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Functional Domains in Nuclear Import Factor p97 for Binding the Nuclear Localization Sequence Receptor and the Nuclear Pore

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The interaction of the nuclear protein import factor p97 with the nuclear localization sequence (NLS) receptor, the nuclear pore complex, and Ran/TC4 is important for coordinating the events of protein import to the nucleus. We have mapped the binding domains on p97 for the NLS receptor and the nuclear pore. The NLS receptor-binding domain of p97 maps to the C-terminal 60% of the protein between residues 356 and 876. The pore complex-binding domain of p97 maps to residues 152–352. The pore complex-binding domain overlaps the Ran-GTP– and Ran-GDP–binding domains on p97, but only Ran-GTP competes for docking in permeabilized cells. The *N*-ethylmaleimide sensitivity of the p97 for docking was investigated and found to be due to inhibition of p97 binding to the pore complex and to the NLS receptor. Site-directed mutagenesis of conserved cysteine residues in the pore- and receptor-binding domains identified two cysteines, C^{223} and C^{228}, that were required for p97 to bind the nuclear pore. Inhibition studies on docking and accumulation of a NLS protein provided additional evidence that the domains identified biochemically are the functional domains involved in protein import. Together, these results suggest that Ran-GTP dissociates the receptor complex and prevents p97 binding to the pore by inducing a conformational change in the structure of p97 rather than simple competition for binding sites.

INTRODUCTION

The localization of nuclear proteins is mediated by loosely conserved amino acid sequences known as nuclear localization sequences (NLSs). Multiple pathways for nuclear localization of proteins have been suggested to exist based on the identification of at least two types of NLSs and unique components required for transport in each pathway. The classical NLS pathway is mediated by sequences comprised of short stretches of basic amino acid residues that direct transport of NLS-containing proteins from the cytoplasm to the nucleus (Dingwall and Laskey, 1991). Recently, a second type of NLS was defined in the hnRNP A1 protein (Siomi and Dreyfuss, 1995). This 35-residue sequence, called the M9 sequence, is required for both import and export of A1 and does not compete for the import of classical NLS-containing proteins (Pollard *et al.*, 1996). Nuclear protein import directed by both types of signals has been dissected biochemically and several required protein factors have been identified.

Classical NLS import begins with recognition of the NLS in the cytoplasm by a protein called the NLS receptor also known as importin α/karyopherin α/PTAC58/SRP1 (Adam and Gerace, 1991; Görlich *et al.*, 1994; Imamoto *et al.*, 1995b; Moroianu *et al.*, 1995a; Weis *et al.*, 1995; Yano *et al.*, 1992). Two homologues of the NLS receptor with only 46% sequence identity have been identified in mammalian cells and both are functional in protein import. Although the functional implication of multiple receptors has not been established, they appear to be expressed in a tissue-specific manner and may show specificity for different NLSs (Adam *et al.*, 1995;

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Prieve *et al.*, 1996). The NLS receptor and its cargo is targeted to the nuclear pore complex by p97, also known as importin β/karyopherin β/PTAC 97/Kap95 (Adam and Adam, 1994; Chi *et al.*, 1995; Görlich *et al.*, 1995a; Imamoto *et al.*, 1995a; Moroianu *et al.*, 1995a). p97 binds to the NLS receptor via a short basic domain in the amino terminus of the receptor called the importin β-binding (IBB) domain (Görlich *et al.*, 1996a; Weis *et al.*, 1996). Transfer of the IBB domain to a cytoplasmic protein causes the fusion protein to be localized to the nucleus. The IBB domain does not contain the NLS-binding domain of the receptor, therefore, NLS binding is not required for p97 to carry the receptor to the nucleus. It has been suggested that the IBB was the archetypal NLS and p97 was the archetypal receptor for transport (Görlich *et al.*, 1996a; Weis *et al.*, 1996). This hypothesis received support recently with the identification of the receptor for the M9 NLS. Surprisingly, M9 mediated targeting to the pore required a single protein, transportin (Pollard *et al.*, 1996). Transportin is a 97-kDa protein with 24% identity to p97/importin β. A yeast homologue to transportin, Kap104p, has been identified which binds to mRNA-binding proteins (Aitchison *et al.*, 1996).

The NLS receptor and p97 are thought to form a cytoplasmic heterodimeric receptor complex that docks the NLS protein to the cytoplasmic side of the nuclear pore complex (Adam and Adam, 1994; Enenkel *et al.*, 1995; Görlich *et al.*, 1995b; Imamoto *et al.*, 1995c). Subsequent accumulation of the NLS protein within the nucleus requires the small nuclear GTPase Ran/TC4 and the small Ran-GDP-binding protein p10/NTF2 (Melchior *et al.*, 1993; Moore and Blobel, 1993, 1994; Paschal and Gerace, 1995). Like other small GTPases, Ran only slowly hydrolyzes GTP in the absence of other factors. GTP hydrolysis by Ran is stimulated by several orders of magnitude by a cytoplasmic GTPase-activating protein RanGAP1 (Bischoff *et al.*, 1994; Becker *et al.*, 1995). Nucleotide exchange of GDP for GTP to complete the Ran cycle is catalyzed by the chromatin-associated guanine nucleotide exchange factor RCC1 (Bischoff and Ponstingl, 1991). Although neither RanGAP1 nor RCC1 need to be added as soluble factors to reconstitute protein import in permeabilized cells, both proteins are essential in intact cells (Tachibana *et al.*, 1994; Corbett *et al.*, 1995). Recently, a novel ubiquitin-like modification that targets RanGAP1 to the nuclear pore was described, and it was shown that antibodies to pore complex-associated RanGAP1 inhibit protein import in the permeabilized cell assay (Matunis *et al.*, 1996; Mahajan *et al.*, 1997).

Docking of the receptor complex to the pore is mediated by the interaction of p97 with a nucleoporin (Nup). A number of nucleoporins bind p97 in vitro, and all of these contain peptide repeats of the sequence XFXFG or GLFG (Iovine *et al.*, 1995; Radu *et al.*, 1995a,b). Which of these nucleoporins provide the actual docking site or sites is unknown. A prime candidate for the initial docking site is Nup358 (Wu *et al.*, 1995; Yokoyama *et al.*, 1995). This protein localizes to the distal ends of the filaments emanating from the cytoplasmic face of the pore at the approximate location where NLS-coated gold particles attach during docking. Nup358 is also a Ran-binding protein (RanBP2) and may provide a site for commitment of the receptor complex to transport (Melchior *et al.*, 1995; Chi *et al.*, 1996). A second docking site over the central transporter of the pore is coincident with the localization of the p62 complex of nucleoporins (Guan *et al.*, 1995). The components of this complex bind the receptor complex, Ran and p10/NTF2 in vitro (Paschal and Gerace, 1995; Hu *et al.*, 1996). The biochemical evidence for these two docking sites on the cytoplasmic side of the pore is supported by electron microscopy localization of NLS-coated gold particles during transport (Panté and Aebi, 1996). The particles can be seen to sequentially occupy discrete regions of the pore coincident with the ends of the filaments and the central transporter. It is possible that the selectivity and direction of transport is ensured by binding of the receptor complex to a discrete number of specific sites as it traverses the pore.

Ran-GTP is an important regulatory factor in protein import because it binds to several import components, including p97 and RanBP2, and modulates the interaction of p97 with other factors (Rexach and Blobel, 1995; Wu *et al.*, 1995; Yokoyama *et al.*, 1995; Lounsbury *et al.*, 1996). Ran-GDP also binds to several import components including p10/NTF2 and p97 (Chi *et al.*, 1996; Nehrbass and Blobel, 1996). Ran-GDP and Ran-GTP binding to p97 have opposite effects on formation of receptor complexes. Whereas Ran-GTP dissociates receptor complexes, the binding of Ran-GDP to p97 in conjunction with p10/NTF2 or RanBP1 stabilizes complexes (Chi *et al.*, 1996; Nehrbass and Blobel, 1996). The different nucleotide bound forms of Ran may have disparate roles in protein import with Ran-GDP escorting the receptor complex through the pore and providing directionality to import and Ran-GTP dissociating the receptor complex after translocation through the pore is complete (Chi *et al.*, 1996; Görlich *et al.*, 1996b; Koepp and Silver, 1996).

To better understand the mechanism of nuclear protein import, we have mapped the binding domains of p97 for interaction with the pore complex and the NLS receptor by deletion analysis and site-directed mutagenesis. The separation of binding domains for transport factors within p97 suggests that conformational changes in the protein rather than simple binding competition may be important for mediating p97 function in protein import.

MATERIALS AND METHODS

Cell Culture

HeLa cells were grown in high glucose DMEM containing 10% neonate bovine serum (Biocell Laboratories, Rancho Dominguez, CA) and penicillin/streptomycin (Life Technologies, Inc., Gaithersburg, MD). Cultures were maintained at 37°C in a humidified incubator with 5% CO_2 atmosphere. Cells were removed from plastic dishes by trypsinization and replated on 18 × 18-mm glass coverslips 18–24 h before use. For all experiments, the culture medium was replaced with fresh medium 2–4 h before use.

Expression and Purification of Recombinant Nuclear Transport Factors

Recombinant S-peptide/His-tag-p97, GST-p97, S-peptide/His-tag NLS receptor, glutathione *S*-transferase (GST)-NLS receptor, Ran/TC4, and RanBP1 were cloned, expressed in bacteria, and purified as described (Chi *et al.*, 1996). GTP and GDP loading of Ran was performed as described (Bischoff *et al.*, 1995).

Subcloning and Site-directed Mutagenesis of p97

p97 deletion constructs were made either by utilizing restriction sites within the cDNA or by amplification of specific regions by polymerase chain reaction (PCR). All constructs were subcloned into the S-peptide/His-tag pET30 expression vector (Novagen, Madison, WI). Constructs used for the NLS receptor-binding domain mapping were also subcloned into pBluescript II SK- (Stratagene, La Jolla CA) for in vitro transcription and translation. Site-directed mutagenesis of cysteines in p97 was performed by overlap extension PCR (Ho *et al.*, 1989). The cysteine of interest was changed to alanine in each case. All sequences were confirmed by restriction digest mapping and DNA sequencing.

Determination of NLS Receptor-binding Domain

For determination of the NLS receptor-binding domain on p97, 1.5 μg of S-peptide/His-tag NLS receptor (mouse pendulin, GenBank accession number U12270) was immobilized on 5 μl of S-protein beads (Novagen). Fragments of the p97 cDNA were subcloned into pBluescript II and expressed in a rabbit reticulocyte lysate in vitro transcription/translation system (Promega Biotech, Madison, WI) and labeled with [^{35}S]methionine. Equal cpm of each translation product as determined by autoradiography were added to receptor beads in 0.5 ml of import buffer containing 2 mM dithiothreitol (DTT), and samples were mixed for 1 h at 4°C. No attempt was made to equalize the concentration of proteins by specific activity. Unbound material was removed by three washes with import buffer, and bound proteins were eluted from the beads with 50 μl of 1× SDS sample buffer. Eluted proteins were resolved by 12.5% SDS-polyacrylamide gels. The Coomassie blue-stained gels were treated with ENHANCE (New England Nuclear Research Products, Boston MA), and labeled proteins were visualized by autoradiography with Kodak XAR-5 film.

Assays in Digitonin-permeabilized Cells

HeLa cells grown on glass coverslips were permeabilized with digitonin as described (Adam and Adam, 1994). For nuclear pore complex-binding assays, bacterially expressed S-peptide p97 constructs were incubated at a concentration of 400 nM with permeabilized cells for 30 min on ice in import buffer containing 0.1 mM TCEP [tris(2-carboxyethyl)phosphine, Molecular Probes, Inc., Eugene, OR] and 1 μg/ml each of aprotinin, leupeptin, and pepstatin A. Unbound protein was removed by washing with import buffer. The cells were then fixed with 2% formaldehyde in import buffer or phosphate-buffered saline (PBS) for 10 min and washed three times in PBS. The nuclear membranes were permeabilized with 0.1% Triton X-100 in PBS for 2 min at room temperature and then washed three times in 0.2% bovine serum albumin in PBS. S-peptide p97 was detected by fluorescence with fluorescein isothiocyanate-S-protein diluted 1:5000 in bovine serum albumin/PBS. After washing in PBS, the coverslips were mounted in 80% glycerol, 50 mM Tris-HCl (pH 8.0), and 0.1% *p*-phenylenediamine. Small differences in affinity between constructs will not be observed in this assay since near-saturating concentrations of each construct were added to the cells. The concentrations used are consistent with the concentrations of full-length p97 used in a typical import assay.

NLS protein docking assays were performed by incubation of 400 nM S-peptide-p97, 400 nM S-peptide NLS receptor, and 1 μM allophycocyanin-NLS (Chi *et al.*, 1996) in 50 μl of import buffer on digitonin-permeabilized cells for 30 min on ice. For competition experiments, a 20-fold M excess of each p97 construct was added to the mixture before incubation with permeabilized cells. For pre-binding block experiments, permeabilized cells were incubated with each construct at 500 nM in import buffer for 20 min on ice. After washing for 10 min in import buffer to remove unbound protein, import and docking reactions were performed as described above. For the Ran competition experiments in Figure 4, 400 nM S-peptide p97, 400 nM S-peptide NLS receptor, and 1 μM APC-NLS were incubated with 800 nM Ran-GDP or Ran-GTP and 800 nM RanBP1 and preincubated for 10 min on ice. The mixture was then incubated on digitonin-permeabilized cells for 10 min on ice. After the incubation, the cells were rinsed in ice-cold import buffer and fixed on ice for 10 min in 2% formaldehyde in import buffer. In all experiments, the cells were observed by epifluorescence illumination with a Zeiss Axioskop microscope equipped with a 63× 1.25 aperture objective. Images were stored by photography on Kodak T-MAX 400 film. For quantification of docking, the negatives were scanned with a Polaroid Sprint Scan 35 slide scanner, and the intensity of the nuclear envelope fluorescence was determined using SigmaScan (Jandel Scientific, San Rafael, CA).

N-ethylmaleimide Inactivation of Transport Components

Twenty micrograms each of S-peptide p97 or S-peptide NLS receptor were diluted in 100 μl of import buffer and treated with 5 mM NEM alone or 10 mM DTT followed by 5 mM NEM (Mock) for 30 min on ice. Unreacted NEM was quenched by the addition of DTT to 10 mM and incubation for 15 min on ice. The treated protein was then used in pore-binding assays or immobilized on S-protein agarose beads at a concentration of 1.5 μg of protein/5 μl of beads in import buffer with 2 mM DTT. After washing in import buffer, the beads were suspended in 500 μl of import buffer. GST-p97 (1.5 μg) was added to the NLS receptor beads and GST-NLS receptor (1.5 μg) was added to the p97 beads. The mixtures were then incubated for 1 h at 4°C with mixing. After three washes with import buffer, the bound material was eluted and resolved by SDS-PAGE.

RESULTS

Identification of the Pore-binding Domain in p97

The proposed role of p97 in the targeting of NLS-containing proteins to the nuclear pore is that of a linker between the NLS receptor and peptide repeat containing nucleoporins (Görlich and Mattaj, 1996). To identify the p97 sequences required for binding to the pore, we prepared deletion constructs of recombinant human p97 and assayed the S-peptide fusion proteins for binding to the nuclear pore in digitonin-permeabilized cells (Figure 1). This binding assay was used because it more realistically recapitulates the actual physiological binding events in protein import, in con-

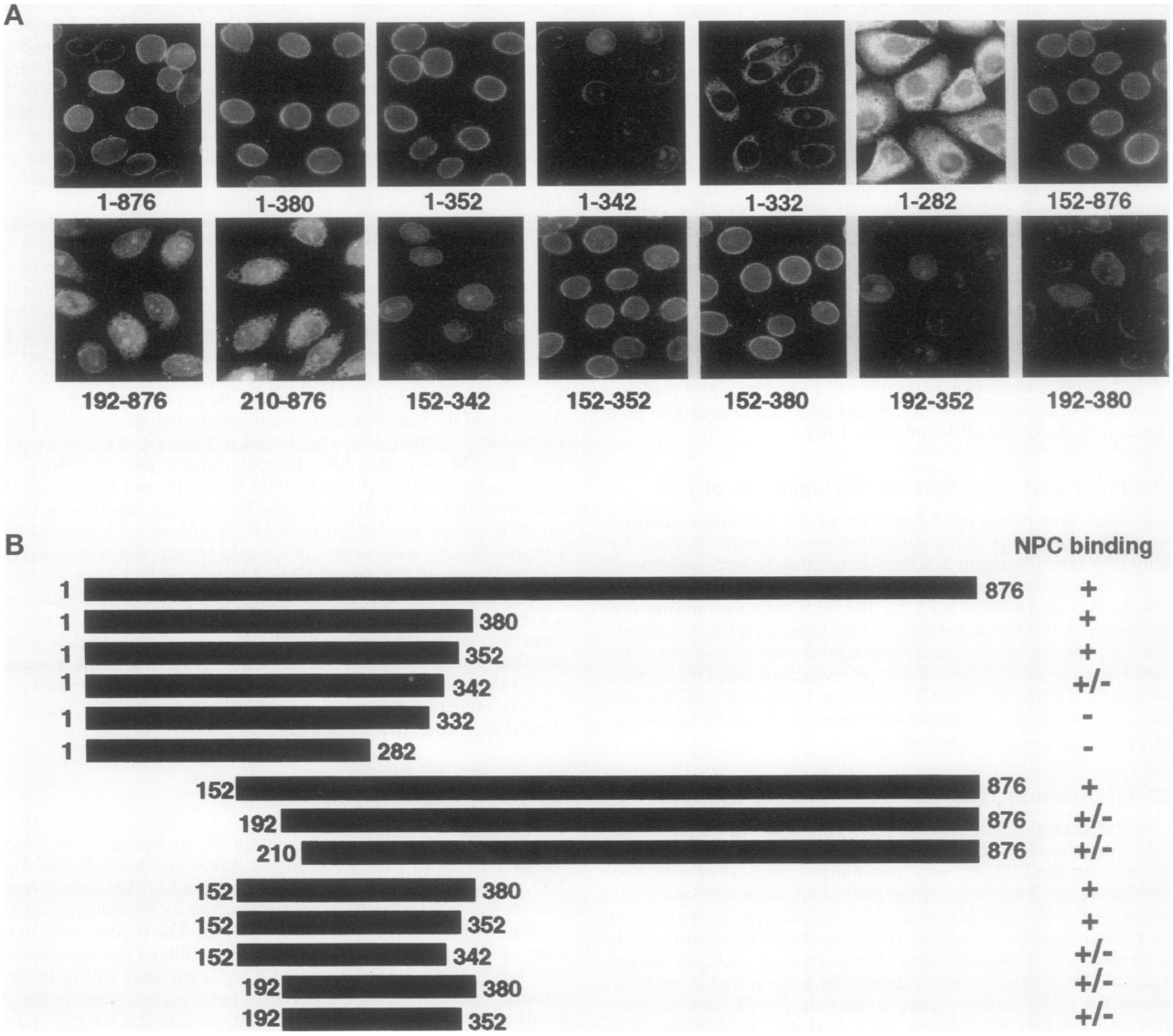

Figure 1. Mapping of the nuclear pore complex-binding domain on S-peptide-tagged recombinant p97. (A) Binding of p97 constructs to digitonin-permeabilized cells detected with FITC S-protein. (B) Structural representation of the p97 fragments and their binding to the pore. + indicates that binding was at least 80% of the full-length protein value. +/− indicates that binding was <80% of the full-length p97.

trast to the binding of p97 to isolated renatured nucleoporins on p97 overlay blots (Moroianu *et al.*, 1995b; Radu *et al.*, 1995a,b). C-terminal deletions of p97 demonstrated that the carboxyl terminus of p97 is dispensable for binding to the pore. Binding of a fusion protein containing amino acid residues 1–352 to the pore was equivalent to the binding of full-length p97 when equimolar amounts of the two proteins were added to the permeabilized cells. The removal of 10 additional amino acids from the C terminus reduced pore binding to <20% of the full-length protein (1–342). Removal of 10 more amino acids completely abolished pore binding (1–332). Therefore, a major determinant for pore binding lies between residues 333 and 352. Interestingly, upon removal of residues required for pore binding, two of the fusion proteins (1–282 and 1–332) then bound to cytoplasmic structures.

Amino terminal deletions of p97 were constructed to define the amino terminal boundary of the pore-binding domain. Removal of residues 1–151 had no effect on pore binding when compared with the full-length protein. Deletion of an additional 40 residues to amino acid 192 decreased binding to <20% of full-length p97. Binding was reduced further to <10% of the binding of the full-length protein by removal of

the first 209 amino acids. Thus, a second major determinant for pore binding lies between residues 153 and 191. Surprisingly, some of the N-terminal deletion constructs accumulated within the nucleus, specifically in association with nucleoli and also associated with cytoplasmic structures.

To verify that the domain between residues 152 and 352 localizes p97 to the pore complex, an S-protein fusion protein was prepared containing these residues and tested for pore binding. This construct bound the pore complex to the same extent as full-length p97. Further deletions from either end of this minimal sequence dramatically reduced binding to the pore (152–342, 192–352, and 192–380). Our earlier cell fractionation and localization of p97 indicated that approximately 20% of the protein is retained in cells after digitonin permeabilization, and this residual protein is tightly associated with the nuclear envelope (Chi *et al.*, 1995). We have been unable to chase this p97 off the pore in permeabilized cells (Chi and Adam, unpublished results). Because p97 does not appear to dimerize in solution, the endogenous pore-associated p97 probably does not influence the binding of fragments of the protein to the pore, other than by blocking available binding sites.

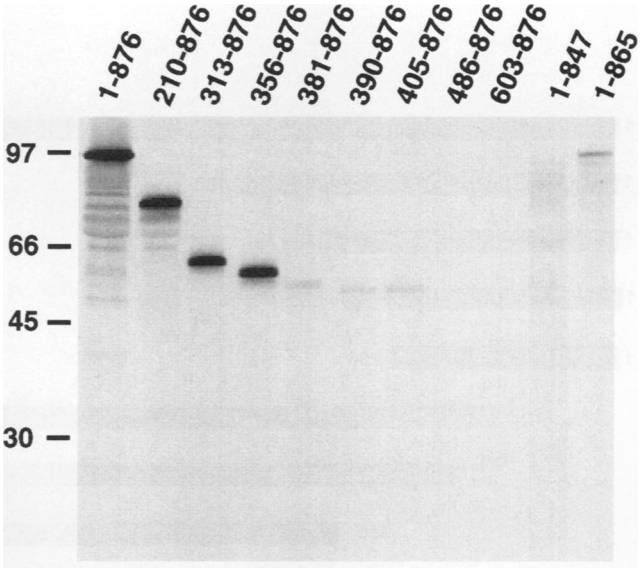

Figure 2. Mapping of the NLS receptor-binding domain on p97. ^{35}S-labeled autoradiograph of in vitro-translated fragments of p97 bound to immobilized NLS receptor (mouse pendulin). + indicates that binding was at least 80% of the full-length protein value. +/− indicates that binding was <80% of the full-length p97. Molecular weights of marker proteins in kilodaltons are indicated to the left.

Identification of the NLS Receptor-binding Domain in p97

The interaction of p97 with the NLS receptor is required for the receptor to dock at the pore and subsequently enter the nucleus. The NLS receptor-binding domain of p97 was identified by creating deletion constructs of p97 and assaying the ability of these constructs to bind the immobilized mouse pendulin homologue of the NLS receptor in a solution-binding assay (Figure 2). Deletion of up to the first 355 amino acids of p97 had no discernible effect on binding. Removal of 25 additional residues dramatically reduced binding (381–876). Further deletion of the first 485 amino acids completely abolished binding. Therefore, a major determinant of p97 for binding to the NLS receptor lies between residues 356 and 381. Surprisingly, deletion of as few as 11 amino acids from the carboxyl terminus dramatically reduced binding of p97 to the receptor. Removal of an additional 18 amino acids to residue 847 completely abolished binding. Thus, the receptor-binding domain resides between residues 356 and 876 of p97.

Functional Analysis of the Pore and Receptor-binding Domains

If the interactions of p97 with the pore and NLS receptor observed in the previous sections were functionally relevant to the import process, the isolated pore-binding domain or receptor-binding domain of p97 should inhibit accumulation of a NLS protein in the permeabilized cell assay by acting as competitive inhibitors with full-length p97 for pore-binding sites or the NLS receptor. This was tested first by using the fusion proteins described in the previous section as inhibitors of the docking reaction in permeabilized cells. The docking inhibition assay was performed in two ways. First, a 20-fold M excess of each construct was preincubated with the permeabilized cells to block pore-binding sites and excess protein was washed away prior to addition of full-length p97, NLS receptor, and APC-NLS. Second, a 20-fold M excess of each construct was added with the other factors during the incubation. The results of these experiments are summarized in Figure 3. When the p97 constructs were preincubated with the permeabilized cells, constructs containing the pore-binding domain (152–352, 1–352, 1–380, and 1–483) inhibited docking of the APC-NLS to the pore, whereas those fusion proteins that did not bind the pore did not inhibit docking. One construct comprising residues 152–876 was unable to inhibit docking, although it contained the pore-binding domain and bound to the pore (Figure 1). This is because the construct contained the NLS receptor-binding domain, also (see below), allowing the receptor and APC-NLS to bind to the predocked p97.

Similar results were obtained when the fusion constructs were mixed with the docking components during the incubation. However, in this experiment, additional constructs lacking a functional pore-binding domain inhibited docking (210–876 and 356–876).

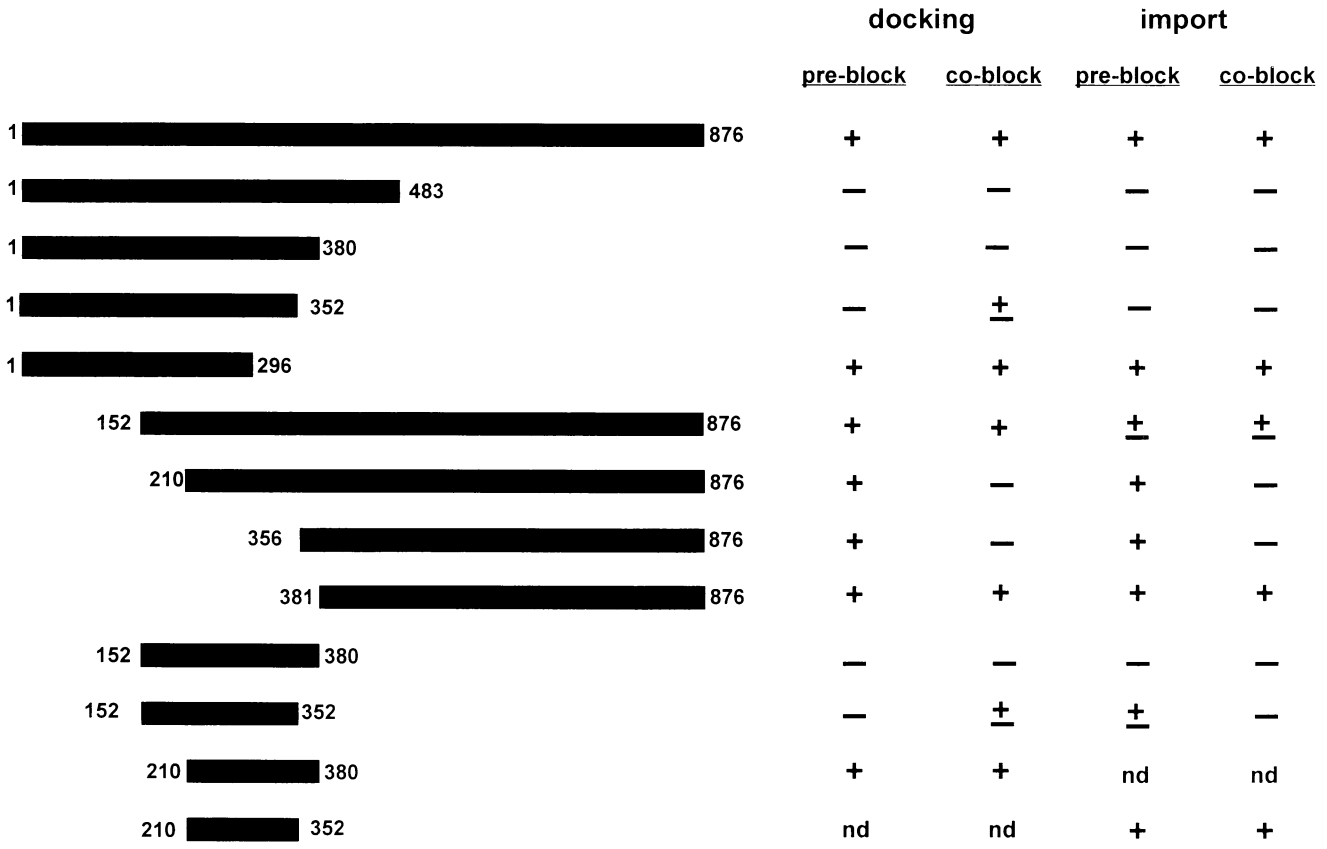

Figure 3. Functional analysis of the NPC-binding domain and NLS receptor-binding domain. Docking and import assays were performed as described in the text. The p97 fragments were either preincubated with the permeabilized cells (preblock) or mixed directly with the transport factors (coblock). + indicates that docking was at least 80% fo the full-length protein value. +/− indicates that docking or transport was <80% of the full-length p97 but was still detectable. nd means not determined.

This was due to competition for the binding of p97 to the NLS receptor rather than inhibition of p97 binding to the pore. Two minimal constructs containing the pore-binding domain (1–352 and 152–352) allowed slight amounts (<10% of control) of docking at the concentration assayed in the coincubation assay. Residues 1–380 completely blocked docking in this assay. A twofold higher concentration of constructs 1–352 and 152–352 completely inhibited docking (Chi and Adam, unpublished results). All three constructs (1–352, 1–380, and 152–352) completely inhibited docking in the preincubation blocking assay. It is likely that the full-length p97 bound to the NLS receptor has a higher affinity for the pore than the shorter constructs alone.

In an import reaction with p97, NLS receptor, Ran/TC4, APC-NLS, and ATP/GTP, the deletion constructs inhibited import in agreement with the binding domain mapping. That is, those constructs containing either a functional pore-binding domain (1–352, 1–380, and 152–352) or a functional NLS receptor-binding domain (152–876, 210–876, and 356–876) inhibited import. One construct comprising residues 152–876 partially inhibited import, although it contained both domains in functional form. This truncated p97 is missing amino terminal sequences necessary for Ran binding. This construct allows docking and may therefore deliver substrate to the pore and concentrate it there, facilitating its transport by the full-length p97 in the assay.

The Ran-GTP–binding domain (1–282) and Ran-GDP/RanBP1–binding domain (1–380) overlap the pore complex-binding domain of p97 (152–352; Chi *et al.*, 1997). If the binding of Ran to p97 dissociates p97 from the pore by simple competition for overlapping binding domains, binding of Ran-GDP/RanBP1 to p97 should also compete for pore binding and inhibit docking. Purified p97, NLS receptor, APC-NLS, RanBP1, and either Ran-GDP or Ran-GTP were combined and incubated on permeabilized cells (Figure 4). RanBP1 was included with both Ran-GTP and Ran-GDP because we have shown that RanBP1 equalizes the binding affinities of Ran-GTP and Ran-GDP for p97 (Chi *et al.*, 1996). Ran and RanBP1 were included at a twofold molar excess over p97 to ensure satura-

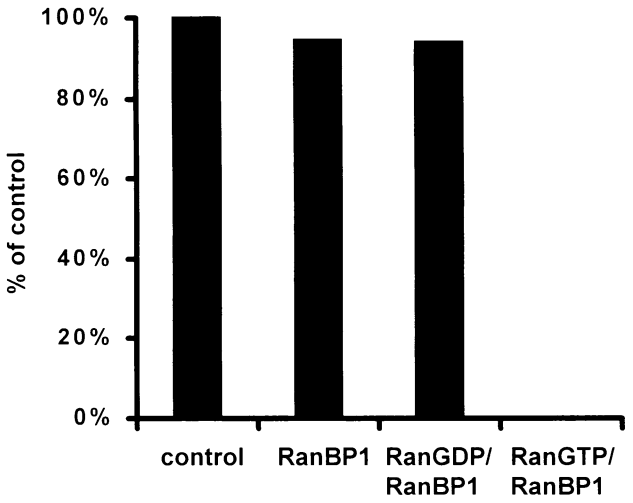

Figure 4. Competition for docking with Ran-GTP and Ran-GDP. Docking assays were performed as described in the text. The control incubation contained only NLS receptor, p97, and APC-NLS. The other incubations contained the indicated proteins. The intensity value of the control incubation was set at 100%.

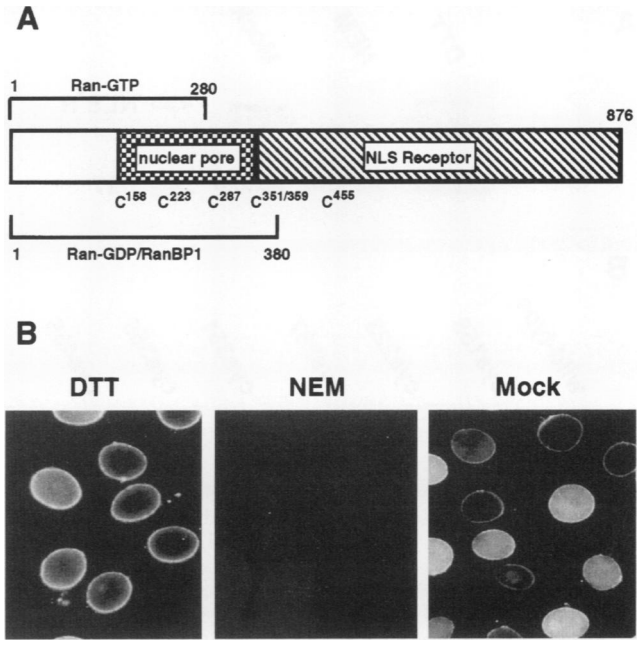

Figure 5. Conserved cysteine residues in p97 and NEM inactivation of pore-binding activity. (A) Schematic diagram of the domain structure of p97 with the positions of the conserved conserved cysteines indicated. (B) Recombinant p97 was treated with NEM as described in the text and incubated on digitonin-permeabilized cells. The bound protein was detected with FITC S-protein as in Figure 1. DTT, treated only with DTT; NEM, treated with NEM and quenched with DTT; Mock, treated with NEM that had first been quenched with DTT.

tion of p97 by Ran. As previously shown, RanBP1 alone had no effect on the docking reaction at 0°C (Chi *et al.*, 1996). Under these conditions, only Ran-GTP/RanBP1 inhibited docking, although both Ran-GTP and Ran-GDP bind p97 with equal affinity. Ran-GTP alone inhibited docking as well as the RanGTP/RanBP1 combination (Chi and Adam, unpublished results).

Identification of Cysteine Residues Required for Pore and NLS Receptor Binding

N-ethylmaleimide treatment of p97 abolishes the ability of the protein to dock NLS-containing proteins to the pore in conjunction with the NLS receptor (Adam and Adam, 1994). Six cysteine residues are conserved between the homologues of p97: C^{158}, C^{223}, C^{287}, C^{351}, C^{359}, and C^{455}. The first five of the conserved cysteines are contained within or are immediately adjacent to the pore-binding domain and the last two are within the receptor-binding domain (Figure 5A). To test the role of these conserved cysteines in p97-mediated docking, we first tested the ability of NEM-treated p97 to bind directly to the pore complex. NEM-treated p97 did not bind the pore in permeabilized cells as demonstrated by detection of the fusion protein with fluoresceinated S-protein (Figure 5B).

NEM-treated p97 was tested also for its ability to bind the NLS receptor in a solution-binding assay (Figure 6A). When S-peptide p97 was treated with NEM prior to immobilization on agarose beads, it no longer bound the GST-NLS receptor. However, NEM treatment of the S-peptide NLS receptor did not affect the ability of the immobilized receptor to bind GST-p97 in solution, indicating that the NEM block of docking is specific to p97.

Single-point mutations were then introduced into the recombinant p97 to change the six individual conserved cysteine residues to alanine. The mutant p97 proteins were immobilized on S-protein agarose beads and mixed with soluble GST-NLS receptor. Surprisingly, none of the cysteine point mutants were defective for NLS receptor binding in the solution even though two of the cysteines, C^{359} and C^{455}, lie within the receptor-binding domain (Figure 6B).

The cysteine point mutants were assayed for their ability to support docking or import of a NLS-containing protein in permeabilized cells (Figure 7). As shown previously, $C^{158} \rightarrow$ A, which binds Ran-GTP but does not bind Ran-GDP/RanBP1, supported docking and was severely deficient for import (Chi *et al.*, 1997). Only one mutant p97, $C^{223} \rightarrow$ A was defective for binding to the pore, but was still able to bind weakly compared with the wild-type protein. Because $C^{223} \rightarrow$ A showed a partial defect, a nearby nonconserved cysteine, C^{228}, was mutated also and was found to be partially defective for pore binding. A double

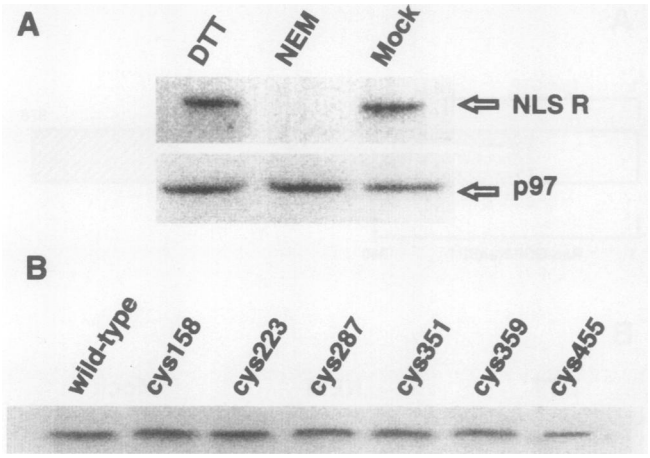

Figure 6. NEM inactivation of the p97-NLS receptor interaction and mapping of cysteine residues. (A) Individual treatment of p97 and NLS receptor with p97. In the top panel, immobilized NEM-treated p97 was used to adsorb soluble NLS receptor. In the bottom panel, immobilized NEM-treated NLS receptor was use to adsorb soluble p97. The bound proteins were eluted in sample buffer and resolved by SDS-PAGE. The gels were stained with Coomassie blue. (B) Point mutants of each of the conserved cysteines in p97 were assayed for their binding to NLS receptor. p97 was immobilized and used to adsorb soluble NLS receptor. Detection of bound receptor is as in A.

mutant converting both C^{223} and C^{228} to alanines was completely defective for binding to the pore. In the import assay, again, $C^{223} \rightarrow A$ and $C^{228} \rightarrow A$, as well as the double mutant, were defective as expected if p97 was unable to interact with the pore. Mutations replacing cysteines 287, 351, 359, and 455 showed no defects in binding of p97 to the pore, docking or import (Figure 7; Chi and Adam, unpublished results).

DISCUSSION

The formation of a receptor complex is an important step in the transport of NLS-containing proteins from the cytoplasm to the nucleus. The minimal receptor complex required for import in digitonin-permeabilized cells contains the NLS protein, the NLS receptor, and p97, but the native composition of the receptor complex is unknown (Görlich and Mattaj, 1996). Other proteins have been observed to copurify or coimmunoadsorb with p97, and their roles in the formation of the receptor complex have not been investigated (Chi *et al.*, 1995; Imamoto *et al.*, 1995c; Saitoh *et al.*, 1996). Ran, p10/NTF2, RanBP1, and RanGAP1 also interact with p97 in solution and the timing of their interaction with the receptor complex is uncertain (Rexach and Blobel, 1995; Chi *et al.*, 1996; Koepp *et al.*, 1996; Lounsbury *et al.*, 1996; Nehrbass and Blobel, 1996; Paschal *et al.*, 1996; Saitoh *et al.*, 1996). By understanding the possible interactions of the various transport factors, we can develop a clearer picture of how they cooperate to move proteins through the pore.

The region of the NLS receptor that binds to p97 has been mapped to a highly conserved amino terminal 41-amino acid motif known as the IBB (Görlich *et al.*, 1996a; Weis *et al.*, 1996). The region of p97 that recognizes the NLS receptor mapped to the carboxyl-terminal 60% of the protein from residues 356–876, a surprisingly large domain given the relatively small size of the IBB domain. Removal of even small segments of p97 from either end of this domain dramatically diminished receptor binding. It seems likely that the recognition of the IBB domain is through multiple sequences in p97 that are spatially oriented by folding of the protein. It will be important to identify the actual contacts between the IBB and p97 to resolve this point. At least 1 and as many as 11 highly degenerate armadillo repeats have been identified in the p97 sequence (Görlich and Mattaj, 1996). The least degenerate of these lies within the receptor-binding domain (residues 400–442). Given that arm repeats are thought to participate in protein–protein interaction, some of the p97 arm repeats may be involved in interactions with the NLS receptor.

Reasoning that the basic IBB domain might interact with an acidic domain of p97, Moroianu *et al.* (1996) used a synthetic peptide corresponding to a highly conserved acidic domain of rat p97 (residues 334–340) to suggest that this domain is a major determinant for binding to the NLS receptor. Our results, however, demonstrate that this acidic domain of p97 is not required for NLS receptor binding because constructs lacking this sequence bind the receptor as well as constructs containing the sequence. Although we cannot rule out that the receptor interacts with this sequence while bound to full-length p97, the acidic domain is not a major determinant for receptor binding to p97.

p97 is a critical factor in protein import, linking the NLS receptor and its cargo with the nuclear pore complex. In solution assays or on overlay blots, p97 binds to nucleoporins containing GLFG or XFXFG peptide repeats (Iovine *et al.*, 1995; Radu *et al.*, 1995b; Rexach and Blobel, 1995). The repeats themselves probably do not directly interact with p97, but surround the binding determinants, possibly performing a structural role. Formation of the receptor complex is not obligatory for the binding of p97 to the nuclear pore or isolated nucleoporins since p97 binding can be demonstrated in the absence of other factors (this study; Görlich *et al.*, 1995b; Iovine *et al.*, 1995; Moroianu *et al.*, 1995b).

Because of the uncertainty over the precise identity of the p97-binding sites on the nuclear pore, we determined the pore-binding domain of p97 by binding recombinant proteins to the nuclear pore in digitonin-permeabilized cells. The pore-binding domain maps

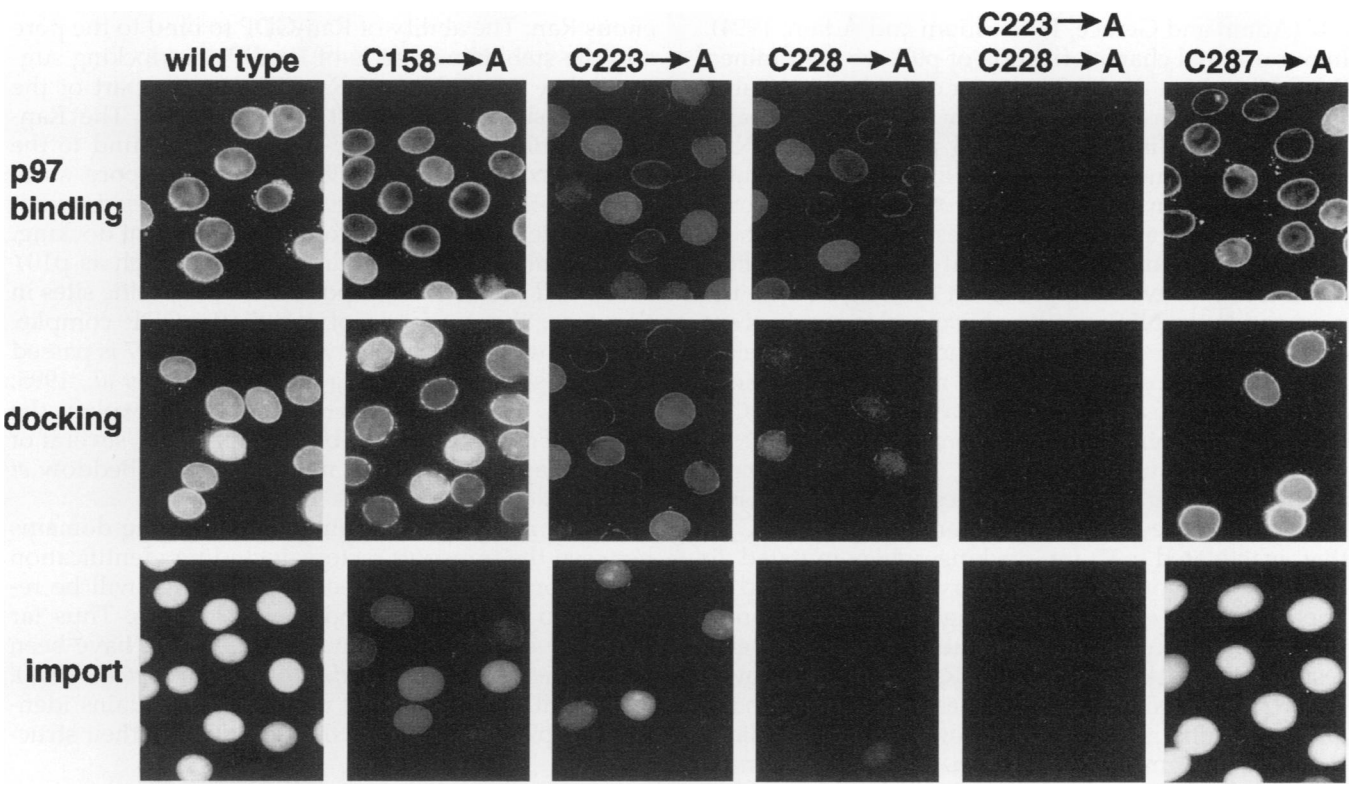

Figure 7. Functional analysis of the cysteine point mutants in p97. The individual point mutants were assayed for binding to the pore, reconstitution of docking, and reconstitution of import in digitonin-permeabilized cells. Detection of pore binding was with FITC S-protein. Docking and import were assayed by accumulation of APC-NLS.

to a relatively small domain between residues 152 and 352 with an additional sequence between residues 352 and 380 possibly strengthening the interaction. This isolated domain blocked both the docking and translocation of a NLS-containing protein mediated by the NLS receptor and full-length p97. This verifies that the binding of the domain to the pore is a functional interaction. The pore-binding domain is the most highly conserved region between human and yeast p97 homologues, with nearly 50% identity between the two proteins compared with only 34% over all of the proteins. The first 130 amino acids of the yeast homologue of p97, Kap95p, interact with the GLFG domain of Nup116p in a yeast two-hybrid assay (Iovine *et al.*, 1995). It seems unlikely that p97 is binding to a GLFG repeat nucleoporin since the pore complex-binding domain of human p97 does not require the same domain of the protein as the interaction of Kap95p with Nup116p. Removal of the GLFG region of Nup116p has a more pronounced effect on RNA export than protein import and may only be required for the export of Kap95p to the cytoplasm during recycling (Iovine *et al.*, 1995).

It has been suggested Ran-GTP displaces the NLS receptor from p97 by competition for overlapping binding sites (Moroianu *et al.*, 1996). Our experiments suggest an alternative mechanism. Although the Ran-GTP–binding domain (1–282) partially overlaps the pore-binding domain on p97 (152–352), the Ran-GDP/RanBP1-binding domain (1–382) completely overlaps the domain (Chi *et al.*, 1997). Yet, only Ran-GTP competes for the binding of p97 to the pore. In the same way, the NLS receptor-binding domain spanning residues 356–876 partially overlaps the Ran-GDP–binding domain but not the Ran-GTP–binding domain. Yet, only Ran-GTP dissociates the NLS receptor from p97. We propose that Ran-GTP dissociates both nucleoporins and the NLS receptor from p97 by inducing a conformational change in the protein. Therefore, the two Ran-binding domains are probably structurally dissimilar. When bound to p97 as a dimer with RanBP1, Ran-GTP and Ran-GDP have similar affinities for p97; therefore, it is unlikely that a difference in affinity for Ran could explain the effects of Ran binding on p97 association with other factors.

The earliest biochemical characteristic determined for protein import was the inactivation of import by NEM (Adam *et al.*, 1990; Newmeyer and Forbes, 1990). Both the docking and translocation steps are sensitive to NEM through inactivation of the NLS receptor and

p97 (Adam and Gerace, 1991; Adam and Adam, 1994). In our original characterization of p97, we determined that p97 and not the NLS receptor could be inactivated for docking by treatment with NEM (Adam and Adam, 1994). Binding of a NLS protein to the NLS receptor also is not sensitive to NEM; therefore, a later step in import involving the receptor must be affected (Adam and Gerace, 1991). The results presented here demonstrate that NEM treatment has a double effect on docking, preventing p97 from binding both to the pore and to the NLS receptor. Six cysteine residues are conserved between the human and yeast p97 proteins. NEM treatment of wild-type p97 and point mutations of these cysteines identified C^{158} as important for RanGDP/RanBP1 binding and accumulation of the NLS protein in the nucleus (Chi *et al.*, 1997). Further mapping of the other cysteine residues identified two residues within the pore-binding domain, C^{223} and C^{228}, that inactivated p97 for docking when mutated to alanine. Mutation of other conserved cysteines had no apparent affect on either docking or accumulation in permeabilized cells. How p97 utilizes these cysteine residues for docking or binding RanGDP is unknown. Given that the conserved cysteines are clustered in the amino-terminal half of p97, they may coordinate a structural feature of the protein. In our preliminary characterization of the protein, we found that p97 bound zinc in a 1:1 M ratio. Perhaps the role of the conserved cysteine residues is the coordination of a metal ion to form a structural determinant for p97 function.

Docking is not observed in permeabilized cells in the presence of Ran mutants that bind but are unable to hydrolyze GTP or in the presence of Ran bound to nonhydrolyzable analogues of GTP (Melchior *et al.*, 1993; Moore and Blobel, 1993; Carey *et al.*, 1996; Schlenstedt *et al.*, 1995). Although it is not clear that these observations have physiological significance, they do illustrate an important property of Ran-GTP interactions with p97. Ran-GTP dissociates the NLS receptor from p97 and prevents p97 from binding to nucleoporins (Rexach and Blobel, 1995). Because of the presence of RanGAP1 in intact cells, it is unlikely that significant concentrations of Ran-GTP would be present in the cytoplasm to prevent formation of a receptor complex under physiological conditions. Indeed, Görlich *et al.* (1996b) recently demonstrated that cytoplasmic Ran-GDP binds to the pore complex and is required for import in permeabilized cells. They concluded that Ran-GDP binding to the pore was independent of p97 and therefore must be associating with some other factor such as p10/NTF2.

We have shown recently that a Ran-GDP/RanBP1 dimer binds to p97 with the same apparent affinity as a Ran-GTP/RanBP1 dimer (Chi *et al.*, 1996). RanBP1 alone does not bind p97 but stabilizes p97-dependent docking in permeabilized cells in the absence of exogenous Ran. The ability of Ran-GDP to bind to the pore and the stabilizing effects of RanBP1 on docking suggests that Ran-GDP and RanBP1 may be part of the docking site on the intact pore complex. The Ran-GDP/RanBP1 dimer could also remain bound to the receptor complex as it moves through the pore, since binding of the dimer to the receptor complex does not release the NLS receptor from p97 or prevent docking. If different Ran-GDP–binding proteins, such as p10/NTF2 and RanBP1, were positioned at specific sites in the pore, the exchange of a p97/RanGDP complex could provide directionality to import as p97 is passed between sites of increasing affinity (Guan *et al.*, 1995; Hu *et al.*, 1996). This mechanism would explain the existence of multiple Ran-binding proteins, several of which may play a role in nuclear import (Beddow *et al.*, 1995).

Higher resolution mapping of the binding domains between the transport factors, including identification of the contact sites between the proteins, will be required to fully understand their functions. Thus far only the structures of Ran and p10/NTF2 have been determined (Scheffzek *et al.*, 1995; Bullock *et al.*, 1996). The small size of some of the binding domains identified in p97 may facilitate the resolution of their structure.

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