**Ran Binding Domains Promote the Interaction of Ran with p97/β-Karyopherin, Linking the Docking and Translocation Steps of Nuclear Import**

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Nuclear protein import is accomplished by two sequential events: docking at the nuclear pore complex followed by ATP-dependent translocation across the nuclear envelope. Docking of nuclear targeted proteins requires a 56-kDa nuclear localization signal receptor (α-karyopherin, importin-α, SRP1α) and a 97-kDa protein (β-karyopherin, importin-β). Components necessary for translocation include the Ran/TC4 GTPase and ATP-dependent translocation of NLS-containing proteins through the nuclear pore complex is mediated by the Ran GTPase and a 10-kDa protein, NTF2/B-2 (11–13). While these components are known to be necessary for the import of NLS-containing proteins, their mechanism of action has not been elucidated. Ran is a 25-kDa Ras-related GTPase that is predominantly localized to the nucleus (14, 15). Expression of a GTPase-deficient mutant of Ran, or depletion of the Ran GTPase-activating protein, Rna1p, blocks nuclear import both in vitro and in intact cells (16, 18, 19),2 demonstrating that GTP hydrolysis is necessary for nuclear transport. After GTP hydrolysis, Ran-GDP may enter the nucleus where guanine nucleotide exchange can be stimulated by the chromatin-associated factor, RCC1 (19). Ran-GTP would then exit the nucleus to complete the cycle (for review see Ref. 20).

Two potential downstream effectors that bind to Ran-GTP have been identified, RanBP1/HTF9A, a 29-kDa cytosolic protein, and RanBP2/NUP358, a 360-kDa nuclear pore protein (21–23). These proteins associate with Ran through one or more conserved Ran binding domains (RanBDs). These domains stabilize Ran in the GTP-bound state and co-activate the GTPase-activating protein, Rna1p (24–26). Additionally, antibodies raised against RanBP2 inhibit nuclear translocation, which suggests that RanBP2 may be a necessary constituent in the nuclear import mechanism (22). No requirement for RanBP1 in nuclear protein import has yet been established.

Removal of the acidic C terminus of Ran reduces binding to RanBDs but enhances binding to two unidentified proteins of 90 and 115/120 kDa (24). In this report we identify the 90-kDa protein as β-karyopherin. Direct interaction of β-karyopherin to wild-type Ran is promoted by association of Ran with RanBP1 or with an isolated Ran binding domain. These results suggest that β-karyopherin links the docking and translocation steps of nuclear import by forming a ternary complex with the Ran GTPase and a RanBP.

**EXPERIMENTAL PROCEDURES**

Production of Recombinant Proteins—Recombinant Ran was expressed in Escherichia coli DH5α bacteria from a pET11a expression vector (Novagen) and purified by DEAE column chromatography as described previously (24). The Δ-DE Ran mutant was created by the removal of the C-terminal sequence of Ran, PDEDDDL (24). GST fusion proteins of Δ-DE Ran and RanBP1 were expressed from pGEX2T (Pharmacia Biotech Inc.) and purified by glutathione-Sepharose chromatography. Truncations of RanBP1 were made using polymerase chain reaction mutagenesis, and the products were subcloned into pGEX2T. The residues expressed by the RanBP1 truncations are RanBP1 Δ-C (residues 1-160), RanBD (residues 27–160), and RanBP1 Δ-N (residues 27–203). The integrity of the constructs was confirmed by DNA se-
Rat brain cytosol proteins were prepared as described previously (3). BHK21 cell proteins were prepared by addition of hot SDS sample buffer to 100-mm plates of confluent BHK21 cells that had been rinsed twice with phosphate-buffered saline. Cell lysate was passed through a 26-gauge syringe needle, and insoluble material was removed by centrifugation at 12,000 × g for 5 min. Protein samples (150 μg) were separated by 8% SDS-PAGE and transferred to nitrocellulose for analysis by the Ran overlay assay, as described previously (24). Equal amounts of Ran-bound [α-32P]GTP (approximately 300,000 cpm) were diluted into 10 ml of binding buffer for the overlay. After 30 min of incubation, overlays were washed and exposed to film (24). Purification of the 90-kDa Protein by Δ-DE Ran-Sepharose Chromatography—All steps were performed at 4 °C. Cytosol was prepared from the brains of 65 rats as described in Ref. 3 in buffer B (20 mM HEPES-KOH, pH 7.3, 2 mM magnesium acetate, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin). The lysate was adjusted to 150 mM sodium acetate and added to 100 ml of DE52 cellulose slurry (Whatman) equilibrated with buffer B plus 150 mM sodium acetate. The mixture was stirred gently for 30 min and then washed with 1 liter of buffer B plus 150 mM sodium acetate. Proteins were eluted with 2 × 50 ml of buffer B plus 500 mM sodium acetate. The eluate was dialyzed overnight against buffer B plus 100 mM sodium acetate. Proteins that bind nonspecifically to GST and glutathione-Sepharose were removed by incubation with 100 μg of GST bound to 100 μl of glutathione-Sepharose beads, and the cleared supernatant was purified by Δ-DE Ran affinity chromatography. The Δ-DE Ran affinity matix was prepared by applying lysate from 1 liter of E. coli expressing GST-Δ-DE Ran (approximately 3 mg) onto 1 ml of glutathione-Sepharose beads. After 30 min, the beads were washed with phosphate-buffered saline and once with 50 mM MOPS, pH 7.1, plus 1 mM EDTA. The beads were resuspended in the same buffer plus 2 mM GTP and incubated for 30 min. Magnesium acetate (20 mM) was then added, and the Δ-DE Ran-Sepharose was centrifuged for 2 min at 2000 × g and washed with binding buffer (24). Cleared supernatant was incubated with the Δ-DE Ran-Sepharose (1–2 h). The mixture was then washed three times with binding buffer plus 0.05% Tween 20 and once with binding buffer and then loaded onto a small column. Purified proteins were eluted with 4 ml of 10 mM glutathione in binding buffer.

Identification of the 90-kDa Protein That Binds to Δ-DE Ran—Proteins purified from the Δ-DE Ran Sepharose column were precipitated with 0.1 M NaClate plus 10% trichloroacetic acid for 16 h and collected by centrifugation. Pellets were washed with acetone, dried, and resuspended in 50 μl of a solution containing 0.1 M NaOH and 0.1% deoxycholate. Proteins were separated by SDS-PAGE and transferred to Immobilon P (Millipore). Proteins were stained with Oncopore S, and the 90-kDa protein was excised. Approximately 70 pmol of the excised protein was generated for sequence identification. Digestion with trypsin and sequence analysis were performed by the Harvard Microchemistry Facility, Cambridge, MA.

Superose 12 FPLC—Approximately 200 μg of brain cytosol proteins, batch-elicuted from DE52 cellulose, were incubated with 2 mM GTP for 10 min on ice with or without 3 μg of GST-Δ-DE Ran that had been preloaded with GTP. This mixture was loaded onto a Superose 12 (Pharmacia Biotech Inc.) column, equilibrated in 20 mM Tris-HCl, pH 7.8 at 4 °C, and 50 mM NaCl. The flow rate was 0.25 ml/min, and 0.5 ml fractions were collected. The 4.5-ml void volume was collected in frac-

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**FIG. 1. A Ran-RanBP1 complex is able to bind to Δ-DE Ran binding proteins.** Six lanes of BHK21 cell extract (150 μg of protein) were separated by 8% SDS-PAGE and transferred to nitrocellulose. Lanes were then incubated separately for 30 min with 300,000 cpm of [α-32P]GTP-GST-Δ-DE Ran alone (lane 5) or with [α-32P]GTP-Ran (wild type) plus the indicated constructs of GST-RanBP1 fusion proteins (lanes 1–4) or plus GST as a negative control (lane 6), each in 10 ml of binding buffer. (Specific activity of the [α-32P]GTP was 3000 Ci/mmol.) RanBP1 consists of residues 27–160 of RanBP1, RanBP1 Δ-Δ of residues 1–160, and RanBP1 Δ-N of residues 27–203. Δ-DE Ran consists of residues 1–197 of Ran. Following incubation, the overlays were washed and exposed to x-ray film overnight. The positions of rainbow molecular weight standards (Amersham Corp.) are noted.

**RESULTS AND DISCUSSION**

We showed previously that deletion of the C terminus of Ran reduces the affinity of Ran-GTP for the Ran-binding proteins RanBP1 and RanBP2 (17). In addition, the C-terminal deletion mutant of Ran, Δ-DE Ran, binds avidly to unidentified proteins of 90 and 115/120 kDa (24). These proteins (Δ-DE RanBP1s) associate specifically with Δ-DE Ran that is in the GTP-bound state; they inhibit release of GTP and are located primarily in the soluble fraction of cell extracts (not shown). The affinity of the proteins for Δ-DE Ran appeared to be higher than that to wild-type Ran, possibly as a consequence of the masking of a region on Ran normally blocked by the C terminus. We reasoned that the formation of a RanBP-Ran heterodimer may present the Ran in a conformation able to bind more efficiently to the 90- and 115/120-kDa proteins.

To test this hypothesis, BHK21 cell extract was separated by SDS-PAGE, transferred to nitrocellulose, and incubated with a complex of [α-32P]GTP-Ran and RanBP1. As predicted, the formation of a Ran-RanBP1 complex dramatically increased the apparent affinity of Ran for the 90- and 115/120-kDa Δ-DE RanBP1s (Fig. 1). The RanBP1 also effectively competed out binding on the nitrocellulose to endogenous RanBP1 and RanBP2 from BHK21 cells. The association of Ran with the 90- and 115/120-kDa proteins was further intensified when Ran was batch-elicuted with an isolated RanBD of RanBP1 (Fig. 1) or of RanBP2 (not shown). Removal of the C terminus from RanBP1 also enhanced the binding of Ran to the Δ-DE RanBP1s (Fig. 1). These results show that the behavior of the Δ-DE Ran mutant can be mimicked by the heteromeric association of full-length Ran with a Ran binding domain. The data suggest that this association causes a change in the conformation of the C terminus of the Ran GTPase that exposes a binding site for the 90- and p115/120-kDa proteins.
was applied to 100 ml of a DE52 anion exchange cellulose slurry, washed with 150 mM sodium acetate, and eluted with 500 mM sodium acetate (as described under "Experimental Procedures"). The eluate was mixed with GST-Δ-DE Ran-GTP coupled to glutathione-Sepharose column beads and eluted with 10 mM glutathione. Shown is a silver-stained gel of 50 μg of DE52 eluate (lane 1), Δ-DE Ran affinity-purified protein (10% of preparation) (lane 2), and proteins present in 100 μl of the GST-Δ-DE Ran matrix before addition of the partially purified Δ-DE RanBP preparation (lane 3). B, autoradiogram of the [γ-32P]GTP-GST-Δ-DE Ran overlay of fractions from the p90 purification. Portions of rat brain cytosol (lane 1), DE52 eluate (lane 2), Δ-DE Ran affinity-purified material (lane 3), and GST-Δ-DE Ran affinity matrix material (lane 4) were subjected to SDS-PAGE and Δ-DE Ran overlay as described in Fig. 1.

These proteins are potentially important effectors of Ran function. We therefore pursued the identification of the 90-kDa protein by affinity purification using a GST fusion of Δ-DE Ran. Fusion to GST did not affect the binding of Δ-DE Ran to the 90- and 115/120-kDa proteins or increase binding to nonspecific proteins in overlay assays (Fig. 1, lane 5). Using the rat brain cytosol fractions defined by Moore and Blobel (3), we observed that Ran is found exclusively in fraction B and the RanBPs and Δ-DE RanBPs were both present in fraction A, the component required for docking of NLS substrates (3, 11). We therefore prepared a modified fraction A from rat brain cytosol as an initial step in the purification of the 90-kDa protein. This partially purified material was then affinity-purified using a glutathione-Sepharose matrix coupled to GST-Δ-DE Ran. The affinity step yielded the 90- and 115/120-kDa proteins, purified nearly to homogeneity (Fig. 2, lane 2). Peptide sequences of two separate trypsin fragments of the 90-kDa protein were identical to sequences within p97/β-karyopherin, an essential component of the 9 S nuclear docking complex (Table I) (4).

To confirm that β-karyopherin is a Ran binding protein, recombinant β-karyopherin carrying a His6-tag (5) was tested for its ability to interact with [α-32P]GTP-Ran in an overlay assay. The conditions that enhance binding of Ran to β-karyopherin (Fig. 3, lower panel) paralleled those that promote binding of Ran to the 90-kDa protein from brain cytosol (upper panel). Wild-type Ran in the presence of GST alone did not detectably bind recombinant β-karyopherin (lane 1). As predicted, however, both Δ-DE Ran (lower panel, lane 5) and a complex of wild-type Ran with the isolated Ran binding domain of RanBP1 (lane 2) did associate with recombinant β-karyopherin. The addition of β-karyopherin to the Ran-RanBP1 complex also abolished binding of the complex to immobilized β-karyopherin and to the 90- and 115/120-kDa brain proteins (lane 4). It did not reduce binding to RanBP2, however (lane 3), confirming that β-karyopherin interacts with a region on Ran distinct from the RanBD binding site. Note that the recombinant β-karyopherin was less efficient in competition for binding to the Δ-DE Ran (lane 6). We do not currently understand the basis for this difference.

Endogenous β-karyopherin is present in the cytosol as part of a multisubunit complex (4), and we explored the possibility that Ran interacts with this complex. Proteins in the modified fraction A from rat brain cytosol were mixed with GST (Fig. 4A) or GST-Δ-DE Ran (Fig. 4B) loaded with GTP. Protein complexes were then separated by size exclusion chromatography. Fractions were analyzed by overlay with GST-Δ-DE Ran to detect the 90- and 115/120-kDa proteins. After exposure to x-ray film, the same nitrocellulose blot was washed to remove the probe and immunoblotted with either anti-Ran antiserum (Fig. 4B, bottom panel) or anti-GST antibody (not shown) to identify fractions containing GST-Δ-DE Ran. Two distinct peaks of Δ-DE RanBPs were detected in fractions that correspond to sizes of approximately 700 and 200 kDa (Fig. 4A). The 700-kDa peak may represent the 9 S complex (4). The addition of GST-Δ-DE Ran-GTP to the cytosol did not affect the elution profile of the Δ-DE RanBPs (Fig. 4B), but the GST-Δ-DE Ran preferentially associated with the 700-kDa peak. One interpretation of this result is that other proteins contained in the large complex increase the affinity of Ran for the Δ-DE RanBPs.

Together these data fit well with the current model for me-

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import: docking and translocation. We propose a model in which β-karyopherin serves as an adapter between the α-karyopherin-NLS substrate complex and a Ran-RanBP heterodimer. RanBP1 may stabilize this complex in the cytosol until it is displaced by RanBP2 at the nuclear pore. Hydrolysis of Ran-GTP, stimulated by the Ran GTPase-activating protein, Rnap, then triggers dissociation of the complex and permits the entry of the NLS substrate-α-karyopherin heteromer into the nucleus, perhaps with NTF2/B-2. The GDP-Ran released from the complex also enters the nucleus where RCC1 catalyzes the exchange of GTP for GDP on Ran such that the cycle can continue. The tools currently available will now allow for a more detailed biochemical analysis of the interactions between the growing number of components that regulate nuclear import.

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