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Yrb4p, a yeast Ran–GTP-binding protein involved in import of ribosomal protein L25 into the nucleus

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Gsp1p, the essential yeast Ran homologue, is a key regulator of transport across the nuclear pore complex (NPC). We report the identification of Yrb4p, a novel Gsp1p binding protein. The 123 kDa protein was isolated from *Saccharomyces cerevisiae* cells and found to be related to importin-β, the mediator of nuclear localization signal (NLS)-dependent import into the nucleus, and to Pse1p. Like importin-β, Yrb4p and Pse1p specifically bind to Gsp1p–GTP, protecting it from GTP hydrolysis and nucleotide exchange. The GTPase block of Gsp1p complexed to Yrb4p or Pse1p is released by Yrb1p, which contains a Gsp1p binding domain distinct from that of Yrb4p. This might reflect an *in vivo* function for Yrb1p. Cells disrupted for *YRB4* are defective in nuclear import of ribosomal protein L25, but show no defect in the import of proteins containing classical NLSs. Expression of a Yrb4p mutant deficient in Gsp1p-binding is dominant-lethal and blocks bidirectional traffic across the NPC in wild-type cells. L25 binds to Yrb4p and Pse1p and is released by Gsp1p–GTP. Consistent with its putative role as an import receptor for L25-like proteins, Yrb4p localizes to the cytoplasm, the nucleoplasm and the NPC.

Keywords: importin/nuclear transport/Ran/YRB1/YRB4

Introduction

Exchange of macromolecules between the nucleus and the cytoplasm requires an efficient transport system across the nuclear envelope. The bidirectional transport of proteins and RNAs is mediated by the nuclear pore complex (NPC) and a number of soluble factors (for review see Görlich and Mattaj, 1996; Panté and Aebi, 1996; Schlenstedt, 1996). While much is already known about the nuclear import of proteins, the putative export pathways are less well understood. Nuclear import of proteins is a highly selective, energy-requiring process which can be divided into several distinct phases (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988). First, the nuclear localization sequence (NLS) within an import substrate is specifically recognized by an import receptor. The receptor–substrate complex then binds to the NPC. The subsequent step is the energy-dependent translocation of this complex across the NPC.

Using an *in vitro* assay to reconstitute nuclear import (Adam *et al.*, 1990), four soluble proteins have been identified which are essential for the transport reaction. These are the two subunits of the NLS-receptor (importin-α and -β), the small GTPase Ran/TC4, and the nuclear transport factor p10/NTF2 (for review see Görlich and Mattaj, 1996; Nigg, 1997). Importin-α binds via an N-terminal domain to importin-β (Görlich *et al.*, 1996a; Weis *et al.*, 1996a) and also binds to proteins containing NLSs consisting of one or two clusters of basic amino acid residues. Importin-β mediates the docking of this trimeric complex to the NPC and the subsequent translocation (for review, see Dingwall and Laskey, 1991; Görlich and Mattaj, 1996; Nigg, 1997).

The small Ras-related GTP-binding protein Ran (termed Gsp1p in yeast) is essential for energy-dependent protein import into the nucleus (Melchior *et al.*, 1993; Moore and Blobel, 1993; Schlenstedt *et al.*, 1995a; Görlich *et al.*, 1996b). Ran is a highly abundant protein that alternates between the GTP-bound and the GDP-bound form. The interconversion between these two nucleotide binding states occurs by GTP hydrolysis and guanine nucleotide exchange. The rates for both processes are very low, but are stimulated by up to five orders of magnitude by the nuclear guanine nucleotide exchange factor RCC1/Prp20p and the cytoplasmic GTPase activating protein RanGAP1/Rna1p (for review see Görlich and Mattaj, 1996; Koepp and Silver, 1996; Schlenstedt, 1996). Given the localization of the nucleotide exchange factor and the Ran–GAP on opposite sides of the nuclear envelope, it is likely that Ran–GTP predominates in the nucleus while Ran–GDP is present in the cytoplasm. This would suggest that Ran shuttles between the nucleoplasm and the cytoplasm, and that the state of the bound nucleotide determines compartment-specific interactions with components of the transport machinery.

One class of Ran–GTP-binding proteins is characterized by a RanBP1-homology domain (for review see Schlenstedt, 1996). RanBP1, the prototype of this family, specifically binds to the GTP-bound form of Ran (Coutavas *et al.*, 1993), rendering it resistant to GTP exchange but facilitating GTPase activation by RanGAP1 (Beddow *et al.*, 1995; Bischoff *et al.*, 1995b). Recently, a functional nuclear export signal (NES) on RanBP1 was identified (Richards *et al.*, 1996, 1997; Zolotukhin and Felber, 1997).

Yrb1p, the yeast homologue of RanBP1, is a cytoplasmic protein essential for growth. *YRB1* mutants exhibit defects in protein import and mRNA export (Schlenstedt *et al.*, 1995b). However, the precise role of RanBP1/Yrb1p in nuclear transport is currently unknown.

Ran–GTP appears to exert two distinct functions during NLS-dependent protein import. First, GTP hydrolysis by Ran is required for transport (Melchior *et al.*, 1993; Moore and Blobel, 1993; Corbett *et al.*, 1995; Weis *et al.*, 1996b). This might represent the energy source for translocation across the NPC. However, it is unclear whether one single or multiple RanGTPase cycles are necessary to complete a single round of import. Second, Ran–GTP mediates import termination in the nucleus (Görlich *et al.*, 1996b) by direct binding to importin-β, which dissociates the importin heterodimer (Rexach and Blobel, 1995; Görlich *et al.*, 1996b). To ensure that this dissociation of the nuclear import receptor is restricted to the nucleus, the cytoplasmic concentration of Ran–GTP is kept low by the Ran-specific GAP. An importin-β mutant deficient in Ran-binding, and therefore unable to be dissociated from importin-α and the NPC, does not promote the complete import reaction. The import substrate was translocated through the nuclear pore but was arrested at the nucleoplasmic side of the NPC (Görlich *et al.*, 1996b). This mutant dominantly inhibits other transport pathways across the NPC as well (Kutay *et al.*, 1997).

Interestingly, the termination of NLS-dependent nuclear import results in an importin-β–Ran–GTP complex in which the Ran-bound nucleotide is protected from exchange and hydrolysis (Floer and Blobel, 1996; Görlich *et al.*, 1996b). This raises the question as to how Ran and importin-β are recycled for the next round of nuclear import.

Recently, an importin-β-independent nuclear import pathway for proteins containing an M9 domain was identified. It is mediated by transportin, a novel transport receptor related to importin-β, which binds directly to M9-containing proteins like hnRNP A1. Kap104p, the transportin homologue in yeast, is involved in import of the mRNA-binding proteins Nab2p and Nab4p. Interestingly, importin-α is not required for this import route (Aitchison *et al.*, 1996; Pollard *et al.*, 1996).

Here we identify and characterize Yrb4p, an importin-β-related protein which binds specifically to the GTP-bound form of Gsp1p. Yeast cells lacking *YRB4* show a defect in nuclear import of a reporter containing the nuclear targeting signal of ribosomal protein L25 but not of proteins containing classical NLSs. Expression of a mutant Yrb4p which is unable to bind Ran–GTP results in a dominant-lethal phenotype indicating that Gsp1p binding is crucial for Yrb4p function. This mutant causes a severe block of bidirectional transport across the NPC. We propose that Yrb4p, which binds to L25, functions like other members of the importin-β family and represents a nuclear import factor recognizing L25-like proteins.

Results

Detection of Ran-binding proteins in Saccharomyces cerevisiae

In an attempt to identify new Gsp1p-binding proteins in *S.cerevisiae*, we performed an overlay blot with [γ-^{32}P]GTP-charged Gsp1p, the yeast homologue of Ran. Using this approach, two major bands of ~34 and 120 kDa were detected in cell lysates (Figure 1A). Competition experiments showed that the corresponding proteins specifically interact with the GTP-bound form of Gsp1p. Binding was prevented by a 100-fold excess of unlabeled Gsp1p–GTP but not Gsp1p–GDP. Human Ran–GTP and Gsp1p–GTP bound equally well to both proteins (not shown). Fractionation of *S.cerevisiae* cells revealed that both Gsp1p-binding proteins are mainly located in the cytoplasm. The 34 kDa band corresponded to Yrb1p. This was confirmed by using extracts from a *yrb1–2* strain (not shown) in which Yrb1p is Ran-binding deficient (Schlenstedt *et al.*, 1995b).

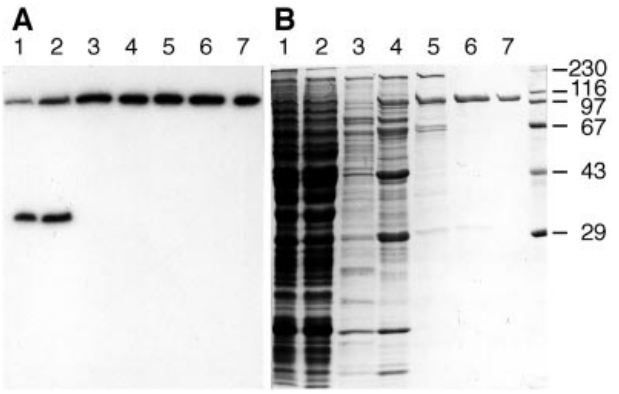

Fig. 1. Detection and purification of Gsp1p-binding protein Yrb4p. Fractions obtained after each purification step (see Materials and methods) were resolved by SDS–gel electrophoresis and assayed with Ran[γ^{32}P]GTP after transfer onto nitrocellulose (**A**), or stained with Coomassie brilliant blue (**B**). Lane numbers correspond to fraction numbers in the enrichment table. Molecular weight markers are shown in kDa.

Table I. Enrichment of Yrb4p

Fraction	Yrb4p (mg)	Total protein (mg)	Purification (fold)	Yield (%)
1. Complete lysate	826	2.4		100
2. 70 000 *g* sup.	430	2.1	1.7	88
3. DMAE fractogel	87	1.9	7.5	79
4. Hydroxylapatite	19	1.6	29	67
5. Hydrophobic chrom.	4.1	1.5	126	62
6. Mono Q	0.53	0.5	325	21
7. Gel filtration	0.45	0.45	344	19

Values refer to 9×10^{10} cells as starting material.

Purification of Yrb4p from yeast cytosol

The 120 kDa Gsp1p–GTP-binding protein, termed Yrb4p, was purified to homogeneity (Table I, Figure 1A and B) from the soluble fraction of *S.cerevisiae*. The purification yielded a 344-fold enrichment. We calculated that there are ~10^5 Yrb4p molecules present in a yeast cell, i.e. Yrb4p represents ~0.3% of total soluble protein. Information obtained from partial peptide sequencing of Yrb4p (Figure 2) showed that it corresponds to an open reading frame located on chromosome V of *S.cerevisiae* (YER110C). The *YRB4* gene encodes a protein of 1113 amino acid residues with a predicted molecular weight of 122.6 kDa. The sequence displays similarity to Pse1p (protein secretion enhancer) (Chow *et al.*, 1992), and to a lesser extent

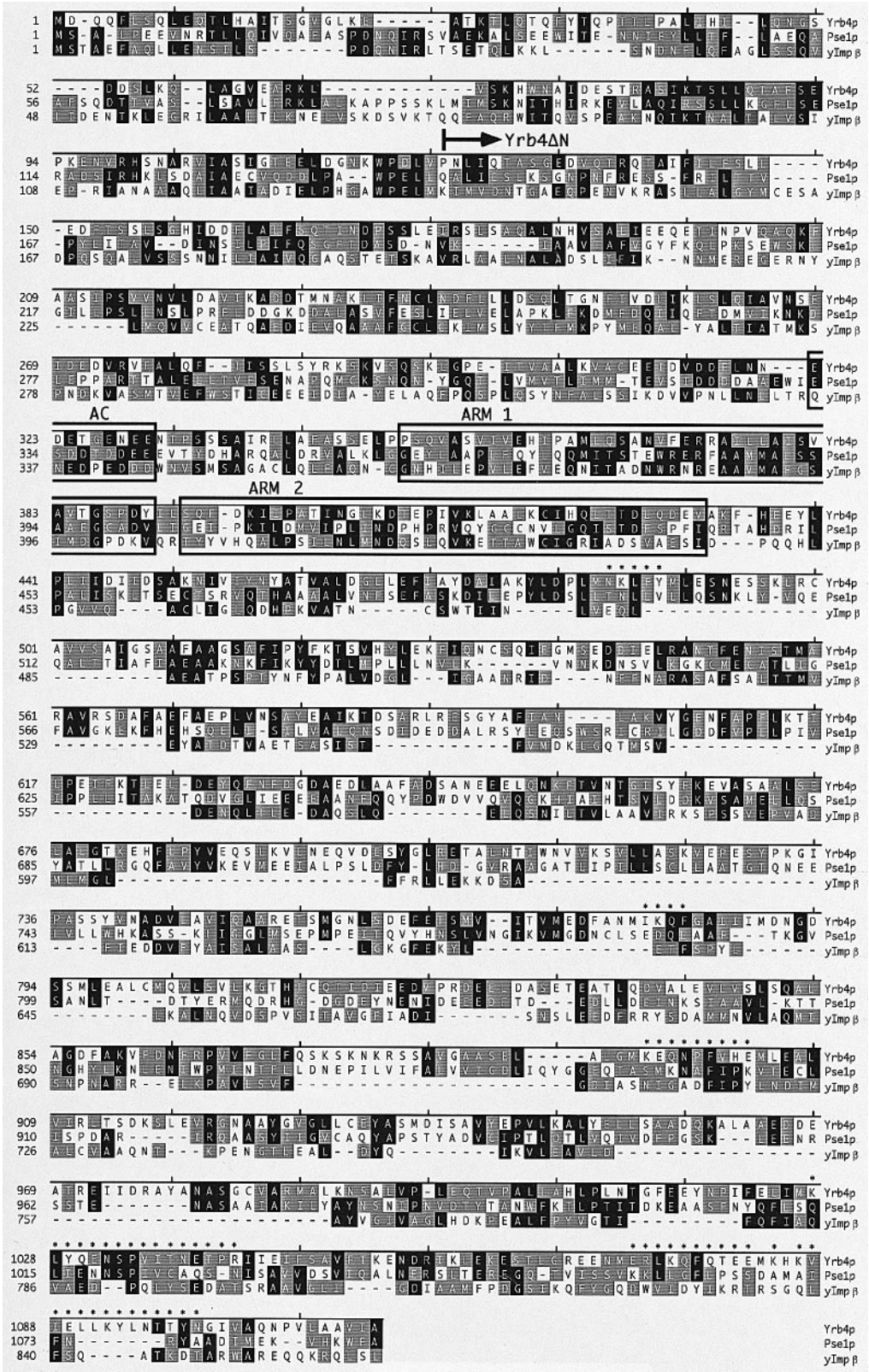

Fig. 2. Amino acid sequence of Yrb4p and alignment with yeast importin-β and Pse1p. The three proteins were aligned using the Clustal megalign program of DNASTAR (Lasergene). Yrb4p is 19% identical (48% similar) to Pse1p (P32337), and 12% identical (35% similar) to yeast importin-β (U19028). Identical residues are shown in black, related residues are shown in grey. The acidic cluster (AC) and the ARM motifs are boxed. Peptide sequences obtained from purified Yrb4p are indicated by asterisks. The translation start of Yrb4ΔNp lacking the 123 N-terminal amino acid residues is indicated. The Yrb4p sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number U75972.

to the *S.cerevisiae* importin-β homologue (yImpβ). An acidic cluster of 9 residues in Yrb4p (positions 322–330) is followed by two 'arm' motifs (domains of 42 amino acid residues) (Peifer *et al.*, 1994) which are also present in importin-β, Pse1p, Kap104p and importin-α/Srp1p (Figure 2). More degenerate 'arm' motifs are found in the C-terminal part of the protein. In addition, Yrb4p shows similarity to a number of importin-β-related proteins from different species. These proteins have recently been described as belonging to a group of Ran-binding proteins which exhibit significant similarity within their N-terminal domains, and which are proposed to represent a family of nuclear transport factors (Fornerod *et al.*, 1997; Görlich *et al.*, 1997).

Nuclear import of a L25-reporter construct is impaired in cells lacking YRB4

The *YRB4* gene was cloned by PCR from genomic DNA. To determine whether *YRB4* is essential, a large fragment of the gene was replaced by the *TRP1* gene in one *YRB4* copy of a diploid strain. After sporulation and tetrad dissection, a four haploid progeny was observed, indicating that *YRB4* is not an essential gene. Strains bearing the marked Δ*yrb4* alleles lack any detectable Yrb4p (see Figures 5E and G and 6B). The growth rate of the null strain in rich media is reduced by ~10–20%. Microscopic examination of Δ*yrb4* cells revealed an increase in cell size in comparison with isogenic wild-type cells (see Figure 3).

We used several assays to determine whether cells lacking *YRB4* display a defect in nucleocytoplasmic transport. *YRB4::TRP1* cells show a normal distribution of poly(A)$^+$RNA, indicating that they do not have a defect in mRNA export. We tested a panel of reporter proteins for proper import into the nucleus. These included endogenous and plasmid-encoded proteins containing different nuclear targeting signals. We detected no mislocalization of Srp1p (yeast importin-α) and yImpβ (Figure 5K). A small fraction (~10%) of the cells showed some cytoplasmic accumulation of normally nuclear localized Npl3p, as well as of a fusion of the green fluorescent protein (GFP) to the C-terminal 139 amino acid residues of Npl3p (Corbett *et al.*, 1995) (not shown). Nuclear reporter constructs consisting of histone H2B fused to GFP (Schlenstedt *et al.*, 1995a), and SV40 NLS of large T antigen fused to cytoplasmic invertase or β-galactosidase (Figure 3D) were correctly targeted to the nucleus. However, a fusion protein consisting of the N-terminal nuclear targeting signal of ribosomal protein L25 and β-galactosidase (Schaap *et al.*, 1991) was dramatically mislocalized. This reporter protein was located exclusively in the nucleus of wild-type cells (Figure 3G), whereas it accumulated in the cytoplasm in the majority of Δ*yrb4* cells (Figure 3J), indicating a defect in nuclear import. The N-terminal domain of L25 was previously shown to contain a complex nuclear targeting signal (Schaap *et al.*, 1991). The L25 reporter protein did not accumulate in the cytoplasm of *rsl1–1* cells, an importin-β mutant which strongly mislocalizes proteins containing classical NLSs (Koepp *et al.*, 1996), even after a prolonged shift to the non-permissive temperature (not shown).

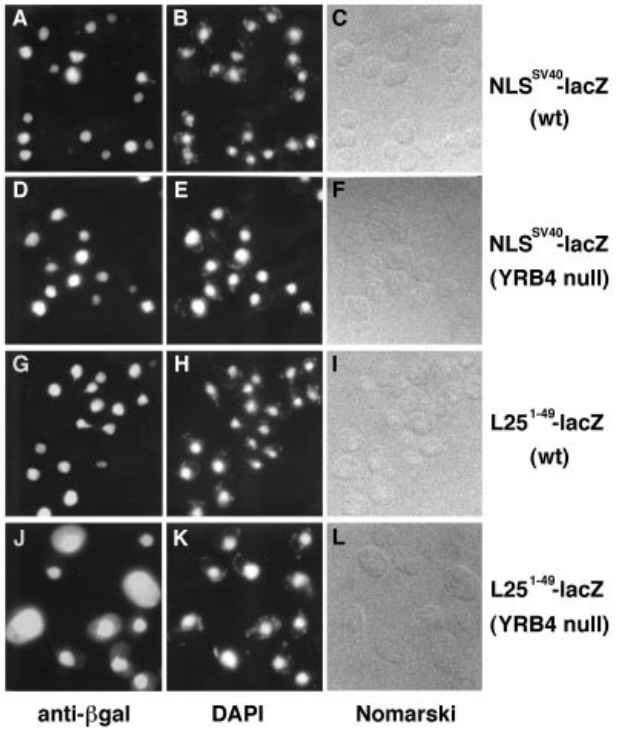

Fig. 3. In cells disrupted for *YRB4*, the nuclear reporter L25$^{1–49}$-β-galactosidase is mislocalized. Wild-type cells (**A**–**C** and **G**–**I**) or cells disrupted for *YRB4* (**D**–**F** and **J**–**L**) carrying plasmids encoding constitutively expressed β-galactosidase fused to the SV40 large T antigen NLS (pGS226, A–F), or fused to the nuclear targeting signal of ribosomal protein L25 (pGS304, G–L), were fixed with formaldehyde for 60 min and prepared for immunofluorescence. Cells were probed with anti-β-galactosidase antibodies (Promega) and FITC-conjugated anti-mouse IgG, and stained with DAPI or viewed by Nomarski optics as indicated. Microscopy was performed using a Zeiss Axioscope at 1000×magnification.

Yrb4p and Pse1p bind to ribosomal protein L25

To date, two types of nuclear import receptors have been described. The first is represented by importin in which the α-subunit serves as an adapter for binding of proteins containing a classical NLS. A prototype of the second is the importin-β related transportin/Kap104p which directly binds to the import substrate (Aitchison *et al.*, 1996; Pollard *et al.*, 1996).

To identify transport substrates for Yrb4p, we expressed Yrb4p and its relatives yImpβ, Pse1p and Kap104p as fusion proteins to glutathione-*S*-transferase (GST) in yeast. Overexpression of these proteins in *S.cerevisiae* is lethal, indicating that they are functional (Figure 4A). After affinity-isolation of the GST fusion proteins from yeast lysates, copurifying proteins were analyzed. Only in the case of yImpβ could an interaction with Srp1p be detected (Figure 4B). This indicates that the used yImpβ was functional and that the yImpβ-related proteins do not bind to the α-subunit of the NLS receptor, which was further suggested by gel filtration experiments (not shown). However, the failure to detect stoichiometric binding partners of Yrb4p in Coomassie-stained gels might indicate that the amount of monomeric transport-competent L25 protein was limiting in this assay. Therefore, we added *in vitro*-synthesized [^{35}S]methionine-labeled L25 as a reticulocyte lysate to the GST fusion proteins bound to glutathione sepharose. The beads were washed and bound proteins

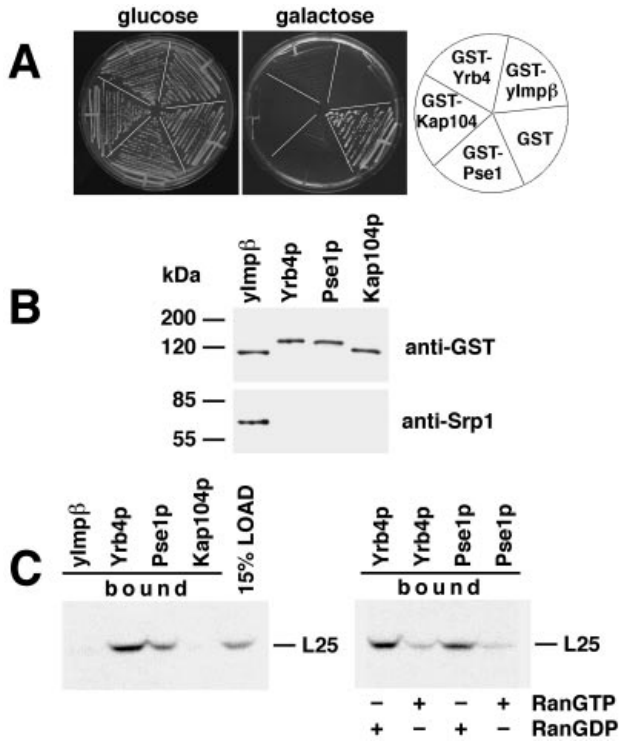

Fig. 4. Ribosomal protein L25 binds to Yrb4p and to Pse1p and is released by Ran–GTP. (**A**) Wild-type yeast cells were transformed with high-copy plasmids encoding GST or GST fused to yeast importin-β, Yrb4p, Pse1p or Kap104p, streaked on selective media containing glucose (repression) or galactose (induction) and incubated at 30°C for 2 days. (**B**) GST fusion proteins were affinity-purified with glutathione sepharose, eluted and analyzed by SDS–PAGE and immunoblotting using anti-GST or anti-Srp1p antibodies. Molecular weight markers are indicated. (**C**) Full-length [^{35}S]methionine-labeled L25 was synthesized in a reticulocyte lysate. Aliquots were incubated with GST fusion proteins bound to glutathione sepharose. Bound proteins were eluted (left) or incubated further with purified RanQ69L (a mutant Ran deficient in GTP-hydrolysis) loaded with GTP or GDP and then eluted (right). Samples and a fraction corresponding to 15% of the load were analyzed by SDS–PAGE and fluorography.

were eluted. As shown in Figure 4C, L25 bound to Yrb4p and Pse1p, but not to yImpβ and Kap104p. Next we added purified Ran–GTP or Ran–GDP to the preformed Yrb4p–L25 or Pse1p–L25 complexes. This resulted in the dissociation of L25 from Pse1p and Yrb4p only in the case of added Ran–GTP (Figure 4C). These results show that Yrb4p and Pse1p could bind to ribosomal protein L25, and that this interaction, in a manner similar to that of importin-α and -β, can be dissociated by Ran–GTP.

Immunolocalization of Yrb4p

In order to determine the intracellular localization of Yrb4p, the full length protein was bacterially expressed, purified and used for the immunization of rabbits. On blots, affinity-purified antibodies recognized both bacterially synthesized Yrb4p and Yrb4p purified from yeast cells. In total yeast cell extracts, a single band corresponding to a molecular size of 120 kDa was detected (Figure 6B). Indirect immunofluorescence was performed on formaldehyde-fixed and methanol-fixed cells. Using the formaldehyde fixation protocol, we detected Yrb4p equally distributed between the cytoplasm and the nucleus (Figure 5C; for comparison see cytoplasmic Yrb1p in Figure 5O). Since the cytoplasmic signal with this fixation method would obscure any signal at the NPC, we also used methanol fixation which results in a loss of soluble proteins and thereby facilitates the detection of structure-bound proteins (Rout and Kilmartin, 1990). Under these conditions, we detected a mainly punctate nuclear rim staining typically observed for nucleoporins, whereas the cytoplasmic signal for Yrb4p was essentially lost (Figure 5A; for comparison see Yrb1p in Figure 5M).

We also localized yImpβ by the same methods. Importin-β is known to localize to the NPC, but it is also present in the cytoplasm (Chi *et al.*, 1995; Koepp *et al.*, 1996). Like Yrb4p, yImpβ localizes to the nuclear envelope in methanol-fixed cells (Figure 5I). In formaldehyde-fixed cells, it is found in the cytoplasm, but compared with Yrb4p, a larger proportion was detected within the nucleus (Figure 5K). Mammalian importin-β, however, was hardly detected in the nuclear interior (Chi *et al.*, 1995; Görlich *et al.*, 1995; Moroianu *et al.*, 1995).

To confirm that Yrb4p localizes to the NPC, we performed double labeling experiments with anti-Yrb4 antibodies and MAb 414, a monoclonal antibody that binds to several NPC epitopes (Davis and Fink, 1990). Figure 5Q–V shows that Yrb4p colocalized with nucleoporins recognized by MAb 414, both in wild-type cells and in *rat2–1* mutants (Heath *et al.*, 1995) which cluster their NPCs into a small region of the nuclear envelope. We conclude that Yrb4p is located both in the cytoplasm and in the nucleus, but is also present at the NPC.

Expression of a Yrb4p mutant deficient in Ran-binding results in a dominant block of nuclear transport

To test the effects of *YRB4* overexpression on nuclear transport, we introduced the *YRB4* coding sequence under the control of the *GAL1* promoter on a centromeric plasmid (pGAL-*YRB4*) into a wild-type strain. When *YRB4* expression was induced by addition of 2% galactose to the culture media, the cells continued to grow at a reduced rate (Figure 6A). It was recently shown that a mutant importin-β lacking the N-terminal 44 amino acid residues was deficient in Ran–GTP-binding. This mutant protein fails to complete the import reaction (Görlich *et al.*, 1996a) and thereby blocks the NPC for subsequent transport events (Kutay *et al.*, 1997). We constructed a similar deletion mutant of Yrb4p which lacks the N-terminal 123 amino acid residues (Figure 2). In wild-type cells, overexpression of this protein, termed Yrb4ΔNp, caused a complete cessation of growth. Cells did not form colonies on plates containing galactose, and in liquid cultures the growth arrest was complete 6 h after galactose addition (Figure 6A).

Immunoblots showed that plasmid-encoded Yrb4p and Yrb4ΔNp are expressed ~3-fold over wild-type Yrb4p levels after a 3 h galactose shift. Gsp1p[γ-^{32}P]GTP-binding was not detected for the faster migrating Yrb4ΔNp in an overlay assay, indicating that Yrb4ΔNp is indeed deficient in Gsp1p-binding (Figure 6B).

In initial *in vivo* studies, we observed a cytoplasmic accumulation of NLS-containing nuclear reporter proteins following overexpression (3 h galactose shift) of Yrb4p and yImpβ (not shown). This indicates that excess amounts of free Yrb4p or yImpβ might compete with NLS–

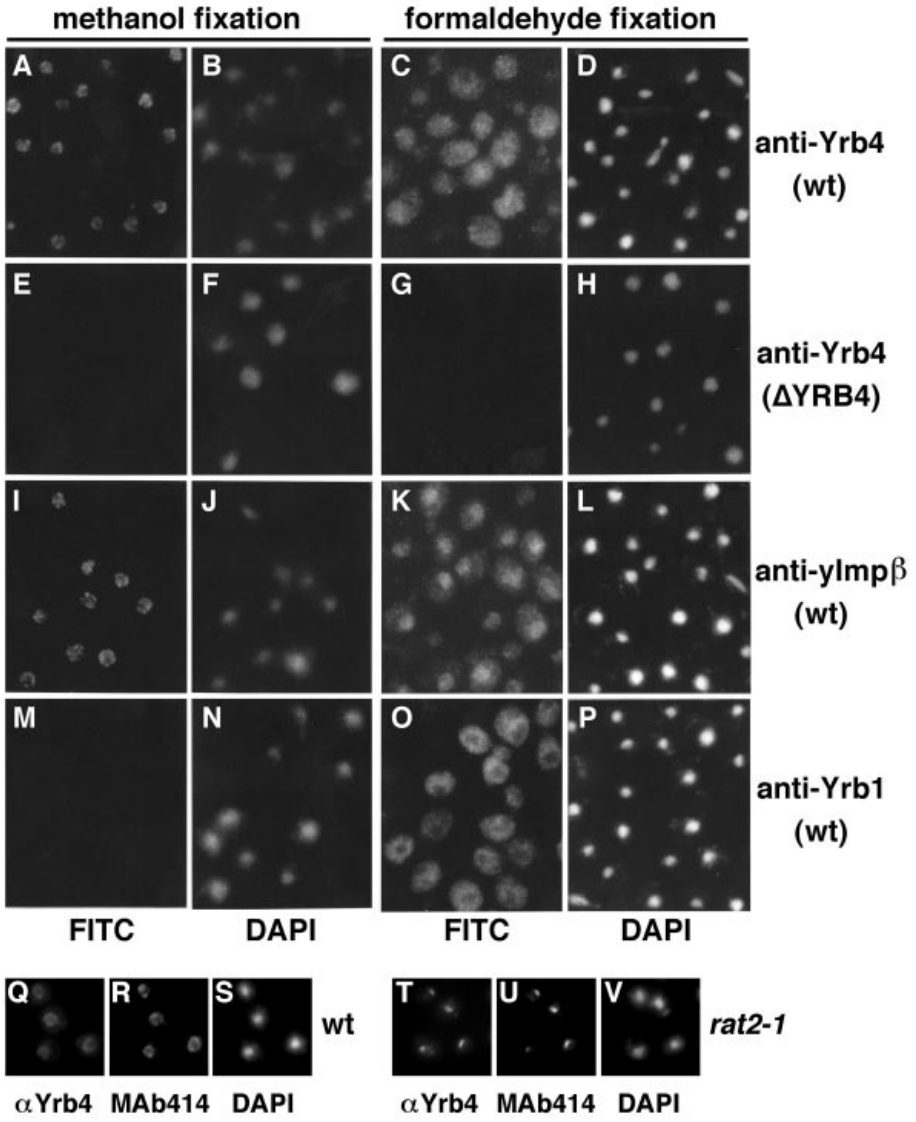

Fig. 5. Yrb4p is located in the cytoplasm, the nucleoplasm and at the nuclear pore complex. Haploid wild-type cells (GSY 154, **A**–**D** and **I**–**S**), cells disrupted for *YRB4* (GSY 393, **E**–**H**) or *rat2–1* cells (GSY 53, **T**–**V**) were grown at 30°C (A–S) or 25°C (T–V) and prepared for immunofluorescence by formaldehyde fixation (15 min) or methanol fixation (see Materials and methods). Cells were probed with the anti-nucleoporin mouse MAb 414 (Berkeley Antibodies Co.), or affinity-purified rabbit antibodies against Yrb4p, yeast importin-β or Yrb1p, and stained with DAPI to visualize DNA as indicated. Cells shown in panels Q–V are double-labeled using FITC-conjugated anti-mouse IgG and Texas Red-conjugated anti-rabbit IgG as secondary antibodies.

receptor–substrate complexes for common NPC docking sites. Next we compared the phenotypes of cells overexpressing the dominant-lethal mutant Yrb4ΔNp or Yrb4p with that of wild-type cells. To analyze nuclear import, wild-type cells containing either the plasmid pGAL without insert, pGAL-*YRB4* or pGAL-*YRB4ΔN* were tested for the localization of a reporter protein consisting of the NLS of SV40 large T antigen fused to cytoplasmic invertase. Cells were grown in the presence of 2% galactose for 3 h and then analyzed by indirect immunofluorescence microscopy. In wild-type cells, NLS-invertase localized to the nucleus (Figure 7A). However, cells overexpressing Yrb4p or Yrb4ΔNp accumulated NLS-invertase in the cytoplasm. Yrb4ΔNp expressing cells are distinguished by a clear reduction of nuclear located NLS-invertase (Figure 7B and C). In addition, Yrb4ΔNp expression also caused mislocalization of the reporter proteins SV40 NLS-GFP-β-galactosidase and $L25^{1–49}$-GFP-β-galactosidase (not shown).

To test for a possible defect in mRNA export, we analyzed the cellular distribution of poly(A)$^+$RNA in cells under the same conditions. As expected, poly(A)$^+$RNA was observed in the cytoplasm of wild-type cells (Figure 7G). Poly(A)$^+$RNA in cells overexpressing Yrb4p is mainly cytoplasmic, but in some cells is concentrated in a small region of the nucleus which corresponds to the nucleolus (Figure 7H). However, essentially all cells expressing Yrb4ΔNp show a dramatic accumulation of poly(A)$^+$RNA in the nucleus, and a concomitant disappearance of cytoplasmic staining (Figure 7I).

Next we tested the effects of overexpression of Yrb4p or Yrb4ΔNp on the distribution of endogenous Srp1p, Yrb1p, Npl3p, Nop1p and nucleolar antigens recognized by MAb 9C4 (Bossie *et al.*, 1992; Tollervey *et al.*, 1993).

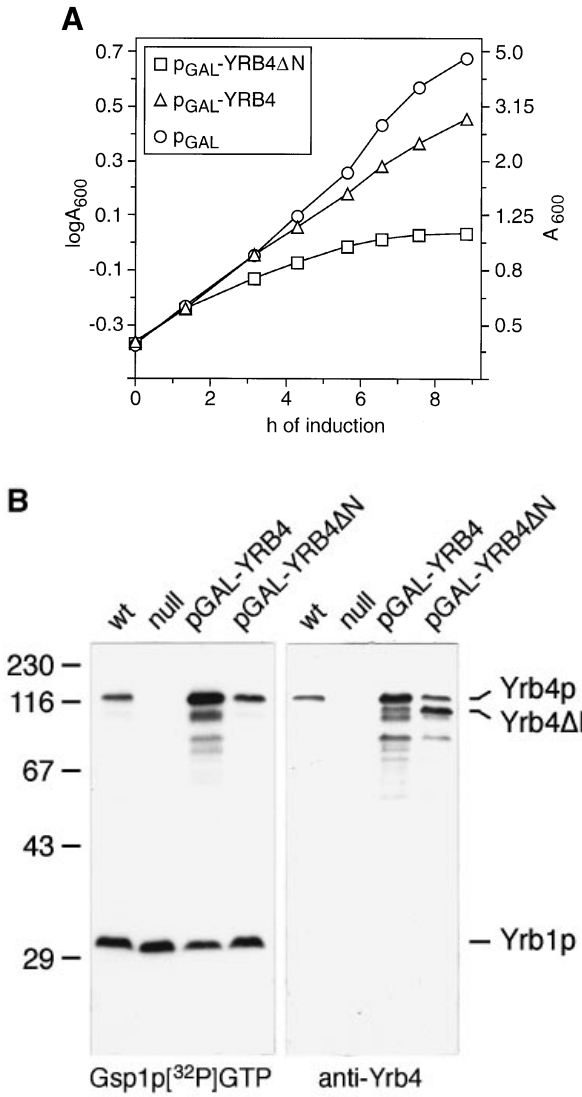

Fig. 6. Yrb4ΔNp is deficient in Ran-binding and causes a dominant-lethal phenotype. Wild-type cells transformed with plasmids pGAL, pGAL-*YRB4* or pGAL-*YRB4ΔN* were grown in raffinose containing media. Two per cent galactose was added (at 0 h) to induce expression. Growth rates were determined by measurement of the optical density at 600 nm (**A**). Cells disrupted for *YRB4* (null) and wild-type cells transformed with plasmids pGAL (wt), pGAL-*YRB4* or pGAL-*YRB4ΔN* were grown in the presence of 2% galactose for 3 h. Extracts were separated by SDS–PAGE, transferred to nitrocellulose and probed with Gsp1p[γ-^{32}P]GTP or with affinity-purified anti-Yrb4 antibodies. Molecular weight markers are shown in kDa (**B**).

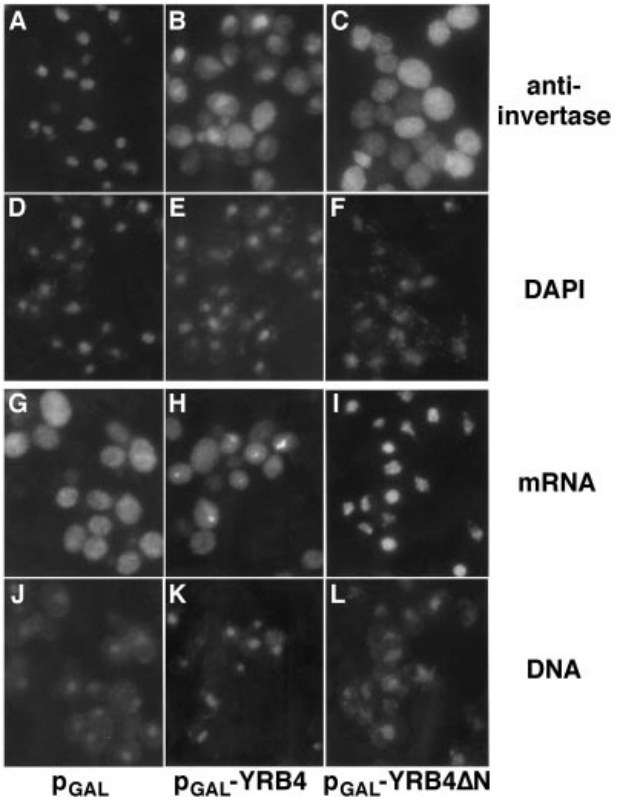

Fig. 7. Overexpression of Yrb4ΔNp causes mislocalization of a nuclear reporter protein and of poly(A)$^+$ RNA. Wild-type cells containing plasmids pGAL (A, D, G, J), pGAL-*YRB4* (B, E, H, K), pGAL-*YRB4ΔN* (C, F, I, L) or pGAL-NLS-*SUC2* (A–F) were grown in the presence of 2% galactose for 3 h. Cells were prepared for immunofluorescence to localize NLS-invertase (**A**–**F**) or for *in situ* hybridization to localize mRNA (**G**–**L**) and stained with DAPI.

Srp1p, the yeast importin-α homologue, shuttles between the nucleoplasm and the cytoplasm. Using standard immunofluorescence conditions, Srp1p and Yrb1p are observed mainly in the cytoplasm of wild-type cells and of cells overexpressing Yrb4p. However, Yrb4ΔNp overexpression resulted in a dramatic accumulation of Srp1p and Yrb1p within the nucleus (Figure 8). Nuclear accumulation might indicate that both proteins could shuttle between the cytoplasm and the nucleus, and that expression of Yrb4ΔNp inhibited their export more strongly than their import into the nucleus.

Using anti-Nop1 antibodies and MAb 9C4, we observed that the corresponding antigens always remained associated with the nucleolus. However in cells expressing Yrb4ΔNp, the nucleolus undergoes fragmentation, a phenomenon often observed in mRNA export mutants (Kadowaki *et al.*, 1994).

Endogenous Npl3p, a soluble protein that in wild-type cells is localized to the nucleus but shuttles between the nucleoplasm and the cytoplasm, accumulated only weakly in the cytoplasm after overexpression of Yrb4ΔNp (not shown), indicating that the integrity of the nuclear envelope was not affected.

In summary, we observed a protein import defect and a minor mRNA export defect in cells overexpressing Yrb4p. However, expression of Yrb4ΔNp results in a severe block of bidirectional transport across the NPC.

Modulation of GTP-exchange on Gsp1p by Yrb4p

In order to characterize the biochemical properties of the Gsp1p-binding proteins Yrb4p, yImpβ, Pse1p and Yrb1p in more detail, we tested the effect of the purified proteins on guanine nucleotide binding and hydrolysis on Gsp1p. First, we examined the stimulation of guanine nucleotide exchange by EDTA, which complexes magnesium ions required for tight binding of GTP and GDP to Gsp1p. Gsp1p[^{32}P]GTP/GDP was preincubated with the Gsp1p-binding proteins. Then EDTA and an excess of unlabeled GDP were added for a 5 min reaction. Figure 9A shows that all tested Gsp1p-binding proteins inhibited GTP exchange whereas the dissociation of GDP was not influenced. This is in agreement with previously described

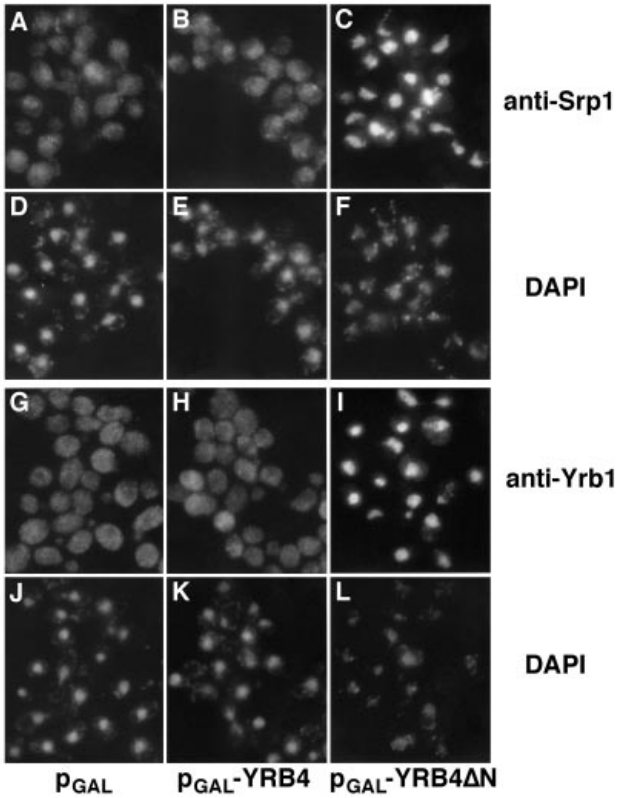

Fig. 8. Overexpression of Yrb4ΔNp causes nuclear accumulation of Srp1p and Yrb1p. Wild-type cells containing plasmids pGAL, pGAL-*YRB4* or pGAL-*YRB4ΔN* were grown in the presence of 2% galactose for 3 h. Cells were fixed with formaldehyde for 60 min and prepared for immunofluorescence using antibodies against Srp1p (**A**–**F**) or Yrb1p (**G**–**L**), and stained with DAPI.

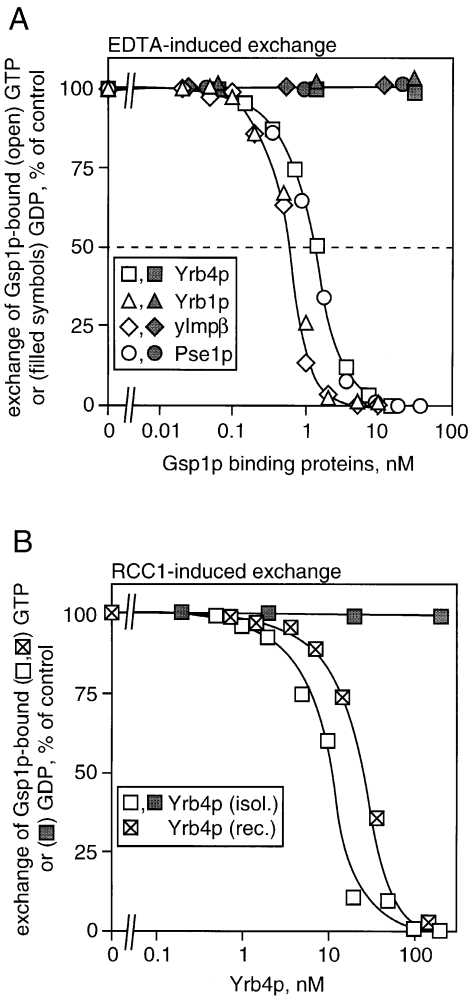

Fig. 9. Inhibition of GTP exchange on Gsp1p by Yrb4p. (**A**) EDTA-induced exchange. Fifty pM Gsp1p[γ-^{32}P]GTP or 1 nM Gsp1p[α-^{32}P]GDP were pre-incubated with Yrb4p, Yrb1p, yeast importin-β or Pse1p. After 20 min, the exchange reaction was started by addition of EDTA and GDP. Nucleotide exchange was determined by measuring protein-bound radioactivity after further incubation for 5 min. The final concentrations of Yrb4p, Yrb1p, yeast importin-β and Pse1p are indicated. (**B**) RCC1-induced exchange. Ten nanomolar Gsp1p[γ-^{32}P]GTP or Gsp1p[α-^{32}P]GDP were incubated for 20 min with Yrb4p isolated from *S.cerevisiae* or with recombinant Yrb4p purified from *E.coli*. Two-hundred μM non-radioactive GDP and 1 nM human RCC1 were added. After 5 min, Gsp1p-bound radioactivity was determined.

data for Yrb1p and importin-β. At the low Gsp1p concentration (50 pM) used in this experiment, the concentration of the added Gsp1p-binding proteins required for inhibition of nucleotide exchange reflects their affinity for Gsp1p. The Gsp1p–GTP dissociation constants were approximately 2 nM for Yrb4p and Pse1p, and 0.7 nM for yImpβ and Yrb1p.

GTP exchange stimulated by the nucleotide exchange factor RCC1 was also efficiently inhibited by Yrb4p (Figure 9B). In this experiment, we used Gsp1p at a concentration above the calculated dissociation constant. Thus, the concentration required to inhibit GTP exchange on Gsp1p reflects the percentage of active Yrb4p. Figure 9B indicates that recombinant Yrb4p had the same effect on RCC1-induced GTP exchange, but was less active than Yrb4p isolated from *S.cerevisiae*. Therefore, we used Yrb4p purified from yeast in all experiments shown below. The ability of human RCC1 to induce guanine nucleotide exchange illustrates the high evolutionary conservation of Gsp1p.

Effect of Yrb4p on GTPase activity of Gsp1p

Next we examined the effect of Yrb4p on hydrolysis of Gsp1p-bound GTP. As shown in Figure 10A, Yrb4p and yImpβ inhibit the intrinsic GTPase activity of Gsp1p in a comparable manner. Yrb1p had no inhibitory effect in this assay. Yrb4p also inhibited the Rna1p-induced GTPase activity of Gsp1p. Binding of yImpβ or Pse1p to Gsp1p–GTP resulted in a similar inhibition. From this experiment we could again calculate the affinities of the Gsp1p-binding proteins for Gsp1p, and obtained similar values as for EDTA-induced GTP exchange (Figure 10B).

Thus Yrb4p, yImpβ and Pse1p behave very similarly with respect to their biochemical activity on Gsp1p. This raises the question as to how complexed Gsp1p becomes sensitized to GTPase activation. In order to address this issue, we tested whether Yrb1p is able to stimulate activation. We incubated Gsp1p[γ-^{32}P]GTP with Yrb4p for 20 min prior to the addition of Yrb1p. After a further incubation for 2 min, Rna1p was added. As shown in Figure 10C, addition of Yrb1p resulted in a dramatic stimulation of GTP hydrolysis on Gsp1p. Similar results were obtained with Pse1p (not shown). The stimulation of GTP hydrolysis was less effective on the Gsp1p–yImpβ complex. An ~5000-fold higher concentration of Yrb1p

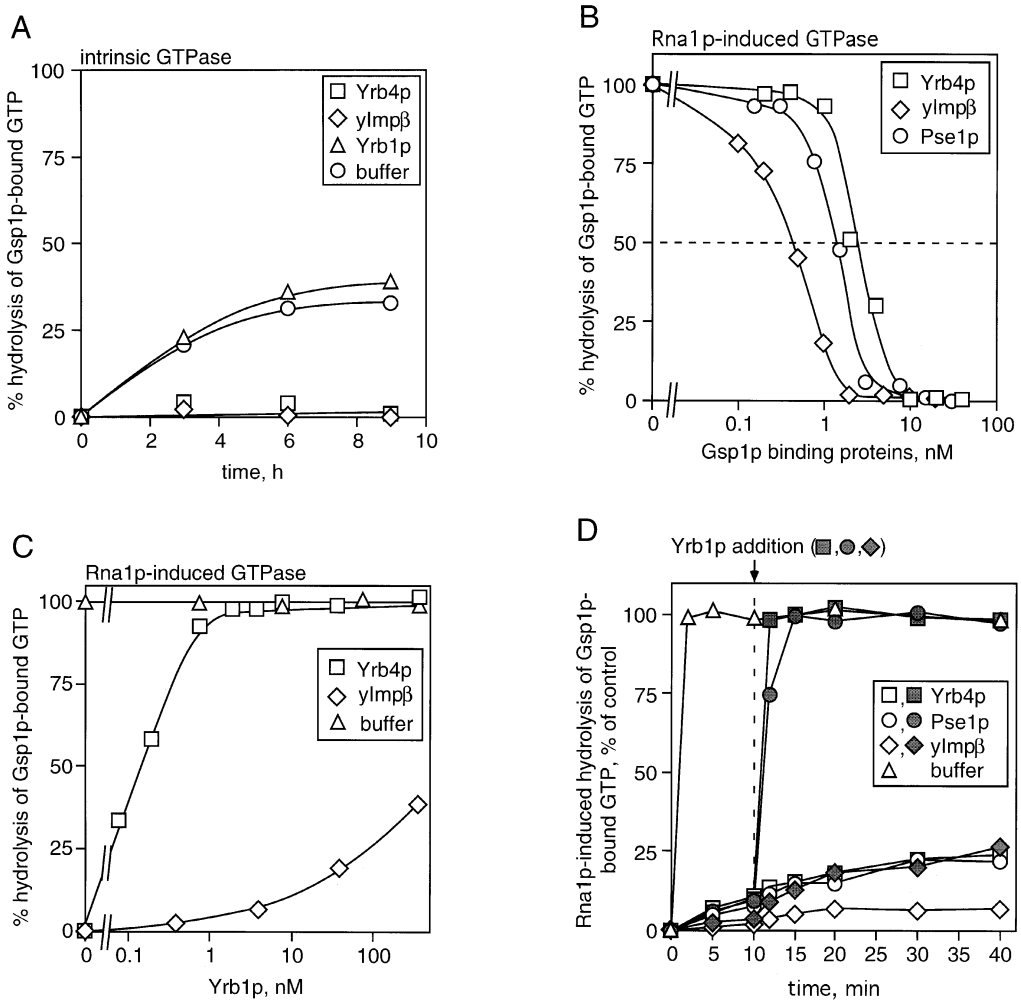

Fig. 10. Effect of Yrb4p on intrinsic and Rna1p-induced GTPase activity of Gsp1p. (**A**) Effect on intrinsic GTPase. Five-hundred µl 50 pM Gsp1p[γ-^{32}P]GTP were incubated with buffer, 20 nM Yrb4p, 20 nM yeast importin-β or 20 nM Yrb1p. After the indicated time, released [^{32}P]phosphate was determined in 50 µl aliquots. (**B**) Effect on Rna1p-induced GTPase. Fifty pM Gsp1p[γ-^{32}P]GTP were preincubated for 20 min with the indicated concentrations of Yrb4p, yeast importin-β or Pse1p. Ten nM Rna1p was added and the reaction was allowed to proceed for 5 min. Hydrolysis of Gsp1p-bound GTP was determined as released [^{32}P]phosphate. (**C**) Yrb1p-induced release of Yrb4p-mediated GTPase block on Gsp1p. Fifty pM Gsp1p[γ-^{32}P]GTP were preincubated for 20 min with buffer, 50 nM Yrb4p or 10 nM yeast importin-β. Then 100 nM Rna1p and indicated amounts of Yrb1p were added. After 5 min, released [^{32}P]phosphate was determined. (**D**) Effect of Yrb1p on GTPase activity of Gsp1p complexed to Yrb4p, Pse1p or yeast importin-β. Ten nM Gsp1p[γ-^{32}P]GTP were preincubated in 500 µl buffer, 150 nM Yrb4p, 150 nM Pse1p or 30 nM yeast importin-β for 30 min. Then 100 nM Rna1p were added. The reactions were incbated in the absence of Yrb1p (open symbols) or 30 nM Yrb1p were added after 10 min (filled symbols). Released [^{32}P]phosphate was determined in 50 µl aliquots after the indicated time intervals.

was required to achieve a similar extent of GTPase activation. A similar effect on the RanGTPase activity had been observed with the human homologues. While RanBP1 strongly induced GTPase activation on Ran–GTP complexed to the Pse1p-related RanBP5, the effect on Ran–GTP–importin-β was only marginal (Görlich *et al.*, 1996b; Deane *et al.*, 1997; Lounsbury and Macara, 1997). However, when RanBP1 was added together with importin-α to the Ran–GTP–importin-β complex, GAP-induced hydrolysis of Ran-bound GTP was highly stimulated (F.R.Bischoff and D.Görlich, unpublished).

In experiments in which GTPase activity was monitored over time, Gsp1p[γ-^{32}P]GTP was preincubated with Yrb4p, Pse1p or yImpβ for 20 min. Then a high concentration of Rna1p was added to ensure that once released, Gsp1p[γ-^{32}P]GTP would be subjected to Rna1p-induced GTP hydrolysis rather than being rebound by the corresponding Gsp1p-binding protein. As with the human homologues, the dissociation rates of the Gsp1p–GTP–Yrb4p, Gsp1p–GTP–Pse1p and Gsp1p–GTP–yImpβ complexes were relatively low (Figure 10D); the half lives were about 1 h and 3 h, respectively. However in samples to which Yrb1p was added, the release of the GTPase block was very pronounced when the GTPase activation was initially inhibited by Yrb4p or Pse1p (50-fold stimulation); only a 3-fold stimulation was observed in the case of the Gsp1p–yImpβ complex.

To summarize, we show that Yrb4p, yImpβ and Pse1p bind tightly to the GTP-bound form of Gsp1p, rendering it resistant to both hydrolysis and exchange of bound GTP. We demonstrate that in the presence of the GTPase activating protein Rna1p, addition of Yrb1p to the Yrb4p–Gsp1p–GTP and the Pse1p–Gsp1p–GTP complex, and to a lesser extent to the yImpβ–Gsp1p–GTP complex, results in an increase in hydrolysis of Gsp1p-bound GTP. This hydrolysis is likely to establish the dissociated state of the complex partners as a result of the lower affinity for the GDP-bound form of Gsp1p.

Discussion

We conclude from a number of observations that Yrb4p represents a protein import receptor. First, a protein containing the nuclear targeting signal of ribosomal protein L25 is mislocalized to the cytoplasm in cells disrupted for *YRB4*. Thus, L25 and related proteins might represent the cargo for Yrb4p. Second, Yrb4p binds to the NPC like other importin-β-related proteins. Third, its overexpression inhibits NLS-dependent protein import, probably by competition with importin-β for the same binding sites at the NPC. Fourth, L25 binds to Yrb4p and is specifically released by Ran–GTP. Fifth, Yrb4p is a Ran–GTP-binding protein, and it interacts with Ran in a similar way to importin-β and other importin-β relatives.

The expression level of Yrb4p in yeast is at least 10-fold higher than that of importin-β and Pse1p. We conclude this from the failure to detect the latter two proteins in standard overlay assays of yeast lysates, whereas both proteins, when purified from *Escherichia coli*, are recognized by Gsp1p[^{32}P]GTP with similar efficiency to Yrb4p. Despite this seemingly high level of expression, cells lacking Yrb4p remain viable. The abundance of this putative transport factor also raises the question regarding its transport cargo. In the absence of Yrb4p, import substrates carrying classical NLSs were correctly localized to the nucleus. However, import of a reporter containing the N-terminal 49 amino acid residues of ribosomal protein L25 was clearly defective. The same reporter protein was previously shown to exhibit different import characteristics compared with similar constructs containing the NLS of Matα2p or Pho2p (Nehrbass *et al.*, 1993). The N-terminal extension of L25 is responsible for nuclear targeting. It consists of two sequence motifs (residues 1–17 and 18–41), each containing a similar cluster of basic and hydrophobic amino acids, which alone showed some nuclear targeting activity, but allowed efficient import only in combination (Schaap *et al.*, 1991). Similar motifs are found in other proteins, e.g. in the N-terminal regions of ribosomal proteins L27, L19, L10, L3 and S7. However, we could not detect a clear consensus sequence. The 75–80 different and very abundant ribosomal proteins are each separately imported into the nucleus where they assemble with rRNA in the nucleolus. Ribosome turnover is very high, and yeast cells are able to respond rapidly to the environment by changing their number of ribosomes (Warner, 1989). Yrb4p could ensure that import of newly synthesized ribosomal proteins does not become rate limiting. Our results further indicate that Pse1p, the nearest relative of Yrb4p, is likely to carry similar cargo to Yrb4p. While our manuscript was under review, a report was published that arrived at a conclusion similar to ours, namely that Yrb4p/Kap123p and Pse1p are involved in the nuclear import of ribosomal proteins (Rout *et al*., 1997).

Yrb4p belongs to a recently identified superfamily of distantly importin-β-related proteins postulated to represent transport receptors with different substrate specificities. Import receptors would probably function by a common mechanism, i.e. they bind their substrate in the cytoplasm, pass the NPC on common tracks and release their cargo in the nucleus by binding to Ran–GTP (Görlich *et al*., 1997). Our results are in agreement with this model. It appears that importin-α is a substrate solely for importin-β, since an interaction with importin-α was not detected for other members of the superfamily (Aitchison *et al.*, 1996; Görlich *et al.*, 1997; this study). These proteins are similar both in their size and secondary structure, and share homologies in their N-terminal regions, which in all tested cases accounted for Ran–GTP-binding (Görlich *et al.*, 1996b; Görlich *et al.*, 1997; Lounsbury and Macara, 1997; this study). The Ran-binding domains of these proteins are different from that of Yrb1p/RanBP1. This is illustrated by the formation of trimeric complexes of importin-β, Ran–GTP and RanBP1 (Chi *et al.*, 1996; Kutay *et al.*, 1997; Lounsbury and Macara, 1997), of Yrb4p, Gsp1p–GTP and Yrb1p, or of yImpβ, Gsp1p–GTP and Yrb1p, which we detected by gel filtration (not shown).

Importin-β, Pse1p and Yrb4p bind specifically to Gsp1p–GTP with high affinity. In the case of importin-β, this triggers the release of importin-α. *In vivo* this probably results in the dissociation of the importin heterodimer in the nucleus where the concentration of Ran–GTP is high (Görlich *et al.*, 1996b). Similarly, we show that the release of the putative transport substrate L25 from Yrb4p is induced by binding of Ran–GTP. The similar behavior of Pse1p indicates that both proteins possess overlapping substrate specificity.

Recently, it was shown that an importin-β mutant deficient in Ran-binding was able to undergo the translocation step through the nuclear pore, but could not be efficiently displaced from the nuclear side of the NPC (Görlich *et al.*, 1996b). This mutant lacking the N-terminal 44 amino acid residues blocks other translocation events as well. It competes with NLS-dependent and M9-dependent protein import, and with export of proteins, mRNA and U snRNA (Kutay *et al.*, 1997). This suggests that transporters with different substrate specificities use common binding sites at the NPC, from which they are released by the interaction with Ran–GTP. We expressed a similar Yrb4p mutant deficient in Gsp1p-binding in yeast cells. As seen with ΔN44importin-β, this leads to a block of bidirectional transport and to a dominant-lethal phenotype. Interestingly, Srp1p and Yrb1p accumulate within the nucleus under these conditions. Assuming that both proteins constantly have to be exported, this can be explained by a general block of protein export. Accordingly, a nuclear accumulation of Srp1p was observed also in other export mutants (Aitchison *et al.*, 1995; Koepp *et al.*, 1996). In the case of a direct inhibition of nuclear import by Yrb4ΔNp, the increase in the nuclear concentration of Srp1p and Yrb1p indicates that the export block must have a faster onset and/or a higher efficiency than the import block. The preferential inhibition of nuclear export is also indicated by the nearly unchanged nuclear localization of the shuttling protein Npl3p, which is involved in mRNA export (Lee *et al.*, 1996). In contrast, when protein import into the nucleus was strongly inhibited, e.g. after expression of a GTPase-deficient form of Gsp1p, Npl3p accumulated in the cytoplasm (Schlenstedt *et al.*, 1995a). The N-terminal truncation mutants of importin-β and Yrb4p probably inhibit traffic across the NPC by the same mechanism. In both cases it could be the failure to perform Ran–GTP-mediated release from the NPC in the nucleus that leads to a block of transport across the NPC.

Like importin-β, Pse1p and Yrb4p form a complex with

Gsp1p–GTP and protect it from GTP exchange and hydrolysis. It is an intriguing possibility that these stable complexes could be exported back to the cytoplasm. The NPC would be able to distinguish these complexes from import complexes which move in the opposite direction. Once transported to the cytoplasmic side of the NPC, the Yrb4p–Gsp1p–GTP complex has to be dissociated. This probably occurs by the concerted action of Yrb1p and the Gsp1p-specific GTPase activating protein Rna1p. We observed that in the presence of both proteins, Gsp1p becomes susceptible to GTPase activation, resulting in Gsp1p–GDP which has a nearly undetectable affinity for Yrb4p. Yrb1p and Rna1p are also sufficient for the displacement of Pse1p from Gsp1p–GTP–Pse1p. However, the efficient dissociation of importin-β from the Gsp1p–GTP–yImpβ complex requires in addition importin-α (F.R.Bischoff and D.Görlich, unpublished).

In summary, a simplified model for Yrb4p-mediated import can be outlined. The Yrb4p–cargo complex is transported by an as yet unknown mechanism through the NPC. In the nucleus, binding of Gsp1p–GTP stimulates the dissociation of the import receptor–cargo complex and probably also its displacement from the NPC. The resulting Gsp1p–GTP–Yrb4p complex is then exported back to the cytoplasm where it is disassembled by Yrb1p and Rna1p. Rna1p-induced hydrolysis of Gsp1p-bound GTP ensures that the released Yrb4p can enter the next import cycle starting with the binding of new cargo.

Materials and methods

Plasmids and strains

The *YRB4* gene was cloned by PCR from genomic DNA using the oligonucleotides 5′-GCGCGTCGACATCCACTTATCCATAGATCC-TTC-3′ and 5′-CCGCGAGCTCGGTCCCTCAGGACACATACATAC-3′ with Pwo polymerase (Boehringer Mannheim) and inserted between the *Sal*I and *Sac*I sites of pBluescript (Stratagene), generating BS-*YRB4*. The disruption plasmid BS-*YRB4::TRP1* was constructed by replacing the 2651 bp *Bam*HI/*Hin*dIII fragment of BS-*YRB4* (a region from 95 bp upstream to 2556 bp downstream of the start codon) by the 960 bp *Bam*HI–*Hin*dIII fragment of pJJ248 (Jones and Prakash, 1990). The centromeric plasmid YCpGAL-*YRB4* contains the *YRB4* coding sequence under control of the inducible *GAL1* promoter. The coding sequence was amplified from BS-*YRB4* using 5′-GCTAGTCTAGACAT ATGGAT-CAACAATTTCTA-3′ and M13 primer (Stratagene) with Pwo polymerase, digested with *Xba*I and *Ecl*136II, and inserted between *Xba*I and blunt-ended *Sal*I of YCpGAL-*URA3* (Schlenstedt *et al.*, 1995a). Plasmid YCpGAL-*YRB4*Δ*N* lacking the first 123 codons of the *YRB4* coding sequence was constructed by PCR amplification using 5′-GCGAGATCTATGCCTAACCTAATCCAAACTGC-3′ and M13 primer (Stratagene), digestion with *Bgl*II and *Ecl*136II, and insertion into YCpGAL-*URA3* which was treated with *Sal*I, Klenow polymerase and *Bam*HI. GST fusion expression plasmids were constructed by subcloning the coding sequences of *YRB4*, *RSL1* and *PSE1* into YEpGAL-GST-*URA3* (Schlenstedt *et al.*, 1995b; Koepp *et al.*, 1996; Görlich *et al.*, 1997). The *KAP104* coding sequence was amplified from genomic DNA using oligonucleotides 5′-GCGGATCCATGGCATCGACATGGAA-GCCC-3′ and 5′-GCGGATCCATTTGCAGTTATGCACC-3′ with Pwo polymerase. The L25 (*RPL25*, YOL127W) coding sequence was amplified with oligonucleotides 5′-GCGGATCCATGGCTCCATCAGCTAA-GGCTACTGCCGCTAAG-3′ and 5′-GGAACCATCGCTGCAGCTA-CGC-3′, cloned into pBluescript/*Bam*HI/*Pst*I and subsequently subcloned between the *Nco*I and *Bgl*II sites of pQE60-T7.

Plasmid YCpGAL-NLS-*SUC2* encoding a fusion of the SV40 large T-antigen NLS to cytoplasmic invertase was constructed by inserting the coding sequence for NLS-invertase as a *Bam*HI fragment into YCpGAL-*LEU2* (Schlenstedt *et al.*, 1995a). Plasmid pGS304 (2μ *LEU2*) containing the first 49 codons of the L25 gene fused to *lacZ* was described before (Nehrbass *et al.*, 1993). Plasmid pGS226 (2μ *URA3*) containing SV40 NLS fused to a GFP-*lacZ* fusion under control of the *ADH1* promoter was a gift of Jim Haselhoff (MRC, Cambridge). For bacterial expression of full length Yrb4p, the coding sequence was PCR-amplified using primers 5′-GCTAGTCTAGACATATGGATCAACAATTTCTA-3′ and 5′-CCTAGTCTAGACCCGGGTCAAGCAATGACGGCAGC-3′, and ligated in frame into pET14b (Novagen).

To create a *YRB4* disruption, the diploid strain GSY158 (*ura3–52*/–, *leu2*Δ*1*/–, *his3*Δ*200*/–, *trp1*Δ*63*/–), a derivative of FY86 and FY23 (Fred Winston, Harvard Medical School, Boston), was transformed with the *Sal*I/*Sac*I fragment of BS-*YRB4::TRP1* generating strain GSY391. Integration of *TRP1* in the heterozygous diploid and in GSY393 (MATa, *ura3–52*, *leu2*Δ*1*, *his3*Δ*200*, *trp1*Δ*63*, *YRB4::TRP1*), a spore after tetrad dissection, was confirmed by Southern blotting. All wild-type experiments were performed with strain GSY154, a MATα spore of GSY158.

Protein purification

Yrb4p was purified from yeast as follows: logarithmically growing *S.cerevisiae* cells (OD_{580} = 1.5) from a 3 l YPD culture were collected by centrifugation for 10 min at 5000 *g*. Cells were resuspended in 100 ml spheroplasting buffer (0.9 M Sorbitol, 10 mM EDTA, pH 7.5) and incubated for 1 h at 25°C with 0.5 mg/ml yeast lytic enzyme (ICN). After centrifugation for 10 min at 3000 *g*, the pellet was washed once in spheroplasting buffer and resuspended in 100 ml lysis buffer [20 mM bis-tris-propane-HCl, pH 7.0, protease inhibitors (Boehringer Mannheim), 10 mM EDTA, 1 mM 2-mercaptoethanol]. Spheroplasts were lysed by 10 gentle strokes with a S-type Dounce homogenizer and centrifuged at 70 000 *g* for 45 min. The resulting supernatant was chromatographed on Fractogel EMD DMAE-650/M (Merck Superformance, 26×115 mm) in 20 mM bis-tris-propane-HCl, pH 7.0, 1 mM 2-mercaptoethanol with a linear NaCl gradient from 0.05 M to 1 M at a flow rate of 5 ml/min. Fractions containing Yrb4p eluting between 300 and 400 mM NaCl were pooled and subsequently applied at 2 ml/min to a hydroxylapatite column (Merck superformance, 10×150 mm). Yrb4p eluted at 100 mM potassium phosphate in a gradient from 20 mM bis-tris-propane-HCl, pH 7.0, 50 mM NaCl, 1 mM 2-mercaptoethanol to 1 M K_2HPO_4/KH_2PO_4, pH 7.0, 1 mM 2-mercaptoethanol. The eluate was brought to 30% saturation with ammonium sulfate, incubated on ice for 30 min and centrifuged at 20 000 *g* for 30 min. The supernatant was chromatographed on a hydrophobic column (Merck Fractogel EMD AFTA 650/S Superformance, 10×20 mm) at 0.5 ml/min in 20 mM Tris–HCl, pH 7.5, 1 mM 2-mercaptoethanol using a gradient from 35% ammonium sulfate to 100 mM NaCl. Yrb4p eluted at 5% ammonium sulfate. The eluate was diluted 10-fold with 20 mM Tris–HCl, pH 7.5, 1 mM 2-mercaptoethanol, and chromatographed in the same buffer on Mono Q (Pharmacia HR 5/5) using a gradient from 0.05 M to 1 M NaCl. Yrb4p eluting at 300 mM NaCl was purified to homogeneity by gel filtration on Superdex 200 (Pharmacia) at 1 ml/min in 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM 2-mercaptoethanol. Aliquots of fractions after each purification step were tested by Ran overlay assays. Yrb4p was quantified by a comparative overlay assay using purified Yrb4p as a standard. The purified protein was cleaved with CNBr as previously described (Bischoff and Ponstingl, 1991b). Expression of recombinant 6×His-tagged Yrb4p was performed in the *E.coli* strain BL21 (DE3) carrying plasmid pLysS (Studier *et al.*, 1990). Cells from a 3 l-culture were induced with 0.1 mM isopropylthio-β-D-galactoside (IPTG) for 3 h, resuspended in 50 mM Tris–HCl, pH 8.0, 200 mM NaCl, 1 mM 2-mercaptoethanol and lysed as described (Bischoff *et al.*, 1994). After centrifugation for 60 min at 70 000 *g*, the supernatant was applied to a 10×50 mm Ni-NTA sepharose column (Qiagen) at a flow rate of 0.5 ml/min; 6×His-Yrb4p was eluted with a step of 500 mM imidazole. Fractions containing the fusion protein were pooled and further purified by chromatography on a Mono Q column (Pharmacia HR 5/5) in 20 mM Tris–HCl, pH 7.5 with a flow rate of 1 ml/min using a gradient of 0.05 M to 1 M NaCl.

The purification of all other recombinant proteins was as described before: human wild-type Ran and RanQ69L (Bischoff *et al.*, 1994), Gsp1p (Corbett *et al.*, 1995), RCC1 (Klebe *et al.*, 1995), *Saccharomyces pombe* Rna1p (Bischoff *et al.*, 1995a,b), Yrb1p (Schlenstedt *et al.*, 1995b), Pse1p (Görlich *et al.*, 1997) and yeast importin-β (Görlich *et al.*, 1996c).

GST fusion proteins were purified from wild-type yeast cells after a 4 h galactose induction as described (Schlenstedt *et al.*, 1995b) using PBS containing 2 mM KCl, 1 mM $MgCl_2$ and 0.1 % Tween-20. L25 was synthesized in the TNT T7 coupled reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine and pQE60-T7-L25. Reticulocyte lysates were diluted 1:100 into reactions containing GST fusion proteins bound to glutathione sepharose (Pharmacia), incubated

for 1 h at 4°C and washed. Bound proteins were eluted with SDS-sample buffer or incubated with 7.5 μM RanQ69L-GTP or RanQ69L-GDP, and then washed and eluted.

Immunoblotting and immunofluorescence

Affinity-purification of antibodies against yImpβ and Srp1p was described before (Görlich *et al.*, 1996c). Antibodies against Yrb4p were generated from rabbits by Immundiagnostica (Eschelbronn, Germany). Antisera were tested by immunoblot analysis using Yrb4p purified from yeast and recombinant Yrb4p. Antibodies were affinity-purified by binding to nitrocellulose-bound Yrb4p and subsequent elution with 0.1 M glycine, pH 2.5, 100 mM NaCl, 0.5% BSA. Eluates were immediately adjusted to pH 7 by addition of 1M Tris–HCl, pH 9.6. Affinity-purified antibodies against Yrb1p were prepared as follows: 3.5 mg recombinant Yrb1p in 5 mM EDTA, 50 mM Tris–HCl, pH 8 were coupled to 1 ml Sulfolink (Pierce) and incubated overnight with 24 ml antiserum. The resin was washed with 10 ml PBS, 20 ml 0.5 M NaCl in PBS and 10 ml PBS. Bound antibodies were eluted with 20 ml 0.1 M glycine, pH 2.2. The eluate was neutralized with 1.45 ml 1.5 M Tris–HCl, pH 8.8. Western blot analysis was carried out as described (ECL guidelines, Amersham) using horse-radish peroxidase conjugated goat anti-rabbit IgG (BioRad) as secondary antibodies.

Immunofluorescence and detection of poly(A)$^+$ RNA by *in situ* hybridization was performed essentially as described (Schlenstedt *et al.*, 1995b) with the following exceptions: for preparation of cells for immunofluorescence by formaldehyde fixation, cells were fixed with 4% formaldehyde (Sigma) for 15 or 60 min. Subsequent spheroplasting was performed with 30 μg/ml zymolyase (ICN). For preparation of cells by methanol fixation, cells were washed twice with 1.1 M sorbitol/PBS and spheroplasted with 50 μg/ml zymolyase in the presence of 0.5% 2-mercaptoethanol for 2 h at 30°C. Cells were washed and applied onto polylysine-coated slides, permeabilized and fixed in cold methanol for 45 min, and dehydrated in acetone for 30 s.

Overlay and enzymatic assays

For overlay assays, proteins were resolved by 12% SDS–PAGE, transferred to nitrocellulose and renatured with 20 mM MOPS, pH 7.1, 100 mM sodium acetate, 5 mM magnesium acetate, 5 mM dithiothreitol, 0.5% bovine serum albumin, 0.05% Tween-20 for 1 h at 4°C (Lounsbury *et al.*, 1994). Blots were incubated with 10 ml buffer containing 100 μM non-radioactive GTP at 25°C. After 10 min, 100 μl containing 1 nM Gsp1p[γ-^{32}P]GTP were added. After incubation for 10 min, blots were rinsed five times with renaturation buffer and autoradiographed. Labeling of Gsp1p with [γ-^{32}P]GTP was performed as described for Ran (Bischoff *et al.*, 1995b).

Enzymatic assays were carried out as described (Bischoff *et al.*, 1994; Bischoff *et al.*, 1995b). Briefly, 30 μl Gsp1p[γ-^{32}P]GDP or Gsp1p[γ-^{32}P]GTP were preincubated at 25°C with 10 μl of the corresponding Gsp1p-binding protein in incubation buffer (20 mM HEPES NaOH, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.02% sodium azide, 0.05% hydrolyzed gelatin). After 20 min, 10 μl 200 mM EDTA, pH 7.5, 2 mM GDP or 1 μl of 50 nM human RCC1 and 10 μl 1 mM GDP were added. After 5 min, Gsp1p-bound radioactivity was determined by the filter-binding assay (Bischoff and Ponstingl, 1991a). The final Gsp1p concentrations are given in the figures. The affinities of Yrb4p, Pse1p and yImpβ for Gsp1p–GTP were calculated by determining the concentration of the respective Gsp1p-binding protein required for 50% inhibition of Rna1p-induced GTP hydrolysis using 30 pM Gsp1p[γ-^{32}P]GTP in a 5 min reaction at 25°C. Following the GTPase reaction, released [^{32}P]phosphate was determined by the charcoal assay (Bischoff *et al.*, 1994).

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