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Identification of Two Novel RanGTP-binding Proteins Belonging to the Importin β Superfamily*

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Nucleo-cytoplasmic transport comprises a large number of distinct pathways, many of which are defined by members of the importin β superfamily of nuclear transport receptors. These transport receptors all directly interact with RanGTP to modulate the compartment-specific binding of their transport substrates. To identify new members of the importin β family, we used affinity chromatography on immobilized RanGTP and isolated Ran-binding protein (RanBP) 16 from HeLa cell extracts. RanBP16 and its close human homologue, RanBP17, are distant members of the importin β family. Like the other members of the transport receptor superfamily, RanBP16 interacts with the nuclear pore complex and is able to enter the nucleus independent of energy and additional nuclear transport receptors.

Transport between the cytoplasm and the nucleus is largely mediated by members of the superfamily of importin β-related nuclear transport receptors (for review, see Refs. 1–3). These receptors drive translocation across the nuclear envelope by direct interaction with the nuclear pore complex (NPC).[1] Importin β-related transport receptors, also referred to as karyopherins (4), can be classified into importins and exportins depending on the direction in which they transport a cargo. Substrate binding to the transport receptors is regulated by the small GTPase Ran that confers directionality to the transport reaction. Ran is a predominantly nuclear protein that is maintained in its GTP-bound form in the nucleus by the chromatin-associated nucleotide exchange factor RCC1 (5). The direct binding of RanGTP to importins upon their entry into the nucleus causes release of the import substrate (6–10). In contrast, exportins are regulated the opposite way and must bind RanGTP to stably associate with their export cargo (11–15). Hence, importins leave the nucleus as complexes with RanGTP devoid of transport substrate, whereas exportins take their cargo along with RanGTP. In the cytoplasm, RanGTP is removed from the transport receptors. Removal requires the concerted action of RanBP1 (and RanBP2) and the RanGTPase-activating protein (RanGAP) that triggers the conversion of Ran into its GDP-bound form (11, 16–18). As a result, importins are liberated from RanGTP so as to bind and import the next substrate. Exportins also release their export cargo and reenter the nucleus on their own to take part in the next export cycle.

The diagnostic features of importin β-related nuclear transport receptors are their similar size (90–130 kDa), an acidic isoelectric point (4.6–5.9; average, 5.1), and, most importantly, the N-terminal RanGTP-binding motif (19, 20). Whereas this domain is well conserved among the different transport receptors and has been instrumental in the identification of most of the 14 yeast members of the superfamily (reviewed in Refs. 2 and 3), the C-terminal regions of these sequences are often diverse and implicated in the recognition of the various transport substrates.

In higher eukaryotes, nine importin β-related, RanGTP-binding proteins have been functionally characterized, although the existence of many more can be anticipated. Characterized importins comprise importin β, transportin, importin 5, importin 7, and transportin SR (21–26). Four exportins have been described in mammals. CRM1 (exportin 1) has a broad substrate specificity and functions in nuclear export of different substrates, many of which contain a leucine-rich nuclear export signal (14). The other known exportins appear to be specialized in the transport of one specific cargo such as CAS, which exports importin α (11), exportin t, which exports tRNA (12, 13), and the recently identified exportin 4, which exports eukaryotic initiation factor 5A (27).

Here we describe the identification of two novel mammalian importin β-like proteins, RanBP16 and RanBP17. They appear to be conserved among higher eukaryotes and belong to an evolutionary distant part of the transport receptor superfamily. RanBP16 is shown to interact with RanGTP directly, albeit with low affinity. It localizes predominantly to the nucleus and the nuclear envelope in living cells. *In vitro*, RanBP16 binds to the nuclear pore complex and, like all importin β-related nuclear transport receptors, RanBP16 can enter the nucleus without the help of any other soluble factor. Although their cargo remain to be identified, we suggest that RanBP16 and RanBP17 function as nuclear transport receptors. Finally, the

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[1] The abbreviations used are: NPC, nuclear pore complex; RanBP, Ran-binding protein; EST, expressed sequence tag; GFP, green fluorescent protein; RanGAP, RanGTPase-activating protein; aa, amino acid(s); PAGE, polyacrylamide gel electrophoresis; ORF, open reading frame; PCR, polymerase chain reaction.

affinity of RanBP16 for RanGTP is intermediate between the high affinity exhibited by nuclear import receptors and the low affinity of export receptors, suggesting the novel possibility that RanBP16 may transport cargo bidirectionally across the nuclear pore complex.

EXPERIMENTAL PROCEDURES

Solution Binding Experiments—Preparation of HeLa extract and affinity chromatography of HeLa extract on immobilized 2zRan and 2zRanQ69L have been described previously (11, 12). In brief, 5×10^9 HeLa cells were lysed in 50 ml of 50 mM Hepes/KOH, pH 7.5, 150 mM potassium acetate, 5 mM magnesium acetate, 1 mM β-mercaptoethanol, and 1 mg/ml digitonin. The cell debris were sedimented at $1,000 \times g$. From the supernatant, a postribosomal supernatant was prepared by centrifugation for 90 min at 50,000 rpm in a Beckman Ti50.2 rotor. For binding experiments, IgG-Sepharose (Pharmacia) was first saturated with 2z-tagged wild type Ran (GDP form) or 2z-tagged RanQ69L (GTP form). The HeLa extract was adjusted to 50 mM Hepes, pH 7.5, 200 mM NaCl, 75 mM potassium acetate, and 5 mM magnesium acetate. Each 0.75 ml of HeLa extract was bound overnight at 4 °C to either 25 μl of immobilized 2zRan or 2zRanQ69L in a final volume of 1.5 ml. After binding, the IgG-Sepharose beads were washed three times with 1.5 ml of 50 mM Hepes/KOH, pH 7.5, 225 mM NaCl, and 5 mM magnesium acetate, and elution was performed as described below.

2z-tagged Nup153(aa898–1200)-His$_6$ was immobilized on IgG-Sepharose to a concentration of 2–4 mg protein/ml Sepharose beads. Binding of proteins from *Escherichia coli* extracts was allowed for 4 h at 4 °C using 10 μl of beads in a final volume of 1.5 ml. Beads were washed twice in binding buffer (50 mM Tris, pH 7.5, 225 mM NaCl, and 2 mM MgCl$_2$). Bound proteins were eluted with 50 mM Tris, pH 7.5, and 1.5 M MgCl$_2$, precipitated with 10 volumes of isopropanol, and analyzed by SDS-PAGE followed by Coomassie staining.

Protein Identification—After affinity chromatography of HeLa lysate on immobilized 2zRanQ69L(GTP), bound proteins were separated by SDS-PAGE followed by Coomassie staining. The RanBP16 band was excised and subjected to tryptic digest. The tryptic peptides were analyzed by Edman sequencing. The resulting peptide sequences are *underlined* in Fig. 2. These sequences were used to search the database. Several matching human ESTs were identified (GenBank™ accession number H71676 was identified by peptides ALVEFTNSPD*X*L and GSS-SYSQLLAAT*X*LTK). In addition, an uncharacterized ORF from *Caenorhabditis elegans* (T19745) revealed homology to peptide TLQLL-NDLSIGYSSVR. The *C. elegans* ORF encodes the hypothetical protein C35A5.8 that shows homology to known importins and exportins in its N-terminal region, suggesting the presence of an importin β-like RanGTP-binding domain.

Using the C35A5.8 protein sequence, we subsequently identified homologous human ESTs. Among them were two nonidentical ESTs (H63478 and H59369) that closely matched the C terminus of the C35A5.8 sequence and appeared to belong to two highly homologous proteins. Based on the H59369 sequence information, we identified other ESTs such as R13716 and T79994, which covered the extreme C terminus of an unknown ORF that later turned out to be RanBP16. Based on H63478, we identified N92666, which covered the C terminus of a homologous unknown ORF (later identified as the C terminus of RanBP17).

Moreover, the search revealed that H71676 encompassed a region close to the N terminus of RanBP16. PCR analysis using primers corresponding to H71676 and T79994 showed that H71676 and T79994 belonged to the same ORF. The deduced protein contained all identified RanBP16 peptides. Subsequently, the N terminus of RanBP16 was determined by 5′ rapid amplification of cDNA ends using Marathon-Ready cDNA (HeLa) from CLONTECH with adaptor primer AP1 and primer GTTGGTAAATTCAACCAAGGC for the first round of PCR and AP2 and TCTCTGCCTGGAGTCGAGTGG for the second round of PCR.

Based on the sequence information of an additional EST (T84433) homologous to C35A5.8 and H63478, 5′ and 3′ rapid amplification of cDNA ends primers were selected to obtain full-length sequence information of RanBP17. Oligonucleotides CAAAAGCCTGGAGTACATTG, CAAAATGGTTGTCCCCATAC, and GCTTCAGAAACCTAATGGAAG, GCTTCAGAAACCTAATGGAAG were used together with the fitting adapter primers for 5′ and 3′ rapid amplification of cDNA ends PCRs, respectively.

Molecular Cloning—The information obtained from sequencing rapid amplification of cDNA ends PCR products was used to generate primers for amplification of the complete coding regions of RanBP16 and RanBP17 by PCR from HeLa cell cDNA. Oligonucleotides were designed such that the coding regions of both ORFs could be inserted into the *Bam*HI/*Xba*I sites of pQE30 (Qiagen) to express both proteins in *E. coli* with an N-terminal His$_6$ tag. Clones were tested for production of full-length protein after *E. coli* expression. Four independent clones of each protein were then analyzed to determine the consensus sequences.

The pEGFP-RanBP16 plasmid was obtained after subcloning the *Bam*HI/*Xba*I insert of pQE30-RanBP16 into the *Bgl*II/*Xba*I sites of the transfection vector pEGFP-C1 (CLONTECH).

To generate an *E. coli* expression vector for 2zNup153(aa898–1200)-His$_6$, the corresponding coding region of Nup153 was PCR-amplified from HeLa cell cDNA and inserted into the *Nco*I/*Bam*HI sites of pQE602z.

Recombinant Protein Expression—The expression and/or purification of importin β, 45–462 importin β, 2zRan (wild type), 2zRanQ69L have been described previously (11, 28).

N-His$_6$-RanBP16 was expressed in the *E. coli* strain XL1(pBS161). The first step of purification was chromatography on Ni-NTA-agarose (Qiagen). The imidazole eluate was then applied to Q-Sepharose FF (Amersham Pharmacia Biotech) and eluted in a step with 50 mM Tris, pH 7.5, and 500 mM NaCl. Then the buffer was exchanged to 50 mM Tris, pH 7.5, and 150 mM potassium acetate by gel filtration on a PD10 column (Amersham Pharmacia Biotech). Before freezing in liquid nitrogen, 250 mM sucrose was added to the purified protein.

2zNup153(aa898–1200)-His$_6$ was expressed in the *E. coli* strain BLR(pREP4) and purified conventionally by chromatography on Ni-NTA-agarose.

Antibodies—The anti-importin β antibody has been described previously (29). Antibodies against RanBP16 were raised in rabbits. For immunization, a C-terminal peptide of RanBP16 (STYGVNSNDMMS) was coupled to different carrier proteins (keyhole limpet hemacyanin, bovine serum albumin, and ovalbumin) by virtue of an additional cysteine at the N terminus of the peptide. These peptide conjugates were used to sequentially immunize the same rabbit. Affinity purification was performed using sulfoLink (Pierce) to which the peptide had been coupled. The resulting antibody was monospecific for RanBP16 on Western blots of total HeLa cells.

RanGTPase assay—The RanGTPase assay was performed essentially as described previously (11, 30), with the exception that the measurements were done at 15 °C in a buffer (20 mM Hepes, pH 7.5, and 120 mM potassium acetate) containing 1 mM magnesium acetate, 0.5% hydrolyzed gelatin, and 0.02% sodium azide.

Immunofluorescence—Indirect immunofluorescence and Triton X-100 extractions were performed as described previously (31).

Alexa Labeling—RanBP16 (in 50 mM Tris, pH 7.5, and 150 mM potassium acetate) was modified with Alexa Fluor 488 C$_5$ maleimide (Molecular Probes) at a 1:1 molar ratio for 60 min on ice. Nonincorporated label was removed by gel filtration on a Sephadex G-25 NAP-5 column (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris, pH 7.5, and 150 mM potassium acetate.

In Vitro Nuclear Transport Assay—*In vitro* nuclear transport assays were carried out with HeLa cells permeabilized with digitonin according to the method described in Ref. 25. Transport reactions were performed as described previously (12, 28). The transport buffer contained 2 mg/ml nucleoplasmin core to block nonspecific binding, 20 mM Hepes/KOH, pH 7.5, 140 mM potassium acetate, 4 mM magnesium acetate, and 250 mM sucrose. The energy-regenerating system was added to a final concentration of 0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, and 50 μg/ml creatine kinase.

RESULTS

A common feature of nuclear transport receptors belonging to the importin β family is their ability to specifically interact with the GTP-bound form of the small GTPase Ran. Thus, to identify additional vertebrate members of this protein superfamily, we used affinity chromatography on immobilized Ran using HeLa cell extracts as starting material (see "Experimental Procedures" and Fig. 1). To identify proteins that specifically interact with the GTP-bound form of Ran, we immobilized RanQ69L, which is a GTPase-deficient mutant of Ran that stays in its GTP-bound form even in the presence of the Ran-GAP present in the HeLa cell extract. Immobilized wild-type Ran in its GDP-bound form served as a negative control. Proteins that specifically interacted with the GTP-bound form of Ran but not with the GDP-bound form of Ran and with an
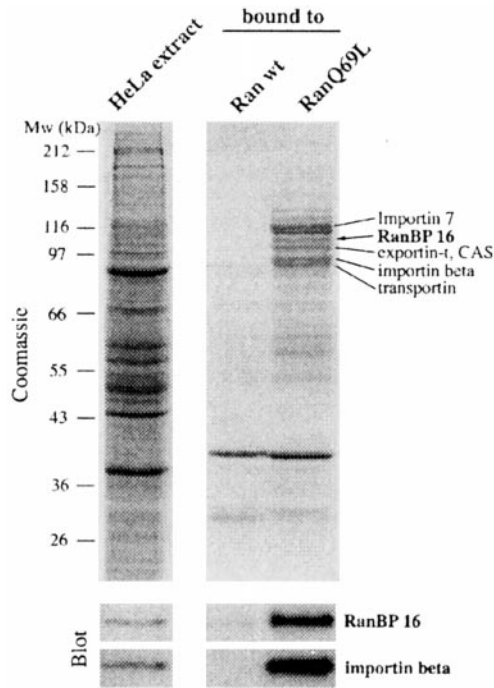
FIG. 1. **Identification of RanBP16 bound to immobilized RanQ69L(GTP).** A HeLa cell extract was subjected to binding to either immobilized RanGDP (wild type Ran) or to the immobilized GTPase-deficient Ran mutant RanQ69L(GTP). Proteins in the bound fractions and the starting material were separated by SDS-PAGE followed by either Coomassie staining or Western blotting. Proteins that were specifically recovered in the RanGTP-bound fraction were subjected to peptide sequencing. Identified proteins include established RanGTP-binding proteins like importin 7, CAS, exportin t, importin β, and transportin (see also Ref. 12). In addition, peptides corresponding to a novel protein named RanBP16 were identified (see Fig. 2). Immunoblotting using peptide-specific antibodies directed to RanBP16 or importin β, respectively, confirmed that RanBP16 was enriched on the RanGTP column, as was importin β.

approximate size between 90 and 130 kDa in SDS-PAGE were identified by peptide sequencing. In addition to a great number of peptides matching already known transport receptors, peptides corresponding to a novel protein were identified. We refer to this protein as RanBP16.

The partial sequence information of the protein matched several expressed sequence tags in the database (see "Experimental Procedures"). These tags showed striking homology to an open reading frame from *C. elegans* (C35A5.8, T19745), which is homologous to known importins and exportins in its N-terminal region, suggesting the presence of an importin β-like RanGTP-binding domain.

The sequence information of the *C. elegans* protein in turn was used to clone a full-length cDNA from HeLa cells coding for RanBP16. Antibodies raised against a C-terminal peptide of RanBP16 were used to confirm by Western blotting that RanBP16 was specifically enriched in the RanGTP-bound fraction (Fig. 1).

In addition to its high homology to the *C. elegans* protein (40% identity), RanBP16 was found to possess a close human homologue. The cDNA corresponding to this human homologue, which we refer to as RanBP17, was amplified and cloned. The consensus sequences of RanBP16 and RanBP17 are shown in Fig. 2. Both human proteins display 67% identity in their primary structure.

Protein orthologues to RanBP16 can be found in many higher eukaryotic organisms, such as *Oryza sativa* (BAA89552), *Drosophila melanogaster* (AF48542), *Xenopus laevis* (EST, AW646417), *Mus musculus* (EST, AW4961112), *Aedes aegypti*

FIG. 2. **Amino acid sequences of human RanBP16 and RanBP17.** RanBP16 and RanBP17 were cloned from HeLa cell cDNA with the aid of the partial peptide information (*underlined*) derived from RanBP16. For details, see "Experimental Procedures." The figure shows the deduced amino acid sequences of both human proteins, which display 67% sequence identity. +, conservative amino acid changes. GenBank™ accession numbers are AF064729 for RanBP16 and AF222747 for RanBP17.

(EST, AI561380), or cotton (EST, AI055049). However, we have not found an obvious homologue in yeast. RanBP17 appears to be present only in vertebrates like human, mouse (EST, AA184068), rat (EST, H33578), and cow (EST, AW659404).

Multiple sequence alignments done with the full sequences of yeast and mammalian members of the importin β family showed that RanBP16 and RanBP17, as well as the recently identified exportin 4 (27), belong to a distant part of the evolutionary tree that is separated from the rest of the family (Fig. 3). The closest homology of both RanBP16 and RanBP17, when compared with the rest of the family, is to CRM1 from all eukaryotes; however, this homology is limited to the N-terminal RanGTP-binding domain. It is well possible that RanBP16 evolved from CRM1 after the divergence into higher and lower eukaryotes.

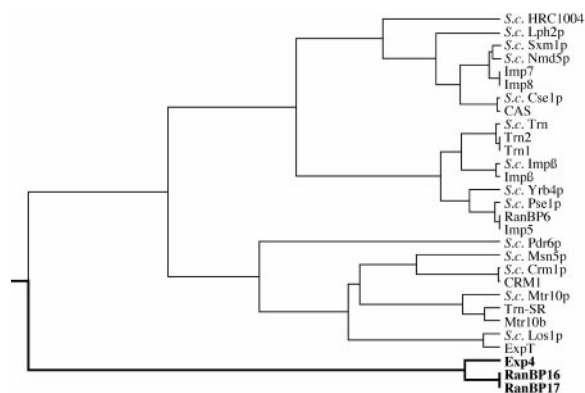Having identified by sequence analysis two new potential members of the importin β family of RanGTP-binding proteins,

FIG. 3. **Dendrogram indicating sequence similarities between RanBP16, RanBP17, and other members of the importin β superfamily.** Dendrogram was calculated from full-length sequences of importin β-like transport receptors using Dialign 2.0 software (37). RanBP16, RanBP17, and the recently identified exportin 4 (27) form a separate subgroup in the phylogenetic tree. Nuclear transport receptors from *Saccharomyces cerevisiae* are indicated by the prefix *S.c.* Higher eukaryotic transport receptors are represented by the human factors.
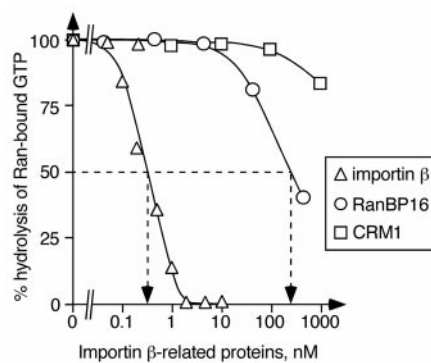

FIG. 4. **The apparent dissociation constant of a RanGTP/RanBP16 complex.** Apparent dissociation constants of complexes between RanGTP and importin β, CRM1, or RanBP16, respectively, were estimated using the RanGTPase assay (11, 30). Ran[γ-^{32}P]GTP (50 pM) was preincubated for 30 min with the indicated concentrations of importin β-related proteins. The GTPase reaction was then started by addition of 40 nM Rna1p (RanGAP from *Schizosaccharomyces pombe*). Hydrolysis of GTP was determined by the charcoal method as released [^{32}P]phosphate after 30 s.

we applied a series of tests to confirm that the biochemical properties of these proteins support this classification. (For technical reasons, only recombinant RanBP16 could be expressed in soluble form and further characterized.) First, we wished to verify that RanBP16 does indeed bind RanGTP; thus, we determined the apparent dissociation constant for recombinant RanBP16 and RanGTP. The assay relies on the observation that complex formation between an importin β-like receptor and RanGTP prevents activation of GTP hydrolysis on Ran by RanGAP. One can calculate the percentage of Ran that is complexed by an importin β-like factor at a given concentration by quantitation of GTP hydrolysis by Ran. From the dose dependence of the protection, it is possible to estimate dissociation constants of the complexes. This dissociation constant can be used as an indication of whether a potential transport receptor functions in import or export. Known importins display a high affinity for RanGTP with dissociation constants in the low nanomolar range (16, 32). In contrast, the affinity of exportins for RanGTP is in the micromolar range, unless their export substrate is present (11, 12, 33, 34). As shown in Fig. 4, RanBP16 is able to protect Ran-bound GTP from hydrolysis in this assay, confirming that RanBP16 is able to interact with RanGTP in a manner that is similar to other importin β-like transport receptors. The apparent dissociation constant of a RanGTP/RanBP16 complex is around 200 nM. This affinity is intermediate and does not allow us to classify RanBP16 as either an importin or exportin based on its affinity for RanGTP.

Next, we determined the intracellular location of RanBP16. Transport receptors of the importin β superfamily are believed to drive translocation of their substrates through the nuclear pore by their ability to directly interact with nucleoporins (for review, see Ref. 35). This is often reflected by their localization in living cells, where they can be found partly associated with the nuclear envelope (9, 12, 19, 21, 29). To address whether RanBP16 is similarly localized, indirect immunofluoresence was performed using a peptide-specific antibody. Fig. 5*A* shows that RanBP16 is a predominantly nuclear protein in HeLa cells. In addition to the nuclear staining, which often appeared speckled in distribution, staining of the nuclear envelope could be observed after Triton X-100 permeabilization of the cells before fixation. We also examined the steady-state localization of a GFP-fusion of RanBP16, which was also observed mainly in the nucleus (Fig. 5*A*).

To examine whether RanBP16 can directly interact with nucleoporins, its binding to the isolated F*X*FG repeat domain of

Nup153 was tested. Nup153 is a component of the nuclear baskets, and its F*X*FG repeats have been shown to interact directly with different importin β-like transport receptors such as importin β, transportin, and CRM1 (36). The Nup153 fragment was immobilized on IgG-Sepharose by virtue of an N-terminal 2z tag, and an *E. coli* lysate from cells expressing recombinant RanBP16 was passed over this column. RanBP16 was specifically recovered on the column, as was importin β from another lysate, which served as a positive control (Fig. 5*B*).

Finally, nuclear entry of RanBP16 was studied *in vitro* using the permeabilized cell system. Fluorescence-labeled RanBP16 entered nuclei rapidly and gave rise to a prominent nuclear rim staining, reflecting its association with nuclear pores (Fig. 5*C*). As in the case of other importin β-like transport receptors, nuclear entry of RanBP16 was independent of additional transport receptors and energy. Nuclear uptake and NPC binding could be abolished by preincubation of the nuclei with a dominant negative mutant of importin β (importin β 45–462) that has been previously shown to prevent active transport through the NPC (28). The addition of RanGTP, in the form of RanQ69L(GTP), to the transport reaction led to a decrease in intranuclear staining and an even more intense staining at the nuclear envelope, suggesting that the association with RanGTP favors association with the NPC.

In summary, the ability of RanBP16 to prevent activation of RanGTPase by RanGAP, the association of RanBP16 with the nuclear pore complex *in vivo*, the direct binding of RanBP16 to a nucleoporin implicated in translocation of transport receptors, and the ability of RanBP16 to enter the nucleus in the absence of other soluble transport factors strongly suggest that RanBP16 is a newly identified member of the importin β family of nuclear transport receptors.

DISCUSSION

Transport through the NPC is mediated largely by members of the importin β family of nuclear transport receptors. Whereas in yeast, transport cargoes have been identified for 13 of the 14 family members, in higher eukaryotes, only 9 of the more than 20 factors that are believed to exist have been functionally characterized (for review, see Ref. 2 and the references herein). There are several possible reasons why there may be more nuclear transport receptors in higher eukaryotes than in yeast. First, in higher eukaryotes, a higher redundancy in nuclear transport pathways may exist. A number of import
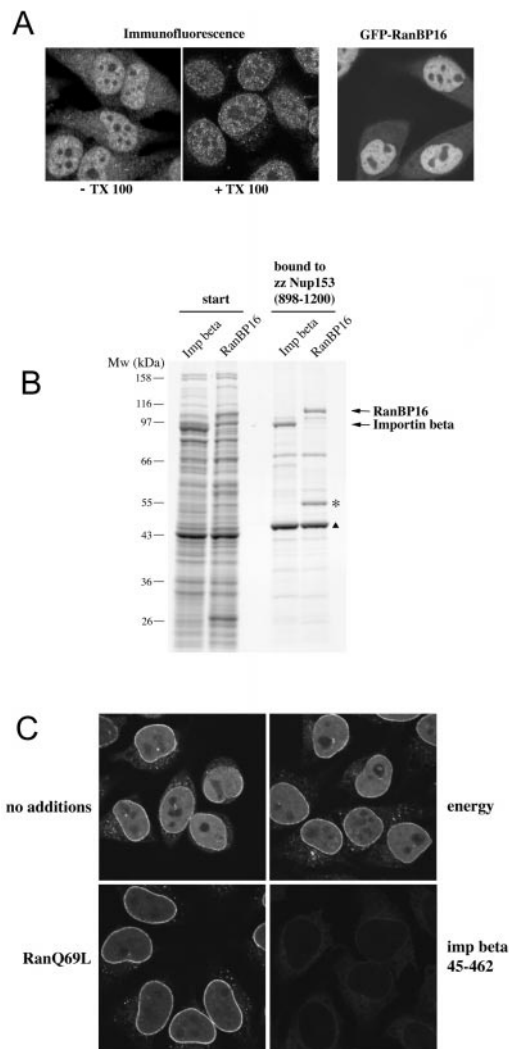
FIG. 5. **RanBP16 is a predominantly nuclear protein that binds to nuclear pore complexes.** *A*, steady-state distribution of endogenous RanBP16 and GFP-tagged RanBP16 in HeLa cells. HeLa cells were grown on coverslips. For immunofluorescence, cells were either fixed directly with formaldehyde, permeabilized with 0.5% Triton X-100, and immunostained (− *TX 100*) or extracted with 0.5% Triton X-100 before fixation (+ *TX 100*). For immunostaining, the affinity-purified anti-RanBP16 antibody was used in conjunction with a fluorescein-conjugated secondary antibody. Note that the anti-RanBP16 antibody was monospecific for RanBP16 on Western blots of total HeLa cells and that the observed staining could be efficiently competed by the peptide against which the antibody had been raised (data not shown). GFP-RanBP16 (*right panel*) was detected in HeLa cells approximately 36 h after calcium phosphate transfection (38) with pEGFP-RanBP16. Whereas GFP on its own was distributed evenly throughout the cytoplasm and the nucleus (data not shown), GFP-RanBP16 displayed a predominantly nuclear localization with the nucleoli being spared. All images were taken by confocal fluorescence microscopy. *B*, direct binding of RanBP16 to isolated F*X*FG repeat domain of Nup153. Total *E. coli* lysates from cells expressing either C-His importin β or N-His RanBP16 were subjected to binding to immobilized 2zNup153 (aa898–1200). Proteins in the starting material and in the bound fractions were separated by SDS-PAGE followed by Coomassie staining. Both importin β and RanBP16 were specifically enriched on the Nup153 fragment. The *asterisk* indicates a RanBP16 fragment that is also efficiently retrieved on the column. The *filled triangle* indicates the position of 2zNup153 (aa898–1200), which partially eluted from the column. *C*, NPC binding and nuclear entry of RanBP16 occur independently of energy and of other soluble transport factors. HeLa cells were grown on coverslips and permeabilized. 0.5 μM Alexa-labeled RanBP16 was incubated for 12 min with the permeabilized HeLa cells in transport buffer at 23 °C without further additions, in the presence of an energy-regenerating system or in the presence of 5 μM RanQ69L. Preincubation of the cells for 2 min with 1 μM dominant negative 45–462 importin β mutant abolished nuclear uptake of RanBP16.

substrates, such as ribosomal proteins (25), histones, and SR proteins, which all use basic patches as import signals, can use different transport receptors to enter the nucleus.[2] For these transport substrates, additional import receptors may have evolved from preexisting ones. The resulting redundancy in the corresponding import pathways may be even more complex than is now anticipated. Secondly, in higher eukaryotic multicellular organisms, novel transport pathways may have emerged for transport of substrates that either do not exist in yeast or whose transport must be specifically controlled.

The overall homology between members of the importin β family is low, which may be due in part to the fact that they have to recognize transport substrates as different as, for instance, basic proteins and tRNA. The defining feature of importin β-like transport receptors, however, is their N-terminal RanGTP-binding motif. We exploited their ability to stably interact with RanGTP to identify novel human family members. Affinity chromatography on immobilized RanGTP allowed us to isolate RanBP16. Based on the RanBP16 sequence, a close homologue, RanBP17, was identified by database searches. RanBP16 and RanBP17, together with the recently identified exportin 4, belong to a distant branch in the phylogenetic tree of the transport receptor superfamily. Their closest homologues are CRM1 proteins from different organisms; however, this homology is restricted to the N-terminal RanGTP-binding domain. RanBP16 and RanP17 do not possess obvious yeast orthologues and may have evolved late in evolution. Whereas proteins with homology to RanBP16 can be found in many higher eukaryotic organisms, RanBP17 thus far appears to be unique to vertebrates.

Although evolutionarily distant, RanBP16 by all applied criteria behaves as a *bona fide* member of the importin β family. RanBP16 can interact directly with the nuclear pore complex and drive its own translocation into the nucleus. As has been shown for other transport receptors of this superfamily, nuclear accumulation is energy-independent and can be inhibited by a dominant negative importin β mutant that blocks receptor-mediated transport through the NPC. Moreover, RanBP16 binds RanGTP directly. In the resulting complex, RanGTP is protected against induction of GTP hydrolysis by RanGAP, a feature that distinguishes importin β-like from RanBP1-related RanGTP-binding proteins.

At present, we do not know which type of cargo is transported by the two novel RanBPs. RanBP16 does not directly support nuclear import of proteins containing basic nuclear import signals such as, for instance, SV40-nuclear localization signal-bovine serum albumin conjugates, ribosomal proteins (*e.g.,* a mixture of fluorescence-labeled ribosomal proteins or ribosomal protein L23a), core histones, or glutathione *S*-transferase fusion with arginine/serine-rich protein domain fusions *in vitro* (data not shown). We assume that it may function in a novel, uncharacterized nuclear transport pathway. Often, the affinity of a transport receptor for RanGTP allows an educated guess as to whether a certain factor functions in nuclear import or export. The apparent dissociation constant of the RanGTP/RanBP16 complex of about 200 nM lies in the middle of what has been described for importins and exportins. Hence, the intermediate affinity of RanBP16 for RanGTP does not exclude either of the two activities. It may even be the case that RanBP16 carries substrates both into and out of the nucleus. In preliminary experiments, we find that the binding of some unidentified proteins in a HeLa cell extract to RanBP16 is enhanced by RanGTP, whereas the binding of others is prevented under the same conditions (data not shown).

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