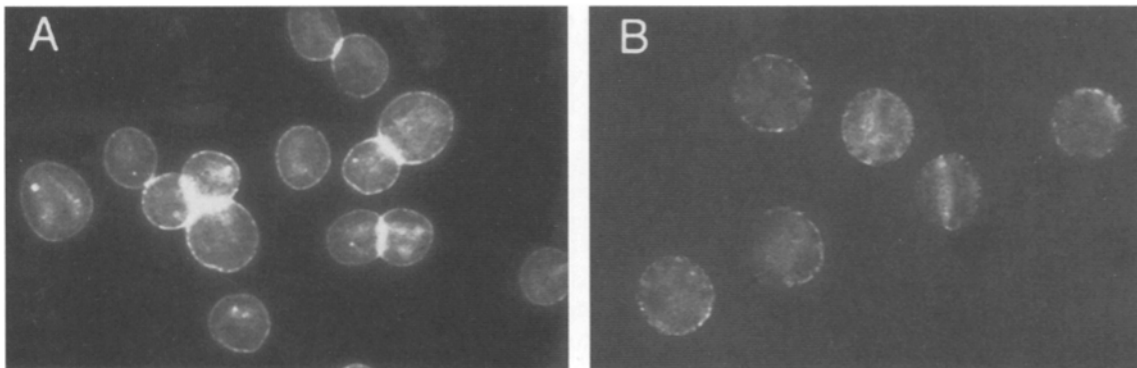


Figure 6. Immunofluorescence localization of p58/p45 and p54. Isolated rat liver nuclei were labeled with affinity purified anti-p58/p45 (A) or anti-p54 (B) antibodies and examined by immunofluorescence microscopy.

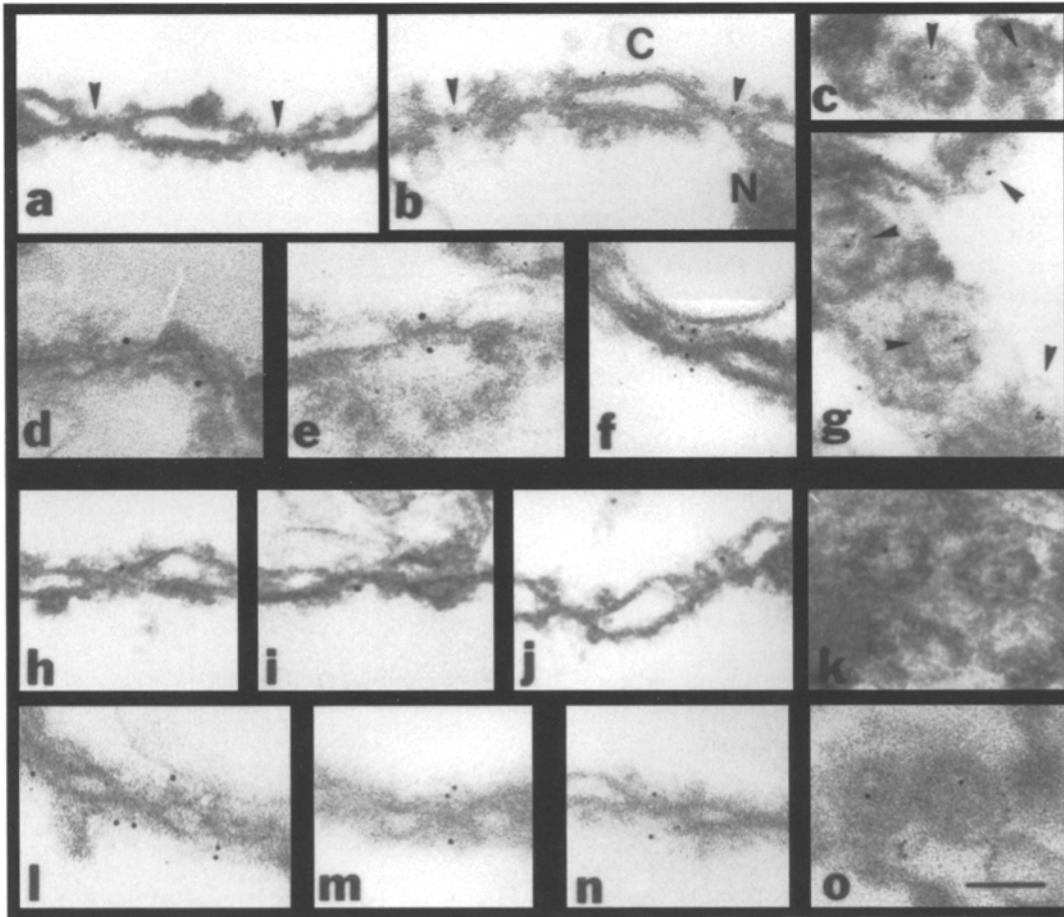


Figure 7. Immunogold localization of p58/p45 and p54 in isolated rat liver NEs. Isolated NEs were incubated with affinity purified anti-p58/p45 (*a-g*) or anti-p54 (*h-o*) antibodies followed by goat anti-rabbit IgG coupled to 5-nm (*a-c, f-o*) or 10-nm (*d* and *e*) gold. Shown are electron micrographs of thin sections showing the NE in cross-section (*a, b, d-f, h-j, i-m*) or grazing sections (*c, g, k, o*). Omission of the primary antibodies yielded essentially no gold labeling (data not shown). C, cytoplasmic surface; N, nucleoplasmic surface. Bar, 100 nm.

treated with any of the three immobilized proteins, compared to control beads containing GST alone (Fig. 8 A). To establish that this effect was not due to the general removal of cytosolic proteins by the p62 complex subunits, we examined the cytosolic proteins bound to beads containing GST-p62, GST-p58, GST-p54, and GST by SDS-PAGE and Coomassie blue staining. We found that the beads containing the p62 complex subunits bound the same complement and relative amounts of nonspecific background bands as the beads containing GST alone (data not shown). This indicates that the p62 complex subunits do not exhibit nonspecific binding under our experimental conditions and that the loss of import activity from cytosol by preincubation with immobilized p62 complex subunits is likely due to specific interactions.

To investigate whether previously described transport factors were depleted from cytosol by these preincubations, we used Western blotting to analyze the distributions of NTF2, Ran, Srp1 α , and p97 in the bound and unbound cytosol fractions in this depletion experiment. We found that NTF2, Srp1 α , and p97 bound to each of the three subunits of the p62 complex, but not to GST alone (Fig. 8 B). In contrast, Ran did not bind to any of the proteins under these conditions (Fig. 8 B). Bound NTF2 was

not detected when extensive washing of the beads was carried out after incubation with cytosol (data not shown), indicating that this interaction has a low affinity or high off rate. To determine the extent of depletion of these factors from cytosol, we examined the levels remaining in the unbound cytosol samples (Fig. 8 C). ~50% of the cytosolic NTF2 was depleted by each of the three subunits of the p62 complex. However, only a relatively small portion of p97 was removed by all of the three polypeptides, and the levels of Srp1 α and Ran were not significantly reduced (Fig. 8 C).

Previous studies have indicated that the NLS receptor (e.g., Srp1 α) and p97 can form a complex with import ligand in cytosol and that this complex may be targeted to an initial docking site at the NPC (Görlich et al., 1995; Imamoto et al., 1995a,b; Radu et al., 1995a). A complex containing these components and other transport factors, such as NTF2, may persist throughout many subsequent transport steps (see Melchior and Gerace, 1995). Thus, the binding of cytosolic factors to subunits of the p62 complex observed in the previous experiment could be either direct or indirect. To investigate whether NTF2, Srp1 α , and p97 can bind directly to the p62 complex subunits, purified recombinant factors were incubated individually with each

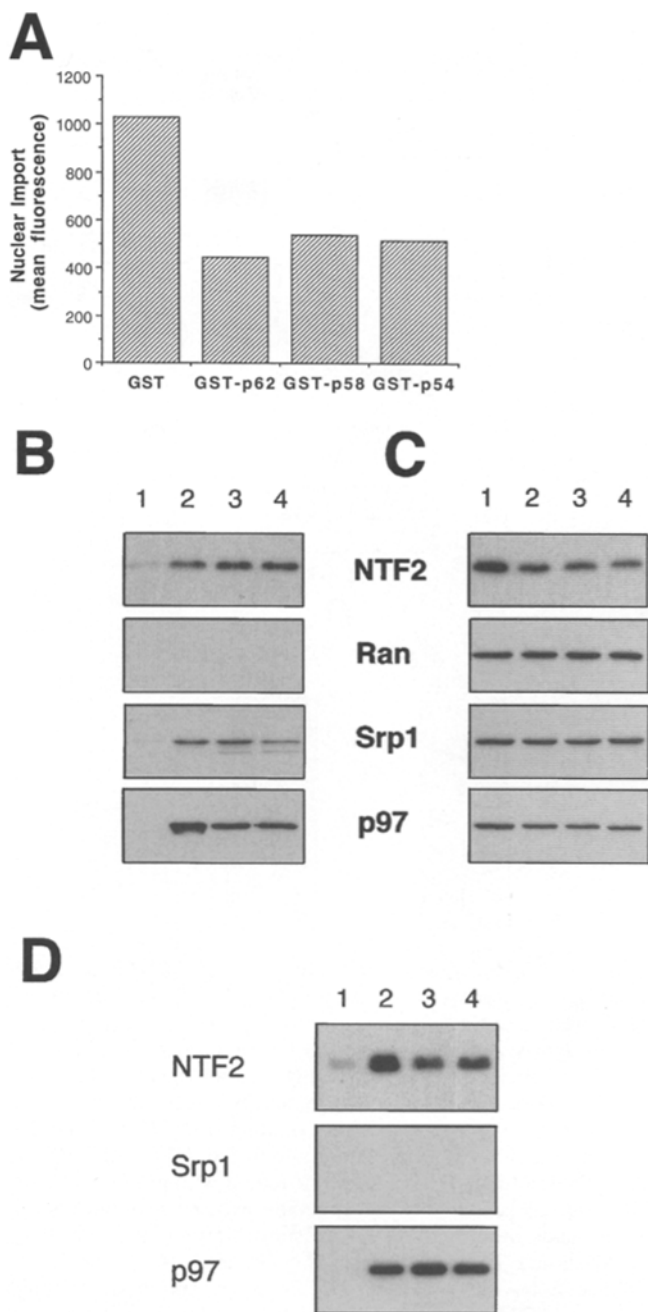


Figure 8. Characterization of the interactions of cytosolic factors with proteins of the p62 complex. (A–C) Immobilized recombinant GST, GST-p62, GST-p58, and GST-p54 were incubated with HeLa cytosol for 2 h at 4°C. (A) Cytosol samples were separated from the beads and tested for their activity in a permeabilized cell nuclear protein import assay. (B and C) The material bound to beads (B) or remaining unbound in the cytosol (C) was analyzed by Western blotting with antibodies against NTF2, Ran, Srp1 α , and p97. Note that the exposure times shown for (B) and (C) are different. (D) Bead-immobilized GST, GST-p62, GST-p58, and GST-p54 were incubated with either NTF2, Srp1 α , or (His)₆-p97, all prepared as recombinant proteins. Proteins bound to the beads were analyzed by Western blotting (see Materials and Methods). Lanes 1, GST; lanes 2, GST-p62; lanes 3, GST-p58; lanes 4, GST-p54. The weak signals of NTF2 on the lanes of GST beads are due to the retention of a small amount of NTF2 solution in the GST beads (see Materials and Methods).

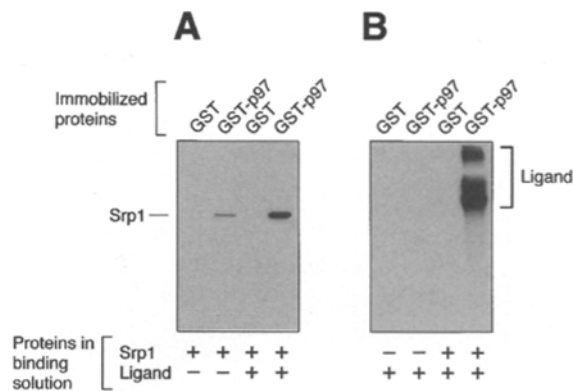


Figure 9. Characterization of the binding of recombinant Srp1 α and transport ligand with p97. Immobilized GST and GST-p97 were incubated with recombinant Srp1 α , biotin-labeled BSA-NLS conjugate (ligand), or a combination of both, as indicated. Western blots of the bound proteins were probed with specific antibodies to detect Srp1 α (A) and streptavidin-HRP to detect transport ligand (B).

of the immobilized p62 complex proteins, and the bound proteins were analyzed by Western blotting (Fig. 8 D). These results showed that NTF2 and p97 bind directly to p62, p58, and p54 but that recombinant Srp1 α does not directly interact with any of these components (Fig. 8 D). This indicates that the binding of Srp1 α to the p62 complex subunits observed in the cytosol depletion experiments (Fig. 8, B and C) is indirect and probably occurs through its interaction with p97 as part of a complex. To determine whether NTF2 and p97 interact with the same or nearby sites on these three subunits of the p62 complex, we incubated immobilized p62, p58, and p54 with 100 nM p97 and a 100-fold molar excess of NTF2. These experiments showed that NTF2 had no detectable effect on the level of p97 binding (data not shown), suggesting that NTF2 and p97 bind to spatially separate sites on subunits of the p62 complex. However, more detailed studies will be required to map the precise binding sites of these proteins.

Interaction of p58 with a Complex of Import Ligand, NLS Receptor, and p97

We next carried out experiments to investigate whether a subunit of the p62 complex is able to interact with a putative transport complex consisting of transport ligand, an NLS receptor (Srp1 α), and p97. To evaluate the biological activity of recombinant Srp1 α and p97 used for these experiments, we examined the binding of Srp1 α to immobilized p97 in the absence and presence of NLS-containing ligand (Fig. 9 A). This experiment showed significant binding of Srp1 α to p97 in the absence of ligand (Fig. 9 A), indicating that the recombinant proteins retain the ability to interact. Interestingly, the presence of import ligand strongly enhanced the binding of Srp1 α to p97 (Fig. 9 A). The import ligand itself was able to bind to p97 only in the presence of Srp1 α (Fig. 9 B), indicating that the ligand bound to p97 through its interaction with Srp1 α and that recombinant Srp1 α was active for binding to ligand as well as p97.

To examine the abilities of complexes of ligand and cy-

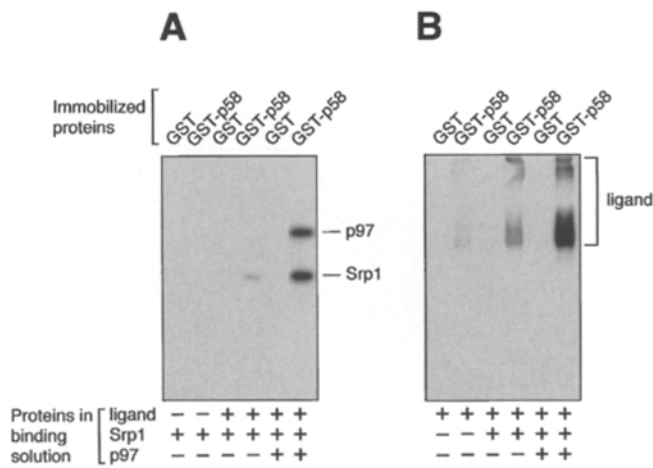


Figure 10. Characterization of the binding of transport ligand, Srp1 α and p97 to p58. Immobilized GST and GST-p58 were incubated with various combinations of transport ligand, Srp1 α , and (His)₆-p97, as indicated. Western blots of the bound samples were probed with antibodies to detect Srp1 α and p97 (A) and streptavidin-HRP to detect transport ligand (B).

tosolic factors to bind to a subunit of the p62 complex, we incubated various combinations of ligand, Srp1 α , and p97 with immobilized GST-p58 and examined the binding of these components to the matrix by Western blotting. Srp1 α did not detectably bind to GST-p58 alone (Fig. 10 A) but associated with the matrix at trace levels in the presence of ligand (Fig. 10 A). This was correlated with a small amount of ligand binding seen under this condition (Fig. 10 B). Because of the low level of this interaction, we believe that it most likely is nonspecific. In contrast, when ligand, Srp1 α , and p97 were incubated together in the presence of immobilized GST-p58, all three components bound at high levels to the matrix. Therefore, p97 seems to strongly stimulate the interaction of the Srp1 α -ligand complex with p58, implying that p58 provides a specific binding site for a trimeric complex consisting of transport ligand, Srp1 α , and p97. The binding of Srp1 α to the p58 matrix in the presence of p97 was substantially increased by the addition of transport ligand to the mixture (data not shown), consistent with the results shown above that ligand stimulates the interaction of Srp1 α with p97 (Fig. 9). Addition of NTF2 to the binding reactions did not have any detectable effect on the binding of a mixture of ligand, Srp1 α , and p97 to p58 (data not shown). Similar incubations of cytosolic factors were carried out with GST-p62 and GST-p54, but for unexplained reasons, we were able to obtain only modest levels of p97-stimulated interaction of Srp1 α and ligand with the immobilized proteins (data not shown), compared to the strong stimulation seen with p58.

Mapping the Regions of p58 That Interact with Cytosolic Factors

Although p62, p58, and p54 all have FG repeat region(s) and a predicted coiled-coil segment, they do not have significant sequence homology with each other. Nevertheless, they all can interact directly with NTF2 and p97. To localize the binding sites for NTF2 and p97 within p58, we dissected p58 into an NH₂-terminal region containing FG

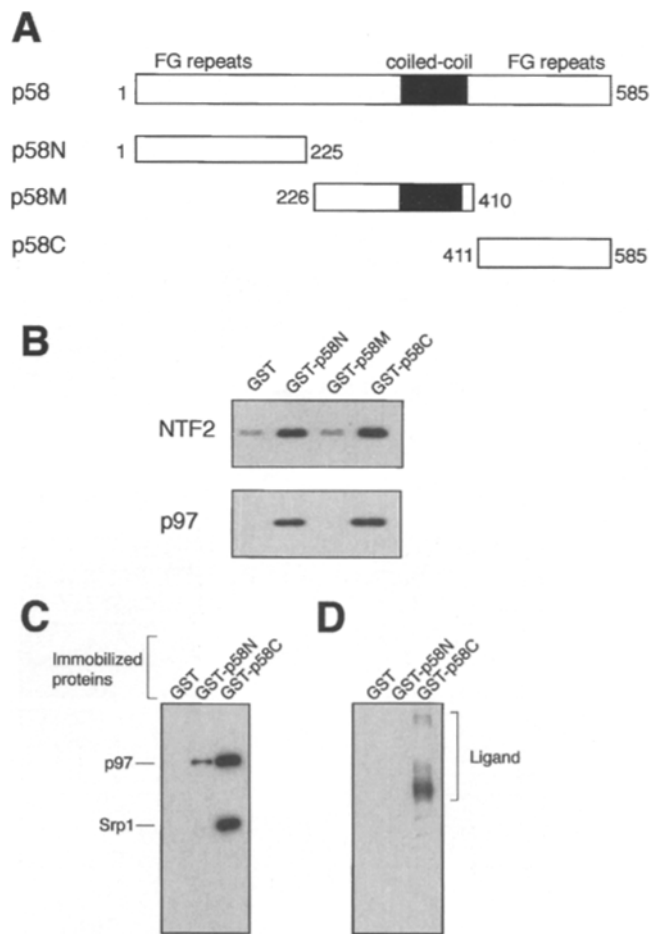


Figure 11. Characterization of binding regions for NTF2 and p97 in p58. (A) Diagrams of full-length p58 and different regions of p58 expressed as GST fusion proteins for in vitro binding assays. The black area indicates the region having a high probability of forming a coiled-coil α -helix (see Fig. 2). Beginning and ending positions of each region are designated by the residue numbers flanking each diagram. (B) Direct binding of NTF2 and p97 to different regions of p58. Bead-immobilized GST, GST-p58N, GST-p58M, and GST-p58C were incubated with either NTF2 or (His)₆-p97, all prepared as recombinant proteins. Proteins bound to the beads were analyzed by Western blotting (see Materials and Methods). The weak signals of NTF2 in the GST and GST-p58M samples are probably due to the retention of a small amount of NTF2 solution in the beads (see Materials and Methods). (C and D) Characterization of the binding of transport ligand, Srp1 α , and p97 to FG repeat regions of p58. Western blots of the bound samples after incubations of immobilized GST, GST-p58N, and GST-p58C with a mixture of transport ligand, Srp1 α , and (His)₆-p97 were probed with antibodies to detect Srp1 α and p97 (C) and streptavidin-HRP to detect transport ligand (D).

and PA repeats (p58N, residue 1–225), a middle region containing the predicted coiled-coil segments (p58M, residue 226–410), and a COOH-terminal region containing FG and PA repeats (p58C, residue 411–585) (Fig. 11 A). These regions were expressed as GST fusion proteins and evaluated for binding of NTF2 and p97. We found that both the NH₂- and COOH-terminal regions of p58 directly bind to both NTF2 and p97 (Fig. 11 B). In contrast, the middle region, which contains predicted coiled-coil seg-

ments of p58, does not bind to either one of the cytosolic factors (Fig. 11 B). These results localize the binding sites for NTF2 and p97 to regions of p58 distinct from the predicted coiled-coil segment and suggest that FG and/or PA repeats may play an important role in the binding of these cytosolic factors.

We then examined the capacity of a complex of ligand, Srp1 α , and p97 to bind to p58N and p58C by mixing a combination of these soluble factors with the GST-p58N and GST-p58C matrices. To our surprise, while the complex strongly bound to p58C, only p97 without the other components bound to p58N under the same conditions (Fig. 11, C and D). A substantially smaller amount of p97 bound to p58N compared to p58C in this experiment (Fig. 11 C), in contrast to the equivalent binding of p97 to each of these matrices when incubated alone (Fig. 11 B). This appears to be due, at least in part, to an increased affinity of p97 for p58C when it is in a complex with ligand and Srp1 α since we detected a higher level of binding of p97 to both full-length p58 and p58C matrices when p97 was incubated together with ligand and Srp1 α than when it was incubated alone (data not shown).

Discussion

Primary Sequence Analysis of p58, p54, and p45

An increasing body of evidence suggests that the p62 complex directly participates in nuclear protein import. Because this protein assembly is localized near the central region of the NPC on both the cytoplasmic and nucleoplasmic surfaces, it provides a useful avenue for studying transport events linked to the central gated channel. To initiate a detailed functional analysis of the p62 complex, we have carried out cDNA cloning of the p58, p54, and p45 subunits in this study and have analyzed their localization and interaction with cytosolic transport factors.

Our molecular cloning studies have shown that p58, p54, and p45 all contain regions with multiple (up to 14) dispersed FG motifs, similar to the previously cloned p62 subunit (Starr et al., 1990). A subset of these FG motifs are found within degenerate versions of the sequence GFxFG that has been previously described for p62 and other FG nucleoporins (discussed by Rout and Wentz, 1994). An additional repeat motif (PA), which was previously described for the p62 subunit (Starr et al., 1990), is also present in the FG regions of p58, p54, and p45. The FG-containing regions of all four subunits of the p62 complex are rich in serine, threonine, and glycine residues and have very few charged amino acids (Starr et al., 1990; this study). Certain FG repeat regions have been implicated in interaction with cytosolic transport factors (Paschal and Gerace, 1995; Radu et al., 1995b; Iovine et al., 1995), but the precise role of the FG or other repeat sequences in this binding is unclear.

Another feature common to all four subunits of the p62 complex is a region with two or more 20–40 amino acid segments predicted to have a high probability of forming α -helical coiled-coils. In principle, these coiled-coil regions could be responsible for homotypic or heterotypic interactions between different subunits of the p62 complex. Consistent with this, a 195 amino acid region of p62 that con-

tains several predicted coiled-coil segments has been shown to bind to p54 in a blot overlay assay (Buss and Stewart, 1995).

Previously it has been uncertain whether p45 is a distinct subunit of the p62 complex present in vivo or whether it is an in vitro degradation product (discussed in Guan et al., 1995). Multiple lines of evidence in this study indicate that p45 is a genuine component of the p62 complex. Analysis of partial length cDNA clones for p45 suggests that mRNAs for p58 and p45 are generated by alternative splicing, a possibility that is further supported by Northern blotting and immunoblotting with antibodies directed against a unique COOH-terminal epitope of p58. The sequence of p45 appears to be identical to that of p58 between residues 40–465, which encompasses the majority of the NH₂-terminal FG repeat region of p58 and the full length of the predicted coiled-coil domain. This common sequence is followed by 120 and 3 residues of unique sequence for p58 and p45, respectively. In the case of p58, this COOH-terminal region encodes its second FG repeat region. We have so far been unsuccessful in cloning the NH₂-terminal sequence of p45, and it remains unclear whether it is identical to that of p58.

The strong similarity between p58 and p45 raises the possibility that two different populations of the p62 complex may exist, one with two copies of p58 and no p45 and a second with two copies of p45 and no p58. If this were the case, one population of complexes could be restricted to the cytoplasmic side of the NPC and the other population to the nucleoplasmic side. This feature could help to specify structural and functional asymmetry of the NPC. It will be interesting to investigate this question in future studies.

Functional Interactions of the p62 Complex with Cytosolic Transport Factors

Previous work showed that immobilized recombinant p62 can deplete nuclear import activity from cytosol by removing NTF2 (Paschal and Gerace, 1995). We have now found that recombinant p58 and p54 also can deplete cytosolic nuclear import activity. Using immunoblot analysis, we showed that p62, p58, and p54 each remove NTF2, p97, and Srp1 α (an NLS receptor) from cytosol. To determine whether these interactions are direct or indirect, we carried out a series of binding assays using recombinant cytosolic factors and bead-immobilized p62, p58, and p54. These experiments showed that NTF2 and p97 interact directly with all three of the p62 complex subunits, while Srp1 binds indirectly through an interaction with p97.

Nuclear import is proposed to involve stepwise, vectorial transfer of an NLS receptor-ligand complex between multiple binding sites in the NPC (discussed by Melchior and Gerace, 1995). Previous experiments have shown that p97 can bind to several mammalian NPC proteins containing FG repeat regions on blot overlays (Moroianu et al., 1995b). These include Nup98, Nup153, and CAN/Nup214, which are located peripherally in the NPC (Sukegawa and Blobel, 1993; Panté and Aebi, 1994; Kraemer et al., 1994; Radu et al., 1995b). Our studies using a solution binding assay show that p97 also binds to FG repeat proteins of the p62 complex that are localized near the central region of

the NPC. Furthermore, we have found that a complex consisting of transport ligand, NLS receptor, and p97 stably binds to p58. This supports the view that p97 may be an adaptor mediating the interaction of a ligand/NLS receptor-containing transport complex with different FG repeat proteins during its stepwise movement across the NPC and that the association of a ligand/NLS receptor/p97 complex with the p62 complex resembles a bona fide intermediate in nuclear import. Whether a ligand/NLS receptor/p97 complex is maintained as a stable entity throughout the import process until the complex dissociates in the nucleus or whether a ligand/NLS receptor complex moves between different p97 molecules bound to distinct FG repeat nucleoporins is unclear.

Consistent with our findings with mammalian p58, Iovine et al. (1995) recently reported that a complex of ligand, Srp1p, and Kap95 (the yeast homolog of p97) binds to several yeast FG-containing nucleoporins in a SDS gel blot overlay assay. This further supports the notion that a complex of ligand, NLS receptor, and p97 can stably associate with discrete NPC sites involving FG repeat nucleoporins during nuclear import. These results differ from data obtained recently by Rexach and Blobel (1995), who found that the FXFG repeat region of yeast nucleoporin Nup1p stimulates the release of the ligand from a complex of ligand/Srp1p/Kap95. Based on this observation, the authors suggested a model for nuclear protein import involving the repeated two-step processes of association of ligand with a complex containing the NLS receptor and p97, and its subsequent dissociation from the latter due to interactions with FG repeat nucleoporins. Our results, together with the data from Iovine et al. (1995), indicate that the results obtained with Nup1 may only reflect an event restricted to Nup1p (e.g., related to transport complex dissociation after it reaches the nucleoplasmic side of the NPC) but cannot be generalized to interactions with all other FG proteins.

Interestingly, we found that *in vitro* binding of Srp1 α to p97 is strongly enhanced by the addition of transport ligand. Thus, binding of transport ligand to the NLS receptor in the cytosol would be expected to promote the formation of a complex containing p97. Since p97 is suggested to function as an adaptor for attaching the NLS receptors to the NPC (see above), this ligand-stimulated formation of a Srp1 α -p97 complex could help promote nuclear import of the population of Srp1 α that is ligand-bound. Complementing our data, it was recently reported that the affinity of transport ligand for the NLS receptor is stimulated by p97 (Rexach and Blobel, 1995). Considered together, these data indicate that the heterotrimeric complex of ligand/NLS receptor/p97 is more stable than any heterodimers formed by these three components. This suggests that the release of any component from the heterotrimeric complex will destabilize the association of the other two. This feature could be important for ligand dissociation inside the nucleus and subsequent factor recycling.

An unexpected finding from our studies was that only the COOH-terminal FG repeat region of p58 showed strong binding to a complex of ligand/Srp1 α /p97, even though both the NH₂- and COOH-terminal FG repeat regions of this protein bound similarly to p97 alone. This indicates that different FG repeat regions of NPC proteins

may have qualitatively and/or quantitatively different interactions with different complexes of cytosolic transport factors. Consistent with this notion, it was found that only the FG repeat region of Nup116p (and not that of other FG repeat nucleoporins) is lethal when overexpressed in yeast (Iovine et al., 1995). These differences in the association of transport complexes with different FG repeat proteins support a model in which distinct transport events occur at different FG repeat proteins of the NPC and in which intrinsic properties of the NPC define the directionality of transport. These findings do not support a model in which movement of a transport ligand across the NPC is a stochastic process involving repeated association/dissociation reactions among functionally equivalent FG repeat docking sites (Rexach and Blobel, 1995).

The functional role of the interaction between NTF2 and proteins of the p62 complex is not understood. Recent results have shown that NTF2 specifically interacts with the GDP-bound form of Ran (Nehrbass and Blobel, 1996; Paschal et al., 1996). With our *in vitro* binding conditions, we were unable to detect any effect of NTF2 or NTF2 plus GDP-Ran on the binding of the ligand/NLS receptor/p97 complex to p58 (Hu and Gerace, unpublished observation). However, for studies on NTF2 and other cytosolic factors, the use of solution-binding analysis with individual subunits of the p62 complex is only a first step towards the characterization of the p62 complex. It is likely that individual subunits of the p62 complex are not able to fully reconstitute interactions occurring at the intact complex. For example, cytosolic factors may have a higher affinity for the intact p62 complex than for individual subunits, and the interactions between the subunits may produce new binding sites for cytosolic factors that are not seen with the isolated subunits. Therefore, both *in vitro* and *in vivo* studies with the intact p62 complex are required to fully understand the functions of this protein assembly in nuclear protein import.

In summary, the interactions of cytosolic factors and subunits of the p62 complex shown in this study strongly support a direct role for the p62 complex as a ligand collection site near the central channel during nuclear import. The molecular tools and insights presented in this study will provide a framework for further analysis of this question and a starting point for investigation of the biochemical nature of the central gated channel of the NPC.

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References

- Adam, E.J., and S.A. Adam. 1994. Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. *J.*

- Cell Biol.* 125:547-555.
- Adam, S.A. 1995. The importance of importin. *Trends Cell Biol.* 5:189-191.
- Adam, S.A., and L. Gerace. 1991. Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. *Cell.* 66:837-847.
- Adam, S.A., T.J. Lobl, M.A. Mitchell, and L. Gerace. 1989. Identification of specific binding proteins for a nuclear location sequence. *Nature (Lond.)* 337:276-279.
- Adam, S.A., R.S. Marr, and L. Gerace. 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* 111:807-816.
- Aebersold, R. 1989. Internal amino acid sequence analysis of proteins after "in situ" protease digestion on nitrocellulose. In *A Practical Guide to Protein and Peptide Purification for Microsequencing*. P.T. Matsudaira, editor. Academic Press, Inc., San Diego. 71-88.
- Akey, C.W., and M. Radermacher. 1993. Architecture of the Xenopus nuclear pore complex revealed by three-dimensional cryo-electron microscopy. *J. Cell Biol.* 122:1-19.
- Breathnach, R., and P. Chambon. 1981. Organization and expression of eucaryotic split genes coding for proteins. *Annu. Rev. Biochem.* 50:349-383.
- Buss, F., and M. Stewart. 1995. Macromolecular interactions in the nucleoporin p62 complex of rat nuclear pores: binding of nucleoporin p54 to the rod domain of p62. *J. Cell Biol.* 128:251-261.
- Carmo-Fonseca, M., H. Kern, and E.C. Hurt. 1991. Human nucleoporin p62 and the essential yeast nuclear pore protein NSP1 show sequence homology and a similar domain organization. *Eur. J. Cell Biol.* 55:17-30.
- Cech, T.R. 1983. RNA splicing: three themes with variations. *Cell.* 34:713-716.
- Chi, N.C., E.J. Adam, and S.A. Adam. 1995. Sequence and characterization of cytoplasmic nuclear protein import factor p97. *J. Cell Biol.* 130:265-274.
- Cordes, V., I. Waizenegger, and G. Krohne. 1991. Nuclear pore complex glycoprotein p62 of *Xenopus laevis* and mouse: cDNA cloning and identification of its glycosylated region. *Eur. J. Cell Biol.* 55:31-47.
- Davis, L.I., and G. Blobel. 1987. Nuclear pore complex contains a family of glycoproteins that includes p62: glycosylation through a previously unidentified cellular pathway. *Proc. Natl. Acad. Sci. USA.* 84:7552-7556.
- Fabre, E., and E.C. Hurt. 1994. Nuclear transport. *Curr. Opin. Cell Biol.* 6:335-342.
- Finlay, D.R., E. Meier, P. Bradley, J. Horecka, and D.J. Forbes. 1991. A complex of nuclear pore proteins required for pore function. *J. Cell Biol.* 114:169-183.
- Frangioni, J.V., and B.G. Neel. 1993. Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. *Anal. Biochem.* 210:179-187.
- Furukawa, K., N. Pante, U. Aebi, and L. Gerace. 1995. Cloning of a cDNA for lamina-associated polypeptide 2 (LAP2) and identification of regions that specify targeting to the nuclear envelope. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:1626-1636.
- Görlich, D., and I.W. Mattaj. 1996. Nucleocytoplasmic transport. *Science (Wash. DC)* 271:1513-1518.
- Görlich, D., S. Prehn, R. A. Laskey, and E. Hartmann. 1994. Isolation of a protein that is essential for the first step of nuclear protein import. *Cell.* 79:767-778.
- Görlich, D., S. Kostka, R. Kraft, C. Dingwall, R.A. Laskey, E. Hartmann, and S. Prehn. 1995. Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. *Curr. Biol.* 5:383-392.
- Guan, T., S. Muller, G. Klier, N. Pante, J. M. Blevitt, M. Haner, B. Paschal, U. Aebi, and L. Gerace. 1995. Structural analysis of the p62 complex, an assembly of O-linked glycoproteins that localizes near the central gated channel of the nuclear pore complex. *Mol. Biol. Cell.* 6:1591-1603.
- Harlow, E., and D. Lane. 1988. *Antibodies: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp. 726.
- Hinshaw, J.E., B.O. Carragher, and R.A. Milligan. 1992. Architecture and design of the nuclear pore complex. *Cell.* 69:1133-1141.
- Imamoto, N., Y. Matsuoka, T. Kurihara, K. Kohno, M. Miyagi, F. Sakiyama, Y. Okada, S. Tsunasawa, and Y. Yoneda. 1992. Antibodies against 70-kD heat shock cognate protein inhibit mediated nuclear import of karyophilic proteins. *J. Cell Biol.* 119:1047-1061.
- Imamoto, N., T. Shimamoto, T. Takao, T. Tachibana, S. Kose, M. Matsubae, T. Sekimoto, Y. Shimonishi, and Y. Yoneda. 1995a. In vivo evidence for involvement of a 58 kDa component of nuclear pore-targeting complex in nuclear protein import. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:3617-3626.
- Imamoto, N., T. Tachibana, M. Matsubae, and Y. Yoneda. 1995b. A karyophilic protein forms a stable complex with cytoplasmic components prior to nuclear pore binding. *J. Biol. Chem.* 270:8559-8565.
- Iovine, M.K., J.L. Watkins, and S.R. Wentz. 1995. The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap95p, an essential yeast nuclear import factor. *J. Cell Biol.* 131:1699-1713.
- Kalderon, D., B.L. Roberts, W.D. Richardson, and A.E. Smith. 1984. A short amino acid sequence able to specify nuclear location. *Cell.* 39:499-509.
- Kita, K., S. Omata, and T. Horigome. 1993. Purification and characterization of a nuclear pore glycoprotein complex containing p62. *J. Biochem.* 113:377-382.
- Kraemer, D., R.W. Wozniak, G. Blobel, and A. Radu. 1994. The human CAN protein, a putative oncogene product associated with myeloid leukemogenesis, is a nuclear pore complex protein that faces the cytoplasm. *Proc. Natl. Acad. Sci. USA.* 91:1519-1523.
- Lupas, A., D.M. Van, and J. Stock. 1991. Predicting coiled coils from protein sequences. *Science (Wash. DC)* 252:1162-1164.
- Melchior, F., and L. Gerace. 1995. Mechanisms of nuclear protein import. *Curr. Opin. Cell Biol.* 7:310-318.
- Melchior, F., B. Paschal, J. Evans, and L. Gerace. 1993. Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J. Cell Biol.* 123:1649-1659.
- Melchior, F., T. Guan, N. Yokoyama, T. Nishimoto, and L. Gerace. 1995. GTP hydrolysis by Ran occurs at the nuclear pore complex in an early step of protein import. *J. Cell Biol.* 131:571-581.
- Moore, M.S., and G. Blobel. 1993. The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature (Lond.)* 365:661-663.
- Moore, M.S., and G. Blobel. 1994. Purification of a Ran-interacting protein that is required for protein import into the nucleus. *Proc. Natl. Acad. Sci. USA.* 91:10212-10216.
- Moroianu, J., G. Blobel, and A. Radu. 1995a. Previously identified protein of uncertain function is karyopherin alpha and together with karyopherin beta docks import substrate at nuclear pore complexes. *Proc. Natl. Acad. Sci. USA.* 92:2008-2011.
- Moroianu, J., M. Hijikata, G. Blobel, and A. Radu. 1995b. Mammalian karyopherin alpha 1 beta and alpha 2 beta heterodimers: alpha 1 or alpha 2 subunit binds nuclear localization signal and beta subunit interacts with peptide repeat-containing nucleoporins. *Proc. Natl. Acad. Sci. USA.* 92:6532-6536.
- Nehrbass, U., and G. Blobel. 1996. Role of the nuclear transport factor p10 in nuclear import. *Science (Wash. DC)* 272:120-122.
- Newmeyer, D.D., and D.J. Forbes. 1988. Nuclear import can be separated into distinct steps in vitro: nuclear pore binding and translocation. *Cell.* 52:641-653.
- Newmeyer, D.D., D.R. Finlay, and D.J. Forbes. 1986. In vitro transport of a fluorescent nuclear protein and exclusion of non-nuclear proteins. *J. Cell Biol.* 103:2091-2102.
- Panté, N., and U. Aebi. 1994. Toward the molecular details of the nuclear pore complex. *J. Struct. Biol.* 113:179-189.
- Paschal, B.M., and L. Gerace. 1995. Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore complex protein p62. *J. Cell Biol.* 129:925-937.
- Paschal, B.M., C. Delphin, and L. Gerace. 1996. Nucleotide specific interaction of Ran/TC4 with nuclear transport factors NTF2, and p97. *Proc. Natl. Acad. Sci. USA.* In press.
- Powers, M.A., and D.J. Forbes. 1994. Cytosolic factors in nuclear transport: what's importin? *Cell.* 79:931-934.
- Radu, A., G. Blobel, and M.S. Moore. 1995a. Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. *Proc. Natl. Acad. Sci. USA.* 92:1769-1773.
- Radu, A., M.S. Moore, and G. Blobel. 1995b. The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex. *Cell.* 81:215-222.
- Rexach, M., and G. Blobel. 1995. Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. *Cell.* 83:683-692.
- Richardson, W.D., A.D. Mills, S.M. Dilworth, R.A. Laskey, and C. Dingwall. 1988. Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. *Cell.* 52:655-664.
- Robbins, J., S.M. Dilworth, R.A. Laskey, and C. Dingwall. 1991. Two interdependent basic domains in nucleoplasmic nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell.* 64:615-623.
- Rout, M.P., and S.R. Wentz. 1994. Pores for thought: nuclear pore complex proteins. *Trends Cell Biol.* 4:357-365.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sharp, P.A. 1981. Speculations on RNA splicing. *Cell.* 23:643-646.
- Shi, Y., and J.O. Thomas. 1992. The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. *Mol. Cell Biol.* 12:2186-2192.
- Snow, C.M., A. Senior, and L. Gerace. 1987. Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. *J. Cell Biol.* 104:1143-1156.
- Starr, C.M., M. D'Onofrio, M.K. Park, and J.A. Hanover. 1990. Primary sequence and heterologous expression of nuclear pore glycoprotein p62. *J. Cell Biol.* 110:1861-1871.
- Sukegawa, J., and G. Blobel. 1993. A nuclear pore complex protein that contains zinc finger motifs, binds DNA, and faces the nucleoplasm. *Cell.* 72:29-38.
- Sweet, D.J., and L. Gerace. 1995. Taking from the cytoplasm and giving to the pore: soluble transport factors in nuclear protein import. *Trends Cell Biol.* 5:444-447.
- Weis, K., I.W. Mattaj, and A.I. Lamond. 1995. Identification of hSRP1 alpha as a functional receptor for nuclear localization sequences. *Science (Wash. DC)* 268:1049-1053.
- Yang, J., and D.B. DeFranco. 1994. Differential roles of heat shock protein 70 in the in vitro nuclear import of glucocorticoid receptor and simian virus 40 large tumor antigen. *Mol. Cell Biol.* 14:5088-5098.