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Human RanBP3, a group of nuclear RanGTP binding proteins

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Abstract A group of novel human Ran-binding proteins, RanBP3, was identified using the yeast two-hybrid system via Ran-mediated interaction with the nucleotide exchange factor RCC1. Several open reading frames, representing putative alternatively spliced products, were established by cDNA cloning. Two of them, RanBP3-a and RanBP3-b, encode nuclear hydrophilic proteins of 499 and 562 amino acid residues. The sequences contain FXFG motifs, characteristic of a subgroup of nucleoporins, and a C-terminal domain showing similarity to the Ran-binding protein RanBP1. These proteins are localized in the nucleus, preferentially bind RanGTP and may be nuclear effectors of the Ran pathway.

Key words: Ran pathway; Ras-related guanosine triphosphatase; Ran-binding protein; RanBP1; yrb2; hba1

1. Introduction

The small Ras-related GTPase Ran plays an essential role in the transport of macromolecules beween the cytoplasm and the nucleus (for a review see [1]). Ran is an abundant soluble protein predominantly localized in the nucleus [2]. Its nucleotide bound state is regulated by the Ran-specific GTPase activating protein RanGAP1 [3] and the specific guanine nucleotide exchange factor RCC1 [4]. RanGAP1 is cytoplasmic, or is attached to the cytoplasmic margin of nuclear pore complexes (NPC) after conjugation to a ubiquitin-like protein [5–7]. In contrast, RCC1 is exclusively nuclear and bound to chromatin [2,8]. The asymmetric subcellular distribution of both Ran regulators is thought to result in a low concentration of RanGTP in the cytoplasm vs. a high concentration of RanGTP in the nucleus. This RanGTP gradient across the nuclear pore complex is believed to be required for the formation and dissociation of complexes involved in import and export from the nucleus [9] (for a review see [10]).

There are two classes of RanGTP-binding proteins which differ in function and in their binding sites on Ran. Members of the importin β related class of transport factors are of similar size (95–130 kDa) and have a weakly conserved consensus sequence in their N-terminal regions involved in Ran binding. Most of them were shown to bind RanGTP but not RanGDP, and to inhibit both GTP hydrolysis and guanine nucleotide exchange on Ran [11,12]. Within this class, import factors like importin β/karyopherin β [13] and transportin [14,15] bind RanGTP with distinctly higher affinity than export factors like CAS [16], CRM1 [17–19], and exportin t/Los1 [20]. The affinity of the export factors for RanGTP is considerably increased by cooperative binding of the cargo to be exported.

Proteins of the second class bind to RanGTP at a different site and inhibit nucleotide exchange but not hydrolysis [21,22]. They differ in size and share one or several regions of homology to RanBP1. This small cytoplasmic protein acts as a dissociation factor for the stable RanGTP·transport factor complex, thereby releasing the transport factor for a new round of transport [16,23–27] and exposing RanGTP to nucleotide hydrolysis stimulated by RanGAP1 in the cytoplasm. A close homologue of RanBP1, Yrb1p, has been identified in yeast as an essential protein involved in chromosome stability and nuclear protein import [28,29]. RanBP2, a protein of 358 kDa [30,31], is a vertebrate nucleoporin located at the short fibres on the cytoplasmic margin of the NPC. It contains four RanBP1 homologous Ran-binding domains and numerous FXFG motifs; the latter are typical for a subgroup of nucleoporins [32]. Yrb2p is a RanBP1-related protein from baker's yeast which is localized to the nucleus [33]. A *yrb2* null-mutant shows synthetic lethality with a mutation in the gene *rna1*, encoding the RanGAP homologue [33]. Its fission yeast homologue is the essential hba1p, which also resides in the nucleus [34]. Here, we present human RanBP3-a and -b as new members of this class of Ran-binding proteins.

2. Materials and methods

2.1. Strains and cDNA cloning

Plasmids were propagated in *E. coli* DH5α. Lambda libraries were transfected into *E. coli* Y1090 *hsdR*. The *E. coli* strain BL21(DE3)-pLysS [35] was used for expression of the 6×His- or GST-fusion proteins. Two human HeLa S3 5′-STRETCH cDNA libraries (Clontech Laboratories GmbH, Heidelberg, Germany), cloned into λgt11 and λDR2 vectors, respectively, were screened for full length RanBP3 cDNAs, using radiolabeled (DECAprimeII kit, Ambion, Austin, TX, USA) RanBP3 DNA probes derived from RanBP3ΔN-217. λgt11 inserts were PCR amplified using λgt11 forward (5′-GGTGGCGACGACTCCTGGAGCCCG) or reverse (5′-CAGACCAACTGGTAATGGTAGCGA) primers and subcloned into the pCRII vector (Invitrogen BV, Leek, The Netherlands). Clones derived from the λDR2 cDNA library were converted into plasmid pDR derivatives by cre-lox mediated recombination in *E. coli* AM1 (Clontech). Several cDNA clones were isolated, of which one with a cDNA insert of 3.2 kb contained the complete RanBP3-a open reading frame (ORF) plus 1.5 kb of 3′-untranslated region (3′-UTR), a second one with 1.8 kb the RanBP3-b ORF except for the first 8 nucleotides.

2.2. Two-hybrid screening

All components of the two-hybrid system were a generous gift from

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The nucleotide and amino acid sequence data reported in this paper have been deposited in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers Y08697, Y08698 and Y08699.

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Dr. Stephen J. Elledge. The human peripheral lymphocyte λACT cDNA library was converted into a plasmid cDNA (pACT) library by in vivo recombination, and the transformation of yeast strains and subsequent selection were done as described [26,36]. The cDNAs encoding human wild-type RCC1 and RCC1 alanine mutants [37], kindly provided by Drs. Takeharu Nishimoto and Yoshiaki Azuma, and the murine RanBP1/HTF9-A cDNA [22,38] were cloned into pAS1-CYH2. Human Ran/TC4 cDNA [39] was cloned into pAS1-CYH2 and pACT-II. Transformation of plasmids into yeast Y190 together with pAS1-CYH2-RCC1 or unrelated 'bait' constructs identified four positive clones, including RanBP3ΔN-217.

2.3. Plasmids for protein synthesis, production and purification of recombinant proteins

The *Bam*HI-*Xba*I DNA fragment of RanBP3-a was subcloned into pBluescript II KS+ (Stratagene GmbH, Heidelberg, Germany) and used for coupled in vitro transcription/translation experiments (TNT T3 Coupled Wheat Germ Extract kit, Promega Corporation, Madison, WI, USA). For synthesis of 6×His-tagged RanBP3 polypeptides in bacteria, cDNA fragments were cloned into the pET-14b vector (AGS, Heidelberg, Germany). For bacterial synthesis of glutathione *S*-transferase-(GST)-RanBP3-a, or for synthesis of RanBP3-a/RanBP3-b in mammalian cell lines, PCR-amplified DNA was cloned into the GST expression vector pGEX-4T-2 (Pharmacia Biotech Europe GmbH, Freiburg, Germany) or the expression vector pcDNA3 (Invitrogen), respectively. Gsp1p was a gift from Dr. Gabriel Schlenstedt. Bacterially synthesized human RCC1, Ran, RanQ69L and Rna1p were purified as described [3,40]. 6×His-tagged proteins were produced in *E. coli* BL21. Cells were lysed by ultrasonication in lysis buffer (20 mM HEPES, 0.5 mM DTT, 1 mM PMSF, pH 7.4 plus protease inhibitors Complete (Boehringer Mannheim)). The recombinant proteins were affinity-purified on Ni-NTA silica resin (Qiagen) under non-denaturing conditions. The mass of recombinant 6×His-RanBP3 was determined on a matrix-assisted laser desorption ionization (MALDI) Reflex II Mass Spectrometer (Bruker/Franzen).

2.4. Northern blot analysis

Total RNA and poly(A)$^+$ RNA isolated from HeLa cells were separated on agarose formaldehyde gels and transferred onto nitrocellulose. Human Multiple Tissue Northern (MTN) blots were from Clontech. A DNA fragment from the coding region of RanBP3-a (nt 668–1305) was labeled by digoxigenin-11-dUTP (Boehringer Mannheim) incorporation by PCR. Human β-actin cDNA was labeled with digoxigenin using the DECAprimeII kit (Ambion). Hybridization conditions were as described [41]. Digoxigenin-labeled DNA was detected by chemiluminescence (CDP-Star, Boehringer Mannheim).

2.5. Protein binding assays in vitro

GST-RanBP3-a (≈25–50 μg)-bound glutathione Sepharose beads in buffer A (20 mM HEPES, 150 mM NaCl, 1 mM $MgCl_2$, 0.5 mM DTT, pH 7.4) were incubated for 30 min at 4°C with 12 μg of RanGTP or RanGDP, or 20 μg of RCC1 in a final volume of 70 μl. Alternatively, GST-RanBP3-a bound glutathione Sepharose beads were incubated with 1 ml of HeLa soluble protein eluted from desalting columns (NAP5, Pharmacia, see below), with or without addition of 60 μg of RanQ69LGTP for 90 min at 4°C. The mixtures were centrifuged, the supernatant was recovered, beads were washed and bound proteins were eluted twice with 10 mM glutathione in buffer A in a final volume of 70 μl. All samples were boiled in SDS sample buffer. Labeling of Gsp1p and human Ran with [γ-^{32}P]GTP and nucleotide exchange assays was done as described [22].

2.6. Antibodies to RanBP3, immunofluorescence studies

Rabbits were immunized with 6×His-RanBP3-ΔN217 protein. In addition, synthetic peptides derived from the sequences 20–32 (QKDKGQKSPAEQK-C) and 100–116 (SPEGGEDSDREDGNYC) in RanBP3-a were coupled to Imject Maleimide Keyhole Limpet Hemocyanin (Pierce, Rockford, IL, USA) and also used for immunization of rabbits. Guinea pigs were immunized with peptide20–32-KLH. The resulting antibodies were affinity-purified either on 6×His-RanBP3-a protein that had been blotted onto nitrocellulose, or on peptide$_{20-32}$ or peptide$_{100-116}$ that had been coupled to SulfoLink coupling gel (Pierce). HeLa S3 and COS-7 cells were transfected with plasmid DNA by electroporation [42]. Twenty-four hours after transfection, cells were plated on coverslips and after 24 h of incubation were fixed in 2% formaldehyde in PBS at 25°C and processed for immunofluorescence microscopy [26]. Secondary antibodies were fluorescein isothiocyanate (FITC)-labeled anti-rabbit or Texas Red-labeled anti-guinea pig IgG antibodies (Dianova GmbH, Hamburg, Germany).

2.7. Immunoblotting and immunoprecipitation

Total protein extracts of mammalian cells were prepared, further separated by SDS-PAGE, transferred onto nitrocellulose and immunodetected as described [3]. For immunoprecipitation experiments, 10^7 cells were lysed for 30 min on ice in 1 ml low salt NP-40 buffer (1% NP-40, 75 mM NaCl, 50 mM Tris-HCl, pH 8.0) containing protease inhibitors. After centrifugation, 5 μg of anti-RanBP3ΔN-217 or 20 μg of anti-peptide$_{20-32}$ antibodies (the latter with or without 20 μg of synthetic peptide$_{20-32}$) were added to the supernatant and incubated for 15 h at 4°C. After addition of 20 μl of protein A beads (Boehringer Mannheim), incubations were continued for 3 h. Supernatant was removed, the beads were washed, resuspended in 50 μl of SDS sample buffer and boiled for 5 min. Ten μl of the samples were analysed by SDS-PAGE and immunoblotting.

3. Results

3.1. RCC1 interacts with RanBP1-type Ran-binding proteins in the yeast two-hybrid system

A plasmid encoding the nuclear guanine nucleotide exchange factor for Ran, pAS1-CYH2-RCC1, was transformed into the yeast strain Y190 together with a pACT-human lymphocyte cDNA library and screened for transactivation of the *LacZ* and *HIS3* reporter genes. Four specifically interacting clones were isolated. Three of them contained cDNA fragments coding for Ran-binding domains of RanBP2/Nup358 [30,31]. The fourth clone contained an insert of 2.7 kb with an incomplete ORF of 345 codons, encoding the C-terminal region of a novel protein which we termed RanBP3 (Ran-binding protein 3), a 3′-untranslated region of 1.5 kb, and a poly(A) sequence. To corroborate that the interaction of this fragment RanBP3ΔN-217, which lacks the 217 amino-terminal residues, with RCC1 is specific, we also analyzed the interaction of the full length RanBP3 (see below) with wild-type RCC1 and with a series of RCC1 mutant proteins in the two-hybrid assay. The full length RanBP3-a interacted with wild-type RCC1 as bait, but did not interact with the RCC1 mutant proteins D182A, H304A, and H410A. These mutants do not interact with Ran in the two-hybrid screen and are unable to complement tsBN2 cells containing a temperature sensitive RCC1 allele, at the restrictive temperature [37]. The lack of interaction in the two-hybrid assay was not due to instability of the mutant RCC1 fusion proteins, since expression of the hybrid proteins was detected in immunoblots (not shown). In contrast, the RCC1 mutant R206A, which does complement tsBN2 at high temperatures [37], interacted both with Ran and RanBP3 in the two-hybrid system. These results showed that binding of RanBP3 to RCC1 in the two-hybrid assay is specific and depends on the ability of RCC1 to interact with its substrate Ran.

3.2. The ranbp3 gene is transcribed ubiquitously in human tissues and codes for proteins that contain FXFG repeats and a RanBP1 homology domain

To obtain cDNAs encoding full length RanBP3, human lambda cDNA libraries were screened with DNA fragments derived from RanBP3ΔN-217. We isolated one clone with a 3.2-kb insert including the entire ORF for RanBP3-a of 563 codons and a 3′-UTR of 1.5 kb that is terminated by a

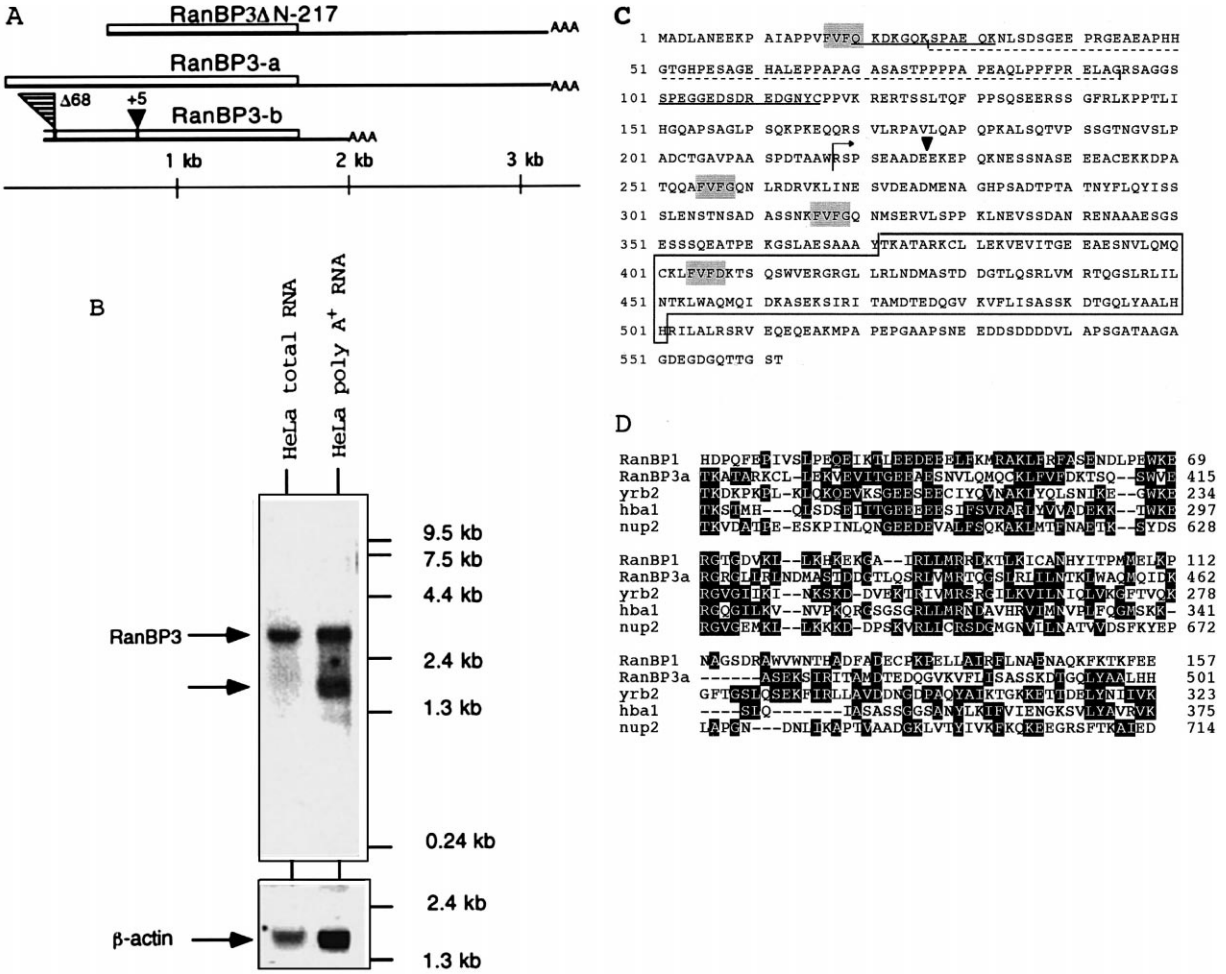

Fig. 1. RanBP3 cDNA cloning, Northern blot analysis, and sequence. A: Schematic representation of cDNA clones. Top: cDNA insert RanBP3ΔN-217 from the pACT clone isolated in the two-hybrid screen. Below: cDNA clones isolated from human lambda cDNA libraries. The complete RanBP3-a ORF is shown as a closed box. Poly(A) sequences are represented by AAA. Putative exon insertions and deletions are indicated: hatched triangle Δ68, deletion of a 204-bp DNA fragment in RanBP3-b, encoding amino acid residues 27–94 of RanBP3-a; black triangle +5, insertion of 15 bp after codon 226 in RanBP3-b. B: Northern blot hybridization using digitonin-labeled DNA fragments from the RanBP3 coding sequence to probe blots of isolated HeLa RNA. The same blot was stripped and re-hybridized with digitonin-labeled human β-actin DNA (lower panel) to control RNA loading in each lane. Molecular size markers are indicated. C: Deduced amino acid sequence of RanBP3-a. Synthetic peptides corresponding to the underlined sequence 20–32 and 101–116 were used to raise antibodies, the carboxy-terminal region RanBP3ΔN-217 is indicated by an arrow. FXFG-type sequence motifs are shown by shaded boxes, the RanBP1-homologous sequence is boxed. The sequence absent in RanBP3-b is underlined by a dashed line. A triangle denotes the position of a five residue (VCALE) insertion after residue 226 in RanBP3-b. D: Sequence alignment of RanBP1 homologous domains. Conserved residues are indicated by black boxes. The EMBL database entries are: human RanBP1 (X83617); human RanBP3a (this work, Y08697); *S. cerevisiae* yrb2 (Z38060); *S. pombe* hba1(U38783); *S. cerevisiae* nup2 (X69964).

poly(A) sequence (Fig. 1A). An in-frame stop codon precedes the first AUG codon that complies with the rule of Kozak for translational initiation sites [43].

Three additional cDNA clones were isolated that show insertions and deletions within the RanBP3-a ORF, flanked by conserved residues of exon-exon boundaries [44]; for example, the coding region of one clone encoding RanBP3-b exhibited a deletion of 68 codons and an insertion of 5 codons as compared to RanBP3-a, resulting in the deduced amino acid sequence comprising 499 amino acid residues and corresponding to a calculated mass of 53 476 Da. The 3′-UTR of this and other clones is 1.2 kb shorter than that for RanBP3-a and may therefore represent a smaller transcript resulting from alternative polyadenylation.

Northern blot analysis on poly(A)$^+$ RNA isolated from HeLa cells (Fig. 1B) and 16 different human tissues (not shown) using labeled RanBP3 cDNA fragments revealed transcripts of approximately 1.8 and 3 kb in all samples, corresponding in size to the isolated cDNA clones for RanBP3-a and RanBP3-b. Therefore, the *ranbp3* gene is ubiquitously transcribed. The signal intensities were particularly strong in testis and heart.

The deduced amino acid sequence of RanBP3-a consists of 562 residues with a calculated mass of 59 643 Da (Fig. 1C). It contains a high percentage of charged residues with a calculated p*I* of 4.5. The protein is rich in serine/threonine residues (16.9%) and the amino-terminal region is characterized by a high content of proline residues. Two FXFG and two FXFX type repeat motifs, previously described for a subclass of NPC proteins [32], are present in the sequence. The C-terminal region shows similarity to the Ran-binding domains of RanBP1 homologues (Fig. 1D). The highest degree of se-

quence identity (34%) within this region was found to the RanBP1-related region of the fission yeast hba1p protein [34]. The overall similarity between these proteins is 47%, with 26% identical residues. The RanBP1 homology domain of RanBP3-a/b also shows 32% sequence identity to the Ran-binding domains of Yrb2p [33] and 20% to that of the nucleoporin Nup2p from baker's yeast [45]. These proteins are clearly divergent from other known RanBP1-homologous proteins that exhibit sequence identities of 38–98% between each other in the homologous regions.

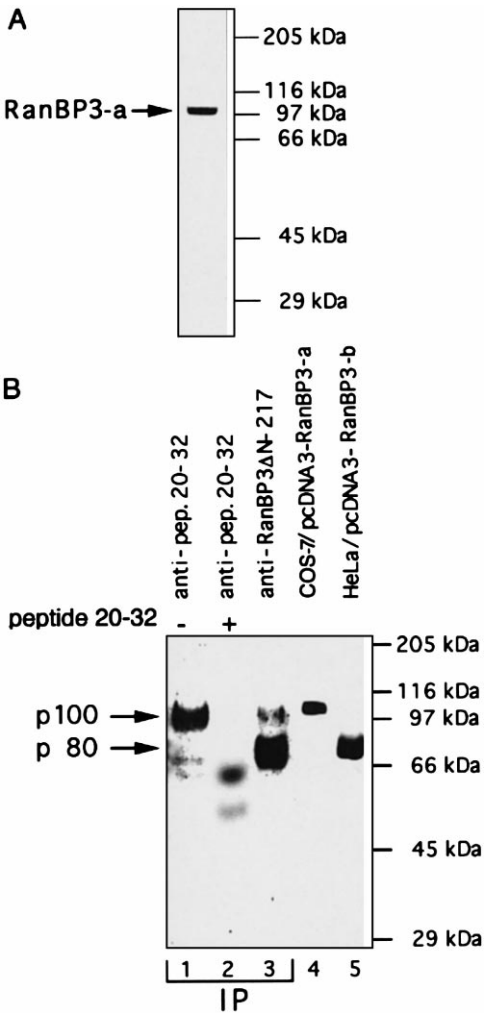

Fig. 2. RanBP3-a and -b encoded proteins. A: Coupled in vitro transcription and translation of RanBP3-a. B: Proteins with apparent molecular masses of 80 kDa and 100 kDa are immunoprecipitated using rabbit anti-RanBP3 antibodies. Lanes 1–3, IP: proteins immunoprecipitated from COS-7 cell total lysates. Rabbit RanBP3 antibodies raised against synthetic peptide$_{20-32}$ (lanes 1 and 2) precipitate a 100-kDa protein in the absence (−) but not in the presence (+) of synthetic peptide$_{20-32}$. Lane 3: rabbit RanBP3ΔN-217 antibodies precipitate proteins of 100 kDa and 80 kDa. The precipitated proteins were immunodetected using guinea pig RanBP3 antibodies. Lanes 4 and 5: immunodetection of overexpressed RanBP3 polypeptides using rabbit antibodies recognizing peptide$_{100-116}$ on total protein extracts from COS-7 (lane 4) and HeLa (lane 5) cells that had been transfected with expression vectors encoding RanBP3-a (lane 4) and the splice variant RanBP3-b (lane 5).

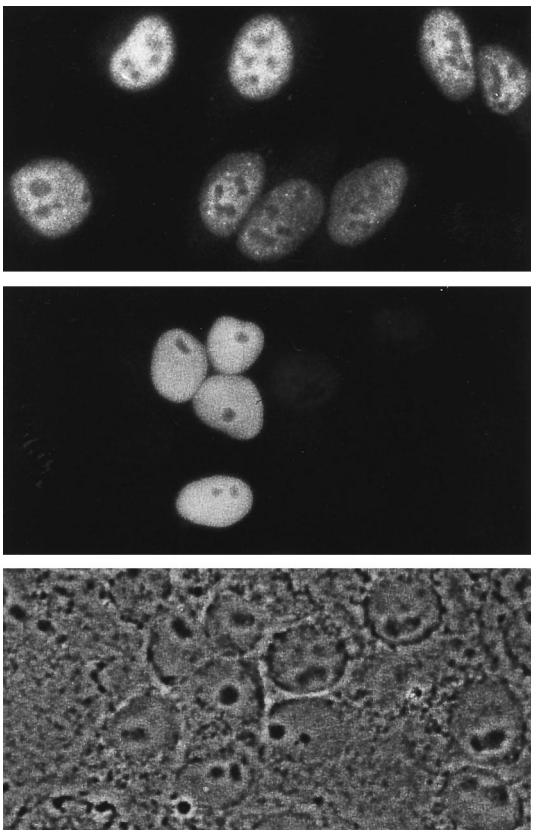

Fig. 3. RanBP3 is localized to the nucleus. Top: Immunofluorescence staining of HeLa cells using rabbit antibodies raised against RanBP3ΔN-217. Center: Staining using rabbit peptide$_{20-32}$ antibodies on African green monkey COS-7 cells transfected with an expression vector encoding RanBP3-b. Although the antibody also reacted with the monkey homologue of RanBP3, only transfected cells containing increased amounts of human RanBP3 are seen due to shortened film exposure. Bottom: Corresponding phase contrast micrograph.

3.3. *RanBP3 proteins are synthesized in low amounts and occur in the nucleus*

In vitro transcription/translation of full length RanBP3-a yielded one major translation product with an apparent molecular mass of 100 kDa as determined by SDS-PAGE, indicating that the 5′-UTR confers successful translational initiation (Fig. 2A). The bacterially synthesized 6×His-RanBP3-a showed the same mobility on SDS-PAGE, whereas 6×His-RanBP3-b migrated at a position corresponding to 80 kDa. Mass spectrometric analysis of 6×His-RanBP3-a revealed a molecular mass of 62 kDa, in agreement with the calculated mass of the 6×His-tagged protein.

We raised rabbit polyclonal antibodies to the carboxy-terminal region (RanBP3ΔN-217), and to synthetic peptides (peptide$_{20-32}$ and peptide$_{100-116}$) deduced from the amino-terminal region (Fig. 1C). All affinity-purified RanBP3 antibodies recognized the bacterially synthesized 6×His-RanBP3-a in immunoblots. Antibodies raised against peptide$_{20-32}$ recognized RanBP3-b with reduced affinity (Fig. 2B), since the epitope recognized by peptide$_{20-32}$ antibodies is in part deleted in the smaller RanBP3-b protein (Fig. 1A and C).

Immunoprecipitation experiments using HeLa, BHK, and COS-7 total cell lysates showed that a 100-kDa protein was specifically immunoprecipitated using the peptide$_{20-32}$ anti-

bodies, whereas the 80 kDa protein was not. Addition of the synthetic peptide$_{20-32}$ abolished immunoprecipitation of the 100-kDa protein (Fig. 2B, lanes 1 and 2). In contrast, antibodies raised against the C-terminal region of the RanBP3 proteins immunoprecipitated both a 100-kDa protein and several polypeptides of approximately 80 kDa (Fig. 2B, lane 3). A similar pattern of immunoreactive proteins was observed in total protein extracts of mammalian cells that had been transfected with RanBP3-a or RanBP3-b encoding expression vectors (Fig. 2B, lanes 4 and 5). We could not detect immunoreactive bands on nitrocellulose blots using total lysate from HeLa or COS-7 cells. Yet, separation of soluble cell protein by ion exchange chromatography yielded fractions in which two proteins of 80 and 100 kDa were immunolabeled (not shown). Using bacterially synthesized purified RanBP3-b as a reference for comparative immunoblotting, we estimated that less than 1×10^5 molecules of the 80-kDa and 1×10^4 molecules of the 100-kDa protein are present per cell.

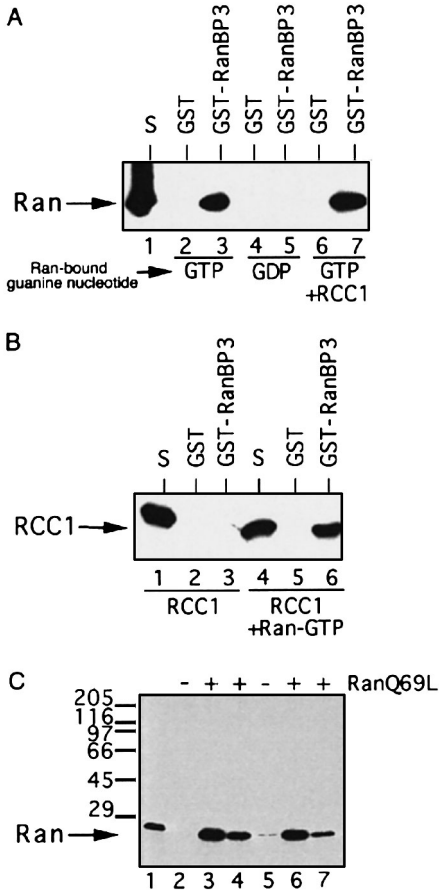

Fig. 4. RanBP3-a specifically binds human RanGTP and forms a Ran-dependent complex with RCC1 in vitro. A: Immunoblot detection of Ran using rabbit anti-Ran antibodies. GST and GST-RanBP3-a were immobilized on glutathione Sepharose beads and incubated with purified human Ran and RCC1. Proteins bound to the beads were eluted with glutathione. S, supernatant after incubation (lane 1); proteins eluted from beads (lanes 2–7). GST or GST-RanBP3-a-bound beads were incubated with RanGTP (lanes 1–3), RanGDP (lanes 4, 5) and RanGTP plus RCC1 (lanes 6, 7). B: Immunoblot detection of RCC1 using rabbit anti-RCC1 antibodies. S, RCC1 in the supernatant (lanes 1, 4); proteins eluted from beads (lanes 2, 3, 5, 6). Incubations were with RCC1 (lanes 1–3) and RCC1 plus RanGTP (lanes 4–6). C: Binding of recombinant RanQ69L (GTP-bound form), the RanGAP1-resistant mutant form of Ran, from HeLa cell lysate to immobilized GST-RanBP3-b. Samples are: HeLa soluble lysate (lane 1), and proteins eluted from beads with buffer containing 20 mM glutathione (lanes 2–7). About 5 μg GST-RanBP3-b (lanes 2–4) or the importin β related GST-RanBP5 [26] (lanes 5–7) were preincubated with 1-ml aliquots of HeLa lysate (lanes 2 and 5), or with HeLa lysate containing 2.5 μM of recombinant RanQ69L (lane 3, 4, 6, 7). Ran is immunodetected using rabbit anti-Ran antibodies.

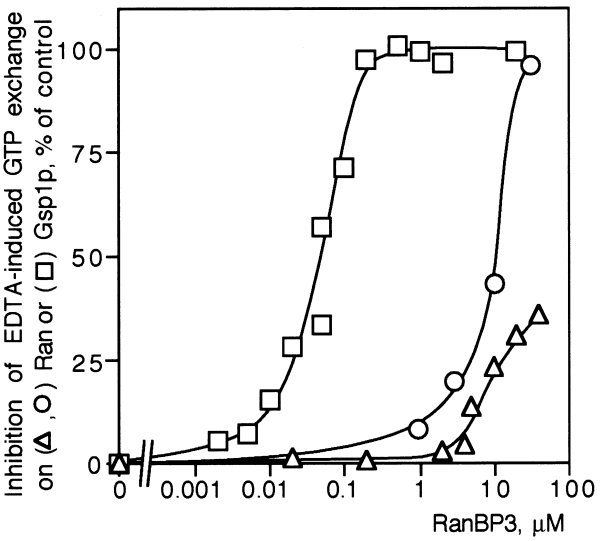

Fig. 5. RanBP3-a and -b proteins inhibit EDTA-induced guanine nucleotide exchange on Ran. Yeast [γ-^{32}P]GTP-Gsp1p (□) and human [γ-^{32}P]GTP-Ran (△,○) were preincubated for 30 min with various concentrations of RanBP3-a (□,△) or RanBP3-b (○). Protein-bound radioactivity was determined 5 min after starting the exchange reaction by addition of EDTA and GDP.

In indirect immunofluorescence studies with different RanBP3 antibodies HeLa, COS-7 and hamster BHK cells gave a diffuse intranuclear staining that excluded nucleoli in interphase cells. The top panel in Fig. 3 shows the staining of HeLa cells with rabbit antiserum to RanBP3ΔN-217. The same pattern was obtained with rabbit peptide$_{20-32}$ antibodies. Likewise, overexpressed RanBP3-a (Fig. 3, center and bottom), RanBP3-b or myc-epitope tagged RanBP3-b in transfected COS-7 cells was exclusively located in the nucleus and excluded from nucleoli. In mitosis, the antigen was dispersed throughout the cell and was not associated with chromatin (not shown).

3.4. RanBP3 proteins preferentially bind RanGTP and form a Ran-dependent trimeric complex with RCC1 in vitro

To analyze the interactions of RanBP3-a and -b with Ran and RCC1, the RanBP3 proteins were expressed as glutathione *S*-transferase (GST) or 6×His-tagged fusion proteins and immobilized on glutathione Sepharose or NiNTA agarose beads. In the example shown in Fig. 4A, GST-RanBP3-b or GST beads were incubated with purified human RanGTP, RanGDP and RCC1. Proteins bound to the beads were analyzed by SDS-PAGE. RanGTP, but not RanGDP binds to GST-RanBP3-b (compare lanes 3 and 5). RCC1 alone does not bind (Fig. 4B, lane 3). Yet in the presence of RCC1 and RanGTP (Fig. 4B, lane 6) or RanGDP (not shown), a trimeric complex of RanBP3-b·Ran·RCC1 is formed. This complex, similar to the RanBP1·Ran·RCC1 complex [22], is likely to exist in a nucleotide free state, as it is also formed in the presence of EDTA. Ran or RCC1 alone or in combination do not bind to immobilized GST.

Next, we analyzed whether RanBP3 could bind Ran in the presence of HeLa lysates. Here, the majority of Ran is converted to the GDP-bound form by the action of RanGAP1. We therefore added GTP-bound RanQ69L, which is resistant to RanGAP1 induced GTP hydrolysis, to HeLa lysate. The final concentration of RanQ69L in lysate aliquots was 2.5 μM, about half of the endogenous Ran concentration. Under these conditions, Ran was efficiently bound to both GST-RanBP3-b (Fig. 4C, lanes 3 and 4) and 6×His-RanBP3-b beads (not shown). Comparable amounts of RanQ69L were bound to the importin β-related RanGTP-binding protein GST-RanBP5 [26], that, with a binding constant of 3 nM, represents an import factor with high affinity for RanGTP and was used as a control in this experiment (lanes 6 and 7). Without the addition of RanQ69L, a barely detectable amount of endogenous Ran was bound to GST-RanBP3-b and GST-RanBP5 (lane2 and 5), reflecting the low concentration of RanGTP in the lysate.

To estimate the affinity of RanBP3 proteins for RanGTP, we measured the ability of soluble RanBP3 proteins to inhibit guanine nucleotide exchange on human RanGTP (Fig. 5), in analogy to RanBP1 [22]. This exchange was induced by addition of EDTA which complexes Mg^{2+} ions required for tight nucleotide binding. High concentrations of RanBP3 proteins were required to obtain inhibition, with apparent half maximal binding at 10 μM RanBP3-a, and even higher concentrations for RanBP3-b, as compared to RanBP1 with half maximal inhibition at 0.1 nM [12]. Interestingly, the nucleoporin Nup2p and the nuclear Ran-binding protein Yrb2p from yeast also show binding constants in the lower μM range (G. Schlenstedt and F.R.B., unpublished). Curiously, the Ran homologue Gsp1p from baker's yeast strongly binds to RanBP3s, showing a half maximal inhibition of EDTA-induced exchange at 40 nM with RanBP3-b (Fig. 5). Inhibition of RCC1-induced nucleotide exchange is significant only at unphysiologically high concentrations of RanBP3 (not shown). Thus, stabilization of bound RanGTP against RCC1-induced nucleotide exchange is probably not an in vivo function of RanBP3. In analogy to RanBP1, stimulation of RanGAP-induced GTPase activity on Ran was not inhibited (not shown).

4. Discussion

We report here the identification of novel human Ran-binding proteins, RanBP3-a and -b, via interaction with RCC1 in the yeast two-hybrid system. Ran-mediated interaction of RCC1 in the two-hybrid system has been reported previously for RanBP1 and RanBP2 [30,33]. In vitro, these proteins bind to the nucleotide-free RCC1·Ran complex, thereby blocking RCC1-induced nucleotide exchange activity on Ran [22]. The interaction of RanBP3 with RCC1 in the two-hybrid assay most likely is also due to formation of a trimeric complex involving the yeast Ran homologue Gsp1p, since in the two-hybrid screen RanBP3 interacts specifically with RCC1, but not with a series of RCC1 mutants that have lost the ability to interact with Ran. Furthermore, in in vitro binding assays RanBP3 did not bind directly to RCC1, but a trimeric RCC1·Ran·RanBP3 complex was formed in the presence of Ran. Yeast Gsp1p also shows high affinity binding to RanBP3 (Fig. 5).

Evidence for the synthesis of different RanBP3 proteins in mammalian cells comes from cDNA cloning, revealing the presence of major alternatively spliced mRNAs, and from the size of the encoded proteins corresponding to that of cellular proteins detected by immunoprecipitation (Fig. 2B).

Immunolocalization showed that the RanBP3 proteins are dispersed through the nucleoplasm. This resembles the location of yeast Yrb2p (327 residues) in *S. cerevisiae* [33], and of hba1p (399 residues) in fission yeast [34]. These two proteins are smaller than RanBP3-a/b (562 and 499 residues) but are grouped by sequence similarity with RanBP3 and Nup2p in a distinct subfamily of the RanBP1-related proteins. They also differ from the other RanBP1-type proteins by their nuclear or nuclear pore complex associated location and by their relatively low affinity for RanGTP or the respective GTP-bound Ran homologues ([34], F.R.B. and G. Schlenstedt, unpublished results).

Yrb2p was found to interact with Gsp1pGTP in vitro and in the two hybrid assay [33]. It is not essential for growth, but a *yrb2* null mutant shows synthetic lethality when combined with a mutation in the yeast RanGAP1 homologue, *rna1-1*, suggesting that both proteins share an essential function in the regulation of the Ran GTPase pathway. Since deletion of the *yrb2* gene does not affect import of NLS protein substrates or mRNA export [46], Yrb2p is unlikely to participate in these two transport pathways.

Instead, the low binding affinities of this group of nuclear RanBP1-related proteins may be a prerequisite for an intranuclear function. In the absence of the exclusively cytoplasmic RanGAP1, a high affinity RanBP·RanGTP complex in the nucleus probably would be unable to dissociate. Indeed, microinjection of RanBP1 into nuclei of *Xenopus* oocytes leads to a block in transport through NPCs [9]. A high concentration of RanGTP in the nucleus (>10 μM) ensures that complexes with low affinity nuclear Ran-binding proteins can be formed and dissociated reversibly. Analysis of interacting proteins and of RanBP3 mutants will help to determine the cellular function for this new group of nuclear RanBP1 related proteins.

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