Yrb2p Is a Nuclear Protein That Interacts with Prp20p, a Yeast Rcc1 Homologue*

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A conserved family of Ran binding proteins (RBPs) has been defined by their ability to bind to the Ran GTPase and the presence of a common region of approximately 100 amino acids (the Ran binding domain). The yeast *Saccharomyces cerevisiae* genome predicts only three proteins with canonical Ran binding domains. Mutation of one of these, *YRB1*, results in defects in transport and nuclear localization of macromolecules across the nuclear envelope (Schlenstedt, G., Wong, D. H., Koepp, D. M., and Silver, P. A. (1995) *EMBO* J. 14, 5367–5378). The second one, encoded by *YRB2*, is a 327-amino acid protein with a Ran binding domain at its C terminus and an internal cluster of FXFG and FG repeats conserved in nucleoporins. Yrb2p is located inside the nucleus, and this localization relies on the N terminus. Results of both genetic and biochemical analyses show interactions of Yrb2p with the Ran nucleotide exchanger Prp20p/Rcc1. Yrb2p binding to Gsp1p (yeast Ran) as well as to a novel 150-kDa GTP-binding protein is also detected. The Ran binding domain of Yrb2p is essential for function and for its association with Prp20p and the GTP-binding proteins. Taken together, we suggest that Yrb2p may play a role in the Ran GTPase cycle distinct from nuclear transport.

Movement of macromolecules into and out of the nucleus is a multistep process crucial for most cellular events, including transcriptional regulation, progression through the cell cycle, and DNA duplication. Studies with both higher eukaryotes and yeast have revealed much about the requirements for this highly conserved process (1, 2). Certain amino acid sequences (termed nuclear localization sequences (NLS)) are recognized by soluble transport factors in the cytoplasm and delivered to the nuclear pore complex (NPC) (3, 4). The NLS receptor is a heterodimer referred to as either importin or karyopherin (5–10). In the yeast *Saccharomyces cerevisiae*, the importin α subunit is encoded by *SRP1* (11) and the β subunit by *RSL1*/*KAP95* (12, 13). Mutations in either essential gene elicit defects in nuclear protein import in *vivo* (13, 14). In addition to these NLS receptors, translocation of substrates through the NPC requires hydrolysis of GTP by Ran, a highly conserved small GTPase (15, 16). Like all GTPases, Ran (Gsp1p in yeast) is regulated by a host of factors including a GTPase activator termed RanGAP1 (18, 19) (Rna1p in yeast) (20–22), a nucleotide exchange factor termed Rcc1 (23) (Prp20p in yeast) (24, 25), and a protein termed Ntf2p (26–28) of unknown function. Mutations in genes encoding Gsp1p and its regulators all cause defects in nuclear transport (13, 21, 26, 29–33).

It has become increasingly clear that the nucleotide-bound state of Ran plays a key role in nuclear protein import and mRNA export. Inhibition of GTP hydrolysis by Ran inhibits nuclear import in well defined *in vitro* reactions (16). Mutants of yeast *Gsp1* (encoding the essential Ran homologue) stabilized in the GTP-bound form block both protein import and mRNA export in *vivo* (33). Additionally, yeast mutants in *Rna1* (encoding the Gsp1p/Ran GAP) are defective for bidirectional nuclear transport of proteins and RNAs (21, 29, 31). The inability to regenerate the GTP-bound form of Ran either by mutation of Prp20p (13, 30, 32) or overexpression of the GDP-bound form of Gsp1p in yeast (15) also results in defects in nuclear transport of proteins and RNAs.

However, Ran and its regulators have been implicated in a number of other nuclear processes. These include chromatin condensation (23, 34), RNA processing (25, 35, 36), cell cycle progression (37–39), DNA replication (40, 41), and nuclear envelope assembly (25, 42). It remains to be determined whether or not these phenotypes are simply a secondary consequence of defects in nuclear transport or reflect processes other than nuclear transport that are directly controlled by Ran.

A conserved protein family has recently been defined by their ability to bind to Ran. These so-called ran binding proteins (RBPs) have in common a stretch of about 100 amino acids that is necessary for Ran binding (43). Mammalian RanBP1 (18, 44, 45) and its yeast homologue, Yrb1p (46, 47), stimulate Ran GTPase by further activating the GAP activity (44, 46). Both proteins bind stably to the GTP-bound but not the GDP-bound form of Ran (18, 44–46). In yeast, *YRB1* is essential for cell growth, and temperature-sensitive mutants display nuclear transport defects (46, 47), consistent with the role as a Ran regulator. Some portion of Yrb1p is located at the nuclear envelope and may provide a “docking” site for Ran at the nuclear pore complex (46). However, Yrb1p was also identified in a screen for mutants of yeast with destabilized chromosomes (47), thus supporting the notion that there may be additional functions for Gsp1p and its regulators.
Yrb2p in Yeast

### EXPERIMENTAL PROCEDURES

#### Yeast Strains and Plasmids—Yeast S. cerevisiae strains used in this work are listed in Table I. Media for cell growth and genetic manipulations were according to standard procedures (54, 55). 5-Fluoroorotic acid (5-FOA) was added at 1 mg/ml if needed. Growth of cells was measured by counting the cell number directly or measurement of absorbance at 600 nm.

All DNA manipulations were according to standard procedures (56, 57). Positions of oligonucleotides used for polymerase chain reaction (PCR) are described by numbers when A of the initiation codon is positioned as +1. The Yrb2p gene was cloned by inserting two PCR fragments generated with yeast genomic DNA as a template into pBluescriptKS+ (Stratagene). Primers used for PCR were: GS59 (5'-AGCTCTAGACGGGGTCCAGGGTGCGACC-3'), GS60 (an antisense primer, 1080 to 1062 with an HindIII site, 5'-CCCTCGGTCTCTTAGGAGTTCC-3'), GS62 (a primer with an XbaI site, 5'-GCTCTAGACGGGGTCCAGGGTGCGACC-3'), GS64 (an antisense primer, 109 to 88, 5'-CATCGTGACGGGTGCGACC-3'), GS66 (a primer with an EcoRI site, 5'-GCGGATCCTGCTGAGTTTGCGACC-3'), GS67 (a primer with an AvaI site which codes codons 30 to 34 of Yrb1p), GS68 (a primer with an SmaI site (5'-TCCCCCGGGGTGGTTGCGTGCGACC-3'), GS69 (a primer with an EcoRI site, 5'-GCGGATCCTGCTGAGTTTGCGACC-3').

#### TABLE I

| Name          | Genotype                        | Source                      |
|---------------|---------------------------------|-----------------------------|
| PSY362        | MATa np3-1 ade2-1 his leu2-3,112 ura3-1 | Laboratory stock           |
| PSY712        | MATa prp20-101 his 3200 leu2Δ3 ura3-1 | Laboratory stock           |
| PSY713        | MATa prp20-1 leu2Δ11 trp1363 ura3-52 | Ref. 13                    |
| PSY714        | MATa leu2Δ11 trp1 ura3-52 rna1-1 | Ref. 21                    |
| PSY878        | MATa/a ade2 ade2 his3 his3 leu2/ trp1/1 trp1 ura3/3 | Ref. 13                    |
| PSY1002       | MATa 92-1 H3IS ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1 | This study                 |
| PSY1003       | MATa Ayrv2-H3IS ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1 | This study                 |
| PSY1004       | MATa YRB2 ade2-1 his3-11,15 leu2-3,112 ura3=1 trp1 | This study                 |
| PSY1005       | MATa YRB2 ade2-1 his3-11,15 leu2-3,112 ura3=1 trp1 | This study                 |
| FY23          | MATa trp1 leu2Δ13 ura3-52         | F. Winston (Harvard Medical School) |
| FY90         | MATa his3Δ2000 leu2Δ1 ura3-52      | F. Winston                  |
| JLY381        | MATa nup2-5 H3IS trp1 leu2Δ3 ura3-52 leu2-3,112 his3Δ200 ade2 | G. Fink (Massachusetts Institute of Technology) |
| I4956        | MATa nup1-2-LEU2 his3Δ200 leu2-3,112 ura3-52 pNUP1( URA3) | G. Fink |
| LG230         | MATa rat7-1 his3Δ200 leu2Δ1 ura3-52 | Ref. 64                    |

Only two additional yeast proteins, Yrb2p and Nup2p, contain predicted Ran binding domains (48, 49). The mammalian RanBP2/Nup95 (50, 51), Nup2p is located at the yeast nuclear pore complex (52). Interestingly, Nup2p is dispensable for normal yeast cell growth (52). One possibility is that Nup2p and Yrb1p play redundant roles as Ran-docking sites. Yrb2p is rich in charged and hydrophilic amino acids with several potential phosphorylation sites and short sequences similar to NLSs. Unlike Yrb1p, cells missing Yrb2p are viable except at low temperatures and show no obvious defects in nuclear transport (53).

Because of its similarity to Yrb1p and the important role of Ran-regulators in nuclear function, we now present results concerning the function of Yrb2p that appear to distinguish it from Yrb1p. Yrb2p is primarily localized in the nucleus. In addition, Yrb2p interacts and functions with Prp2p, based on both biochemical and genetic experiments.

#### Additional Primers

TT17, TT21, TT7, TT18, TT8, TT6, TT21-GS60, TT7-TT14, TT7-TT17-GS60, TT7-TT17, TT7-TT14, and TT7-TT14 were generated with primers a) TT7 (5'-CAATGG-3') and c) TT8 (5'-CCCTCGGTCTCTTAGGAGTTCC-3') were generated with primers b) TT7-GS60, a primer with an EcoRI site (5'-CGGGATCCTGCTGAGTTTGCGACC-3'), d) TT7-TT14, a primer with a HindIII site and the first 17 bases of the Yrb2p coding region, and e) TT7-TT14 was cloned into pPS293 using BamHI and XhoI to generate pPS1092. A 1.08-kb YRB2 fragment generated for the construction of pPS1084 was placed on the 3' end of GST (encoding glutathione S-transferase gene) of pPS892 (13) by using BamHI-XbaI sites to yield pPS1083.

YRB2::HA plasmids with various deletion mutations were constructed as follows. A 0.68-kb PCR fragment was generated with pPS842 as a template and with primers GS60 and TT13 (430 to 447 with a ClaI site, 5'-CATCGTGACGGGTGCGACC-3'), digested with ClaI and XhoI and then ligated with ClaI-XhoI digested pPS1081 to replace the full-length YRB2 with YRB2 lacking codons 27 to 143 (ΔN) to yield pPS1084. To obtain mutants of YRB2 lacking codons 94 to 149 (ΔNup, pPS1085) or codons 198 to 327 (ΔC, pPS1086), we carried out PCRs with pPS842 as a template and primers of TT7/T21 and TT7/T17 and TT17/GS60 (for pPS1085) or TT7/T14 and TT17/GS60 (pPS1086). Descriptions of these primers are following: TT7/Anti; 21 anti-anti-coding of 282 to 257 with a ClaI site (5'-CGGGATCCTGCTGAGTTTGCGACC-3'), TT7/TT17, anti-coding of 351 to 451 with an EcoRI site (5'-CGGGATCCTGCTGAGTTTGCGACC-3'), TT7/T17, an EcoRI fragment of 0.5 kb (5'-CGGGATCCTGCTGAGTTTGCGACC-3').

After treatment of the PCR fragments with proper restriction enzymes (Avec and EcoRI for the TT7-TT21 fragment, EcoRI and XhoI for the TT7-TT17-GS60, Avec for the TT7-TT14, Avec and XhoI for TT7-TT15-GS60), fragments were ligated into XhoI-XbaI digested pPS1081 to replace YRB2 with the truncated genes. For construction of a YRB2 chimeric gene with the Yrb1p Ran binding domain, we produced two PCR fragments. A 0.50-kb fragment, carrying HA followed codons 7 to 157 of YRB2, was generated with primers TT7 and TT18 (anti-coding of 471 to 541 with an EcoRI site, 5'-CGGGATCCTGCTGAGTTTGCGACC-3') and with pPS842 as a template. A 0.53-kb PCR fragment was obtained with TT19 (an EcoRI primer which codes codons 30 to 34 of YRB1, 5'-CGGGATCCTGCTGAGTTTGCGACC-3').

#### Strains used in this study

| Strain            | Source                                      |
|-------------------|---------------------------------------------|
| JLY381            | Reference to Table I                        |
| FY90              | MATa his3Δ2000 leu2Δ1 ura3-52               |
| JLY381            | MATa nup2-5 H3IS trp1 leu2Δ3 ura3-52 leu2-3,112 his3Δ200 ade2 |
| I4956            | MATa nup1-2-LEU2 his3Δ200 leu2-3,112 ura3-52 pNUP1( URA3) |
| LG230            | MATa rat7-1 his3Δ200 leu2Δ1 ura3-52         |

Additional primers TT7 (5'-CCGGATCCTGAGTTTGCGACC-3'), which carries a XhoI site and the HA coding sequence followed by 19 to 34 nucleotides of YRB2 coding sequence, and TT8 (5'-CCGGATCCTGAGTTTGCGACC-3'), a primer carrying a XhoI site followed by an anti-coding sequence of 18 to 2 pPS842 was used as a template for PCR with GS59/TT8 and TT5/GS60 to produce a 0.67- and 1.10-kb fragments, respectively. After digestion with KpnI and AseI (for GS59-TT7 fragment) or AseI and XhoI (for TT7-GS60), the fragments were inserted into the KpnI-XhoI sites of pRS316 to yield pPS1081. YRB2::HA fragment amplified from pPS1081 with T99 (5'-CCGGATCCTGAGTTTGCGACC-3'), a primer with a BamHI site and the first 17 bases of the Yrb2p coding region, and GS60 was cloned into pPS293 using BamHI and XhoI to generate pPS1092. A 1.08-kb YRB2 fragment generated for the construction of pPS1084 was placed on the 3' end of GST (encoding glutathione S-transferase gene) of pPS892 (13) by using BamHI-XbaI sites to yield pPS1083.
Yrb2p in Yeast

Construction of YRB2/Δyrb2 Diploid Strain—YRB2 was deleted as described by Baudin et al. (60) with the following modifications. A 1.10-kb DNA fragment, which carries the HIS3 gene as well as 46 bases flanking regions of the YRB2 open reading frame, was generated by PCR. The resulting fragment was introduced into diploid cells (PSY78; Ref. 13), and HIS3 transformants were selected. Colonies carrying HIS3 integrations at YRB2 were screened by PCR with GS59 and a HIS3 internal primer (5'-GCCCTCATCCAAAAGGCGC-3'). The integration was then confirmed by Southern blotting.

Protein and mRNA Localization—Yeast cells were grown in complete media or selective media containing 2% (w/v) glucose to a density of 1–5 × 10⁸ cells/ml. For expression of genes under control of the GAL promoter, cells were grown in selective media containing 2% glucose to a density of 0.5–2 × 10⁸ cells/ml, then galactose was added to 2% (w/v), and induction was conducted for 2–4 h. Cells were fixed by treatment with 1/10 volume of 37% formaldehyde for 60–90 min and prepared for immunofluorescence as described previously (46). Mouse monoclonal antibody 12CA5 was used at 1/4000 dilution in 5% bovine serum albumin. Immunofluorescence as described previously (46) with modifications and carried out at 4 °C if not otherwise mentioned.

Effects of Mutation of YRB2 on Cell Growth—To begin to understand the function of YRB2 in vivo, a deletion mutant was constructed by replacement of the entire YRB2 coding region with HIS3 in a diploid by homologous recombination (see “Experimental Procedures”). The proper integration of HIS3 into one of the YRB2 loci on chromosome IX was confirmed by Southern blot analysis (data not shown). The YRB2/Δyrb2::HIS3 diploid was sporulated, and the resulting tetrads were analyzed for growth at various temperatures. Consistent with the previous observation by Noguchi et al. (53), we also found that YRB2 is not essential for cell viability. However, YRB2 is necessary for the growth at 15 °C (data not shown, and Ref. 53).When cultivated in liquid media, the growth of yrb2 strains was impaired after shifting from 30 to 15 °C, and the viability of the deletion mutant was lower as compared with similarly treated wild-type cells (data not shown).

Overexpression of YRB2 also slowed cell growth significantly (Fig. 2). YRB2 was placed under control of the regulatable strong GAL1 promoter. When grown on glucose where the promoter is repressed, cells bearing the pGAL-YRB2 plasmid grew normally. However, when placed on plates with galactose as the sole carbon source, the pGAL-YRB2 containing cells failed to grow (pGAL YRB2 in Fig. 2). Because Yrb2p contains a Ran/Gsp1p binding domain and Gsp1p and Yrb1p have been implicated in regulation of nuclear transport, we examined yrb2 cells for their nuclear transport activity. In agreement with the previous report (53), neither localization of both a nuclear RNA binding protein, Npl3p, and an NLS-containing protein nor distribution of poly(A)⁺ RNA were affected up to 6-days shift to 15 °C (data not shown). In addition, no defects on nuclear protein import or mRNA export were observed when Yrb2p was overproduced by the GAL1 promoter (data not shown).

Localization of Yrb2p—To determine the intracellular localization of Yrb2p, the influenza HA epitope was inserted between amino acids 6 and 7 of Yrb2p. Intact Yrb2-HA protein was detected by immunoblotting with the 12CA5 mouse monoclonal antibody specific for the HA tag (data not shown). The epitope-tagged Yrb2p is functional because, when present on a low copy CEN plasmid in the Δyrb2 strain, normal growth at 15 °C was achieved (data not shown), and when overexpressed, the value is less than the identity between Yrb1p and mammalian RanBP1, which are 56% identical. The middle region of Yrb2p contains two FGF and three FG amino acid repeats (between residues 98–149).

Yrb2p contains a Ran/Gsp1p binding domain and analogous to other Ran binding proteins, we were interested in exploring the possibility that Yrb2p could also function as a regulator of Ran-GTPase and nuclear transport.
the tagged gene causes a growth defect as does the wild type (YRB2::HA in Fig. 2). The intracellular distribution of Yrb2p-HAp was determined by immunofluorescence microscopy with the 12CA5 antibody. We expressed YRB2::HA at physiological levels from the YRB2 promoter or overexpressed it from the GAL1 promoter. In both cases, Yrb2::HAp was located exclusively within the nucleus (e.g. see Fig. 4C). The same conclusion has been reached by Noguchi et al. (53) although they used an alternatively tagged YRB2.

**Genetic Interactions of YRB2 and Other GSP1 Regulators**—To examine whether Yrb2p may function in the regulation of Gsp1p, we tested for genetic interactions with other nuclear transport factors and regulators of Gsp1p by creating double mutants with Yrb2. In the cases of nup1 (63), nup2 (52), rat7/nup159 (64) (all encoding nucleoporins), rna1 (65) (a yeast GAP homologue), and npl3 (66), the double mutants were able to grow at 25 °C. However, when the yrb2 mutation was combined with two different temperature-sensitive alleles of prp20 (prp20-1 (25) and prp20-101 (12)), the resulting double mutant could not grow unless a functional copy of YRB2 was maintained in the cells at 25 °C. However, when the yrb2 mutation was combined with two different temperature-sensitive alleles of prp20 (prp20-1 (25) and prp20-101 (12)), the resulting double mutant could grow at 25 °C.

**Interaction of Yrb2p with Prp20p**—The genetic relationship and co-nuclear localization of Yrb2p and Prp20p suggests that these proteins may function together in a complex. To test this possibility, we constructed a GST-YRB2 fusion gene where GST is fused to the 5’ end of YRB2, and the resulting fusion is expressed from the GAL1 promoter. Cell lysates were prepared from cells expressing the GST-Yrb2 fusion protein and incubated with agarose beads conjugated to glutathione, and the bound proteins were analyzed by silver staining and immunoblotting. Silver staining did not reveal any proteins that co-purified in near stoichiometric amounts. However, by immunoblotting, Prp20p was found to co-purify with GST-Yrb2p (Fig. 4B, GST-Yrb2) and not with GST alone (data not shown). This interaction is weakened by the presence of the non-hydrolyzable GTP analog, GMPPNP, which should stabilize Gsp1p in the GTP-bound form during the incubation with glutathione-agarose beads. However, we did not find any interaction of Yrb2p and Gsp1p under these conditions, and neither addition of GMPPNP nor GDPpS affected the result (Fig. 4B, GST-Yrb2).

We originally discovered Yrb1p because it co-purified with GST-Gsp1G21V mutant protein, whose mutation stabilizes the protein in the GTP-bound form (46). We repeated the solution binding assay as described in Fig. 4B with lysate from a strain expressing wild-type GST-Gsp1p and Yrb2::HAp. Yrb2::HAp was not found in the bound fraction even in the presence of any analogs, whereas Yrb1p co-purified with the GST-Gsp1p fusion only in the presence of the GMPPNP as expected (data not shown).

Gsp1p/Ran binds in its GDP-bound form to the small Ntf2p/p10 nuclear transport factor (67, 68). Recently, a protein referred to as Nup36p was shown to bind in solution to Ntf2p (68). Nup36p is identical to Yrb2p. We re-examined the binding of Yrb2::HAp to Ntf2p. Cell lysates containing Yrb2::HAp were incubated with agarose conjugated with Ntf2p (62). The resulting bound proteins were determined by immunoblotting (Fig. 5). We found a slight decrease in binding when the nonhydrolyzable analog GMPPNP was included, consistent with NTF2 preference for the GDP-bound form of Ran/Gsp1p. However, Yrb2::HAp was not observed in the Ntf2p bound fraction regardless of the nucleotide bound state of Gsp1p. Our results may differ because we used crude cell lysates, whereas the previous experiments were done with purified recombinant proteins where much higher concentrations can be achieved to overcome low affinities.

**Yrb2 Interacts with a Novel GTP-binding Protein**—The marginal affinity of Yrb2p binding to Gsp1p leads to the possibility that the Yrb2p/Prp20p complex might be involved in the regulation of a distinct GTPase. This idea is supported by the observation that Prp20p appears to interact with multiple GTP-binding proteins (69). To search for additional GTPases that may interact with Yrb2p, we carried out an [α-32P]GTP overlay assay. Proteins co-purified with GST-Yrb2p were reanalyzed on the membrane and probed with [α-32P]GTP (Fig. 6). A
single polypeptide of approximately 150 kDa was found to co-purify with Yrb2p and to bind GTP. A co-purifying protein of the correct size was only marginally detectable when the corresponding gel was silver-stained. No binding of [$\alpha^32P$]GTP was observed when high amounts of cold GTP were included to compete for binding, but inclusion of cold ATP had no effect (data not shown). Moreover, the amount of the 150-kDa GTP-binding protein was decreased when the nonhydrolyzable GDP analogue was included in the preparation of the GST-Yrb2p binding proteins. Also, the Ran binding domain of Yrb2p is required for this interaction as no GTP-binding proteins were observed when a GST-Yrb2p fusion deleted for this domain was used (see below).

The Yrb2p Ran Binding Domain Is Required for Function and Determines Its Binding Specificity—Other than its Ran binding domain, Yrb2p has no significant similarity to other known Ran binding proteins. Another possibility we considered is that the other regions of Yrb2p are inhibiting its ability to bind proteins.
The Yrb2-Yrb1 chimeric protein does not function in yrb1 temperature-sensitive mutants (Ref. 46, and data not shown). Taken together, these data suggest a distinct function for each Ran binding domain that cannot be exchanged.

The intracellular localization of the various Yrb2 mutant proteins was determined by immunofluorescence with the 12CA5 antibody (Fig. 4C). ΔC remained located inside the nucleus. On the other hand, most of ΔN and some of ΔNup were found in the cytoplasm, suggesting that information for Yrb2p nuclear localization resides in the N-terminal half. Two putative NLSs, KRPREK and KKEEGKKDQEPSHKKIK, can be found in the region at amino acids residues 33–38 and at 61–77 of Yrb2p, respectively. The partial function of the ΔN construct described above may be explained, in part, by a fraction of the protein entering the nucleus by passive diffusion since nuclear exclusion is not observed.

All mutant proteins were examined as GST fusions for their ability to bind to Gsp1p and its exchange factor (Fig. 4B). We were interested in the possibility that the inability of Yrb2p to strongly bind Gsp1p might be explained by conformational obstruction by the N terminus. However, we found little or no binding of Gsp1p to GST-ΔN, which contains only the Ran binding domain (Fig. 4B ΔN). Moreover, the GST-chimera still binds Gsp1p in the GTP-bound form, indicating that the Yrb1 Ran binding domain can still function properly when fused to the Yrb2 N terminus (Fig. 4B, Chimera). In addition, the level of GST-chimera is much lower than for the other test proteins (compare silver-stained bands in Fig. 4B). When cell lysate of increased protein concentration (20–30 times higher than lysate used in Fig. 4B) was used, we found a trace amount of Gsp1p that co-purified with GST-Yrb2p and was diminished by inclusion of nucleotide analogs (Fig. 7, GST-ΔC). The binding specificity to GTP-bound Gsp1p, which was previously reported (53), was not observed. This discrepancy may be generated by the presence of “high-affinity competitors” such as Yrb1p in a crude lysate. It is notable that the preference for GTP-Gsp1p of the chimeric protein is diminished under these conditions (compare two GST-Chimeras in Fig. 7).

In contrast to the results obtained for Gsp1p binding, the ability of Yrb2p to bind Prp20p was lost by the replacement of the Yrb2 Ran binding domain with that of Yrb1p. Similarly, deletion of the Yrb2p Ran binding domain resulted in loss of interaction with Prp20p while ΔN and ΔNup were still able to properly bind to Prp20p (Fig. 4B). Taken together, it appears that the ability to restore normal growth to the cold-sensitive Δyrb2 cells is linked with the ability of Yrb2p to bind to Prp20p, indicating that the interaction with Prp20p is essential for Yrb2 function.

3 G. Schlenstedt and P. A. Silver, unpublished observation.

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**Fig. 5. Binding assay of Yrb2::HAp to Ntf2p.** Lysate from cells expressing YRB2::HA was prepared and incubated with Ntf2p-conjugated agarose beads at 4 °C in the presence of GMPPNP or GDPβS at 1 mM. Preparation of samples for immunoblotting was carried out as described in Fig. 4B.

**Fig. 6. A 150-kDa GTP-binding protein is co-purified with Yrb2p.** GST-Yrb2 fusion protein or the C-terminal deleted mutant was expressed and purified with Glutathione-Sepharose beads in the presence of nonhydrolyzable GTP or GDP analogues as in Fig. 4B. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane, and GTP-binding proteins were screened by [α-32P]GTP overlay assay. After the overlay, the membrane was dried and subjected to autoradiography at −80 °C.

**Fig. 7. Detection of low affinity interaction of Yrb2p-Gsp1p by liquid phase binding assay.** Binding assay of GST-YRB2 derivatives to Gsp1p were carried out as in Fig. 4B with the indicated concentration of lysate.
DISCUSSION

The sequencing of the yeast genome has revealed only three proteins with similarity to the growing family of Ran binding proteins. We find that one of these, Yrb2p, is essential for growth only at low temperatures, and yrb2 mutant cells show no obvious defects in nuclear transport. However, we did find that Yrb2p interacts both functionally and physically with Prp20p, the Rcc1 homologue of yeast. Interestingly, any detectable interactions between Yrb2p and Gsp1p, the essential yeast Ran homologue, were marginal under the conditions in which we observe interactions with Prp20p. These findings lead us to propose that Yrb2p may be involved in displacement of Gsp1p from Prp20p and/or interaction with a GTPase other than Gsp1p.

Given that mutation of Yrb1p (the yeast homologue of mammalian RanBP1) resulted in defective nuclear transport (46), we might have expected a similar situation with Yrb2p. Instead, we find that unlike YRB1, YRB2 is dispensable for cell growth except at low temperature and is not required for normal nuclear transport. The same observations were recently reported by Noguchi et al. (53). These findings suggest that Yrb2p may be redundant with the function of some other proteins or that the process it is involved in is not slowed enough except at low temperature to cause an effect on growth. Moreover, this could suggest that the primary role of Yrb2p is in some process involving Gsp1p but separate from nuclear transport. Alternatively, any effect on transport may be too small to be observed by our in vivo assays. Since the discovery of the role of Ran/Gsp1p in nuclear transport, there has been a debate as to whether or not this is the primary cellular function of Ran. Ran and its regulators have historically been implicated in regulating nuclear events other than transport (70, 71). One possibility is that Yrb2p defines a regulatory pathway distinct from transport that impacts on other nuclear processes.

When compared with the Ran binding domains of other Ran binding proteins, the sequence of Yrb2p is very similar except for a region between amino acids 271 and 303. In this region, there is only similarity to Nup2p and a Schizosaccharomyces pombe protein Hba1p (72). Interestingly, all of these proteins have a lower affinity for Gsp1p or Spip1, an S. pombe Ran homologue (49, 72). In agreement with our results, Noguchi et al. (53) have recently also reported a lowered affinity of Yrb2p for Gsp1p. Nuclear localization of Hba1p (72) and similarity of its primary sequence with Yrb2p suggest that Hba1p is the functional equivalent of Yrb2p in S. pombe.

Yrb2p is concentrated inside the nucleus (53) as opposed to Yrb1p and Nup2p, which are concentrated at the nuclear pore (46, 52). Deletion of the N terminus results in a failure of Yrb2p to localize within the nucleus. Our preliminary data4 indicate that a region which is similar to a bipartite NLS is important for nuclear localization of Yrb2p. However, deletion of the N terminus or just the “Nup” domain does not affect the ability of Yrb2p to restore normal growth at low temperature to a strain deleted for yrb2 or to allow Δγrb2 prp20Δ mutants to grow. Thus, this region does not have an essential function for activity though it is required for full nuclear localization. One possibility is that, even when missing a potential NLS, enough protein enters the nucleus to allow it to function. There is precedent for this as in the case of GAL4 (73).

However, the Yrb2p Ran binding domain is essential for its function. Deletion of the Ran binding domain results in a non-functional protein in that it cannot rescue either the cold-sensitivity of the yrb2 null or synthetic lethality of Δγrb2 and prp20Δ. This domain is also required for the interaction of Yrb2p with Prp20p because, if it is deleted or replaced with the Yrb1p Ran binding domain, Prp20p no longer co-purifies. Although it is still not clear if formation of the Yrb2p/Prp20p complex is mediated by Gsp1p, it is likely that each Ran binding domain has a specific function besides simply interacting with Ran.

Both the biochemical and genetic data presented here suggest that the intracellular function of Yrb2p is tightly linked to that of Prp20p. Both proteins are located in the nucleus and can be isolated together. Synthetic lethality between Δγrb2 and prp20 indicates that the proteins may function in the same reaction “pathway.” Since Prp20p is the major yeast homologue of Rcc1 (24), it is reasonable to suspect that it is the major regulator of nucleotide exchange by Gsp1p. This is supported by genetic (17, 36) and physical5 interactions between Gsp1p and Prp20p. One possibility is that Yrb2p may regulate the exchange reaction by stabilizing the Gsp1p-Prp20p complex. However, we do not detect significant amounts of Gsp1p in the Yrb2p Prp20p complex, and others observed no effect of Yrb2p on the GDP-GTP exchange reaction (53). Another possibility is that Yrb2p and Gsp1p might compete for interaction with Prp20p in the cell.

Another proposal is to assume an additional GTPase exists besides Gsp1p. In this model, the unknown GTPase would mediate the Yrb2p-Prp20p interactions. This GTPase cycle might be required for nuclear functions distinct from transport. Prp20p may serve two different GTPase cycles as a GDP/GTP exchanger. One candidate for an additional GTPase would be Gsp2p, a non-essential, second Ran homologue in S. cerevisiae (17, 36). However, we have found no genetic evidence for interactions between YRB2 and GSP2.4 And it is generally believed that the function of Gsp2p is the same as Gsp1p because GSP2 can rescue a gsp1 mutation. Recently, a putative GTPase termed Gtr1p has been identified as a suppressor of PRP20/MTR1 temperature-sensitive mutants (74). The suppressor mutant also shows cold-sensitive growth as is the case for Δγrb2. Although Gtr1p is localized predominantly in the cytoplasm, it may be sufficient to transiently interact with Prp20p (perhaps at the NPC), and Yrb2p may be involved in such a complex. However, we did not detect any genetic interactions with Gtr1p.4 Instead, we find a novel 150-kDa GTP-binding protein associated with Yrb2p, and this association is sensitive to the nucleotide-bound state.

In summary, we conclude that Yrb2p interacts with Prp20p and is required for its proper function. Future experiments will be focused on understanding more about how this interaction interfaces with other GTP-mediated nuclear processes.

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