Several proteins are required for the transport of nuclear proteins from the cytoplasm to the nucleus, including the nuclear location sequence receptor (NLS receptor), p97, the small nuclear GTPase Ran/TC4, and several nucleoporins. The interaction of Ran with p97 is thought to regulate the interaction of these transport components. Ran-GTP alone binds p97, but Ran-GDP binds p97 only in conjunction with RanBP1. Using site-directed mutagenesis and deletion analysis, we have identified two distinct but overlapping binding domains for Ran-GTP and Ran-GDP/RanBP1 on p97. A short acidic sequence in p97 is part of the Ran-GDP/RanBP1 binding domain, possibly functioning in a similar manner as the C-terminal acidic sequence in Ran. A conserved cysteine residue in p97, Cys-158, is required for binding Ran-GDP/RanBP1, but not for binding of Ran-GTP to p97. In a permeabilized cell protein import assay, a mutant p97 with alanine substituted for Cys-158 is unable to support import in the presence of NLS receptor and Ran. These results support a direct active role for Ran-GDP in the receptor complex and provide evidence that the activity of downstream effectors of small GTPases may be regulated by both GTP- and GDP-bound forms of the protein.

The transport of karyophilic proteins from the cytoplasm to the nucleus involves multiple steps and several protein factors. Proteins destined for the nucleus associate with the nuclear import machinery via short basic peptide sequences termed nuclear localization sequences or NLSs1 (1). The NLS is recognized by a specific NLS-binding protein in the cytoplasm, the NLS receptor and karyophage, then bind to the nuclear pore via a second factor p97/importin β/karyopherin β/PTAC90 (6–9). p97 mediates interaction with the pore by direct association with a subset of a peptide repeat-containing family of nuclear pore complex proteins (nucleoporins) (10–12). Subsequent translocation of the receptor complex through the pore requires the action of the small nuclear GTPase Ran/TC4 and GTP hydrolysis (13, 14). Like other small GTPases, Ran requires a GTPase-activating protein Ran-GAP1, which is localized to the cytoplasm and a nuclear guanine nucleotide exchange factor, RCC1 (15–18).

Several other Ran-binding proteins have been identified that are important for Ran function in protein import. The Ran-GTP binding proteins RanBP1 and RanBP2 were identified on overlay blots as specifically binding the GTP form of Ran (19, 20). RanBP2/Nup358 is a nucleoporin that localizes to filaments extending from the cytoplasmic surface of the pore (21, 22). RanBP2 binds both p97 and Ran-GTP (11, 21–23) and has been proposed to be the initial docking site for the receptor complex on the pore and the site of GTP hydrolysis during transport (23). RanBP1 localizes to the cytoplasm, is essential for protein import in yeast (24), and stimulates protein import in a permeabilized cell assay (25). In solution, RanBP1 co-activates Ran-GTPase through an interaction with Ran-GTP and Ran-GAP1 (26–28) and inhibits GTP dissociation from Ran-GTP (26). Another Ran-associated protein, p10/NTF2, is also required in yeast and stimulates import in vitro (29, 30). p10 may coordinate the association of Ran-GDP with other transport factors and nucleoporins (31).

A heterodimer of the NLS receptor and p97 is thought to act as a receptor complex that targets a karyophile from the cytoplasm to the nuclear pore (32). The subsequent interaction of Ran-GTP with p97 mediates the dynamics of the receptor complex and the interaction of the complex with the pore. In solution assays with recombinant factors, association of a receptor complex with the peptide repeats of the nucleoporins dissociates the karyophile from the receptor-p97 dimer. Binding of Ran-GTP to p97 dissociates the receptor complex and releases the karyophile from the NLS receptor (33). These results are the basis for a model of transport in which multiple assembly/disassembly cycles of the receptor on an array of nucleoporins leads to facilitated diffusion of the karyophile into the nucleus. However, these solution binding results are inconsistent with functional experiments in permeabilized cells that reconstitute docking of the receptor complex to the pore (4, 6, 8, 34) and other solution binding experiments that demonstrated the formation of a stable nucleoporin-receptor complex with the mammalian nucleoporin p62 (35).

Recently, we demonstrated that the docking step of transport is stabilized and overall import is stimulated in a permeabilized cells assay by RanBP1 (25). These stabilizing and stimulatory effects are due to the binding of a Ran-GDP/RanBP1 dimer to p97. Unlike the binding of Ran-GTP to p97, the binding of Ran-GDP/RanBP1 to p97 does not dissociate the NLS receptor from p97. Therefore, Ran-GDP may be an integral part of the receptor complex during translocation through the pore. In the presence of RanBP1, Ran-GTP shows a 3-fold increase in binding affinity for p97. The affinity of Ran-GDP for p97 is increased by at least 10-fold to the same level as Ran-GTP by the presence of RanBP1. Formerly, it was thought that the effects of Ran on nuclear protein import were mediated by the action of Ran-GTP on its downstream effectors such as p97.
These results provided evidence that Ran-GDP is also an active participant in the translocation process.

In order to understand how the binding of Ran-GTP and Ran-GDP/RanBP1 to p97 affect protein import, we have mapped the binding domain for Ran on p97 by deletion analysis. Site-directed mutagenesis of specific amino acid residues was used to identify a specific cysteine residue required for Ran-GDP/RanBP1 binding. These results provide additional evidence for a role for Ran-GDP in protein import.

**EXPERIMENTAL PROCEDURES**

**Preparation of Cell Lysates and Affinity Adsorption**—Suspension cultures of human erythroleukemia cell line K562 were maintained in RPMI 1640 supplemented with 10% newborn calf serum and penicillin/streptomycin. For ^35S-labeling of amino acids, cells were harvested by centrifugation at 100 × g for 10 min and resuspended at 1 × 10^6 cells/ml in methionine and cysteine-free Dulbecco's modified Eagle's medium containing 5% newborn calf serum. L-[35S]ProMix (Amersham Corp.) was added to 20 μCi/ml, and the cells were incubated for 3–4 h at 37 °C. The cells were collected by centrifugation, washed twice with cold phosphate-buffered saline, and extracted for 5 min on ice in import buffer (20 mM HEPES, pH 7.35, 150 mM potassium acetate, 2 mM magnesium acetate, 0.1 mM EDTA, and 2 mM dithiothreitol) containing 50 μg/ml digitonin at 2 × 10^6 cells/ml. The lysate was cleared by centrifugation at 100,000 × g for 30 min, and Tween 20 was added to the supernatant to a final concentration of 0.1%.

Recombinant human p97, Ran, and RanBP1 were prepared and purified as described previously (25). Recombinant S-tag p97 was immobilized by adsorption to S-protein-agarose (Novagen, Madison WI) directly from bacterial lysates at a concentration of 2 μg of p97/10 μl of beads followed by washing in import buffer with 0.1% Tween 20. MAb3E9 or mouse IgG was immobilized on goat anti-mouse agarose beads as described by Chi et al. (7). Each adsorption from cell lysate contained 5 × 10^6 cell equivalents and 2 μg of p97 or MAb3E9 and was carried out for 60 min at 4 °C. After several washes in import buffer/Tween 20, bound proteins were eluted with 1 × SDS-sample buffer and resolved by 12.5% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250 or impregnated with ENHANCE (DuPont NEN) for autoradiography. Ran and RanBP1 were detected in Fig. 1 by immunoblotting after transfer to nitrocellulose and blocking with 5% non-fat dry milk in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) (7). Rabbit antiserum to RanBP1 was the generous gift of Takeharu Nishi- moto. Affinity-purified rabbit antibodies to Ran were the generous gift of Mary Moore and Frauke Melchior. Solution binding assays with recombinant proteins were performed in 20 mM HEPES, pH 7.0, 150 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol, 0.1% Tween 20, and 0.1% casamino acids. N-Ethylmaleimide (NEM) treatment of p97 was performed as described previously (6).

**Site-directed Mutagenesis and Deletion Analysis of p97**—The conserved cysteine residues of p97, as determined by the Genetics Computer Group Bestfit program, were mutated to alanine residues by polymerase chain reaction site-directed mutagenesis (36). Deletion constructs of p97 were created by polymerase chain reaction or by restriction enzyme digests and subcloned into an S-tag pET30 expression vector. S-tag constructs were immobilized on S-protein-agarose beads as were the wild-type p97. The RanDE deletion mutant was created by deletion of the ^21^DEDDDL^216 carbonyl sequence by polymerase chain reaction, purified, and loaded with nucleotide as was done with wild-type Ran.

**In Vitro Docking and Transport Assays**—Digitonin permeabilization of cells, docking, and import reactions were performed as described previously (25). 400 mM mutant or wild-type p97, 400 mM NLS receptor, and 1 μM allopophycocyanin (APC)-NLS were used for APC-NLS docking assays. Import experiments were performed using 1 μM Ran-GDP or Ran-GTP, 1 μM of RanBP1, 400 nM p97, 400 mM NLS receptor, 1 μM APC-NLS, 1 mM ATP, and 1 mM GTP in import buffer at 25 °C. Porcine complex-bound p97 was detected with fluorescein isothiocyanate-S-protein (Novagen).

**RESULTS**

In order to understand how the binding of the different nucleotide-bound forms of Ran regulates the activity of p97, we mapped the Ran-GTP and Ran-GDP/RanBP1 binding domains of p97. The binding domains were mapped by truncating recombinant p97 from the N and C termini and assaying the ability of the truncated proteins to bind Ran-GTP or Ran-GDP/RanBP1 in a solution binding assay (Fig. 1). The major binding determinant for Ran-GTP is contained in the first 282 amino acids of p97. A shorter construct containing residues 1–192 bound Ran-GTP with significantly lower affinity than the longer constructs, suggesting that a major binding determinant lies between residues 192 and 282. Removal of the first 7 amino acids from p97 decreased the affinity of Ran-GTP to levels undetectable by immunoblotting (construct 8–380). When Ran-GTP was added at 10-fold higher concentrations to 8–380 in the binding assay, Ran-GTP binding still could not be detected by protein staining, but could be detected weakly by immunoblotting for Ran (data not shown). Removal of the first 71 amino acids completely abolished the binding of Ran-GTP to p97. The Ran-GDP/RanBP1 binding domain in p97 was contained within a construct containing amino acids 1–352, although binding comparable to full-length p97 was achieved with a slightly longer construct containing residues 1–380. As with Ran-GTP binding to p97, Ran-GDP/RanBP1 binding was abolished by the removal of the first 7 amino acids of p97. Therefore, the Ran binding domain lies between residues 1 and 282, while the binding domain for Ran-GDP/RanBP1 lies between residues 1 and 380.

The interaction of RanBP1 with Ran is through the C-terminal domain of Ran that contains a conserved acidic domain (DEDDDL). Removal of the acidic domain lowers the affinity of RanDE-GTP for RanBP1, but has no effect on protein import in permeabilized cells (28, 37). However, RanDE expressed in cultured cells has a dominant negative phenotype for both nuclear protein import and RNA export (28, 37, 38). RanDE-GTP binds p97 with higher affinity than wt Ran in an overlay blot assay, although the increase in affinity has not been quantified (39). In order to determine if removal of the acidic terminus of Ran affected the ability of RanDE-GDP/RanBP1 to bind p97, we constructed recombinant Ran deleting the terminal DEDDDL sequence (RanDE). In solution binding assays with saturating amounts of Ran added (Fig. 2), RanDE-GTP bound p97 to the same extent as full-length Ran-GTP, but less RanBP1 bound to the p97-RanDE-GTP complex than to the p97-full-length Ran-GTP complex. RanDE-GDP/RanBP1, in contrast to Ran-GDP/RanBP1, did not bind p97. In a permeabilized cell transport assay, RanDE had less than 10% of the import activity of wild-type Ran in contrast to earlier published results (data not shown) (37).

Treatment of p97 with the sulfhydryl alkylating reagent NEM blocks the ability of p97 to participate in the docking step of transport (6). The stability of the docked receptor complex is modulated by Ran and RanBP1 (25), therefore we wanted to determine if NEM treatment of p97 affected Ran binding. As an initial experiment, recombinant p97 immobilized on agarose beads was treated with NEM and then incubated with cytosol.
the presence or absence of RanBP1 in the assay.

To demonstrate directly the effect of NEM treatment of p97 on the interaction with Ran and RanBP1 in the absence of other cytosolic proteins, we tested the capacity of NEM-treated recombinant p97 to bind recombinant Ran and RanBP1. Ran-GTP or Ran-GDP was mixed with NEM-treated or mock-treated immobilized recombinant p97 (Fig. 3B). In the absence of RanBP1, Ran-GDP did not bind to either mock-treated or NEM-treated p97, but Ran-GTP bound to both mock-treated and NEM-treated p97. When RanBP1 was added with Ran-GDP or Ran-GTP to p97, Ran-GDP and Ran-GTP both bound with RanBP1 to untreated or mock treated p97 with similar affinities; however, Ran-GDP/RanBP1 was unable to bind to NEM-treated p97 while Ran-GTP/RanBP1 bound with high affinity. RanBP1 alone did not bind p97 (data not shown) (25).

Six of the 23 cysteine residues in human p97 are conserved in the sequences of the homologous proteins from rat, mouse and Saccharomyces cerevisiae: Cys-158, Cys-223, Cys-287, Cys-351, Cys-359, and Cys-455 (4, 7, 8). Five of these residues lie within the binding domains for Ran-GTP and Ran-GDP/RanBP1, therefore we investigated the role of these residues in Ran binding to p97. Point mutagenesis of each conserved cysteine to alanine was carried out to identify the specific residues affected by NEM treatment of p97. The Cys-158 → Ala mutant was the only one of six point mutants that was defective for adsorbing Ran and RanBP1 from cell lysates, but NEM-treated p97 was unable to bind Ran and RanBP1 in the cell lysate (Fig. 3A) (25).

The Cys-158 → Ala mutant is defective for nuclear protein import. A, 400 nM Cys-158 → Ala mutant or wild-type p97 was incubated on digitonin-permeabilized HeLa cells, and bound p97 was detected with fluorescein isothiocyanate-S-protein (fitc; a and b). Docking reactions were performed on digitonin permeabilized HeLa cells with the NLS receptor, APC-NLS conjugate, and Cys-158 → Ala p97 mutant or wild-type p97. Binding of APC-NLS substrate was observed by fluorescence microscopy (APC-NLS; c and d). B, the Cys-158 → Ala mutant is defective for nuclear protein import. Nuclear import assays were performed on digitonin-permeabilized HeLa cells with recombinant NLS receptor, Ran, RanBP1, and p97. Accumulation of APC-NLS was detected by fluorescence microscopy.

RanBP1 is required for nuclear protein import and RNA export in vivo (24). In permeabilized cells, RanBP1 stimulates nuclear protein import and inhibits the temperature-dependent release of docked receptor complex from the pore (25). As we have shown recently, the addition of RanBP1 to the import assay containing the NLS receptor, wild-type p97, and Ran stimulated import (Fig. 5B, panels d and h). When wild-type p97 was substituted with Cys-158 → Ala, import with Ran-GDP or Ran-GTP was dramatically diminished (Fig. 5B, panels a and e). Interestingly, Ran-GTP did not support significant amounts of import in the presence of Cys-158 → Ala even though the binding of Ran-GTP to p97 was not affected by the mutation. The addition of RanBP1 had no effect on import with

FIG. 3. Binding of Ran and RanBP1 to Nem-treated p97. A, immobilized p97 was treated with 2 mM dithiothreitol (DTT), 5 mM NEM, or 10 mM dithiothreitol, followed by 5 mM NEM (Mock), and used to adsorb Ran and RanBP1 from 35S-labeled K562 cell lysates. Bound proteins were detected by autoradiography. B, recombinant p97 binding to recombinant Ran-GTP or Ran-GDP in the presence or absence of RanBP1 after NEM treatment of p97 as in A. Bound proteins were detected by Coomassie Blue staining.

FIG. 4. Mutational analysis of conserved cysteine residues in p97. A, immobilized recombinant p97 containing point mutants of each of the six conserved cysteines in p97 individually was used to adsorb Ran and RanBP1 from 35S-labeled K562 lysates. B, mutated recombinant p97 was used to adsorb recombinant Ran-GTP or Ran-GDP and RanBP1 in solution as in A. Proteins were detected by Coomassie Blue staining.

FIG. 5. The Cys-158 → Ala mutant is defective for nuclear protein import. A, 400 nM Cys-158 → Ala p97 mutant or wild-type p97 was incubated on digitonin-permeabilized HeLa cells, and bound p97 was detected with fluorescein isothiocyanate-S-protein (fitc; a and b). Docking reactions were performed on digitonin permeabilized HeLa cells with the NLS receptor, APC-NLS conjugate, and Cys-158 → Ala p97 mutant or wild-type p97. Binding of APC-NLS substrate was observed by fluorescence microscopy (APC-NLS; c and d). B, the Cys-158 → Ala mutant is defective for nuclear protein import. Nuclear import assays were performed on digitonin-permeabilized HeLa cells with recombinant NLS receptor, Ran, RanBP1, and p97. Accumulation of APC-NLS was detected by fluorescence microscopy.

FIG. 2. Binding of p97 and RanBP1 to RanDE. Ran-GDP, Ran-GTP, RanDE-GDP, RanDE-GTP, and RanBP1 were added in various combinations to immobilized p97 in solution binding assays. The bound proteins were detected by Coomassie Blue staining. + and − indicate the presence or absence of RanBP1 in the assay.
Ran-GDP and stimulated import to barely detectable levels with Ran-GTP when the Cys^{358} → Ala p97 was used (Fig. 5B, panels c and g). Only very low levels of docking are seen in these assays because Ran-GTP dissociates the docking complex in the absence of import (14, 33).

**DISCUSSION**

The nuclear Ras-like GTPase Ran/TC4 regulates multiple nuclear events, including nuclear protein import, RNA processing and export, nuclear growth, and cell cycle progression (40). How a single nuclear GTPase independently regulates peripherally related nuclear functions remains unclear. Ras-like GTPases regulate a variety of cellular events by acting as molecular switches; Ras-GTP is the active form and Ras-GDP is inactive (41). Ran mutants that bind, but are unable to hydrolyze, GTP have a dominant negative effect on Ran-dependent pathways, indicating that cycling between the GTP and GDP forms is important for Ran function (24, 27, 38). Similar phenomena are seen with GTPases involved in vesicular traffic, indicating that they may have similar mechanisms of action (42).

Several lines of evidence support a role for Ran-GDP in nuclear protein import. First, RanBP1 stimulates protein import in vitro and stabilizes docked receptor complexes against Ran-dependent release (25). Since RanBP1 does not bind p97 directly, this stabilization is probably due to RanBP1 binding Ran-GDP associated with the docking site on the pore. Second, RanBP1 increases the affinity of Ran-GDP for p97 by more than 10-fold to the same affinity as Ran-GTP (25). Third, the results presented here suggest that the NEM sensitivity of p97 could be partially due to the failure of p97 to bind Ran-GDP/RanBP1. Mutation of a specific cysteine residue in the Ran-GDP binding domain of p97 allows normal docking of the receptor complex and high affinity binding of Ran-GTP to p97, but the mutant protein is defective for transport. Mutation of the other cysteine residues leads to defects in binding of p97 to the pore complex and the NLS receptor. It is interesting to note, also, that neither NEM-treated p97 nor the Cys^{358} → Ala mutant adsorb Ran from cell lysates, suggesting either that the state of the Ran in the cytosol in vivo may be predominantly Ran-GDP or that Ran-GTP is hydrolyzed rapidly upon cell lysis. The difference in Ran-GTP and Ran-GDP/RanBP1 binding domains in p97 is probably due to changes in the structure of Ran upon exchange of nucleotide. p97^{ras} is known to undergo substantial structural changes upon exchange of GTP for GDP and many similar changes are likely to occur in Ran (41, 43). Because of the difference in binding domains for Ran-GTP and Ran-GDP/RanBP1, it is likely that binding different forms of Ran will have significant structural implications for p97. A model for displacement of the NLS receptor from p97 by the binding of Ran-GTP to an overlapping binding site has been proposed (28). The Ran-GDP/RanBP1 binding domain is more extensive than the Ran-GTP binding domain, yet Ran-GDP/RanBP1 does not displace the receptor from p97 (25), suggesting that the two Ran-binding domains are structurally very different or that the displacement of the receptor by Ran-GTP is due to induced conformational changes in p97, not competition for overlapping binding sites.2

The acidic C terminus of Ran is involved in regulating the interaction of Ran with other regulatory factors. This acidic sequence is required for the high affinity interaction of RanBP1 with Ran and affects the activity of RanBP1 on other Ran-related activities. It has been suggested that this region folds into the nucleotide binding pocket, stabilizing the binding of the GDP and preventing the binding of RanBP1. Upon exchange of GTP for GDP, the domain is displaced from the nucleotide pocket allowing the binding of RanBP1 to the acidic sequence (28). A similar sequence in p97 (DEQDEDDDDW) is immediately adjacent to sequences required for binding Ran-GDP/RanBP1, but not Ran-GTP. The failure of construct 1–341, which contains the acidic domain, to bind Ran-GDP/RanBP1 suggests that additional residues between 342 and 352 may provide sequence or structural determinants required to stabilize the acidic p97 sequence for Ran-GDP binding. In addition, the failure of RanDE-GDP/RanBP1 to bind p97 suggests that the acidic sequences of p97 and Ran are both required to form the complex. We propose that this sequence in p97 substitutes for the C-terminal Ran sequence in the nucleotide binding pocket of Ran when GDP is bound. This rearrangement would expose the C terminus of Ran for binding to RanBP1, stabilizing the trimeric complex. It was recently reported by Moroianu et al. (44) that major determinants for Ran-GTP binding to p97 are contained between residues 329–382 in a region proposed to form loops of antiparallel amphipathic helices. In our experiments, these sequences can be removed from p97 without affecting Ran-GTP binding and N-terminal deletions of p97 containing these sequences do not bind Ran-GTP.

The GTP-dependent affinities of GTPases for effector molecules determines their cellular functions (41). It is likely that the interaction of Ran with unique effectors for each different nuclear function is the key to specificity of action. In a mechanism thus far unique to the small GTPase Ran, both the GTP and GDP-dependent interactions of Ran with p97 appear to be important for regulating nuclear protein import. The stable association of both the GTP and GDP bound forms of Ran with the nuclear transport complex would dramatically increase the efficiency of the transport process by abrogating the necessity of re-assembling a transport complex at each step in translocation through the pore. The identification of additional effectors of the Ran-dependent nuclear pathways will help to further distinguish the diverse mechanistic aspects of Ran function.

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