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Ran-Binding Protein 5 (RanBP5) Is Related to the Nuclear Transport Factor Importin-β but Interacts Differently with RanBP1

RÓISÍN DEANE,[1] WOLFGANG SCHÄFER,[1] HANS-PETER ZIMMERMANN,[1] LIZ MUELLER,[1] DIRK GÖRLICH,[2] SIEGFRIED PREHN,[3] HERWIG PONSTINGL[1] AND F. RALF BISCHOFF[1]*

Abteilung Molekulare Biologie der Mitose, Deutsches Krebsforschungszentrum,[1] and Zentrum für Molekulare Biologie der Universität Heidelberg,[2] 69120 Heidelberg, and Institut für Biochemie, Humboldt-Universität Berlin, D-10115 Berlin,[3] Germany

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We report the identification and characterization of a novel 124-kDa Ran binding protein, RanBP5. This protein is related to importin-β, the key mediator of nuclear localization signal (NLS)-dependent nuclear transport. RanBP5 was identified by two independent methods: it was isolated from HeLa cells by using its interaction with RanGTP in an overlay assay to monitor enrichment, and it was also found by the yeast two-hybrid selection method with RanBP1 as bait. RanBP5 binds to RanBP1 as part of a trimeric RanBP1-Ran-RanBP5 complex. Like importin-β, RanBP5 strongly binds the GTP-bound form of Ran, stabilizing it against both intrinsic and RanGAP1-induced GTP hydrolysis and also against nucleotide exchange. The GAP resistance of the RanBP5-RanGTP complex can be relieved by RanBP1, which might reflect an in vivo role for RanBP1. RanBP5 is a predominantly cytoplasmic protein that can bind to nuclear pore complexes. We propose that RanBP5 is a mediator of a nucleocytoplasmic transport pathway that is distinct from the importin-α-dependent import of proteins with a classical NLS.

Ran/TC4 is a highly abundant, small GTP-binding and -hydrolyzing protein that is located predominantly in the nucleus (8, 12). Ran has the biochemical properties of a GTPase switch cycling between two conformational states, the GTP-bound state and the GDP-bound state. The intrinsic rates of nucleotide exchange and GTP hydrolysis are very low and can be increased up to five orders of magnitude by the regulatory proteins RCC1 and RanGAP1, respectively (4, 8, 22). The first indications concerning the functions of Ran came from the analysis of mutants of these Ran regulators in both mammalian and yeast cells. These studies implicated Ran in a variety of processes, including the onset of mitosis, initiation of S phase, exit from mitosis, maintenance of nuclear structure, and pre-mRNA processing and mRNA export into the cytoplasm (for reviews, see references 41 and 43).

Furthermore, in vitro studies with permeabilized cells showed that Ran is an essential factor for the nuclear localization signal (NLS)-dependent nuclear protein import (30, 31). Macromolecular transport across the nuclear envelope occurs at the nuclear pore complexes (NPCs) and involves the import of proteins into the cell nucleus and the export of RNAs and proteins. Four soluble cytosolic factors are required to reconstitute nuclear import of NLS substrates in vitro. In addition to Ran, these are importin-α and importin-β (also known as karyopherin-α and karyopherin-β) (17, 19, 36) and NTF2 (alternatively known as pp15 or p10) (32, 34). Together, importin-α and importin-β comprise the import receptor complex, where importin-α binds proteins bearing an NLS and importin-β mediates the interaction with the NPC. Ran appears to be required for at least two steps in nuclear import. First, translocation through the nuclear pore requires GTP hydrolysis by Ran, probably even as the sole source of energy (47). Second, the disassembly of the importin-α/-β–NLS protein complex following translocation into the nucleus is very probably mediated by the interaction of RanGTP with importin-β (14, 18, 27, 37). Although not yet measured directly, a high nuclear concentration of free RanGTP is expected since RCC1, the GDP-GTP exchange factor for Ran, is found exclusively in the nucleus (7, 33). In contrast, a prevalence of the GDP-bound form of Ran in the cytoplasm is supposed by free cytoplasmic RanGAP1 and RanGAP1 attached to the cytoplasmic fibrils of the NPC by virtue of a ubiquitin-like modification (28, 29, 42). High levels of RanGTP in the cytoplasm may otherwise result in the premature dissociation of the import complex.

In vitro, the importin-β interaction with RanGTP results in a block of both the hydrolysis and exchange of Ran-bound GTP (14, 18). In addition to importin-β, other RanGTP-binding proteins have been characterized. By using an overlay assay with radioactively labelled RanGTP, two classes of Ran-binding proteins were defined. RanBP1 is a small cytoplasmic protein which specifically binds to the GTP-bound form of Ran (6, 11, 25). It blocks the exchange of Ran-bound GTP but increases GTP hydrolysis induced by RanGAP1 by approximately 1 order of magnitude (6). RanBP2, a 358-kDa protein located at the cytoplasmic face of the NPC (48, 49), contains four domains homologous to RanBP1, which each specifically bind RanGTP and behave biochemically like RanBP1 (3). RanBP1 and RanBP2 compete for binding to Ran, whereas importin-β binds to a different site on Ran. This allows the formation of a trimeric RanBP1–RanGTP–importin-β complex (9, 18, 27). A similar complex resulting from the cooperative binding of RanBP1 and importin-β to the GDP-bound form of Ran has also been observed (9). A requirement of RanBP1 for nuclear transport is suggested by the finding that the yeast homolog Yrb1p is essential for nucleocytoplasmic transport in vivo (45).

Recently, an increasing number of importin-β-like proteins have been identified; these include hCRM1 (15) and Kap104p (1) and its human homologue transportin, which is required for the import of the A1 hnRNP (35) and which might therefore indicate that a family of transport factors exists (15, 16). We

* Corresponding author. Mailing address: Division for Molecular Biology of Mitosis, Deutsches Krebsforschungszentrum Heidelberg, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany. Phone: 49-6221-423408. Fax: 49-6221-423460. E-mail: r.bischoff@dkfz-heidelberg.de.

report here on a novel importin-β-related Ran-binding protein, RanBP5, which specifically binds the GTP-bound form of Ran. Similar to importin-β, RanBP5 blocks both GTP exchange and hydrolysis on Ran. However, in contrast to importin-β, the RanBP5-mediated block on GTPase activation can be relieved by the addition of RanBP1 alone, which might be an in vivo function for RanBP1. RanBP5 binds to the NPC but not to importin-α. We propose that RanBP5 might represent a novel transport factor with a substrate specificity different from that of the importin-α/β import receptor.

MATERIALS AND METHODS

Strains and media. The yeast strain Y190 [*MAT***a** *gal4 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 + URA3::GAL→lacZ, LYS2::GAL(UAS)→HIS3 cyh*^{r}] was used for the two-hybrid selection. Cultures were grown at 30°C in YPAD (1% yeast extract, 2% Bacto-Tryptone, 0.004% adenine sulfate, 2% glucose) or in synthetic minimal medium with the appropriate supplements (39). Plasmids were propagated in *Escherichia coli* DH5α. Glutathione-*S*-transferase (GST) fusion proteins were expressed in *E. coli* BL21(DE3)pLysS (46). *E. coli* Y1090hsdR was used for transfection of the lambda libraries.

Two-hybrid screen. All components were a kind gift from S. Elledge, Houston, Tex. (20). A plasmid expressing the target protein, RanBP1, fused to the GAL4 DNA-binding region, was prepared by inserting the full-length cDNA of RanBP1 (6) into the yeast expression vector pAS1-CYH2 and introduced by electrotransformation into the yeast strain Y190 (13). The pACT cDNA library prepared from human lymphocytes as a source of interacting proteins was introduced into the Y190 pAS1-RanBP1 strain by the LiCl procedure (44). Transformants were selected for tryptophan, leucine, and histidine prototrophy in the presence of 25 mM 3-aminotriazole. Isolated colonies were tested for β-galactosidase activity. Clones that tested positive were selected for cycloheximide curing to remove the "bait" plasmid. Plasmid DNA was isolated from positive clones, and false-positives were eliminated by retransforming the host Y190 strain containing the bait pAS1-RanBP1 plasmid or other nonspecific bait fusions with the isolated pACT plasmids.

cDNA cloning of human RanBP5. A human λDR2 cDNA library derived from HeLa cells (Clontech) was screened with the 420-bp PCR-derived fragment of RanBP5 (see below) labelled with [α-^{32}P]dCTP by using the DECAprimeII DNA-labelling kit (Ambion). Following plaque purification of λDR2 cross-hybridizing clones, the corresponding pDR2 plasmids were excised by *cre-lox*-mediated recombination in the bacterial strain AM1. DNA minipreparations were carried out and plasmids were transformed into DH5α for subsequent analysis. DNA sequencing was performed by the dideoxy chain termination method with the T7 Sequencing Kit (Pharmacia). The 5′ end was determined by the RACE method (rapid amplification of cDNA ends) with the 5′ Ampli-FINDER RACE kit (Clontech).

Expression and purification of recombinant proteins. For bacterial expression, the full-length RanBP5 DNA was PCR amplified from two different overlapping pDR2 clones (100 ng each of pRD2-2 and pRD2-25) with a forward primer including the start ATG and an appropriate reverse primer spanning the 3′ end of the open reading frame. The overlap between each template DNA served as a primer in the first extension step. In subsequent cycles, the 5′ and 3′ primers were used to amplify the full-length cDNA.

The N-terminal fragment of RanBP5 was PCR amplified with the forward primer described above and 5′-CCCGGATCCTTAGAAAGGGCACAACTTC ACTCTTCT-3′ as the reverse primer. To generate a myc epitope-tagged full-length protein for immunofluorescence analysis, a myc tag was incorporated into the 5′ primer, 5′-CGCGGATCCAGCGCA**ATG**GAGCAGAAGCTTATAAGC GAGGAGGACCTGATGGCGGCGGCCGCGGCGGAGCAG-3′. The amplified products were treated with the Klenow fragment of DNA polymerase in the presence of 2 mM deoxynucleoside triphosphates and ligated into *Eco*RV-cleaved pKS Bluescript for confirmatory sequencing. For the expression of recombinant proteins, the full-length DNA was ligated in frame into pGEX-4T-2 (Pharmacia). Recovery of the expressed GST fusion proteins was as follows. Briefly, a 3-liter culture induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was lysed in 50 mM Tris-HCl (pH 8.0)–200 mM NaCl–2.5 mM EDTA with protease inhibitors as described previously (4). After centrifugation for 60 min at 70,000 × *g*, the supernatant was applied to a glutathione-Sepharose 4B column at a flow rate of 0.5 ml/min and eluted with 20 mM glutathione. To remove the N-terminal GST domain, fractions containing the expressed fusion protein were pooled and incubated with thrombin at an enzyme-to-substrate ratio of 1:20 for 15 h at 4°C. Final purification of full-length RanBP5 was achieved by separation on Mono Q (Pharmacia). Human Ran and RCC1 proteins were expressed and purified as described previously (4, 6, 21). Recombinant *Schizosaccharomyces pombe* Rna1p and recombinant murine RanBP1 were prepared as described previously (5, 6).

Cell culture, transfections, and immunostaining. The N-terminal fragment of RanBP5 was used for immunization (Immundiagnostica). Serum was tested by immunoblot analysis with HeLa lysates, purified HeLa RanBP5, and both the N-terminal and full-length RanBP5 bacterially expressed proteins. RanBP5 specific antibodies were affinity purified by binding to nitrocellulose-bound purified RanBP5 and subsequent elution with 100 mM glycine (pH 2.5)–100 mM NaCl–0.5% bovine serum albumin. The eluate was immediately adjusted to pH 7.5. The monoclonal anti-human myc antibody 9E10 was a gift from V. C. Cordes (DKFZ, Heidelberg, Germany). For expression in mammalian cells, both the myc-tagged and untagged full-length RanBP5 were cloned into the mammalian expression vector pcDNA3 (Invitrogen). COS-7 cells maintained in minimal essential medium supplemented with 2 mM L-glutamine and 10% fetal calf serum were electrotransfected as described previously (2). At 24 h posttransfection, the cells were plated on multiwell slides for immunofluorescence analysis. One day later, the slides were rinsed in 1× phosphate-buffered saline (PBS)–2 mM $MgCl_2$ and fixed in 100% ice-cold methanol for 7 min at −20°C followed by 100% ice-cold acetone for 30 s. Alternatively, the slides were fixed in 3% paraformaldehyde–1× PBS for 20 min at room temperature and then given a short wash in 1× PBS–50 mM NH_4Cl. The slides were then incubated in 1× PBS–0.004% digitonin for 3 min on ice and washed briefly in 1× PBS. The primary antibody (or preimmune serum) was added to each well, and the mixture was incubated for 60 min at 25°C. Three 5-min washes in 1× PBS–2 mM $MgCl_2$ were followed by the addition of the secondary antibodies, either the fluorescein isothiocyanate-labelled anti-rabbit or anti-mouse immunoglobulin G (Dianova) for 30 min at 25°C. The slides were rinsed three times in 1× PBS–2 mM $MgCl_2$, briefly in distilled water, and finally for 3 min in 96% ethanol before being subjected to air drying and mounting.

Purification of RanBP5 from HeLa cells. Packed mitotic HeLa cells (25 ml, 10^{10} cells) were lysed as previously described (4). After centrifugation for 60 min at 70,000 × *g*, the soluble fraction was chromatographed on Fractogel EMD DMAE-650/M (Superformance [Merck], 26 by 115 mm) in 20 mM Bis-Tris-propane-HCl (pH 7.0)–1 mM dithiothreitol (DTT) with a linear gradient of NaCl from 0.05 to 1 M at a flow rate of 5 ml/min. Fractions containing RanBP5 eluting between 300 and 400 mM NaCl were identified by an overlay assay with [^{32}P]GTP-bound Ran. Pooled fractions were brought to 30% saturation with ammonium sulfate and stored for 30 min on ice. Following centrifugation for 20 min at 20,000 × *g*, the pellet was dissolved in 20 mM potassium phosphate (pH 7.0)–1 mM DTT. The sample was applied to a hydroxylapatite column (Superformance [Merck], 10 by 50 mm) and chromatographed at 1 ml/min in the same buffer with a potassium phosphate gradient from 0 to 1 M. RanBP5 fractions eluting between 200 and 300 mM phosphate were then diluted threefold with 20 mM Tris (pH 7.5) and chromatographed on Mono Q (HR 5/5; Pharmacia) in the same buffer with an NaCl gradient from 0 to 1 M. Purified RanBP5 was eluted at 350 mM NaCl. Cleavage with CNBr, separation of the peptides by reversed-phase chromatography, and sequence determination were performed as previously described (4).

Overlay assay. Protein fractions were resolved by polyacrylamide gel electrophoresis (PAGE) on 12% sodium dodecyl sulfate (SDS) Laemmli gels. Electrotransferred, nitrocellulose-blotted proteins were renatured by incubation with a renaturation buffer (20 mM morpholinepropanesulfonic acid [MOPS; pH 7.1], 100 mM sodium acetate, 5 mM magnesium acetate, 5 mM DTT, 0.5% bovine serum albumin, 0.05% Tween 20) for 1 h at 4°C (25). The blots were subsequently incubated at 25°C with 100 μM nonradioactive GTP in renaturation buffer. After 10 min, 100 μl of 1 nM Ran[γ-^{32}P]GTP or a mixture of 5 μM murine RanBP1 or 5 μM importin-β and 1 nM Ran[γ-^{32}P]GTP was added to 10 ml of incubation solution for a further 10 min. The blots were rinsed five times with renaturation buffer and autoradiographed. Labelling of Ran with [γ-^{32}P]GTP was done as described previously (6).

Enzymatic assays. Enzymatic assays were carried out essentially as described previously (4, 6). In summary, 30-μl aliquots of either 80 pM Ran[γ-^{32}P]GTP or 1.7 nM Ran-[α-^{32}P]GDP were preincubated at 25°C with 10-μl aliquots of RanBP5 in incubation buffer (20 mM HEPES-NaOH [pH 7.4], 100 mM NaCl, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.02% sodium azide, 0.05% hydrolyzed gelatin). After 30 min, 10-μl aliquots of 1 mM GDP and 10 μl of either 100 mM EDTA (pH 7.5) or 10 nM RCC1 were added for another 5 min. Ran-bound radioactivity was determined by a filter-binding assay as described previously (7). The affinities of RanBP5 and importin-β for RanGTP were calculated by determining the concentrations of the respective Ran-binding protein required for 50% inhibition of Rna1p-induced GTP hydrolysis on 50 pM Ran[γ-^{32}P]GTP during a 5-min reaction at 25°C. After the GTPase reaction, released [^{32}P]phosphate was recovered by the charcoal adsorption assay as described previously (6).

Import assay. Recombinant RanBP5 was labelled with fluorescein-5′-maleimide at a 1:1 molar ratio as previously described (24). Labelled RanBP5 was used at 300 nM. The importin-β concentration for competition was 3 μM. The binding buffer contained 20 mM HEPES-KOH (pH 7.5), 140 mM potassium acetate, 4 mM magnesium acetate, 1 mM DTT, 250 mM sucrose, and 2 mg of nucleoplasmin core per ml plus 2 mg of bovine serum albumin per ml to block nonspecific binding. Also present were 1.5 μM Ran (GDP form), 150 nM RanBP1, 150 nM Rna1p, 150 nM NTF2, and an energy-regenerating system. Binding to the NPC was allowed to continue for 10 min at room temperature. The nuclei were fixed with paraformaldehyde-glutaraldehyde, centrifuged onto coverslips, and analyzed by confocal microscopy.

ESTs. Searching of the databases identified the following expressed sequence tags [ESTs] as having greater than 100-bp homology to the full-length RanBP5: Aa040825, W52931, H29071, W40371, N42306, N93878, W03230, W66752, H04223, Aa014259, Aa004784, R84930, H13499, T31653, W86324, H10013, R58885,

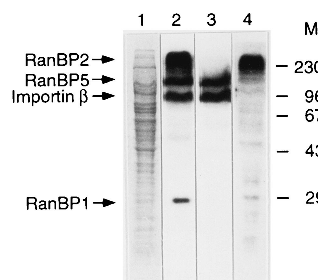

FIG. 1. Detection of two classes of Ran-binding proteins in HeLa cells. The soluble fraction of HeLa cell lysates was separated by SDS-PAGE and transferred to nitrocellulose. Renatured blots were incubated with Ran[γ-^{32}P]GTP (lane 2) or with preformed complexes of RanBP1 (lane 3) or importin-β (lane 4) and Ran[γ-^{32}P]GTP. Lane 1 shows the soluble fraction stained with Coomassie brilliant blue. The positions of the molecular weight standards (in thousands) are indicated.

N69555, R11819, R06779, N94046, Aa068107, T92819, W60445, W16948, R16461, R18480, D31395, W16853, H13447, R48037, F00275, Aa040031, Aa043342, N35180, H05851, Z19201, W10252, T10424, N56331, N78664, R14964, T11236, R47929, H12249, R95432, N87727, Aa054692, R45689, G06290, Z38856, Aa071721, S45357, Z11538, Z49212, W31869, W43739, W36629, R19226, Z43830, R17986, R19344, D68946, T01748, W15152, and D69578.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession no. Y08890.

RESULTS

Detection of two classes of Ran-binding proteins in HeLa cells. By using overlay assays, a number of putative effectors of the Ran pathway have previously been described. Essentially, Ran was loaded with radioactively labelled GTP and used to probe blots on which total-cell lysates had been resolved by SDS-PAGE (6, 11, 25, 38). By using a modified protocol of Lounsbury et al. (25) for the renaturation of the nitrocellulose-bound proteins, we could detect at least four bands of approximately 350, 120, 90, and 23 kDa (Fig. 1, lane 2). Binding to these proteins was shown to be specific for the GTP-bound form of Ran. Purification and amino acid sequence analysis showed that two of these proteins had been previously described as RanBP1 (23 kDa) (11) and importin-β (90 kDa) (17). The 350-kDa band is likely to represent RanBP2 (48, 49).

In competition experiments, Ran[γ-^{32}P]GTP was preincubated with a 1,000-fold molar excess of RanBP1 or importin-β. As can be seen in Fig. 1, lane 3, preincubation with RanBP1 inhibited the labelling of the bands corresponding to RanBP1 and RanBP2 on blots with total HeLa cell lysates. This competition shows that RanGTP binds in a similar manner to both factors (3) and also correlates with the sequence homology of RanBP1 and RanBP2 defining the Ran-binding domain (6, 11, 17). However, RanGTP binding to both importin-β and the 120-kDa protein band was still observed in the presence of RanBP1. This is in agreement with the findings of Lounsbury et al. (27) and indicates that the Ran-binding domains of RanBP1 and RanBP2 cover a different site on RanGTP than importin-β or the 120-kDa protein(s). This is further indicated by the existence of a trimeric complex consisting of RanBP1, RanGTP, and importin-β (9, 18). Preincubation of Ran with importin-β inhibited binding both to the 120-kDa band and also to importin-β itself. Interestingly, binding to RanBP1 was clearly reduced, indicating that the order of addition of Ran-binding proteins to Ran may influence complex formation. RanBP2 was still strongly detected (lane 4). This may indicate that binding of RanGTP to RanBP2 may also occur via importin-β. Similarly, as is the case for the nuclear pore protein p62 (10a), the additional bands visible in the overlay assay (lane 4) may also represent proteins that bind the importin-β–RanGTP complex.

TABLE 1. Enrichment of RanBP5[a]

Fraction (no.)	Total amt of protein (mg)	Amt of RanBP5 (μg)	Purification (fold)	Yield (%)
70,000 × g supernatant (1)	552	850		100
DMAE (2)	34	710	13.6	84
30% $(NH_4)_2SO_4$ (3)	3.20	320	64.9	38
Hydroxylapatite (4)	0.72	240	216	28
Mono Q (5)	0.095	95	649	11.2

[a] Values refer to 10^{10} cells as starting material. The values given take into consideration those proteins of approximately 120 kDa that were separated from RanBP5 during the DMAE and hydroxylapatite separation steps.

Purification of RanBP5 from HeLa cells. We purified to homogeneity one of the 120-kDa Ran-binding proteins from 10^{10} HeLa cells as the starting material (for details, see Materials and Methods). The enrichment table (Table 1) shows the purification scheme. The corresponding Coomassie blue-stained fractions are shown in Fig. 2A. Purification was monitored by analysis of the resulting fractions in an overlay assay with Ran[γ-^{32}P]GTP (Fig. 2B). During chromatography on dimethylaminoethane (DMAE) and hydroxylapatite, other bands around 120 kDa, which probably represent additional Ran-binding proteins (25, 27) such as RanBP7 and RanBP8 (16) or possibly posttranslationally modified forms of these proteins, were also detected. However, for this study we focussed on the predominant Ran-binding activity at 120 kDa, which we named RanBP5. From the enrichment table, we would estimate that a HeLa cell contains roughly 4 × 10^5 molecules of RanBP5, which compares to 10^7 molecules of Ran.

Cloning of RanBP5 cDNA. Based on a comparison of partial peptide sequences (Fig. 3), obtained from the purified HeLa RanBP5, with sequences in the databases, a number of expressed sequence tags were identified and used to design oligonucleotide primers. These were in turn used to amplify a fragment of 420 bp from a HeLa λgt11 cDNA library. Screening a λDR2 HeLa cDNA library (Clontech) with this 420-bp fragment allowed the identification of two classes of cDNA clones. Sequencing of these clones revealed the presence of

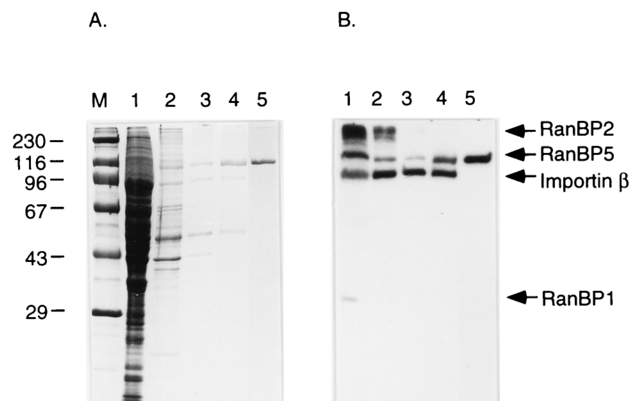

FIG. 2. Purification of RanBP5 from HeLa cells. (A) Fractions obtained after each step of purification were resolved by SDS-PAGE and stained with Coomassie brilliant blue. (B) The same fractions after transfer to nitrocellulose and overlay with Ran[γ^{32}P]GTP. Fraction numbers correspond to those in Table 1.

FIG. 3. Amino acid sequence of RanBP5 and alignment with yeast Pse1p and human importin-β (IMP β). RanBP5 (ACC Y08890), human importin-β (ACC L39793), and PSE1 (ACC S45357) were aligned with the Clustal Megalign program of DNA star (Lasergene). Identical amino acids are shown in black, while related amino acids are shown in grey. Dashed lines represent gaps introduced into the sequences to optimize the alignment. Asterisks mark the positions of peptide sequences obtained from RanBP5 purified from HeLa cells.

two distinct polyadenylation signals. These clones are 3,583 and 2,993 bp, respectively, and represent the 3′ region of the RanBP5 cDNA, where 2,259 bp each belong to the coding region.

Interestingly, the 5′ region of the RanBP5 cDNA was obtained in a search for proteins that interact with human RanBP1 in the yeast two-hybrid system. The cDNA coding for human RanBP1 was fused to the DNA-binding domain of GAL4 and used as a bait to screen a pACT cDNA library prepared from human lymphocytes (13) for clones coding for interacting proteins. A total of 114 cDNA clones were isolated and classified into four independent groups based on their nucleotide sequences. The first group (107 clones) represented Ran. Subsequent analysis of the second class of positive clones revealed an incomplete open reading frame of 368 codons lacking the start ATG. Sequence analysis revealed homology to approximately 30 human ESTs present in the databases (see Materials and Methods). The 5′ end of the RanBP5 cDNA including the translation start codon was cloned by screening a λDR2 HeLa cDNA library (Clontech) with one of the pACT clones. It was further confirmed by the RACE method. The nucleotide sequence at the putative N-terminal region of the coding sequence correlates well with a Kozak sequence (23). The initiation codon is preceded by an in-frame termination codon.

The polypeptide encoded by the original pACT clone represents amino acid residues 5 to 372 of the complete open reading frame of RanBP5. It was also shown to interact with Ran in the two-hybrid system, indicating that the N-terminal region of RanBP5 is sufficient for Ran binding (data not shown); this then raises the question whether the RanBP5 interaction with RanBP1 is Ran/Gsp1p dependent (see below).

Comparison of the sequence encoding the carboxy-terminal region of RanBP5 obtained from the purified HeLa protein with the sequence encoding the N-terminal fragment obtained by the two-hybrid method revealed a 76-bp overlap between the 3′ end of the sequence encoding the N-terminal fragment and the 5′ end of the sequence encoding the C-terminal fragment. Further confirmation that both sequences represented the full-length cDNA for RanBP5 was provided by PCR from HeLa cDNA with 5′- and 3′-specific primers to amplify the region of overlap. The combined sequence contains an open reading frame encoding 1,097 amino acid residues corresponding to a calculated molecular mass for the protein of 123,549 Da. This is in agreement with the size of the native protein purified from HeLa cells. Northern blot analysis of RanBP5 gene expression was carried out, and in HeLa cells a single transcript of 4.5 kb was detected (data not shown).

The RanBP5 sequence was found to be homologous (30% identity, 56% similarity) to that of PSE1 from *Saccharomyces cerevisiae* (Fig. 3). In addition, the sequence displayed a significant similarity to importin-β (21% identity, 46% similarity), which might suggest that RanBP5 is also involved in nuclear transport (Fig. 3). Furthermore, recent results have indicated that RanBP5 is likely to belong to a new family of proteins characterized by the presence of a common N-terminal motif (15, 16).

RanBP5 is a predominantly cytoplasmic protein. To further characterize RanBP5, both the sequence encoding the N-terminal fragment and the full-length cDNA of RanBP5 were amplified by PCR, expressed in *E. coli*, and confirmed to bind Ran[γ-^{32}P]GTP in an overlay assay (Fig. 4A). The additional bands in lanes 1 and 2 are likely to reflect degradation products of RanBP5. In addition, polyclonal antibodies were raised against the N-terminal region of the recombinant RanBP5 protein. The affinity-purified antibodies (AP-3/3) were shown

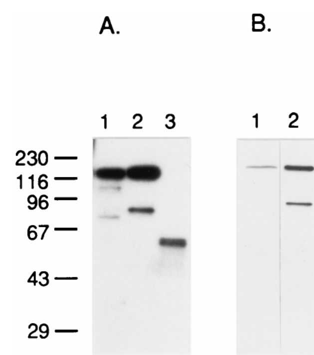

FIG. 4. Both the N-terminal domain and full-length RanBP5 bind RanGTP. (A) Purified HeLa RanBP5 (lane 1), full-length recombinant RanBP5 (lane 2), and the recombinant N-terminal RanBP5 fragment (lane 3) were separated by SDS-PAGE (12% polyacrylamide) and transferred to nitrocellulose. An overlay assay with Ran[γ^{32}P]GTP was carried out as described in Materials and Methods. (B) Affinity-purified antibodies against RanBP5 recognize both the recombinantly expressed RanBP5 protein (lane 2) and a single band with the same mobility in HeLa total-cell lysates (lane 1).

to recognize both the full-length expressed RanBP5 recombinant protein and a single band with the same mobility in a HeLa total-cell lysate (Fig. 4B). Indirect immunofluorescence microscopy was carried out on both methanol-acetone-fixed and formaldehyde-fixed cultured mammalian COS-7 cells. Immunostaining with the AP-3/3 antibodies showed a diffuse cytoplasmic pattern of staining with more intense staining around the nucleus (Fig. 5A). These results were further supported by immunofluorescence analysis of transfected COS-7 cells in which a recombinant form of RanBP5 was overexpressed (Fig. 5B). In these cells, it was possible to see a staining of the endogenous RanBP5 in all cells and a more pronounced staining in the transfected cells (Fig. 5B). Furthermore, the overexpression of a myc-tagged form of RanBP5 and detection with the anti-myc epitope monoclonal antibody showed that the overexpressed RanBP5 has the same intracellular distribution as the endogenous antigen (Fig. 5C).

Inhibition of GTP exchange and hydrolysis on Ran by RanBP5. We then determined, with importin-β as a reference, the effect of RanBP5 on both guanine nucleotide exchange and GTP hydrolysis on Ran. Ran has a high affinity for guanine nucleotides, and once bound, GTP and GDP are exchanged very slowly. The nucleotide exchange can be accelerated by the addition of EDTA, which complexes the magnesium ions that are required for tight nucleotide binding, or by the addition of the physiological exchange factor RCC1. In experiments where the guanine nucleotide dissociation from Ran was examined, RanBP5 shows essentially the same inhibition of EDTA- and RCC1-mediated GTP exchange as previously observed for importin-β (Fig. 6) (18). The extent of inhibition of RCC1-mediated nucleotide exchange was not changed after the concentration of RCC1 was increased from 1 to 100 nM, although 1 nM is already sufficient to exchange all Ran-bound GTP within the 5-min period of the reaction. This indicates that RanBP5 hinders RanGTP sterically from interacting with the exchange factor RCC1. We calculated the half-lives of the complexes of RanGTP with RanBP5 or importin-β at 25°C to be in the range of 3 and 4 h, respectively. At the low RanGTP concentration of 50 pM used in this experiment, the concentration of RanBP5 and importin-β required for 50% inhibition of nucleotide exchange directly reflects the affinity of binding for the corresponding complex partners. For the RanBP5-RanGTP com-

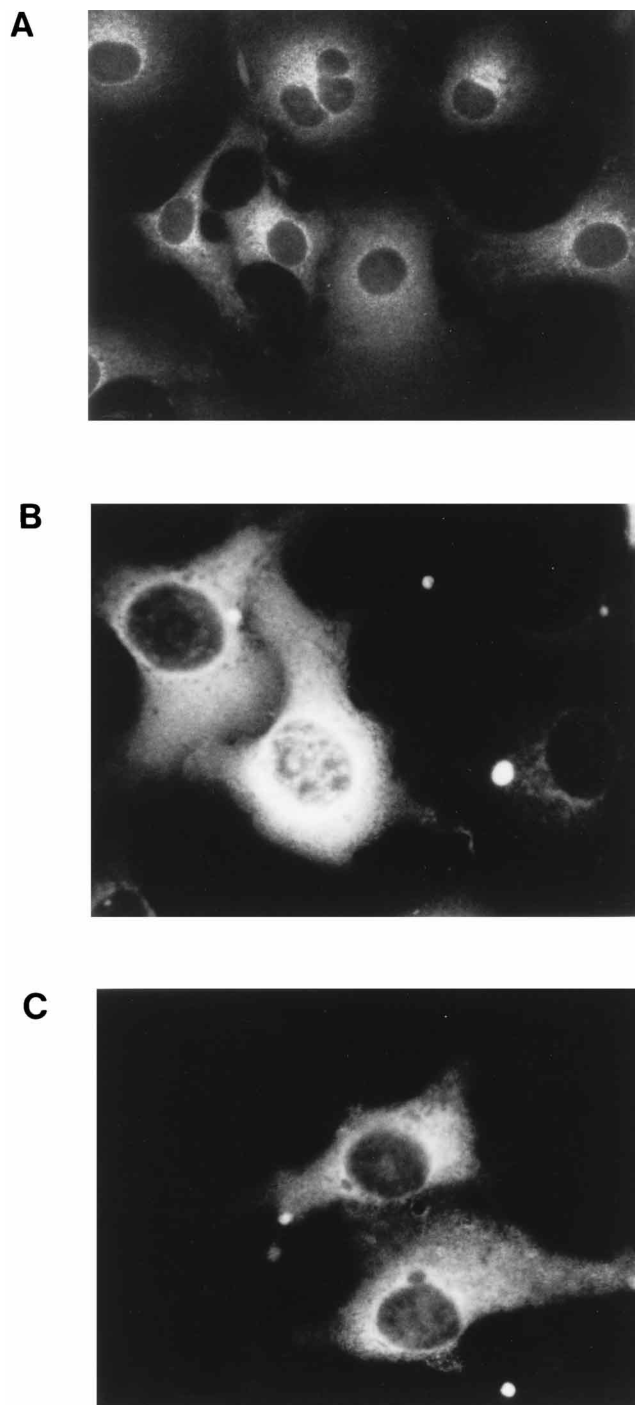

FIG. 5. Localization of RanBP5 in COS-7 cells by immunofluorescence. COS-7 cells were fixed in either paraformaldehyde (A) or methanol-acetone (B and C) prior to immunofluorescence analysis. (A) Immunodetection of endogenous RanBP5 with antibody (AP 3/3) raised against the N-terminal region of the protein. (B) Cells overexpressing a 5′-myc-tagged form of RanBP5 after transient transfection with the pcDNA3-myc-RanBP5 expression vector. The cells on the right have a stronger fluorescence and reflect the detection of the overexpressed RanBP5 relative to the endogenous protein, which is seen in the nontransfected cells to the left. (C) Cells, transfected as in panel B, stained with the anti myc-epitope antibody.

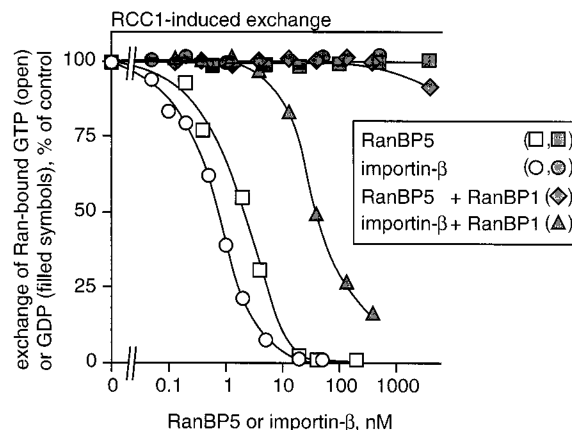

FIG. 6. Inhibition of GTP exchange on Ran by RanBP5. Ran[γ-^{32}P]GTP (50 pM) (open symbols) or 1 nM Ran[α-^{32}P]GDP (shaded symbols) were preincubated with either recombinant RanBP5 or importin-β. After 15 min, RanBP1 was added (where indicated) to a final concentration of 400 nM. At 1 min later, nonradioactive GDP was added to 200 μM, and a 5 min exchange reaction was started by the addition of 10 nM RCC1. Nucleotide exchange was determined by measuring protein-bound radioactivity. The final concentrations of RanBP5 and importin-β were as indicated.

plex, we calculated a K_d of 3 nM, which is about fivefold higher than that of the importin-β–RanGTP complex.

Next, we examined the effects of RanBP5 on the hydrolysis of Ran-bound GTP. As was described previously for importin-β (14, 18), RanBP5 inhibits both the intrinsic (not shown) and the Rna1p/RanGAP-induced (see Fig. 8A) GTPase activity of Ran. The concentrations of importin-β and RanBP5 required for 50% inhibition of Rna1p-induced hydrolysis of Ran-bound GTP are comparable to those required for inhibition of GTP exchange. However, the N-terminal fragment of RanBP5 binds with a lower apparent affinity to RanGTP (see Fig. 8A), and in experiments in which time-dependent GTPase inhibition was measured (data not shown), the dissociation rate was found to be 60-fold higher than for the full-length protein. This may indicate that additional regions are required for Ran binding or for the correct folding of the protein.

In an in vitro binding assay with GST-RanBP5 immobilized on glutathione-Sepharose, we were able to enrich RanBP1 from a lysate of *E. coli* cells expressing RanBP1, provided that RanGTP was also present (data not shown). This indicates that RanBP5 binds to RanBP1 as part of a trimeric complex with Ran. This trimeric RanBP5-RanGTP-RanBP1 complex was also detected in gel filtration experiments (Fig. 7A). This suggests that the observed interaction of RanBP5 and RanBP1 in the two-hybrid system is mediated by the conserved Ran homologs Gsp1p and Gsp2p provided by the yeast cell.

We have also determined the characteristics of the binding of both RanBP5 and importin-β to RanGDP and found that both proteins fail to inhibit nucleotide exchange on RanGDP. Furthermore, RanBP1 has no measurable effect on RCC1-induced GDP dissociation under the conditions tested. However, when added with a molar excess of RanBP1 over RanGDP, importin-β shows a clear inhibitory effect, indicating a cooperative binding of RanBP1 and importin-β to the GDP-bound form of Ran and the formation of a RanBP1–RanGDP–importin-β complex (Fig. 6). We were also able to detect such a complex by gel filtration (Fig. 7C). As was the case for the GTP-bound form of Ran, this inhibition was still observed in the presence of a high RCC1 concentration (100 nM), indicating a low dissociation rate and indicating that RCC1 does not

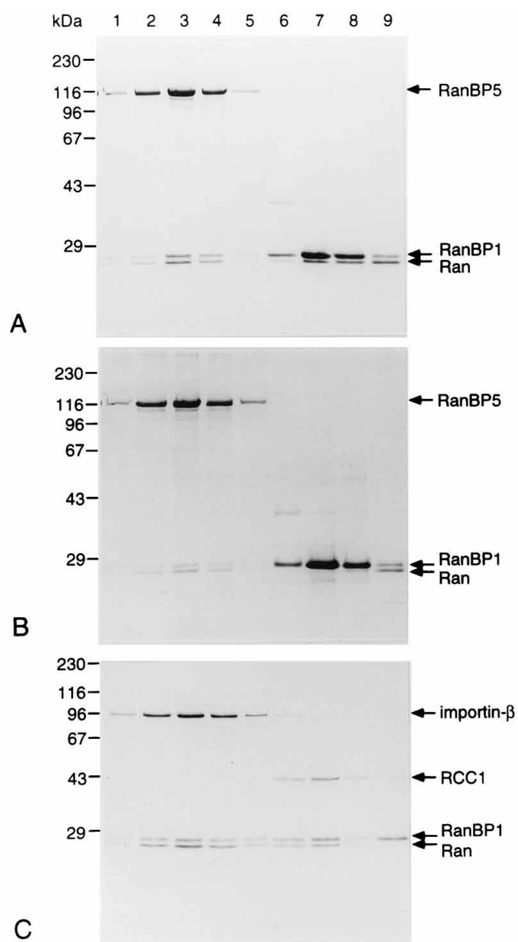

FIG. 7. Ran-dependent binding of RanBP1 by RanBP5 and importin-β. (A) Recombinant RanGTP, RanBP1, and RanBP5 (40 μg each were) was incubated in 200 μl of 20 mM HEPES-NaOH (pH 7.4)–100 mM sodium acetate–1 mM magnesium acetate–0.02% sodium azide. After 15 min, the sample was applied to a Superdex 200 column (HR 10/60; Pharmacia), and gel filtration was performed in the same buffer at a flow rate of 1 ml/min. The samples were analyzed by SDS-PAGE. The proteins were stained with Coomassie brilliant blue. The fractions were numbered in the order of their elution. (B) RanGDP was used instead of RanGTP. (C) RanGDP, RanBP1, importin-β, and RCC1 (40 μg each) were incubated and gel filtered as above.

directly influence the stability of the RanBP1–RanGDP–importin-β complex. Consequently, when added to a mixture of RanGDP, RanBP1, and importin-β, RCC1 fails to bind to the resulting RanBP1–RanGDP–importin-β complex (Fig. 7C). Thus, measuring the concentration of importin-β to reveal half-maximal inhibition of nucleotide exchange in the presence of a high concentration of RanBP1 is sufficient to calculate the affinity of a RanBP1–RanGDP–importin-β complex. The calculated constant for the dissociation of importin-β from this complex is with 40 nM about 50-fold lower than in the case of the importin-β–RanGTP complex. We also could observe a cooperative binding of RanBP5 and RanBP1 to the GDP-bound form of Ran. However, micromolar concentrations of RanBP5 were required for an inhibition of RCC1-induced GDP exchange (Fig. 6). Similarly, only a small population of added RanGDP and RanBP1 was associated with RanBP5 after gel filtration, indicating a low affinity of this complex.

RanBP1 removes the RanBP5-mediated block on RanGTP hydrolysis. We have shown that RanBP5 binds to RanGTP, resulting in a complex with a half-life of approximately 3 h. In an attempt to identify a mechanism by which Ran would become susceptible to GTPase activation in the presence of RanBP5, we tested the effects of RanBP1 as a stimulating factor. The reasons for this were twofold. First, according to the overlay assay and gel filtration experiments, RanBP1 should be able to interact physically with the RanGTP-RanBP5 complex, and second, the heterodimeric complex of RanGTP and RanBP1 is susceptible to RanGAP-induced GTP hydrolysis, a property that could also be retained by the trimeric complex.

Ran[γ-^{32}P]GTP was preincubated with HeLa-purified and bacterially expressed RanBP5 for 30 min. Hydrolysis of the Ran-bound GTP following the addition of Rna1p is then blocked by the formation of the RanBP5-RanGTP complex (Fig. 8A). However, addition of RanBP1 prior to or together with Rna1p resulted in a dramatic stimulation of hydrolysis of Ran-bound GTP (Fig. 8B). In contrast, for the importin-β–RanGTP complex, only a slight stimulatory effect was evident.

To test whether this difference in behavior simply reflects a different off-rate of the RanGTP-RanBP5 and the RanGTP–importin-β complexes, we determined their rates of dissociation. Following the formation of the corresponding complexes, GTPase activation was started with the addition of Rna1p (Fig. 8C). The use of a very high concentration of Rna1p should ensure that the dissociation of the complexes involving

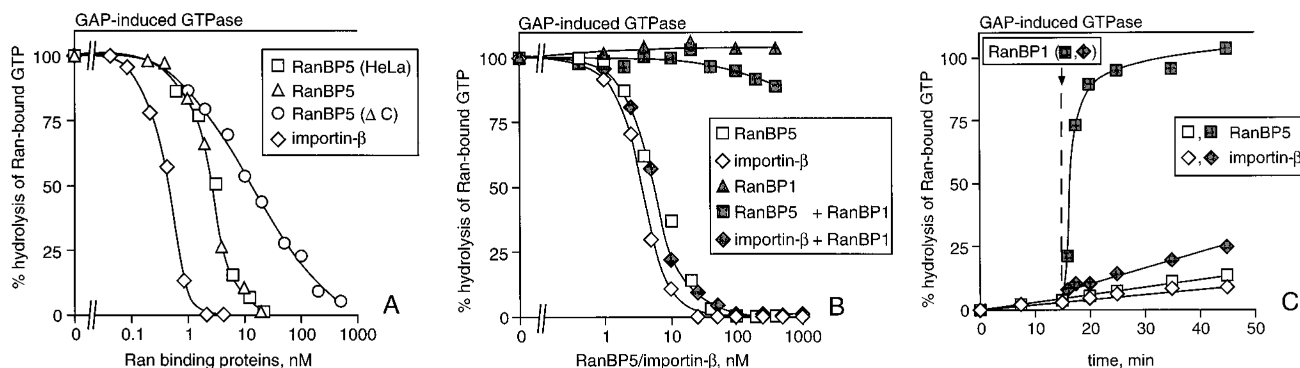

FIG. 8. Effect on GAP-induced GTPase and removal of the RanBP5-mediated block on GTP hydrolysis by RanBP1. (A) Ran[γ-^{32}P]GTP (50 pM) was preincubated with either HeLa-derived, bacterially expressed full-length RanBP5, the N-terminal fragment of RanBP5 or importin-β for 30 min. Then 5 nM Rna1p was added, and the reaction was allowed to proceed for 5 min. Hydrolysis of Ran-bound GTP was determined as released [^{32}P]phosphate. (B) Ran[γ-^{32}P]GTP (10 nM) was preincubated with bacterially expressed RanBP5, importin-β, or incubation buffer for 30 min. Immediately before the addition of 100 nM Rna1p, RanBP1 was added to a final concentration of 40 nM where indicated and the reaction was allowed to proceed for 5 min. Hydrolysis of Ran-bound GTP was determined as released [^{32}P]phosphate. (C) Ran[γ-^{32}P]GTP (10 nM) was preincubated in a 500-μl volume with 250 nM RanBP5 or 50 nM importin-β for 30 min. Then 500 nM Rna1p was added. After 15 min, 30 nM RanBP1 was added as indicated. Released [^{32}P]phosphate was determined in 50-μl aliquots at the indicated time intervals.

RanGTP and RanBP5 or importin-β immediately leads to the hydrolysis of the Ran-bound GTP and not to rebinding of the corresponding Ran-binding protein. The calculated half-lives of the RanGTP-RanBP5 and the RanGTP–importin-β complexes are in both cases on the order of 3 to 4 h. However, when RanBP1 was added, hydrolysis of Ran-bound GTP was more than 100-fold stimulated in the sample containing RanBP5. In contrast, a considerably lower GTPase activation with a factor of only twofold stimulation was seen in the presence of importin-β.

In summary, RanBP5 and importin-β strongly inhibit both intrinsic and RanGAP-induced GTP hydrolysis, as well as nucleotide exchange on RanGTP. Both RanBP5 and importin-β can form a trimeric complex with RanBP1 and either the GDP- or GTP-bound form of Ran. Binding of either RanBP5 or importin-β to RanGTP could result in an irreversible sink for the GTP-bound form of Ran and the respective Ran-binding protein, which would culminate in a disruption of the nuclear import pathway. We show that in contrast to the importin-β–RanGTP complex, binding of RanBP1 to the RanBP5-RanGTP complex allows efficient stimulation of hydrolysis of Ran-bound GTP by RanGAP.

RanBP5 binds to the NPC. To test if RanBP5 would bind to NPCs, we labelled the full-length RanBP5 with fluorescein-5-maleimide and added it to permeabilized HeLa cells in the presence of Ran and an energy-regenerating system. After fixation, the distribution of RanBP5 was determined by confocal fluorescence microscopy. Figure 9A clearly shows that RanBP5 gave the typical NPC staining pattern of narrow punctate rims. When an excess of unlabelled importin-β over RanBP5 was added to the import reaction mixture, such binding to the nuclear rim was no longer detected (Fig. 9B). This would suggest that RanBP5 binds to the NPC and competes with importin-β for the same binding sites.

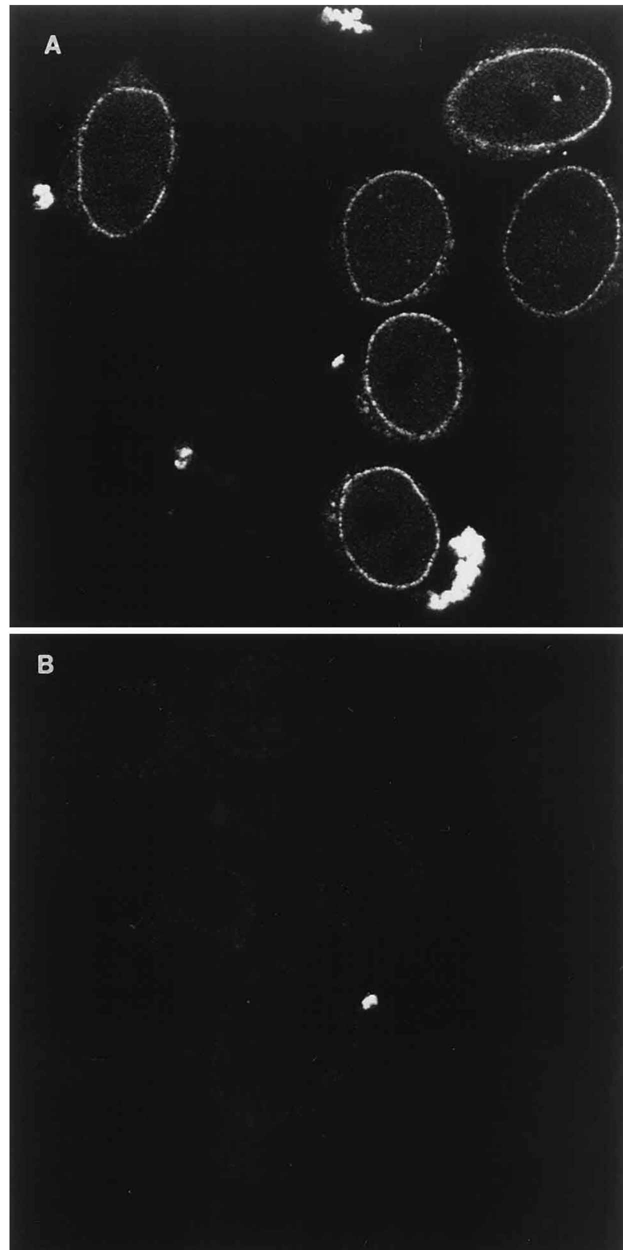

FIG. 9. Fluorescein-labelled RanBP5 competes with importin-β for binding to the nuclear envelope. (A) Confocal scanned images through nuclei of permeabilized HeLa cells after import reactions with fluorescein-labelled RanBP5. (B) The same import reaction in the presence of excess unlabelled importin-β shows a competition for binding.

DISCUSSION

The multitude of processes that are affected in cells with defects in the Ran system prompts the question whether this is due to a dysfunction of one common central process such as NLS-dependent nuclear protein import, where a role for Ran has been clearly documented, or whether this could indicate an independent function for Ran in the regulation of additional cellular processes. One way of addressing this key issue is the characterization of factors which directly interact with Ran in either its GTP- or GDP-bound form. In an overlay assay, a number of RanGTP-interacting proteins have been detected (11, 25). Further characterization by competition analysis revealed two distinct types of RanGTP-binding proteins, one comprising RanBP1 and RanBP2 and the other comprising importin-β and a number of uncharacterized proteins of approximately 120 kDa ((25, 27); this study). Sequence analysis of RanBP5, a 124-kDa protein which we isolated from HeLa cells, revealed a significant level of similarity to importin-β (21% identity, 46% similarity). However, the high degree of homology between RanBP5 and *S. cerevisiae* Pse1p (30% identity, 56% similarity) would suggest that RanBP5 is a structural homolog of the yeast Pse1p. In *S. cerevisiae*, overexpression of PSE1 results in an increase in protein secretion (10). It is unknown whether this could be an indirect effect resulting from an increase in nuclear transport of certain macromolecules. As suggested for a RanBP5 homolog, Pse1p biochemically interacts with the GTP-bound form of Gsp1p, a yeast homolog of Ran (16) (45a). In addition to RanBP5, further importin-β-related proteins, including transportin and its yeast homolog Kap104p (1), hCRM1 (15), RanBP7, and RanBP8 (16) have been identified, suggesting a new family of RanGTP-binding proteins which may play a role in nucleocytoplasmic transport (15, 16).

That RanBP5 could be involved in nuclear transport we assume first from its homology to importin-β. More direct evidence for a possible role of RanBP5 in nuclear transport came from an in vitro transport assay in which fluorescently labelled RanBP5 was found to bind to NPCs. This, together with the finding that binding to the nuclear envelope was competed by the addition of excess importin-β, indicates that both proteins are likely to bind at the same sites at the NPC and that RanBP5 could function as an import factor mediating docking

to the NPC. At present, we have no clear indication about the in vivo substrate that is transported by RanBP5. However, the RanBP5-related yeast proteins Pse1p and Kap123p (29 and 23% identity, respectively) were recently shown to be involved in the nuclear import of ribosomal proteins (40). Since we were unable to detect any binding of RanBP5 to importin-α (data not shown), we would assume an importin-α independent substrate recognition. Importin-α-independent transport mechanisms have recently been described for transportin, an importin-β-related protein required for the import and export of the A1 heterogeneous nuclear ribonucleoprotein particle (35), for the import of Nab2p and Nab4p by Kap104p (1), and for the Kap123p-mediated import of ribosomal proteins into the nucleus (40).

As is evident with importin-β, RanBP5 inhibits both the RCC1-mediated GTP exchange and the RanGAP-induced GTP hydrolysis on Ran. Given that the RanBP5-RanGTP complex has a half-life of 3 h in vitro, this raises the question how such a complex can dissociate in vivo. To address such issues, we tested whether RanBP1, a cytoplasmic protein that was identified by its specific binding to the GTP-bound form of Ran (11), could destabilize the RanBP5-RanGTP complex. In contrast to the heterodimeric complexes with importin-β and RanBP5, the complex of RanGTP and RanBP1 is highly susceptible to the action of RanGAP1 (6). This, together with the ability of RanBP1 to bind to the RanBP5-RanGTP complex, makes it an excellent candidate for a recycling factor that would promote the disassembly of the RanBP5-RanGTP complex. Indeed, the addition of RanBP1 to the performed RanBP5-RanGTP complex, in the presence of the RanGAP Rna1p, resulted in a dramatic increase in the GTPase activity. The conversion of the released RanGTP to the GDP-bound form would then prevent re-formation of the Ran-RanBP5 complex.

The induction of GTPase activation by RanBP1 has also been shown between complexes of RanGTP and all importin-β related proteins tested to date (RanBP5 [this study], transportin [32a], and RanBP7 and RanBP8 [16]). However, GTP hydrolysis on Ran involved in the importin-β–RanGTP interaction was only weakly stimulated by RanBP1 (18, 26), indicating that another factor is required for efficient dissociation. In general, RanBP1 could represent a recycling factor that would stimulate the in vivo disassembly of complexes between RanGTP and at least some of the putative importin-β-like transport factors that reappear in the cytoplasm after one round of nuclear import. A similar role could also be proposed for RanBP2, which has four RanBP1 homology domains that bind RanGTP and behave like RanBP1 biochemically (3, 48, 49). In particular, its location at the cytoplasmic fibrils of the NPC make it an excellent candidate to ensure efficient recycling of import receptors.

Recently Chi et al. (9) reported on a different aspect of RanBP1 function. In addition to the trimeric RanBP1–RanGTP–importin-β complex, they could detect a similar complex with the GDP-bound form of Ran. From their biochemical analysis, they propose that this complex is involved in the docking of a cargo to the NPC. Our results also show a clear cooperative binding of RanBP1 and importin-β to RanGDP. We calculated the affinity of the resulting complex to be about 50-fold lower than the affinity of importin-β for RanGTP. However, taking into consideration the cellular concentrations of these proteins, this affinity is still high enough to permit an in vivo formation of an importin-β–RanBP1–RanGDP complex. We were also able to detect a similar trimeric complex with RanBP5, RanBP1, and RanGDP. However, the calculated affinity of this complex is low, and complex formation cannot be expected at the given cellular concentrations of the proteins. This could indicate that a possible synergistic action of RanBP1 and RanGDP at the docking step, as suggested by Chi et al. (9) might be restricted to importin-β-mediated import.

We suggest here that RanBP5 is a nuclear import factor for an as yet unknown import substrate. By analogy to importin-β, we propose that RanGTP dissociates the RanBP5-import substrate complex following translocation to the nucleoplasmic side of the NPC. The resulting RanGTP-RanBP5 complex would have to be returned to the cytoplasm, where RanBP1, together with RanGAP1, would disassemble it. RanBP5 could then bind another substrate molecule and participate in a further round of nuclear import.

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ADDENDUM IN PROOF

During the review process of the manuscript, Yaseen and Blobel published the sequence of karyopherin-β3, which corresponds to that of RanBP5. Karyopherin-β3 was shown to bind to several nucleoporins and to ribosomal proteins in an overlay assay (N. R. Yaseen, and G. Blobel, Proc. Natl. Acad. Sci. USA **94:**4451–4456, 1997).

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