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Andreas Braunwarth‡, Micheline Fromont-Racine§, Pierre Legrain¶, F. Ralf Bischoff‖, Thomas Gerstberger‡, Ed Hurt‡, and Markus Künzler‡‡**

*From ‡Biochemie-Zentrum Heidelberg, Ruprecht-Karls-Universität, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany, §Departement des Biotechnologies, Institut Pasteur, 25,28 rue du Docteur Roux, 75724 Paris CEDEX 15, France, ¶Hybrigenics SA, 3–5 impasse Reille, 75014 Paris, France, ‖Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany, and **Mikrobiologisches Institut, Eidgenössische Technische Hochschule, Schmelzbergstrasse 7, 8092 Zürich, Switzerland*

The small Ras-like GTPase Ran plays an essential role in the transport of macromolecules in and out of the nucleus and has been implicated in spindle (1, 2) and nuclear envelope formation (3, 4) during mitosis in higher eukaryotes. We identified *Saccharomyces cerevisiae* open reading frame YGL164c encoding a novel RanGTP-binding protein, termed Yrb30p. The protein competes with yeast RanBP1 (Yrb1p) for binding to the GTP-bound form of yeast Ran (Gsp1p) and is, like Yrb1p, able to form trimeric complexes with RanGTP and some of the karyopherins. In contrast to Yrb1p, Yrb30p does not coactivate but inhibits RanGAP1(Rna1p)-mediated GTP hydrolysis on Ran, like the karyopherins. At steady state, Yrb30p localizes exclusively to the cytoplasm, but the presence of a functional nuclear export signal and the localization of truncated forms of Yrb30p suggest that the protein shuttles between nucleus and cytoplasm and is exported via two alternative pathways, dependent on the nuclear export receptor Xpo1p/Crm1p and on RanGTP binding. Whereas overproduction of the full-length protein and complete deletion of the open reading frame reveal no obvious phenotype, overproduction of C-terminally truncated forms of the protein inhibits yeast vegetative growth. Based on these results and the exclusive conservation of the protein in the fungal kingdom, we hypothesize that Yrb30p represents a novel modulator of the Ran GTPase switch related to fungal lifestyle.

GTPases of the Ras superfamily act as molecular switches in a number of cellular processes (5). The two states of the switch are the GDP- and the GTP-bound forms of the GTPase where the GTP-bound state is the "on"-state based on its interaction with downstream effectors or target proteins. These target proteins represent the molecular links between a given GTPase and the regulated cellular process.

The Ras-like GTPase Ran (Gsp1p in *Saccharomyces cerevisiae*) is an abundant, soluble protein shuttling between the cytoplasm and the nucleoplasm with a predominant localization in the nucleoplasm at steady state. Typical for all members of this superfamily, Ran has low intrinsic GTP hydrolysis and guanine-nucleotide exchange activities, which are activated by a specific cytoplasmic GTPase-activating protein (GAP)[1] (RanGAP1/Rna1p) and a nuclear guanine-nucleotide exchange factor (RanGEF) (RCC1/Prp20p), respectively. Besides RanGAP1 and RanGEF, several Ran-binding proteins have been identified (6). Binding of most of these proteins is restricted to either the GDP- or the GTP-bound state of the GTPase. The major classes of RanGTP-binding proteins are the RanBP1 homologous proteins, which act as coactivators of RanGAP1-mediated GTP hydrolysis on Ran, and the family of nuclear transport receptors or karyopherins. Binding of RanGTP to latter class of proteins is the basis of the essential role of Ran in nucleocytoplasmic transport (6–8). Ran has also been implicated in spindle and nuclear envelope formation in higher eukaryotes, and recent reports (1, 2, 9) suggest that karyopherins are also the target proteins in these two processes.

Here we report the results of a two-hybrid screen with the aim to identify novel Ran-binding proteins in the yeast *S. cerevisiae* and the characterization of a yeast ORF, YGL164c, which was found to encode a novel RanGTP-binding protein.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—The yeast strains used in this work are listed in Table I. Chromosomal tagging of *YRB30* with ProtA or GFP was performed in the RS453 strain according to Longtine *et al.* (10) and confirmed by PCR and immunoblot analysis.

Transformation of yeast cells with DNA was performed using a modified version of the lithium acetate method (11). Unless indicated otherwise, yeast cells were cultivated at 30 °C. Preparation of standard yeast media was described previously (12). Induction with galactose was performed by adding an equal volume of YPGal to cells grown in selective medium containing 2% (w/v) raffinose as sole carbon source (SRC). Leptomycin B treatment of CRM1T539C cultures was done as described (13). Growth of yeast and *Escherichia coli*, plasmid recovery, mating, and tetrad analysis were done as described previously (14).

Plasmid Constructions—Standard techniques were used for the manipulation of recombinant DNA (15). PCR amplifications were performed using standard conditions (16) and Vent DNA polymerase (New England Biolabs, Beverly, MA). Fusions between wt and mutant *GSP1* and the DNA-binding domain of *E. coli* LexA protein were constructed by inserting PCR-generated *Stu*I-*Pst*I *GSP1* fragments into the *Sma*I-*Pst*I sites of the two-hybrid vector pBTM116 (17).

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‡‡ To whom correspondence should be addressed: Mikrobiologisches Institut, Eidgenössische Technische Hochschule (ETH), ETH-Zentrum LFV E23, Schmelzbergstr. 7, CH-8092 Zürich, Switzerland. Tel.: 41-1-632-4925; Fax: 41-1-632-1148; E-mail: markus.kuenzler@micro.biol.ethz.ch.

[1] The abbreviations used are: GAP, GTPase-activating protein; GEF, guanine-nucleotide exchange factor; GST, glutathione *S*-transferase; NES, nuclear export signal; ORF, open reading frame; ProtA, protein A; RBD, RanGTP-binding domain; wt, wild-type; GFP, green fluorescent protein; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; TEV, tobacco etch virus protease cleavage site; NLS, nuclear localization signal; TB, thrombin cleavage site.

TABLE I
List of yeast strains used

Yeast strains	Characteristics	Source
L40Δgal4	*MAT***a** *his3-delta200 trp1–901 leu2–3,112 ade2 lys2–801 am gal4::KanMX6 LYS2*::(*lexAop*)*4-HIS3 URA3*::(*lexAop*)*8-lacZ*	Ref. 38
Y187	*MATα ura3–52 his3–200 ade2–101 trp1–901 leu2–3,112 met-gal4Δ gal80Δ URA3*::$GAL1_{UAS}$-$GAL1_{TATA}$-*lacZ*	Ref. 38
W303–1A	*MAT***a** *ura3–1 trp1–1 his3–11,15 leu2–3,112 ade2–1 can1–100 GAL*	R. S. Fuller
W303–1A rna1–1	*MAT***a** *ura3–1 trp1–1 his3–11,15 leu2–3,112 ade2–1 can1–100 GAL rna1–1*	M. Künzler
YAB20	*MAT***a** *ura3–1 trp1–1 his3–11,15 leu2–3,112 ade2–1 can1–100 GAL yrb30*::*HIS3*	This study
YAB21	*MATα ura3–1 trp1–1 his3–11,15 leu2–3,112 ade2–1 can1–100 GAL yrb30*::*HIS3*	This study
YAB22	*MAT***a**/*α ura3–1*/*ura3–1 trp1–1*/*trp1–1 his3–11,15*/*his3–11,15 leu2–3,112*/*leu2–3,112 ade2–1*/*ade2–1 can1–100*/*can1–100 GAL*/*GAL yrb30*::*HIS3*/*yrb30*::*HIS3*	This study
CRM1T539C	*MAT***a** *ura3–1 trp1–1 his3–11,15 leu2–3,112 ade2–1 can1–100 GAL xpo1*::*KAN pRS315-CRM1T539C*	Ref. 13
RS453	*MAT***a** *ura3–52 trp1–1 leu2–3 ade2–1 his3–11,15 can1–100*	Ref. 14
RS453 YRB30-ProtA	*MAT***a** *ura3–52 trp1–1 leu2–3 ade2–1 his3–11,15 can1–100 YRB30-ProtA*::*TRP1*	This study
RS453 YRB30-GFP(S65T)	*MAT***a** *ura3–52 trp1–1 leu2–3 ade2–1 his3–11,15 can1–100 YRB30-GFP*::*HIS3*	This study
YAB15	W303–1A *msn5*::*TRP1*	This study
AJH54	*MAT***a** *cse1*::*LEU2 ade2–101 trp1-delta901 ura3–52 leu2–3,112 his3–11,15 pRS314-cse1–2*	Ref. 39
Y0546	RS453 *los1*::*HIS3*	K. Hellmuth
Y1717	W303–1A *xpo1*::*KAN pRS313-xpo1–1*	F. Stutz/M. Künzler
NMD3 shuffle	*MAT***a** *his3 leu2 lys2 ura3 TRP1 nmd3*::*KanMX4* (*ARS/CEN URA3 NMD3*)	Ref. 33

For pNOPPATA1L-*YRB30* and pGALPATG1L-*YRB30* the whole ORF of *YRB30* was amplified from genomic DNA as a *Nco*I-*Bam*HI fragment and inserted into the corresponding sites of pNOPPATA1L (pUN100-*NOP1p*::ProtA-TEV::*ADH1t*) (17) or pGALPATG1L (pUN100-*GAL1p*::ProtA-TEV::*GAL4t*) (17), respectively.

The *GFP-YRB30* fusion gene was generated by subcloning the ORF of *YRB30* as a 1.4-kb *Pst*I fragment from pNOPPATA1L-*YRB30* into pNOPGFP2L (pRS425-NOP1p::GFP(S65T)) (17). Recloning of the *NOP1p*::GFP(S65T)::*YRB30*::*GAL4t* fusion gene as a *Sac*I-*Kpn*I fragment from pNOPGFP2L-*YRB30* into the corresponding sites of pRS424 or pRS426 yielded analogous plasmids with *TRP1* or *URA3* marker genes, respectively.

pET9d-GST-TEV-*YRB30* was generated by replacing a *Nco*I-*Sac*I fragment containing the *MAD2* gene from pET9d-GST-TEV-*MAD2* (G. Stier, European Molecular Biology Laboratory (EMBL) Heidelberg, Germany) with the corresponding fragment from pGALPATG1L-*YRB30*. For expression of a His_6-tagged fusion protein in *E. coli* the entire *YRB30* ORF was ligated as a *Nco*I-*Bam*HI fragment (see above) into pET9d-His_6-TB adjacent to the Thrombin cleavage site (G. Stier, EMBL). The various plasmids containing deleted or mutated *YRB30* genes were constructed in an analogous way.

Fusions between the bipartite NLS of ribosomal protein L25 under control of its own promoter and *GFP-YRB30* were constructed by inserting *YRB30* as *Pst*I fragment from pNOPGFP2L-*YRB30* into pRS314-L25NLS-GFP-*ARC1*[2] between GFP and the coding sequence of *ARC1*. The various plasmids containing deleted or mutated *YRB30* genes were constructed by PCR in an analogous way.

For assessing the functionality of the C-terminal NES in the *YRB30* ORF various PCR-generated *Bam*HI fragments of *YRB30* were introduced into the *Bcl*I site of pRS315-NMD3(ΔNES1+2)-eGFP, which will be described elsewhere.[3] To check the correct orientation of the insert a *Nco*I site was introduced immediately upstream of the 3′-*Bam*HI site.

For overexpression studies, *YRB30* was expressed under control of the galactose-inducible *GAL1*-promoter from high copy number vector pRS426GAL1 (18). For this purpose, full-length and truncated *YRB30* ORFs were cloned as PCR-generated *Spe*I-*Xho*I fragments. Expression of all proteins was verified by Western blot analysis using the anti-Yrb30p antiserum or commercially available anti-ProtA, anti-GFP, anti-His_6, or anti-GST antibodies.

Plasmids pNOPPATA1L-*GSP1wt* and pNOPPATA1L-*GSP1(G21V)* were described previously (19). pRS315-*NMD3*-eGFP and pRS315-*NMD3*(ΔNES1/2)-eGFP were kindly provided by T. Gerstberger. Plasmids for expression of GST, His_6-Gsp1wt, His_6-Yrb1, His_6-Rna1, GST-Yrb1, His_6-Xpo1, and GST-Kap95 were described previously (20, 21). *E. coli* expression plasmids for His_6-Gsp1wt (T. Gerstberger) and His_6-Gsp1ΔDE (this study) were derived from pTrcHisA-*GSP1(G21V)* (22) by replacing the *Nhe*I-*Hin*dIII *GSP1(G21V)* fragment by analogous PCR-generated fragments coding for Gsp1wt and Gsp1p (1–212). A plasmid for expression of His_6-Kap95p in *E. coli* was constructed by inserting the *KAP95* ORF as *Bam*HI fragment from pGEX4T3-KAP95 (1–861) (20) into the *Bam*HI site of pPROEX1.

Disruption of YRB30—The *YRB30* gene was disrupted by replacing the entire open reading frame of *YRB30* with a PCR-generated *HIS3*-cassette in the diploid strain W303-D. The disruption was verified by PCR, and haploid *yrb30*::*HIS3* strains were obtained by sporulation and tetrad dissection of the heterozygous diploid strain. Mating of haploid disruption strains of opposite mating type led to homozygous diploid disruption strains.

Preparation of Rabbit Polyclonal Anti-Yrb30p Antiserum—A rabbit polyclonal antiserum against Yrb30p was raised against His_6-Yrb30p fusion protein expressed in BL21(DE3) cells (23) and affinity purified over Talon beads (Clontech, Palo Alto, CA). Immunization of two rabbits was performed by a commercial antibody service (J. Pineda, Berlin, Germany). For detection of Yrb30p in immunoblots, the resulting antisera were used at a dilution of 1:4000.

Two-hybrid Screen—The two-hybrid screen was performed as described previously (24) but using L40Δgal4 cells for transformation of the bait-plasmids.

In Vitro Protein Interaction/Pull-down Assays—*In vitro* interaction between recombinant proteins was assayed as described previously (25).

For the pull-down assay from yeast extract a culture from wt strain W303–1A was grown at 30 °C to an A_{600} of 1.5. Cells were harvested by centrifugation and lysed using a Fritsch Pulverisette 6 (Fritsch GmbH, Idar-Oberstein, Germany). Endogenous glutathione in the supernatant was removed by gel filtration using G25-fine-Sepharose (Amersham Biosciences). Loading of extract with GTPγS was done according to loading of Gsp1p with GTP (25). Purified GST-Yrb30p (~15 μg) was rebound to 50 μl of GSH-Sepharose in universal buffer (25) containing 5 mM β-mercaptoethanol and one tablet of Complete EDTA-free protease inhibitor mix (Roche Diagnostics) per 50 ml, washed, and incubated with ~800 μg of protein of the corresponding supernatant. After washing the bound proteins were eluted in sample buffer and analyzed with SDS-PAGE and Coomassie Blue staining, as well as mass spectrometry.

Purification of ProtA-TEV-Yrb30p from Yeast—Affinity purification of ProtA fusion proteins was performed essentially as described (26).

Quantification of RCC1-induced GTP Exchange and RanGAP-induced GTP Hydrolysis on Gsp1pGTP—Assays were described previously (27).

Release Assay to Determine Inhibition of RanGAP-induced GTP Hydrolysis—GST-Yrb30p or GST-Yrb1p as a control were incubated in universal buffer (25) with Gsp1pGTP. After washing, the complexes were incubated for 12 min at 4 °C with His_6-Rna1p or buffer alone. The released proteins were collected. After washing the bound fraction was eluted by boiling with sample buffer. Human Ran loaded with GTP and Rna1p from *Schizosaccharomyces pombe* were kindly provided by R. Bischoff (Deutsches Krebsforschungszentrum, Heidelberg, Germany).

Fluorescence Microscopy—Classical and confocal fluorescence microscopy of living cells expressing GFP(S65T) fusion proteins was done as described previously (28).

Miscellaneous—SDS-PAGE, immunoblot, and preparation of yeast cell extracts were performed according to standard protocols.

[2] K. Galani and E. Hurt, unpublished data.
[3] T. Gerstberger and E. Hurt, manuscript in preparation.

TABLE II
List of identified two-hybrid preys

Bait	Prey	Number of clones (number of independent fusions)	Function
Gsp1 wt	KAP123 (YRB4)[a]	1	Karyopherin (importin)
Gsp1 wt	KAP109 (CSE1)[a]	1	Karyopherin (exportin)
Gsp1 wt	NUP42 (RIP1)[b]	29 (6)	Nucleoporin
Gsp1 wt	NUP159[b]	6 (3)	Nucleoporin
Gsp1 wt	SLZ1[c]	19 (7)	Meiosis
Gsp1 wt	SHE1 (YBL031W)[c]	2 (1)	Unknown
Gsp1 wt	SEC3	1	Cell polarity
Gsp1 wt	FRS2	4 (1)	Protein synthesis
Gsp1 wt	YER139C[c]	1	Unknown
Gsp1 wt	YEL043W[c]	2 (1)	Unknown
Gsp1 T26N	MOG1[a]	5 (3)	Multicopy suppressor of gsp/ts mutations
Gsp1 T26N	YPL009C[c]	3 (1)	Unknown
Gsp1 T26N	YBR225W[c]	1	Unknown
Gsp1 G21V	YRB1[a]	10 (3)	RanBP1
Gsp1 G21V	YGL164C (YRB30)[c]	3 (1)	Unknown

[a] Already known Yrb.
[b] Proteins that bind to yeast Ran probably via three-hybrid interactions involving karyopherins.
[c] ORFs selected for further analysis.

RESULTS

Two-hybrid Screen for Novel Yrb (Yeast Ran Binders)—To identify novel yeast Ran-binding proteins a two-hybrid screen was performed using both wt (40 million of interactions tested) and two dominant mutant forms (G21V and T26N; 29 million or 46 million of interactions tested, respectively) of yeast Ran, Gsp1p. These mutant forms are locked in the GTP-bound and the GDP-bound state, respectively (29). Among the preys identified, we pulled out a number of already known Ran-binding proteins, such as members of the karyopherin family, the yeast homolog of RanBP1 (Yrb1p), and Mog1p (Table II). In addition, we identified a number of candidate novel Ran-binding proteins (Yrbs), most of them in the screen with the wt form of Gsp1p. Comparably few preys were identified with the mutant forms of Gsp1p.

Yrb30p Interacts with Gsp1p in Vivo—As a first approach to confirm a physical interaction between these candidate Ran-binding proteins and Gsp1p, we fused selected ORFs (see Table II) to a TEV-cleavable ProtA moiety and expressed the fusion proteins under control of the *NOP1* or the *GAL1* promoter. After affinity purification over IgG-Sepharose we probed the column loads and the TEV eluates with a specific antiserum against Gsp1p. In none of the cases we could detect an enrichment of Gsp1p in the eluate (data not shown).

The fact that one of the ORFs, YGL164C (*YRB30*), was found as the only prey besides Yrb1p in the two-hybrid screen with the G21V mutant, suggested that the protein bound specifically to the GTP-bound form of Gsp1p, which may be underrepresented in our cell lysate because of RanGAP1(Rna1p)-mediated GTP hydrolysis. We hypothesized that a putative Yrb30p-Gsp1pGTP complex would be stabilized in the *rna1–1* mutant strain, in which GTP hydrolysis is inhibited. We therefore expressed and affinity-purified the ProtA-tagged Yrb30p driven from a galactose-inducible *GAL1* promoter from both wild-type and *rna1–1* mutant yeast cells. As predicted, we could enrich Gsp1p using ProtA-Yrb30p when purified from the *rna1–1* background (Fig. 1). In contrast, no Gsp1p could be copurified with ProtA-Yrb30p from the wt strain or with ProtA alone. A similar result was obtained if Yrb30p was expressed from the constitutive *NOP1* promoter (data not shown).

In an inverse approach, we coexpressed GFP-tagged Yrb30p with ProtA-tagged Gsp1p in its wt form or in its G21V mutant form, respectively, under control of the *NOP1* promoter in wt cells. Proteins were purified over ProtA-Sepharose and eluted by TEV cleavage. The eluates were analyzed by immunoblotting using a GFP antiserum. In agreement with the two-hybrid data we detected GFP-Yrb30p coeluting with ProtA-Gsp1(G21V) but not with the wt form of Gsp1p (Fig. 1). We conclude that Yrb30, initially found in a two-hybrid screen with Gsp1(G21V), interacts specifically with the GTP-bound form of Gsp1p *in vivo*.

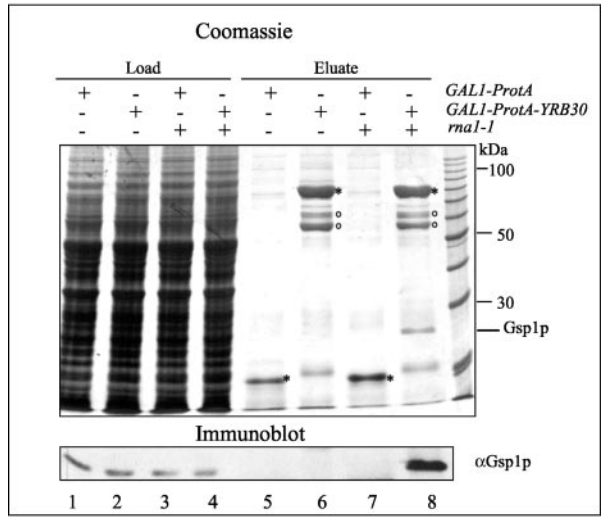


FIG. 1. **Gsp1p and Yrb30p interact *in vivo*.** *yrb30*::*HIS3* (*rna1–1*; −) and *yrb30*::*HIS3 rna1–1* (*rna1–1*; +) cells containing a plasmid expressing ProtA-Yrb30p or ProtA alone under control of the *GAL1* promoter were grown in SRC-Leu at 23 °C. After induction with YPGal the cells were shifted for 2 h to 37 °C. The ProtA fusion proteins were affinity-purified on IgG-Sepharose and eluted using acetic acid. Loads and eluates were analyzed by SDS-PAGE, Coomassie Blue staining, and immunoblotting using a αGsp1p antiserum. Prominent bands were identified by mass spectrometry. The relative mobility of Gsp1p is indicated. *Asterisks* indicate the purified ProtA fusion proteins, and degradation products of ProtA-Yrb30p are indicated by *open circles*.

Yrb30p Binds Directly to Gsp1p—To test whether Yrb30p could bind to Gsp1p directly, we performed pull-down assays using recombinant proteins produced in *E. coli*. We immobilized GST-Yrb30p, as well as GSTp alone, as a negative control and GST-Yrb1p (yRanBP1) and GST-Kap95p (yImpβ) as positive controls on GSH-Sepharose beads and analyzed binding of His_6-tagged Gsp1p, loaded either with GTP or GDP, to these beads. Like the already known RanGTP-binding proteins, Yrb30p showed clear binding of Gsp1pGTP but not of Gsp1pGDP in this assay, which indicates that these proteins interact directly and specifically (Fig. 2*A*). Moreover, we could demonstrate that Yrb30p binds human Ran with the same specificity (data not shown).

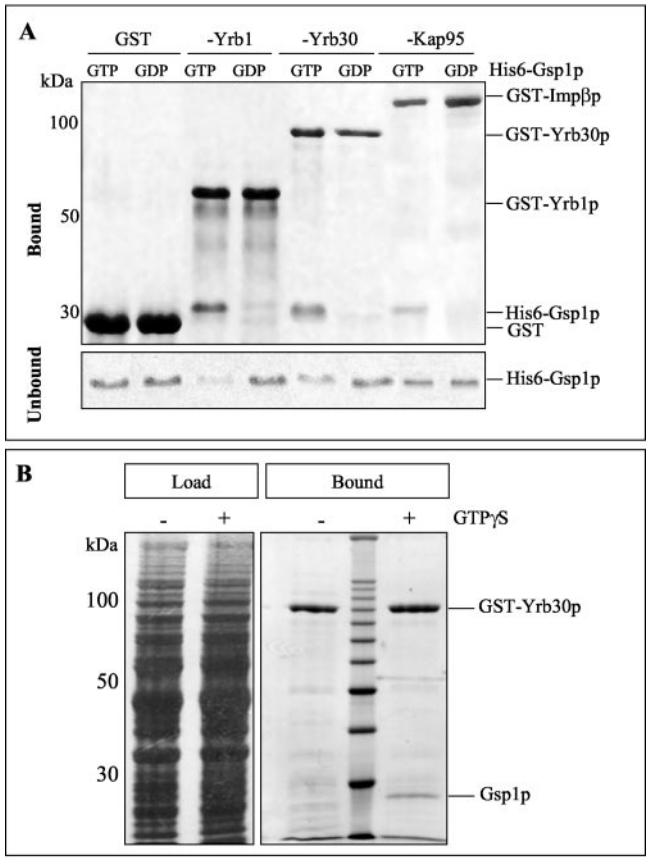


FIG. 2. **Yrb30p binds directly and specifically to the GTP-bound state of Gsp1p.** *A*, *in vitro* binding of recombinant Ran-binding proteins to the GTP-bound state of Gsp1p. GST fusions of Yrb1p, Yrb30p, Kap95p, and GST alone as a negative control were analyzed in pull-down assays using GSH-Sepharose for binding to recombinant His_6-Gsp1pGTP and His_6-Gsp1pGDP (see "Experimental Procedures"). Bound (*upper panel*) and unbound fractions (*lower panel*) were analyzed by SDS-PAGE and Coomassie Blue staining. *B*, pull-down of Gsp1pGTP from a yeast extract using recombinant Yrb30p. GST-Yrb30p was immobilized on GSH-Sepharose and incubated with yeast extract that had been loaded with GTPγS or mock-treated (see "Experimental Procedures"). Loaded and bound fractions were analyzed by SDS-PAGE, followed by Coomassie Blue staining and mass spectrometric analysis of protein bands.

As an alternative approach to test the specificity of the interaction between Yrb30p and Gsp1pGTP, we tested whether we could pull down endogenous Gsp1p from a yeast extract using an excess of immobilized GST-Yrb30p. To convert all GTPases in the yeast extract to their GTP-bound state, half of the extract was loaded with GTPγS whereas the other half was mock-treated (see "Experimental Procedures"). Indeed, Yrb30p was able to pull down Gsp1p that was verified by mass-spectrometry but only from the GTPγS-treated extract (Fig. 2*B*), suggesting a high specificity and affinity for Gsp1pGTP.

Identification of the RanGTP-binding Domain of Yrb30p—Having established the direct interaction between Yrb30p and Gsp1pGTP we were interested in delimiting the domain of Yrb30p required for binding to Gsp1pGTP. For this purpose we created various N- and C-terminal truncations of GST-Yrb30p and tested them for binding to His_6-Gsp1pGTP as outlined above.

As shown in Fig. 3*A* only the N-terminal 127 amino acids of Yrb30p were dispensable for RanGTP binding. Hence, the minimal RBD of Yrb30p comprises residues 128 to 440. This domain is much larger than the RBD of RanBP1 (~130 residues) and does not show any sequence similarity to the RBDs of RanBP1 or importin β, suggesting a novel motif for RanGTP binding (Fig. 3*B*, *RBD*).

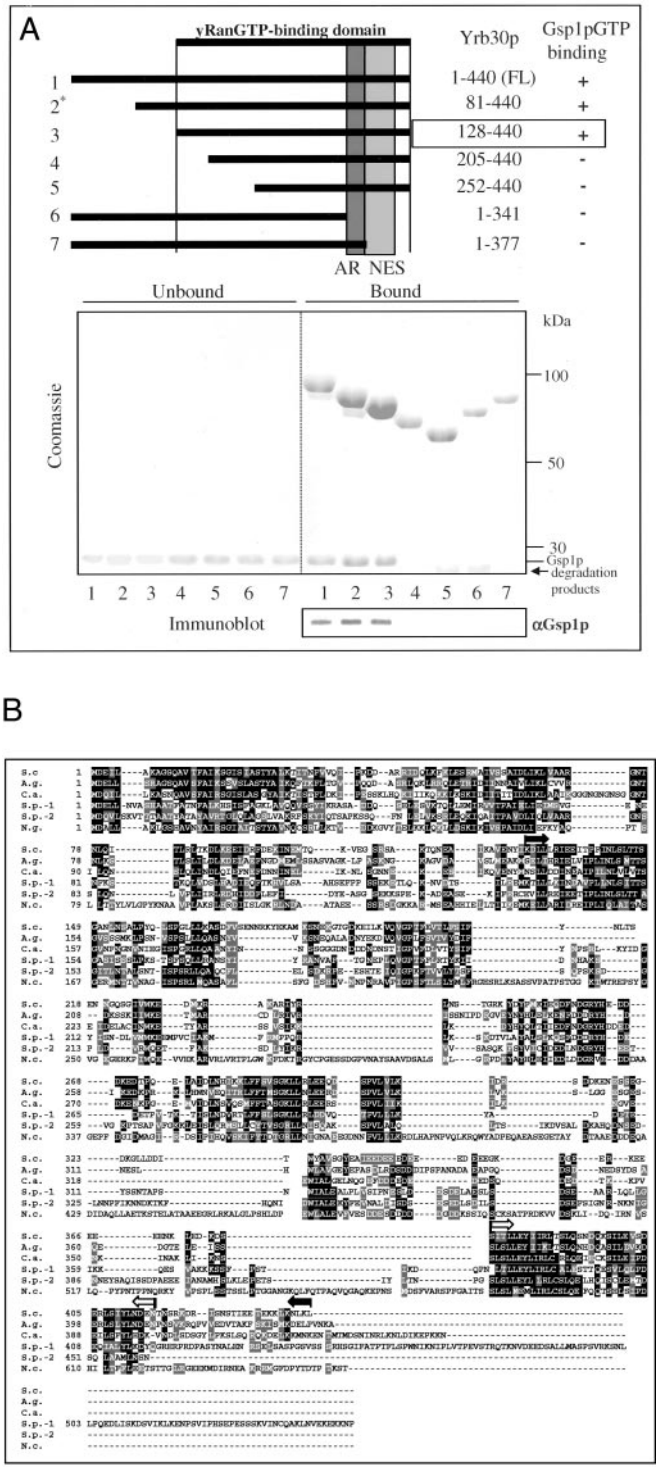
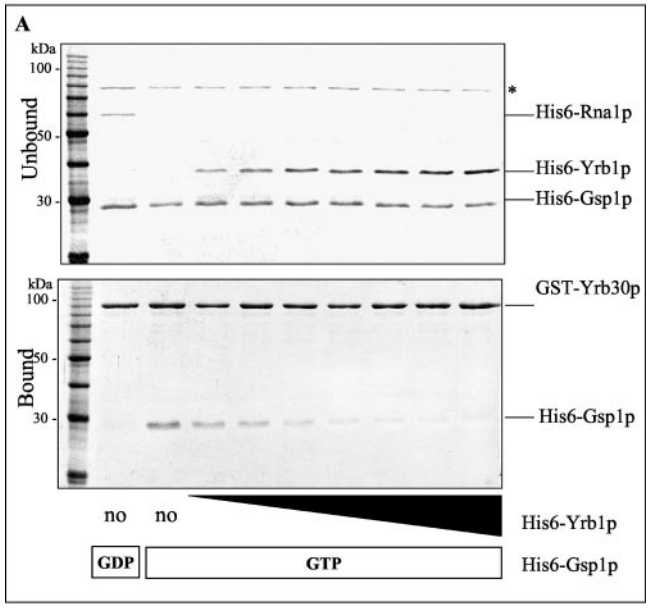


FIG. 3. **Domain structure of Yrb30p.** *A*, delimiting the RBD of Yrb30p. GST-Yrb30p fusion proteins comprising the indicated amino acids were bound to GSH-Sepharose and incubated with His_6-Gsp1pGTP. Unbound and bound fractions were analyzed by SDS-PAGE and Coomassie Blue staining. The bound fraction was further analyzed by immunoblotting using αGsp1p antiserum. A region rich in acidic residues (*AR*) and a sequence with homology to an NES are indicated. The *asterisk* indicates the portion of Yrb30p identified in the two-hybrid screen. *B*, amino acid sequence alignment of Yrb30p from *S. cerevisiae* (*S.c.*; GenBank™ accession number CAA96876.1), *S. pombe* (*S.p.-1* and *S.p.-2*; GenBank™ accession numbers CAA21820.3 and CAA90468.2, respectively), *C. albicans* (*C.a.*; Candida data base accession number CA5261), *A. gossypii* (*A.g.*) (see Footnote 4), and *N. crassa* (*N.c.*; GenBank™ accession number CAC04441.1). The limits of the identified RBD of Yrb30p are indicated by *filled arrows*, and the limits of the NES-like sequence are indicated by *open arrows*.



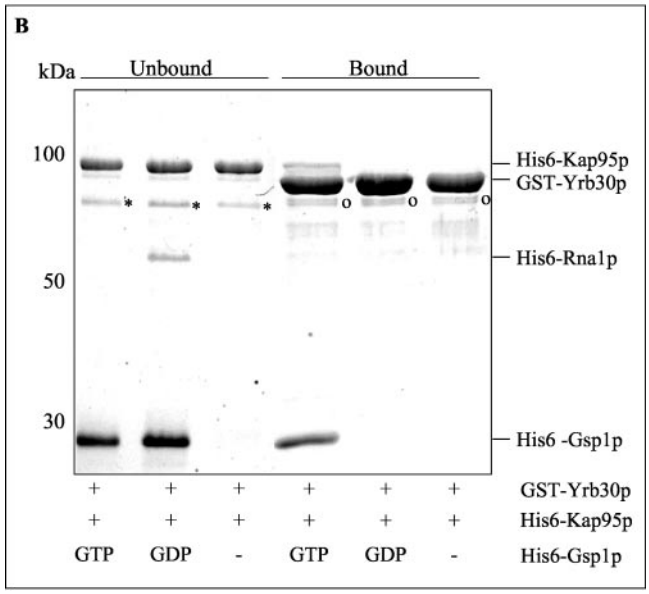


FIG. 4. **Comparison between RanGTP-binding properties of Yrb30p and known RanGTP-binding proteins.** *A*, competition between Yrb1p and Yrb30p for an overlapping binding site on Gsp1pGTP. GST-Yrb30p was bound to GSH-Sepharose and incubated as indicated with His_6-Gsp1pGDP or His_6-Gsp1pGTP, together with increasing concentrations of His_6-Yrb1p. The GDP-bound form of His_6-Gsp1p had been produced by preincubation of His_6-Gsp1GTP with recombinant RanGAP (*His_6-Rna1p*) (see "Experimental Procedures"). Unbound and bound fractions were analyzed by SDS-PAGE and Coomassie Blue staining. The *asterisk* marks a prominent *E. coli* protein present in the His_6-Gsp1p preparation. *B*, formation of trimeric complexes among Yrb30p, Kap95p, and Gsp1pGTP. GST-Yrb30p was bound to GSH-Sepharose and incubated with His_6-Kap95p alone or with His_6-Kap95p and His_6-Gsp1p in its GTP- or GDP-bound form. Unbound and bound fractions were analyzed by SDS-PAGE and Coomassie Blue staining. The *asterisk* indicates a prominent *E. coli* protein present in the His_6-Kap95p preparation. Degradation products of GST-Yrb30p are indicated by *circles*.

A BLAST search with the *YRB30* coding sequence reveals homologues of Yrb30p in *S. pombe* (GenBank™ accession numbers CAA90468.2 and CAA21820.3), *Ashbya gossypii*,[4] *Neurospora crassa* (GenBank™ accession number CAC04441.1), *Candida albicans* (Candida data base (genolist.pasteur.fr/CandidaDB) number CA5261) (Fig. 3*B*), and *Candida glabrata*

[4] F. Dietrich, T. Gaffney, and P. Philippsen, personal communication.

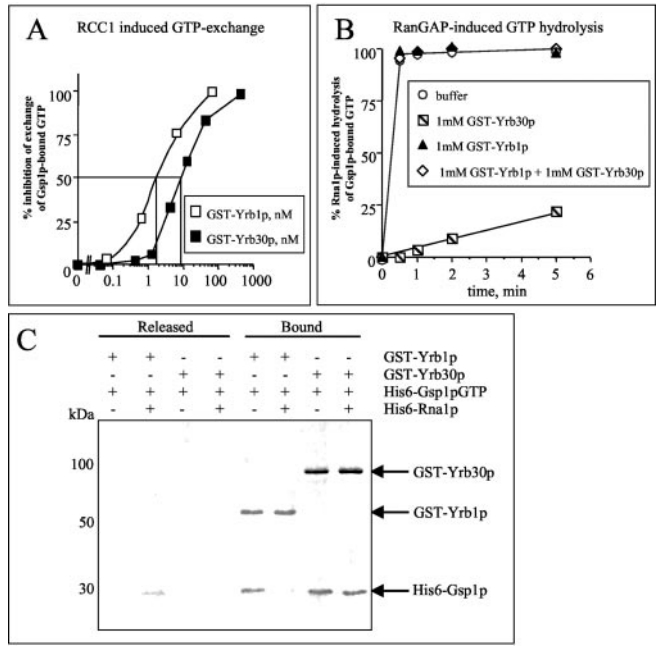


FIG. 5. **Modulation of the RanGTPase cycle by Yrb30p.** *A*, inhibition of RCC1-mediated GTP exchange. The *graph* shows % inhibition of RCC1-induced GTP exchange on Gsp1pGTP as a function of the concentration of the indicated GST fusion proteins of Yrb30p and Yrb1p (see "Experimental Procedures"). *B*, inhibition of Rna1p-mediated GTP hydrolysis. The *graph* shows % Rna1p-induced GTP hydrolysis on Gsp1pGTP with time in the presence of 1 mM of the indicated GST fusion proteins (see "Experimental Procedures"). *C*, Rna1p dissociates Yrb1p-Gsp1pGTP but not Yrb30p-Gsp1pGTP complexes. Preformed complexes between His_6-Gsp1pGTP and GST-Yrb1p or GST-Yrb30p, respectively, on GSH-Sepharose were incubated with His_6-Rna1p (see "Experimental Procedures"). Released and bound fractions were analyzed by SDS-PAGE and Coomassie Blue staining.

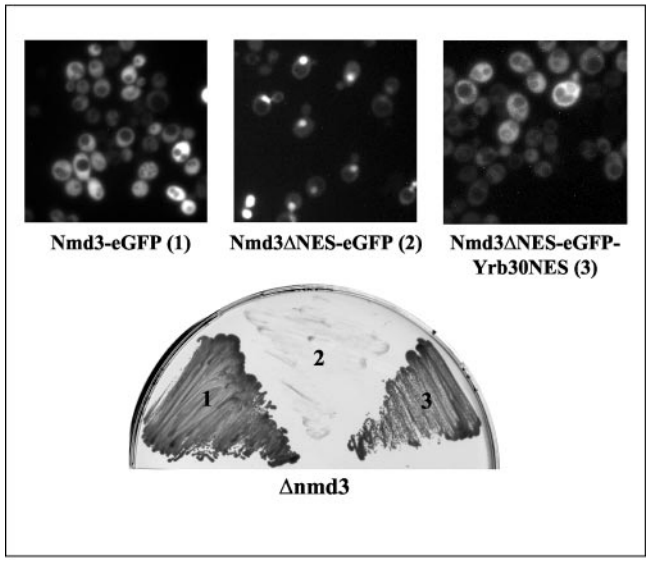


FIG. 6. **The NES of Yrb30p is functional in a heterologous context.** The two NESs of Nmd3p-eGFP (*1*) were deleted (*2*) or replaced by the NES of Yrb30p (*3*) and the corresponding proteins were analyzed *in vivo* for their steady-state localization in a wt background (*upper panel*) and for complementation of a lethal *nmd3* null mutation (*lower panel*). For the latter experiment, the plasmids encoding proteins 1–3 were introduced in a *NMD3* shuffle strain, and the transformants were analyzed for growth on 5-fluoro-orotic acid-containing medium, which selects against the plasmid carrying the essential *NMD3* wt gene.

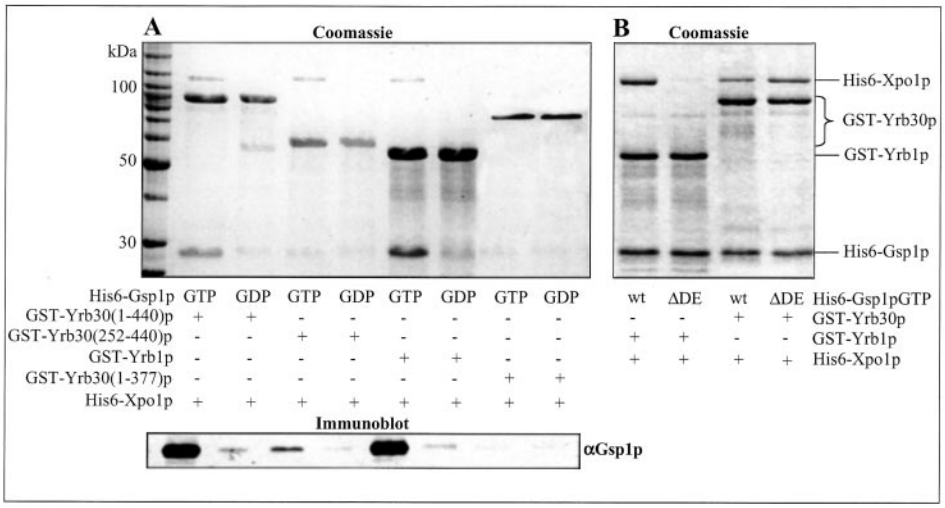


FIG. 7. **Complex formation among Yrb1p, Xpo1p, and Gsp1pGTP.** *A*, the NES but not the RBD of Yrb30p is necessary for complex formation. Recombinant GST fusions of full-length Yrb30p and Yrb1p, as well as N- and C-terminally truncated forms of Yrb30p, were tested for complex formation with His_6-Xpo1p in the presence of His_6-Gsp1pGTP and His_6-Gsp1pGDP. The bound fractions were analyzed by SDS-PAGE and Coomassie Blue staining, as well as immunoblotting using αGsp1p antiserum. *B*, complex formation of Yrb30p is, in contrast to Yrb1p, not dependent on the integrity of the acidic C terminus of Gsp1p. Recombinant GST-Yrb30p, and GST-Yrb1p as a control, were tested for complex formation with His_6-Xpo1p in the presence of full-length His_6-Gsp1pGTP or a version lacking the last seven residues (Δ*DE*). Shown are the bound fractions of the pull-down assays using GSH-Sepharose analyzed by SDS-PAGE and Coomassie Blue staining.

(GenBank™ accession number AAA35271.1). These proteins show sequence identities of 30 to 40% and similarities of 50 to 60% with highly conserved sequence blocks that are spread over the entire sequence and not restricted to the RanGTP-binding domain.

Characterization of the Yrb30p-binding Site of Gsp1pGTP—It was shown previously (30) that importin β and RanBP1 bind to different surfaces on RanGTP thus enabling the formation of ternary complexes. To get an idea about the epitope on RanGTP recognized by Yrb30p, we tested how His_6-Kap95p (yeast importin β) and His_6-Yrb1p (yRanBP1) would interfere with the *in vitro* interaction between GST-Yrb30p and His_6-Gsp1pGTP. In a first experiment, GST-Yrb30p was bound to GSH-Sepharose beads and incubated with a mixture of His_6-Gsp1pGTP and His_6-Yrb1p. With increasing concentrations of His_6-Yrb1p the amount of His_6-Gsp1pGTP bound to GST-Yrb30p was decreasing suggesting that Yrb1p and Yrb30p compete for an overlapping binding site on Gsp1GTP (Fig. 4*A*). To test whether Yrb30p could build trimeric complexes, like Yrb1p, with karyopherins, we incubated GST-Yrb30p pre-bound GSH-Sepharose with His_6-Kap95p and His_6-Gsp1p either in its GTP- or GDP-bound form. As shown in Fig. 4*B* Yrb30p was able to build trimeric complexes with Kap95p and Gsp1pGTP. In contrast to Yrb1p, no trimeric complexes were observed with His_6-Gsp1pGDP. In summary, the binding site for Yrb30p on RanGTP appears to overlap with the one for RanBP1 but is distinct from the one for importin β.

Modulation of the RanGTPase Cycle by Yrb30p—All RanGTP-binding proteins known so far, including the karyopherins and the RanBP1-related proteins, inhibit RanGEF (RCC1)-mediated GTP-to-GDP exchange on Ran, presumably by competition with RCC1 for binding to RanGTP. Accordingly, GST-Yrb30p also had an inhibitory effect on RCC1-mediated guanine nucleotide exchange (Fig. 5*A*). The dissociation constant of the Yrb30p-Gsp1pGTP complex derived from the concentration of GST-Yrb30p necessary for half-maximal inhibition was 10 nM, which is ~five times higher than the value for Yrb1p (21) but still significantly lower than for importin β, suggesting a rather high affinity.

Because Yrb30p showed similar binding characteristics like Yrb1p, the coactivator of RanGAP-mediated GTP hydrolysis on Ran, we checked whether Yrb30p would show a similar biochemical behavior. RanGAP-mediated GTP hydrolysis on RanGTP was measured as release of ^{32}P from Ran loaded with [γ-^{32}P]GTP as described earlier (24). Addition of GST-Yrb30p did not coactivate the reaction but acted as an inhibitor like karyopherins (Fig. 5*B*). We were able to confirm this result in pull-down assays where we incubated pre-formed complexes between His_6-Gsp1pGTP and GST-Yrb1p or GST-Yrb30p, respectively, with recombinant His_6-Rna1p (Fig. 5*C*). Under the applied conditions, we observed release of Gsp1p only from Yrb1p but not from Yrb30p.

Evidence for a Shuttling Mechanism of Yrb30p—One of the conserved sequence blocks (Fig. 3*B*) in the C terminus of Yrb30p (residues 377 to 413) resembles a canonical NES for the nuclear export receptor CRM1 (L-X_{2-3}-(F,I,L,V,M)-X_{2-3}-L-X-(I,L)) (31). Because this finding suggests that Yrb30p shuttles between nucleus and cytoplasm, like importin β and RanBP1, we tested this sequence for functionality.

First, we tested whether the Yrb30p NES would function in a heterologous context. For this purpose we replaced both NESs in Nmd3p by the NES-containing C terminus of Yrb30 (residues 341–440). Nmd3p is an adaptor in the Crm1p-mediated nuclear export of the large ribosomal subunit and shuttles between nucleus and cytoplasm (32, 33). Deletion of the NESs in Nmd3p is lethal and leads to nuclear accumulation of a GFP-Nmd3p fusion protein, which is cytoplasmic at steady state (Fig. 6). Insertion of the Yrb30p C terminus rescued both cell growth and cytoplasmic localization of GFP-Nmd3p devoid of its own NESs (Fig. 6). This result demonstrates the functionality of the Yrb30p NES in a heterologous context.

Next, we tested whether the sequence was recognized *in vitro* by yeast Crm1p/Xpo1p. For this purpose, we performed pull-down assays as described above using GST-Yrb30p, His_6-Gsp1pGTP, and His_6-yCrm1p. We were able to form trimeric complexes suggesting that the NES was indeed recognized by yCrm1p (Fig. 7). Complex formation was cooperative, because no binding of His_6-yCrm1p to GST-Yrb30p was observed in the absence of His_6-Gsp1pGTP (data not shown), and yCrm1p does not bind Gsp1pGTP alone (21). Similar complexes are formed by GST-Yrb1p where the formation is dependent on the integrity of the acidic C terminus of Gsp1p and the integrity of the

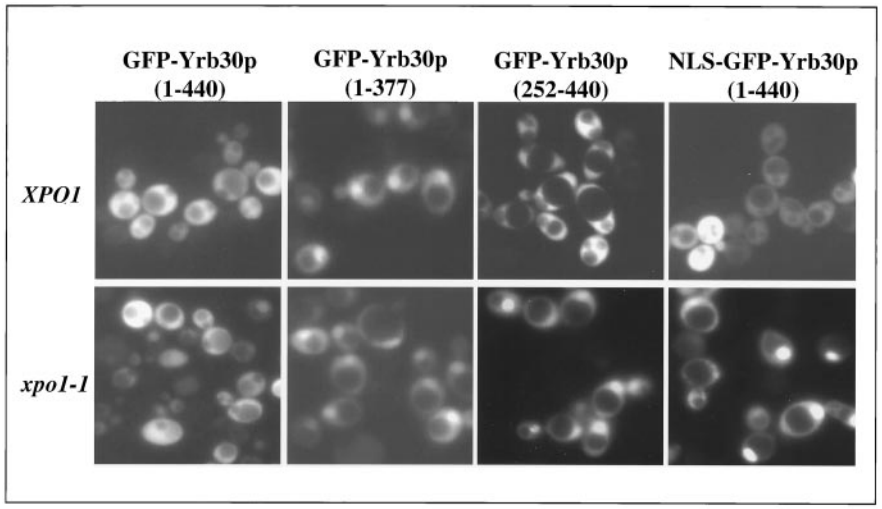


FIG. 8. **Subcellular localization of GFP-Yrb30p.** The *xpo1–1* strain Y1717 was transformed with plasmids encoding the indicated GFP-Yrb30p fusion proteins, together with a plasmid carrying *XPO1* (*XPO1*) or empty vector (*xpo1–1*). Transformants were grown at 23 °C, shifted for 15 min to 37 °C, and examined by fluorescence microscopy. The NLS-GFP-Yrb30p fusion protein contains the bipartite NLS of ribosomal protein Rpl25p (see "Experimental Procedures").

RBD of Yrb1p. To show the NES dependence of the interaction we tested a C-terminally truncated Gsp1pGTP lacking the last seven residues (1–212; ΔDE), an N-terminal truncation of Yrb30p defective in Gsp1pGTP binding (252–440) but retaining the NES, and a C-terminal truncation of Yrb30p (1–377), devoid of both NES and Gsp1pGTP binding, for complex formation. Complex formation was possible in all cases except the C-terminally truncated Yrb30p, suggesting that the NES is necessary and sufficient for complex formation (Fig. 7).

Third, we examined the localization of various GFP-Yrb30p fusion proteins in a wt background and in various export mutants. In a wt background and in the four export mutants, *xpo1–1*, *cse1–2*, Δ*msn5*, and Δ*los1*, the protein localizes exclusively to the cytoplasm (Fig. 8) (data not shown). However, deletion of residues 378 to 440 including the NES and part of the RBD leads to a mislocalization of the protein to the nucleoplasm of wt cells suggesting that this region also acts as a NES in the homologous context (Fig. 8). Interestingly, impairment of yCrm1p function either by mutation (*xpo1–1*) or inhibition by leptomycin B does not result in nuclear accumulation of GFP-Yrb30p unless a strong heterologous NLS is fused to the protein, or, alternatively, RanGTP binding is compromised (GFP-Yrb30 (252–440)p) (Fig. 8). In these cases, however, nuclear accumulation occurs very rapidly *i.e.* within 5 min upon switching to the restrictive conditions (data not shown). These results suggest that Yrb30p shuttles between nucleus and cytoplasm and is exported from the nucleus by two alternative pathways, one dependent on its NES and Crm1p and another one on its ability to bind RanGTP.

Cellular Function of Yrb30p—Interestingly, there are two proteins in *S. pombe* that are homologous to Yrb30p. These two proteins bind specifically to the GTP-bound form of Gsp1p *in vitro* suggesting that they are conserved not only on a structural but also on a functional level (data not shown). Thus far, we did not find any homologue in higher eukaryotes suggesting that the function of Yrb30p is restricted to fungi.

To explore the function of this novel RanGTP-binding protein, we created complete deletions of the corresponding ORFs in *S. cerevisiae* (W303–1A), *S. pombe* (single and double knock-outs), and *A. gossypii*. Neither of these mutations had any obvious phenotype under normal growth conditions. For the *S. cerevisiae* null mutant we tested in addition growth at different temperatures and on different media formulations, as well as mating and sporulation, haploid invasive growth, and localization of various nucleocytoplasmic transport cargoes. None of these processes was affected by the deletion of *YRB30*. Neither could we find any synthetic

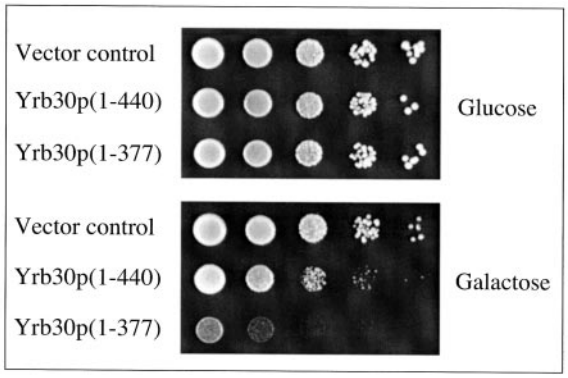


FIG. 9. **Dominant-negative effect of C-terminally truncated forms of Yrb30p on yeast vegetative growth.** wt strain W303–1A was transformed with high copy number plasmids producing Yrb30p(1–440) (full-length) or Yrb30p(1–377), respectively, under control of the galactose-inducible *GAL1* promoter. The plasmid (pRS426GAL1) containing the empty expression cassette was used as vector control. Transformants pre-grown in selective medium with raffinose as sole carbon source were spotted in serial dilutions on selective plates containing either glucose or galactose as sole carbon source and grown for 2 or 3 days, respectively.

growth phenotype of the deletion with other mutations in the Ran GTPase cycle or the nucleocytoplasmic transport machinery. Mutations tested included *gsp1–1*, *rna1–1*, *prp20–1*, *yrb1–51*, Δ*mog1*, Δ*yrb4*, *srp1–31*, *xpo1–1*, *crm1–1*, *-2*, *-3*, Δ*los1*, Δ*msn5*, *cse1–2*, Δ*nup2*, Δ*yrb2*, and Δ*nup42*. On the other hand, galactose-induced overproduction of the full-length protein from a high copy number plasmid under control of the *GAL1* promoter did not suppress the temperature sensitivity of *gsp1–1*, *rna1–1*, *prp20–1*, or *yrb1–51* strains. However, the *GAL1-YRB30* construct was able to partially suppress the toxicity of overproduced Gsp1(G21V)p (data not shown). Because the same effect is also seen with a *GAL1-YRB1* construct (data not shown), this phenotype confirms again the *in vivo* interaction between Yrb30p and RanGTP. During these overproduction studies we noticed a slight inhibition of vegetative growth by overproduction of full-length Yrb30p independent of the genetic background. This phenotype was much more pronounced in case of the overproduction of C-terminally truncated forms of Yrb30p, such as Yrb30p (1–377), which strongly inhibits the vegetative growth of a wt strain (Fig. 9). It remains to be seen whether this phenotype is dependent on the nuclear accumulation of these constructs (see Fig. 8).

DISCUSSION

Effector or target proteins of small Ras-like GTPases are the molecular links between these molecular switches and the cellular processes regulated by them. Given that the variety of cellular processes Ran had been implicated (6, 34), it seemed not unlikely that there were several effectors of Ran, each one specific for a given cellular process. However, today we know that probably all three processes that are controlled by Ran, nucleocytoplasmic transport, microtubule aster formation, and nuclear envelope formation, are mediated by one type of effector proteins, the karyopherins (2, 9). This family of proteins thus represents, in a strict sense, the only effector or target protein of Ran. Besides, however, there is a family of RanGTP-binding proteins, RanBP1 and its relatives (Yrb1p, Yrb2p, and Nup2p in *S. cerevisiae*), that plays an important role as modulators of the Ran GTPase system (6).

In this study, we set out to identify further RanGDP- or RanGTP-binding proteins in the yeast *S. cerevisiae* using a two-hybrid approach. Among the proteins identified were already known Ran-binding proteins such as karyopherins, RanBP1 and Mog1p. Interestingly, latter Ran-binding protein was identified using as a bait the Gsp1p(T26N) mutant form of yeast Ran, which is supposed to be locked in the empty or RanGDP-bound state (35). This finding is in agreement with recent publications (36, 37) where Mog1p is implicated in the release of RanGDP from its import receptor NTF2 and in the subsequent guanine-nucleotide exchange on RanGDP to RanGTP by RanGEF (RCC1 in metazoans, Prp20p in *S. cerevisiae*). The only novel protein among the identified preys whose physical interaction with Ran could be confirmed by various means was YGL164c, which was termed Yrb30p. Consistent with its identification in the screen with Gsp1p(G21V), where yeast RanBP1 (Yrb1p) was also identified, Yrb30p binds specifically and with high affinity (10^{-7} M) to the GTP-bound form of yRan (Gsp1p).

Its binding site on Gsp1pGTP overlaps with the one for yRanBP1 (Yrb1p) and is distinct from the one recognized by importin β. Despite these similarities, Yrb30p does not coactivate RanGAP-mediated GTP hydrolysis on Ran but rather inhibits this reaction, as well as RanGEF-mediated guanine-nucleotide exchange, like karyopherins do. The existence of a functional NES for the major nuclear export receptor, Exportin 1 (CRM1) in metazoans or Xpo1p in *S. cerevisiae*, suggests that the protein, despite its exclusive cytoplasmic localization at steady state, shuttles between nucleus and cytoplasm. This suggestion is confirmed by the nuclear accumulation of a C-terminally truncated form of the protein, which is defective for RanGTP binding and devoid of the NES, in a wt background, and by the nuclear accumulation of a N-terminally truncated Yrb30p, harboring the NES but unable to bind to RanGTP directly, upon inhibition of Crm1p-dependent nuclear export. The lack of nuclear accumulation of full-length Yrb30p upon impairment of yCrm1p function may be explained by the existence of two alternative nuclear export pathways for Yrb30p, one dependent on the NES and Crm1p and another one on the integrity of the RanGTP-binding domain. In latter case, Yrb30p may leave the nucleus as piggy-bag of any RanGTP-karyopherin complex. Alternatively to such a shuttling mechanism, the cytoplasmic localization of Yrb30p could be explained by a strong retention mechanism and diffusion of the protein into the nucleus upon loss of this retention. In this model, the nuclear export pathways for Yrb30p would be needed as a kind of safeguard mechanism to avoid the occurrence of Yrb30p in the nucleus, which may be detrimental for the cell. At the moment we cannot distinguish between these two possibilities, but the very rapid kinetics of the nuclear accumulation of GFP-Yrb30 (252–440)p upon impairment of yCrm1p function rather argues for a shuttling mechanism.

Regardless of its interesting binding behavior and localization, the cellular function of Yrb30p is unclear. Despite the fact that Yrb30p and RanBP1 (Yrb1p) see the same epitope on RanGTP, they differ in their effect on the cytoplasmic RanGAP1 (Rna1p in *S. cerevisiae*)-mediated GTP hydrolysis on RanGTP in that Yrb30p inhibits the reaction whereas Yrb1p coactivates it. Based on this behavior and the formation of ternary complexes among Yrb30p, RanGTP, and karyopherins, we hypothesize that Yrb30p may stabilize specific export complexes toward dissociation by Yrb1p and Rna1p in the cytoplasm to allow their transport to further destinations in the cell. Such a hypothesis of a pathway-specific rather than a general function of Yrb30p in the Ran GTPase cycle is in agreement with the cellular abundance of Yrb30p, which is estimated to be only [1/20] of Yrb1p. This estimation is based on immunoblotting of whole cell extracts derived from strains containing ProtA-tagged versions of the chromosomal *YRB30* and *YRB1* genes (data not shown). However, the hypothetic modulation of the RanGTPase cycle or nuclear export appears to be dispensable for vegetative growth, because a deletion of *YRB30* does not result in any obvious phenotype, neither in *S. cerevisiae* nor in *S. pombe* or *A. gossypii*. The dominant-negative effect of overproduced, C-terminally truncated forms of the protein on yeast vegetative growth may result from the sequestration of an essential protein whose interaction with Yrb30p is stabilized by the truncation of Yrb30p. Ran is an unlikely candidate for this interaction partner, because these C-terminal truncations abolish the interaction of Yrb1p with Gsp1pGTP (see Fig. 3*A*). The non-essential function of Yrb30p might be related to the exclusive conservation of Yrb30p in the fungal kingdom as there is no evidence for homologues of Yrb30p in higher eukaryotes. A prominent feature of fungi is their closed mitosis *i.e.* the integrity of the nuclear envelope during nuclear and cellular division. At the moment, however, we have no evidence for a role of Yrb30p in this context.

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