The Karyopherin Kap122p/Pdr6p Imports Both Subunits of the Transcription Factor IIA into the Nucleus

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Abstract. We discovered a nuclear import pathway mediated by the product of the previously identified Saccharomyces cerevisiae gene PDR6 (pleiotropic drug resistance). This gene product functions as a karyopherin (Kap) for nuclear import. Consistent with previously proposed nomenclature, we have renamed this gene KAP122. Kap122p was localized both to the cytoplasm and the nucleus. As a prominent import substrate of Kap122p, we identified the complex of the large and small subunit (Toa1p and Toa2p, respectively) of the general transcription factor IIA (TFIIA). Recombinant GST-Kap122p formed a complex with recombinant His-Toa1p and His-Toa2p, peptide repeat–containing fragments of Nup1p and Nup2p. In wild-type cells, Toa1p and Toa2p were localized to the nucleus. Consistent with Kap122p being the principal Kap for import of the Toa1p-Toa2p complex, we found that deletion of KAP122 results in increased cytoplasmic localization of both Toa1p and Toa2p. Deletion of KAP122 is not lethal, although deletion of TOA1 and TOA2 is. Together these data suggest that Kap122p is the major Kap for the import of Toa1p-Toa2p into the nucleus like other substrate–Kap complexes, the Toa1p/Toa2p/Kap122p complex isolated from yeast cytosol or reconstituted from recombinant proteins, was dissociated by RanGTP but not RanGDP. Kap122p bound to nucleoporins, specifically, to the peptide repeat–containing fragments of Nup1p and Nup2p.

Key words: yeast • karyopherin β family • nuclear import • transcription factor IIA • RanGTP dissociation

The karyopherins (Kaps) are structurally related family of proteins that function in transporting proteins, nucleic acids, and nucleoproteins into and out of the nucleus (for reviews see Pemberton et al., 1998; Wozniak et al., 1998). Comparative sequence analysis of the yeast genome indicated that this organism may have as many as 14 members of the karyopherin β family but only one representative of the karyopherin α family. Not all members of the Kap β family have been definitively shown to function as such (Pemberton et al., 1999; for reviews see Mattaj and Englmeier, 1998; Pemberton et al., 1998; Wozniak et al., 1998). Each of the Kap β family members binds to a cognate signal in a transport substrate, and then docks the resulting complex to a subset of nucleoporins (Nups, collective term for nuclear pore complex [NPC] proteins). In contrast, Kap α functions as an adapter that binds to a classical nuclear localization sequence (NLS) (Conti et al., 1998) and to a member of the Kap β family, termed Kap95p or Kap β1. The small GTPase Ran and factors that regulate the GDP- or GTP-bound form of Ran are involved in transport across the NPC (Melchior et al., 1993; M oore and Blobel, 1993; Rexach and Blobel, 1995; Schwoebel et al., 1998; Englmeier et al., 1999; Ibbel et al., 1999). Although several reactions that are likely to be relevant for transport have been reconstituted in vitro, the sequence of reactions leading to import or export of substrates remains to be elucidated.

The members of the Kap β family that have been identified so far function either in import or in export (for reviews see Pemberton et al., 1998; Wozniak et al., 1998), although it has not yet been excluded that a given Kap could function in both processes. The directionality of transport appears to depend on at least two nucleocytoplasmic asymmetries. First, the localization of RanGAP (GTPase-activating protein) in the cytoplasm and of RanGEF (GDP/GTP exchange factor) in the nucleus (for reviews see Corbett and Silver, 1997; M oore, 1998) is likely to yield a high ratio of RanGTP/RanGDP in the nucleus and a high ratio of RanGDP/RanGTP in the cytoplasm (Izaurralde et al., 1997; for review see Melchior and Gerace, 1998). Second, certain nucleoporins that serve as docking

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sites for Kap s are asymmetrically located on either the cytoplasmic or the nucleoplasmic fibers of the NPC (Yang et al., 1998; for review see Ohno et al., 1998). Structural asymmetry of the NPC is a likely determinant for the directional transport of, as certain Kap s appear to interact preferentially with certain Nups (Fornerod et al., 1997; Marelli et al., 1998).

All members of the Kap β family show a low level of sequence homology to each other ( Görlich et al., 1997). Those that have been characterized so far can function without an adapter and bind directly to both a substrate and to a Nup (Aitchison et al., 1996; Pemberton et al., 1997; Ronsen et al., 1997; Rout et al., 1997; Ibertini et al., 1998; Senger et al., 1998). Even Kap β1 can bind to certain substrates directly, without the Kap α adapter (J. Akel and G. Görlich, 1998; More et al., 1999; Palmeri and Malim, 1999; Truant and Cullen, 1999). Several of the identified Kap s are not essential for viability, although often they transport substrates that are essential for viability (Rout et al., 1997; Schlenstedt et al., 1997; Pemberton et al., 1999). The explanation for this apparent paradox lies in overlapping substrate specificities, i.e., a given substrate can be transported by more than one Kap.

Here, we have characterized a putative Kap β homolog, Pd6p. PDR6 was previously classified as a member of a gene family that is involved in pleiotropic drug resistance. Overexpression of PDR6’s wild-type allele conferred sensitivity to cycloheximide, borrelidin, and hygromycin B in some drug-resistant strains (Aitchison et al., 1991). Pdr1p is a Zinc finger-containing transcription factor that regulates the expression of several ATP binding cassette transporter–encoding genes, including YOR1, YOL1, and YOR1 (M. Ahe et al., 1996). How Pd6p overexpression suppresses mutations in the transcription factor Pdr1p is not known. Pd6p is not essential for growth (Chen et al., 1991) and no function has yet been assigned to Pd6p. Here, we show that Pd6p functions as a Kap. In agreement with previously proposed nomenclature we, therefore, designated it Kap122p (because of Pd6p’s calculated M, of 123,529 D, it might have been designated either Kap123p or Kap124p; however, these terms had already been assigned to two other Kap s [for review see Pemberton et al., 1998]; therefore, we propose the name Kap122p for Pd6p, knowing that the molecular masses of other putative Kap β s are not in this range). We have identified a complex of the large subunit (Toa1p) and the small subunit (Toa2p) of the general transcription factor II A (TFII A) as import substrate for Kap122p.

**Materials and Methods**

**Yeast Strains and Methods**

All strains used were derived from Saccharomyces cerevisiae wild-type D FO5a (Finley et al., 1987) and its derivative kap122a (MATα, lys2-801, leu2-3, 112, ura3-52, his3-200, trp1-1 (am), pdr6::URA3). Yeast strains were grown at 30°C in yeast extract/potato/glucose (YPD) medium containing 60 μg/ml kanamycin and 100 μg/ml ampicillin to a density of 0.7 at 37°C.

**Gene Replacement and Protein A Fusion Constructs**

For deletion of PDR6 in wild-type strain D FO5a, the HIS3 gene was used as a selective marker to replace the PDR6 open reading frame by integrative transformation in a haploid DF50 strain. HIS3 replacement cassette was generated by PCR amplification of markers from pRS S06 with primers that contained 60 nucleotides flanking the PDR6/KAP122 open reading frame from 5’ and 3’ ends (Aitchison et al., 1995). HIS3 marker was switched to URA3 marker by recombination (plasmids were gifts of Dr. F.R. Cross, Rockefeller Univ.). Deletion of genes was confirmed by PCR on total yeast DNA with internal primers.

Carboxy-terminal genomic KAP122-PRA fusion constructs were created by integrative transformation of PCR-amplified constructs with yeast genomic DNA by PCR using synthetic oligonucleotide primers with overlapping substrate specificities, i.e., a given substrate can be transported by more than one Kap.

**Cell Fractionation and Immunolocalization**

Fractionation and immunolocalization of protein A fusion proteins were performed as described (Aitchison et al., 1996). For a typical isolation, 500 ml of postribosomal supernatant (cytosol) was prepared from a 6-liter YPD culture with a density of 1.7 at A600. Cytosol was incubated with 200 μl of rabbit IgG-Sepharose beads at 4°C overnight. After washing with transport buffer (TB: 20 mM Hepes-KOH, pH 7.5, 110 mM KAc, 2 mM MgCl2, 1 mM DTT, 0.1% Tween 20), bound proteins were eluted with a step gradient of MgCl2 from 50 to 4,500 mM. Proteins were precipitated, resolved by SDS-PAGE on a 4–20% acrylamide gel (Novex), and stained with Coomassie blue. Proteins of interest were excised and prepared for MALDI-TOF spectrometry and/or sequencing.

**Immunofluorescence Microscopy**

Yeast cells were fixed in 3.7% formaldehyde for 15 min and cell walls were digested. Indirect immunofluorescence was carried out according to published protocols (Wente et al., 1992). Rabbit polyclonal antibodies to mouse IgG were detected with rabbit IgG-Sepharose beads at 4°C overnight. After washing with transport buffer (TB: 20 mM Hepes-KOH, pH 7.5, 110 mM KAc, 2 mM MgCl2, 1 mM DTT, 0.1% Tween 20), bound proteins were eluted with a step gradient of MgCl2 from 50 to 4,500 mM. Proteins were precipitated, resolved by SDS-PAGE on a 4–20% acrylamide gel (Novex), and stained with Coomassie blue. Proteins of interest were excised and prepared for MALDI-TOF spectrometry and/or sequencing.

**Recombinant Protein Expression**

**Blot Overlay Assay**

A Nup3p fragment containing the FXFG repeat region (amino acids 432-519) and a Nup2p fragment containing the FXFG repeat region (amino acids 186-561) were expressed as glutathione S-transferase (GST) fusion proteins as described (Einenkel et al., 1995; Rexach and Blobel, 1995). Proteins of bacterial lysates were separated by SDS-PAGE and transferred to nitrocellulose. Overlay assays were performed as described (Aitchison et al., 1996). Yeast cytosol from the KAP122-PRA-expressing strain was diluted 1:1 with TB-5% milk and incubated on the blot overnight at 4°C. Bound proteins were detected with rabbit antibodies to mouse IgG and HRP-conjugated donkey anti-rabbit antibodies and enhanced chemiluminescence.

**Yeast Strains and Methods**

All strains used were derived from Saccharomyces cerevisiae wild-type D FO5a (Finley et al., 1987) and its derivative kap122a (MATα, lys2-801, leu2-3, 112, ura3-52, his3-200, trp1-1 (am), pdr6::URA3). Yeast strains were grown at 30°C in yeast extract/potato/glucose (YPD); all yeast manipulations were performed according to described protocols (Sherman et al., 1986).

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A~600~ Protein expression was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 10 h at 17°C. E. coli were lysed in TB with added protease inhibitors (Boehringer Mannheim) using the French pressure cell. The bacterial lysate was incubated with Talon™ resin (CLONTech Laboratories) for 30 min at room temperature (RT). Talon™ beads were washed with lysis buffer (TB-0.1% Tween 20) and TB buffer containing 2 mM ATP, 10 mM Mg(OAc)₂, and 10 mM imidazole. For protein binding assays, the His$_{6}$-Toa1p/Toa2p complex was used either still bound to Talon™ beads or was eluted with TB containing 80 mM imidazole.

KAP122 was subcloned into BamHI-EcoRI sites of the derivative of pGEX 4T3 (Pharmacia Biotech) with additional Tev protease cleavage site (Chook, Y. M., and G. Blobel, 1999), to allow expression of KAP122 with a potentially cleavable amino-terminal GST tag. Kap122p-expressing E. coli cells were grown in LB medium containing 100 μg/ml ampicillin to a density of 0.7 at A$_{600}$ and GST-Kap122p expression was induced by 1 mM IPTG for 3 h at 30°C. GST-K Kap122p fusion protein was purified from bacterial lysates according to the manufacturer’s protocol (Pharmacia Biotech).

**Solution Binding Assays**

**Interaction of Immobilized GST-Kap122p with Purified Recombinant Toa1p–Toa2p.** Binding assays were performed in TB with the addition of 10% glycerol and 0.1% casamino acids (Invitrogen Corp.) to prevent nonspecific interactions. 10 μl of glutathione-Sepharose beads containing either 2 μg immobilized GST-K Kap122p or 2 μg immobilized GST ST were incubated with 5 μg of purified His$_{6}$-Toa1p/Toa2p complex in a total volume of 100 μl for 1 h at RT. Beads were collected by centrifugation at 1,000 g for 30 s, washed five times with mixing by 1 ml of TB followed by sedimentation, and were resuspended in 15 μl of sample buffer. Proteins in one half of each sample were resolved by SDS-PAGE on 4–20% acrylamide gel (Novex, Inc.) and stained with Coomassie blue.

Interaction of Immobilized His$_{6}$-Toa1p/Toa2p with KAP122p. 200 μl of GST-K Kap122p E. coli lysate was incubated with 10 μl Talon™ resin at RT for 1 h to deplete the lysate of endogenous bacterial proteins that interact nonspecifically with Talon™ beads. Beads were sedimented at 1,000 g for 30 s, and the lysate was passed through a Micro Bio-Spin® chromatography column (Bio-Rad Laboratories) to exclude any remaining Talon™ beads. 50 μl of TB containing 50% glycerol and 0.5% casamino acids was added to 200 μl of precleared lysate and the mixture was incubated for 1 h at RT with 2 μg of His$_{6}$-Toa1p/Toa2p complex immobilized on 10 μl of Talon™ beads or with 10 μl of empty Talon™ beads as a control. At the end of incubation, beads were sedimented and washed five times with 1 ml of TB. Talon™ beads with bound proteins were resuspended in sample buffer and half of each sample was resolved by SDS-PAGE on 4–20% acrylamide gel (Novex, Inc.) and stained with Coomassie blue.

**In Vitro Dissociation of the Isolated Yeast KAP122p/Toa1p/Toa2p Complex by RanGTP**

Recombinant Saccharomyces cerevisiae Ran was prepared and loaded with GDP or GTP as described (Floer and Blobel, 1996). Protein A–tagged Toa2p with bound KAP122p was immunosolated from a postribosomal supernatant as described above. A fter washing, IgG-Sepharose beads were resuspended in TB and divided into several equal fractions. These fractions were incubated either with TB alone, or with 4 μM RanGDP or RanGTP for 60 min at room temperature. 1 mM GTP was included, except when using RanGDP. Beads were transferred to a column, and the drained liquid, together with a subsequent 100-μl TB wash, was collected; this constituted the released material. After washing columns with TB, remaining bound proteins were eluted with 1,000 and 4,500 mM MgCl₂, step gradient. Fractions eluted at 1,000 and 4,500 mM were combined and comprised the bound material. Proteins were precipitated, resolved by SDS-PAGE, and stained with Coomassie blue.

**In Vitro Dissociation of the Recombinant KAP122p/Toa1p/Toa2p Complex by RanGTP**

15 μl of Talon™ beads with 3 μg of immobilized His$_{6}$-Toa1p/Toa2p complex were washed five times with 1 ml of TB, incubated with precleared GST-K Kap122p lysate, as described above, to obtain a GST-K Kap122p/His$_{6}$-Toa1p/Toa2p complex. Three equal aliquots of beads were incubated with either 4 μM RanGTP, 4 μM RanGDP, or TB alone for 2 h at RT in a final volume of 40 μl. 1 mM GTP was included, except when using RanGDP. Beads were sedimented by centrifugation and 30 μl of supernatant were collected and passed through Micro Bio-Spin® chromatography column (Bio-Rad Laboratories) to exclude any contaminating Talon™ beads. The drained liquid represented the released material. Beads were washed five times with 1 ml of TB and resuspended in 15 μl of sample buffer. Proteins in one half of each bound and released sample were resolved by SDS-PAGE on 4–20% acrylamide gel (Novex, Inc.) and stained with Coomassie blue.

**Results**

In all cases described here, we found that the genomic replacement of a gene by its protein A–tagged version did not result in any apparent changes in growth rates under the conditions used in our experiments and when compared with those of an otherwise isogenic strain. This indicated that PrA tagging did not apparently interfere with the function of the essential proteins that were tested here; however, this cannot be confirmed for the Kap122p–PrA strain because of the absence of a discernible phenotype of the KAP122 deletion strain.

**Immunolocalization of Kap122p–PrA**

Immunofluorescence microscopy of a KAP122-PrA haploid strain showed that Kap122p–PrA is located both in the cytoplasm and the nucleus (Fig. 1), which is consistent with the localization of other protein A–tagged Kaps and their function in shuttling between the nucleus and the cytoplasm.

**Interaction of Kap122p with Repeat-containing Fragments of Nucleoporins Nup1p and Nup2p**

Karyopherins have been previously shown to interact with peptide repeat-containing nucleoporins (R exach and Blobel, 1995; Aitchison et al., 1996; Fornerod et al., 1997; Rout et al., 1997; Pemberton et al., 1997; Rosenblum et al., 1997). Kap122p is diffusely located in both the cytoplasm and the nucleus. A haploid strain where endogenous Kap122p was replaced by protein A–tagged Kap122p (KAP122-PrA) was examined by Nomarski (left), by indirect immunofluorescence of the protein A tag (middle), and by staining of the DNA with DAPI (right).

![Figure 1](Image)
To test whether Kap122p interacts with nucleoporins, we expressed FXFG repeat-containing fragments of the nucleoporins Nup1p and Nup2p in E. coli, separated proteins of bacterial lysates by SDS-PAGE, transferred them to nitrocellulose, and performed overlay assays with Kap122p-PrA cytosol. Kap122p-PrA interacted strongly and specifically both with Nup1p and Nup2p fragments (Fig. 2). This strongly suggests that Nup1p and Nup2p are among the nucleoporins that bind to Kap122p.

Identification of Proteins Interacting with Kap122p-PrA

Kaps that function in protein import form stable complexes with their import substrates in the cytoplasm (Aitchison et al., 1996; Pemberton et al., 1997; Rosenblum et al., 1997; Rout et al., 1997; Albertini et al., 1998). To isolate such complexes, we prepared a postribosomal cytosol fraction from the KAP122-PrA haploid strain and incubated it with IgG-Sepharose. Bound proteins were eluted with a step gradient of MgCl₂, separated by SDS-PAGE, and stained with Coomassie blue (Fig. 3). The Kap-bound substrates usually elute at MgCl₂ concentration between 100-1,000 mM MgCl₂, whereas the PrA-tagged Kap elutes at 4.5 M MgCl₂ (Aitchison et al., 1996). Indeed, a major band with an apparent Mₐ of 150 kD, which likely represented Kap122p-PrA, eluted at 4.5 M MgCl₂. The numerous minor bands in this fraction, as well as in fractions eluting at lower MgCl₂ concentrations, are likely degradation products of Kap122p-PrA retaining their PrA moiety, as confirmed by immunoblotting with rabbit IgG (data not shown). A number of proteins were present primarily in the 100- and 250-mM MgCl₂ eluates and these are putative substrates for Kap122p. One of these bands, migrating like a protein of ∼14 kD, was identified by mass spectrometry as the small subunit (Toa2p) of the TFIIA. The major band eluting at 4.5 M MgCl₂ is Kap122p-PrA. Relative molecular mass standards are indicated on the left.

Figure 2. Kap122p interacts with FXFG repeat-containing nucleoporins Nup1p and Nup2p. Overlay assay demonstrates specific binding of Kap122p-PrA to Nup1p and Nup2p repeat-containing fragments expressed in E. coli (Nup1* and Nup2*). Total bacterial lysates transferred to nitrocellulose were incubated with crude yeast cytosol from Kap122p-PrA strains. Bound proteins were detected via the PrA moiety (Overlay). A mid blue-stained nitrocellulose strips with whole bacterial lysates from Nup1p- and Nup2p-expressing E. coli, respectively (Total).

Figure 3. Immunoisolation of cytosolic proteins interacting with Kap122p-PrA. Cytosol from a haploid KAP122-PrA strain was incubated with IgG-Sepharose. The last wash fraction and fractions subsequently eluted with a step gradient of MgCl₂ were analyzed by SDS-PAGE and Coomassie blue staining. Proteins eluting in the 100- and 250-mM MgCl₂ fractions are potential binding partners for Kap122p. One of these bands, migrating like a protein of ∼14 kD, was identified by mass spectrometry as the small subunit (Toa2p) of the TFIIA. The major band eluting at 4.5 M MgCl₂ is Kap122p-PrA. Relative molecular mass standards are indicated on the left.

Figure 4. Mislocalization of Toa2p-PrA in a kap122Δ strain. Haploid wild-type or kap122Δ cells, both with a genomic Toa2p-PrA fusion, were visualized by Nomarski (left), or stained by indirect immunofluorescence of the protein A tag (middle) and by DAPI (right). Note the primarily nuclear localization of Toa2p-PrA in wild-type cells and the shift to a diffuse cellular staining in kap122Δ.
and peptide microsequencing after digestion with trypsin (Gharahdaghi et al., 1996; Fernandez et al., 1998), and thereby identified as the small subunit (Toa2p) of the general transcription factor IIA (TFIIA). These data suggested that Toa2p might be an import substrate for Kap122p.

Defective Nuclear Import of Toa2p-PrA in a kap122Δ Strain

To determine whether Toa2p is indeed a transport substrate for Kap122p, we examined the localization of Toa2p in wild-type (WT) and KAP122 deletion (kap122Δ) strains. There were no apparent differences in growth rates between kap122Δ and an isogenic wt strain in YPD medium at 30°C. For these experiments, TOA2 was genomically tagged with PrA in isogenic wt and kap122Δ strains. Immunofluorescence microscopy showed that in wild-type cells Toa2p-PrA was located primarily in the nucleus, whereas in the kap122Δ cells Toa2p-PrA was mislocalized largely to the cytoplasm (Fig. 4). The diminished nuclear localization of Toa2p-PrA in the kap122Δ strain is consistent with Kap122p representing the principal Kap for import of Toa2p.

Localization of several other substrates, whose transport is mediated by distinct Kaps, was not affected by KAP122 deletion. The nuclear import of Npl3p, mediated by Kap111p (Pemberton et al., 1997; Senger et al., 1998) and Nab2p, mediated by Kap104p (Aitchison et al., 1996), appeared similar in both wild-type and kap122Δ strains (Fig. 5), showing that deletion of KAP122 does not generally affect nucleocytoplasmic transport.

Toa2p and Toa1p Form a Cytoplasmic Complex that Binds to Kap122p

Yeast TFIIA has been shown to consist of two subunits: a small subunit (Toa2p, calculated Mr 13.5 kD) and a large

![Figure 5](image_url). Nab2p and Npl3p nuclear import is not affected by KAP122 deletion. Haploid wild-type or kap122Δ cells were visualized by Nomarski (A and B, left), or stained with polyclonal antibodies to Nab2p (A, middle), or monoclonal Abs to Npl3p (B, middle). Nuclei were visualized with DAPI (A and B, right).
subunit (Toa1p, calculated Mr 32 kD) that migrates as a 43-kD protein in SDS-PAGE (Hahn et al., 1989; Ranish and Hahn, 1991; Ranish et al., 1992). Separately expressed Toa1p and Toa2p are insoluble and unable to complement transcription systems unless both subunits are renatured together (Ranish et al., 1992); this suggests that both subunits of TFIIA are unlikely to exist as separate entities in the cell (Ranish et al., 1992; Geiger et al., 1996). Crystallographic data of TFIIA bound to a DNA–TATA-binding protein (TBP) complex revealed that the two subunits are intertwined with each other to form a heterodimer, and that the heterodimer interacts with TBP and also with the phosphate backbone of DNA (Geiger et al., 1996; Tan et al., 1996). Hence, it is clear that in the nucleus Toa1p and Toa2p interact with the DNA–TBP complex as a heterodimer.

Our data above (Fig. 3) suggested that, in the cytoplasm, Toa2p existed in a complex with Kap122p-PrA and it was possible that Toa1p was part of this complex. As Toa2p and Toa1p bind as a dimer to the DNA–TBP in the nucleus, we investigated whether a Toa1p-Toa2p complex existed also in the cytoplasm. Therefore, we analyzed a postribosomal cytosol from a haploid TOA2-PrA strain by IgG-Sepharose affinity chromatography. A band migrating at ~110 kD was eluted between 100 and 1,000 mM MgCl₂ (Fig. 6), and this band was confirmed to be Kap122p by mass spectrometry analysis. A band migrating at ~45 kD, which coeluted with Toa2p-PrA (Fig. 6), was another major band, migrating at ~45 kD, which coeluted with Toa2p-PrA (Fig. 6). By mass spectrometry analysis this band was identified as Toa1p. The elution of Toa1p at 4.5 M MgCl₂ suggested that Toa1p and Toa2p form a tight cytoplasmic complex that interacts with Kap122p.

To determine the cytoplasmic binding partners of Toa1p-PrA, we analyzed the postribosomal cytosol of a TOA1-PrA strain by IgG-Sepharose affinity chromatography. As expected, immunofluorescence microscopy of Toa1p-PrA in a wt and a kap122Δ strain gave similar results to those obtained for Toa2p-PrA (Fig. 4): the primarily nuclear localization of Toa1p-PrA in the wt strain is diminished in the kap122Δ strain in favor of a diffuse cytoplasmic localization (Fig. 8).

Search for Alternative Import Pathways

Toa1p and Toa2p are essential for viability, whereas Kap122p is not. One solution to this apparent paradox is that one or several Kap(s) other than Kap122p can import these proteins into the nucleus in a kap122Δ strain. How-
ever, the immunofluorescence data of Figs. 4 and 8 suggested that if Toa1p-Toa2p were imported by alternative Kaps, this import would be less efficient than that mediated by Kap122p. Nevertheless, to search for alternative Kaps, a cytosol from a \textit{kap122}D/TOA2-PrA strain was analyzed by IgG-Sepharose affinity chromatography (Fig. 9). As expected, Toa2p-PrA was eluted together with Toa1p (and its numerous degradation products) in the 4.5-M MgCl$_2$ fraction (Fig. 9). However, fractions eluted between 100 and 1,000 mM MgCl$_2$ did not show any visible bands in the 100-kD region and above, where Kaps migrate. Hence, if Kaps other than Kap122p can import the Toa1p-Toa2p, they might do so with lower efficiency than Kap122p and, therefore, are likely to be below the limits of detection of this assay.

**Dissociation of Kap122p from Toa2p-Toa1p by RanGTP**

Import complexes of substrate–Kap are dissociated by RanGTP (Rexach and Blobel, 1995; Schlenstedt et al., 1997; A Ibertini et al., 1998; Jakel and Gorlich, 1998; Kaffman et al., 1998; Senger et al., 1998). To investigate whether this is also the case for the Toa1p/Toa2p/Kap122p complex, we used postribosomal cytosol from a TOA2-PrA strain to prepare an IgG-Sepharose–bound complex of Toa2p-PrA/Toa1p/Kap122p. This complex was incubated with either transport buffer alone, RanGDP, or RanGTP, and the material that was released from the IgG-Sepharose after completion of incubation was collected. Thereafter, the remaining IgG-Sepharose–bound proteins were eluted at 1.0 and 4.5 M MgCl$_2$. Proteins were analyzed by SDS-PAGE and Coomassie blue staining (Fig. 10). Incubation with RanGTP clearly led to the release of most of the Kap122p (compare lanes 1–3). In contrast, incubation with RanGDP did not result in dissociation of Kap122p (lanes 4–6). These data indicated that the cytoplasmic Toa2p-PrA/Toa1p/Kap122p complex, like other import substrate–Kap complexes is sensitive to dissociation by RanGTP but not by RanGDP.

**Recombinant Kap122p Interacts Directly with Recombinant Toa1p–Toa2p Complex**

To determine whether Kap122p is able to interact directly with a Toa1p–Toa2p complex, we coexpressed both His$_6$-Toa1p and Toa2p in \textit{E. coli}. The TFIIA subunits formed a soluble complex and were purified from bacterial lysate via the His$_6$ affinity tag at the amino terminus of Toa1p. Kap122p with an amino-terminal GST tag was also expressed in \textit{E. coli}. We tested for binding between GST-
Kap122p and the His$_6$-Toa1p/Toa2p complex when either was immobilized on GSH-Sepharose or Talon™ beads, respectively.

We found that purified and immobilized His$_6$-Toa1p/Toa2p complex bound GST-Kap122p present in a preincubated bacterial lysate depleted of its Talon™-binding proteins (Fig. 11, lane 1). Incubation of empty Talon™ resin with E. coli lysate containing GST-Kap122p did not result in any Kap122p binding (Fig. 11, lane 2). Likewise, GST-Kap122p, immobilized on GSH-Sepharose, bound the purified soluble His$_6$-Toa1p/Toa2p complex (Fig. 11, lane 3). A control using immobilized GST alone showed no binding of His$_6$-Toa1p/Toa2p (lane 4).

These experiments using E. coli-expressed Toa1p–Toa2p and Kap122p show that Kap122p interacts directly with Toa1p–Toa2p complex.

Dissociation of Recombinant Kap122p and Toa1p–Toa2p complex by RanGTP

We investigated whether the complex of recombinant GST-Kap122p/His$_6$-Toa1p/Toa2p is sensitive to dissociation by RanGTP but not RanGDP. To this end, we prepared a Talon™-bound complex of GST-Kap122p/His$_6$-Toa1p/Toa2p recombinant proteins (Fig. 11, lane 1). Beads were divided into three equal fractions and incubated with RanGTP, RanGDP, or TB alone. At the end of incubation, released material was collected, beads were extensively washed with TB, and bound and released proteins were analyzed by SDS-PAGE and Coomassie blue staining (Fig. 12). Incubation with RanGDP did not release GST-Kap122p from the Toa1p–Toa2p complex (Fig. 12, lanes 3 and 4), neither did control incubation with TB in the absence of Ran (Fig. 12, lanes 5 and 6). These results confirm the specificity of the interaction between recombinant Kap122p and the recombinant Toa1p–Toa2p complex as well as the sensitivity of this interaction to dissociation by RanGTP.

Discussion

Based on sequence similarity with karyopherin βs, the product of the PDR6 gene of S. cerevisiae was previously suggested to be a member of the Kap β family (for review see Pemberton et al., 1998). In this paper, we report that the hitherto uncharacterized product of the PDR6 gene does indeed function as a Kap and, therefore, named it Kap122p. We show that Kap122p functions in the nuclear import of the complex of large and small subunits, Toa1p, and Toa2p, of TFIIA. The relationship between the observed drug resistant phenotype of PDR6 and the function of Kap122p/Pdr6p in nuclear import of TFIIA (or of other proteins) remains to be elucidated.

We found that Kap122p is localized both in the cytoplasm and the nucleus, which is consistent with its function of shuttling between these two compartments. Cytosolic Kap122p exists as a complex with the small and large subunit of TFIIA. The precise stoichiometry of this complex remains to be determined. Based on biochemical and crys-
tallographic data, it is unlikely that the two subunits exist as separate entities (Ranish et al., 1992; Geiger et al., 1996; Tan et al., 1996). Therefore, one possibility is that Kαp122p also functions as a chaperone, and that immediately after synthesis in the cytoplasm, each subunit associates with Kαp122p. In this scenario, each subunit would contain a Kαp122p cognate NLS. Each of the subunit/Kαp122p heterodimers would associate, via interaction between the two subunits, to form a tetramer, which is imported into the nucleus. A ternary, only one of the subunits may contain a Kαp122p-cognate NLS. A fatter synthesis, this subunit could associate with Kαp122p and with the other subunit to form a heterotrimer that would be imported into the nucleus. A fatter import, in each of these two scenarios, RαnGTP (Figs. 10 and 12) would dissociate the TFIIA heterodimer from Kαp122p. Our data here argue against a third possibility, namely that a subunit/Kαp122p heterodimer is imported separately because we found a stable interaction between the two subunits in the cytoplasm. In fact, while the Toa1p-Toa2p complex was dissociated from Kαp122p by MgCl₂ concentrations between 100 and 1,000 mM, the interaction between the two subunits resisted dissociation at these MgCl₂ concentrations (Figs. 6, 7, 9, and 10).

PrA -tagged Kαp122p was found to be associated in the cytoplasm with other proteins (Fig. 3). We do not yet know the identity of these proteins and whether they represent contaminants or alternative import substrates for Kαp122p. However, it is clear that these other proteins are not part of the Toa1p/Toa2p/Kαp122p complex as they were not copurified in a reverse pullout with PrA-tagged Toa1p or Toa2p (Figs. 6 and 7). As in the case of other PrA -tagged Kαps, several degradation products of Kαp122p-PrA retaining their protein A moiety and eluting predominantly in the 4,500 mM MgCl₂ fraction were observed and confirmed by immunoblotting (data not shown).

We were able to reconstitute the Kαp122p/Toa1p/Toa2p complex from recombinant proteins (Fig. 11). Moreover, this complex was sensitive to dissociation by RαnGTP but not RαnGDP (Fig. 12). These findings support the conclusion that Kαp122p interacts directly with the Toa1p-Toa2p complex and that this interaction, like Kαp122p/Toa1p/Toa2p interaction in the yeast cytosol, is sensitive to dissociation by RαnGTP but resists dissociation by RαnGDP.

It is known that RαnGTP dissociates import substrates from Kαps by binding to the Kαp (Rexach and Blobel, 1995). The X-ray crystal structure of RαnGTP complexed to Kap β2 (transportin) and Kap β1 (importin) has been determined (Chook and Blobel, 1999; Vetter et al., 1999). Rαn binding to Kαp122p/Pdr6p has been previously investigated and no Rαn binding was detected (Görlich et al., 1997). Consistent with this report, we have so far not been able to demonstrate binding of RαnGTP to Kαp122p in overlay or solution binding assays (data not shown). This might indicate that RαnGTP binds Kαp122p with very low affinity. Stable binding of RαnGTP to Kαp122p may require additional proteins. However, the Kαp122p/Toa1p/Toa2p complex isolated from yeast (Fig. 10) or reconstituted from recombinant proteins (Figs. 11 and 12) could be dissociated by RαnGTP but not RαnGDP. These data confirm that RαnGTP is directly involved in dissociating Kαp122p from the Toa1p-Toa2p complex and provide support for the existence of functionally relevant interaction between RαnGTP and Kαp122p.

It appears that Kαp122p is the principal Kαp dedicated to the import of TFIIA because in a strain where Kαp122 had been deleted there was a significant mislocalization of the two TFIIA subunits from the nucleus to the cytoplasm (Figs. 4 and 8). Surprisingly, Kαp122 deletion is not lethal, whereas deletion of either of the two TFIIA subunits is. Therefore, Kαps other than Kαp122p are likely to be involved in nuclear import of the TFIIA subunits. However, so far we have failed to identify alternative Kαps by cytosolic pullout experiments with Toa2p-PrA in a kap122Δ strain (Fig. 9). It is likely that import of the TFIIA subunits by these putative alternative Kαps proceeds with much lower efficiency than import by Kαp122p, based on the significant reduction in the nuclear localization of the TFIIA subunits observed in a kap122Δ strain. Nevertheless, import of these two essential proteins in the absence of Kαp122p appears to be sufficient, as there is no apparent difference in the growth rate between the kap122Δ and an isogenic wt strain. A ternary Kαp(s) may be difficult to detect in an immunosoliation assay as they may bind to the two TFIIA subunits with lower affinity. There are precedents for essential proteins being imported by several Kαps of which the principal one is not essential. For example, ribosomal proteins have been shown to be imported by the abundant Kαp123p (Rout et al., 1997; Schlenstedt et al., 1997). However, Kαp123p is not essential, but the es-

Figure 12. Dissociation of the recombinant GST-Kαp122p/His5-Toa1p/Toa2p complex by RαnGTP. Immobilized His5-Toa1p/Toa2p complex was incubated with GST-Kαp122p. coli lysate to obtain a GST-Kαp122p/His5-Toa1p/Toa2p complex. Three equal aliquots of beads were incubated with either RαnGTP, RαnGDP, or buffer alone. A fatter incubation, released and still bound proteins were separated by SDS-PAGE and stained with Coomassie blue. Note that RαnGTP almost completely dissociated Kαp122p from immobilized Toa1p-Toa2p complex (lanes 1 and 2), while Kαp122p remained in the complex with Toa1p-Toa2p after incubation with RαnGDP (lanes 3 and 4) or buffer alone (lanes 5 and 6).
sential Kap121p can back ribosomal protein import in a kap121a strain (Rout et al., 1997; Seedofor and Sil-ver, 1997). A alternatively, the Toa1p-Toa2p heterodimer is small enough (<60 kD) that it might diffuse through the NPC.

Kap122p is the third Kap that so far has been shown to be dedicated to the import of a general transcription factor, the others being Kap119p, which is involved in the nuclear import of the nonessential transcription factor TFIIS (A Ibertini et al., 1998), and Kap114p, which is involved in the nuclear import of the TATA binding protein (Pemberton et al., 1999). The advantages of maintaining dedicated Kap for the import of specific transcription factors are likely to be in the regulatory realm and remain to be elucidated.

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References
Aitchison, J.D., M.P. Rout, M. Marelli, G. Blobel, and R.W. Wozniak. 1995. Two novel related yeast nuclear transporters Nup170p and Nup157p: complementation with the vertebrate homologue Nup155p and functional interaction with the yeast nuclear pore-membrane protein Pomp152p. J. Cell Biol. 131:1339–1348.

Aitchison, J.D., G. Blobel, and M.P. Rout. 1996. Kap104p: a karyopherin involved in the nuclear transport of messenger RNA binding proteins. Science. 274:624–627.

A Ibertini, M.-L. Pemberton, J.S. Robinson, and G. Blobel. 1996. A novel nuclear import pathway for the transcription factor TFIIS. J. Cell Biol. 134:1447–1455.

Bailz, E., W. Chen, S. Ulaszewski, E. Capieaux, and A. Goffeau. 1987. The multidrug resistance gene PDR1 from Saccharomyces cerevisiae. J. Biol. Chem. 262:18671–18679.

Chen, W.N., E. Balzi, E. Capieaux, M. Choder, and A. Goffeau. 1991. The ATE1 loci on chromosome VII from Saccharomyces cerevisiae. J. Biol. Chem. 266:20920–20927.

Conti, E., U. L. Leighton, G. Blobel, and J. Kuriyan. 1998. Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import receptor importin alpha. Cell. 94:237–247.

De Jong, J., and R.G. Roeder. 1993. A single cDNA, hTFIIA/TFIIB, encodes a subunit of the nuclear transport complex containing the subunits of the yeast transcription factor IIA. J. Cell Biol. 121:845–855.

Dujon, J., and R. G. Roeder. 1997. The human homologs of yeast CR M1 is in a dynamic subcomplex with CAl N/Nun2 and a novel nuclear pore component Nup88. EMBO J. (Eur. Mol. Biol. Organ.) 16:807–816.

Geiger, J.H., S. Hahn, S. Lee, and P.B. Sigler. 1996. Crystal structure of the yeast TATA box binding protein (TBP) and TATA complex. Science. 272:830–836.

Gharaighthi, F., M. Kirchner, J. Fernandez, and S.M. Mische. 1996. Peptide mass profiles of polyvinylidene difluoride-bound proteins by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry in the presence of nonionic detergents. Anal. Chem. 68:934–940.

Görlich, D., M. Dabrowski, F.R. Bischoff, U. Kutay, P. Bork, E. Hartmann, S. Prehn, and E. Izaurralde. 1997. A novel class of RanGTP binding proteins. J. Biol. Chem. 272:139–145.

Jakkal, S., and D. Görlich. 1998. Importin beta, transportin, RanBPS, and RanGAP mediate nuclear transport of ribosomal proteins in mammalian cells. EMBO J. (Eur. Mol. Biol. Organ.) 17:4491–4502.

Kaffman, A., J.D. Aitchison, and G. Blobel. 1992. Phosphorylation regulates association of the transcription factor Pho4 with its import receptor Pse1p. J. Biol. Chem. 267:2673–2683.

Mahe, Y., Y. Lemoine, and K. Kuchler. 1996. The ATP binding cassette transporters Pdr5p and Sng2p of Saccharomyces cerevisiae can mediate transport of steroids in vivo. J. Biol. Chem. 271:25167–25172.

Marelli, M., J.D. A. Ibertini, and R.W. Wozniak. 1996. Specific binding of the karyopherin kaap121p to a subunit of the nuclear pore complex containing nup53p, nup53p, and nup170p. J. Cell Biol. 134:1813–1819.

Mattaj, I.W., and L. Egner. 1998. Nucleocytoplasmic transport: the soluble phase. Annu. Rev. Biochem. 67:265–306.

Melchior, F., and L. Gerace. 1998. Two-way trafficking with Ran. Trends Cell Biol. 8:175–179.

Melchior, F., B. Paschal, J. Evans, and L. Gerace. 1998. Inhibition of nuclear protein import by nonhydrolyzable analogs of GTP and identification of the small G protein RanCdc4 as an essential transport factor. J. Cell Biol. 132:1649–1659.

Moore, M.S. 1998. Ran and nuclear transport. J. Biol. Chem. 273:22857–22860.

Moore, M.S., and G. Blobel. 1993. The GTP-binding protein Ran/Tcf4 is required for import protein into the nucleus. Nature. 365:661–663.

Moore, J.D., J. Yang, R. Truant, and S. Kornbluth. 1999. Nuclear import of CDC25C/cdc25Cp: recombination of import-competent mutants. J. Cell Biol. 144:213–224.

Ohno, M., M. Fornerod, and I.W. Mattaj. 1998. Nucleocytoplasmic transport: the last 200 nanometers. Cell. 92:327–336.

Palmer, D., and M.H. Alm. 1999. Importin beta can mediate the nuclear import of a arginine-rich nuclear localization signal in the absence of importin alpha. Mol. Cell. Biol. 19:1218–1225.

Pemberton, L.F., J.S. Robinson, and G. Blobel. 1997. A distinct and parallel pathway for the nuclear import of an mRNA-binding protein. J. Cell Biol. 139:1645–1653.

Pemberton, L.F., J.S. Robinson, and G. Blobel. 1998. Transport routes through the nuclear pore complex. Curr. Opin. Cell Biol. 10:392–399.

Pemberton, L.F., J.S. Robinson, and G. Blobel. 1999. Nuclear import of the TATA binding protein: mediation by the karyopherin Kap114p and a possible mechanism for intranuclear targeting. J. Cell Biol. 145:1407–1417.

Ranish, J.A., and S. Hahn. 1991. The yeast general transcription factor TFIIA is composed of two polypeptide subunits. J. Biol. Chem. 266:1920–1927.

Ranish, J.A., W.S. Lane, and S. Hahn. 1992. Isolation of two genes that encode subunits of the yeast importin/iIA. Science. 255:1127–1129.

Reach, M., and G. Blobel. 1995. Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. Cell. 83:683–692.

Ribbeck, K., U. Kutay, E. Paraskeva, and D. Görlich. 1999. The translational control of transportin-cargo complexes through nuclear pores is independent of borealin and augmin. J. Cell Biol. 145:1407–1417.

Rosenblum, J.S., L.F. Pemberton, and G. Blobel. 1997. A nuclear import pathway for a protein involved in trk N MATuration. J. Cell Biol. 139:1655–1661.

Rout, M.P., G. Blobel, and J.D. A. Ibertini. 1997. A distinct nuclear import pathway used by ribosomal proteins. J. Cell Biol. 139:715–725.

Schwoebel, E.D., B. Talcott, I. Cushman, and M.S. Moore. 1998. Ran-dependent signal-mediated nuclear import does not require GTP hydrolysis by Ran. J. Biol. Chem. 273:35170–35175.

Schliephake, G., E. Smirnova, J. Solisbacher, U. Kutay, D. Görlich, H. Poznanski, and F.R. Bischoff. 1997. Yrb4p, an yeast ran-GTP-binding protein involved in import of ribosomal protein L25 into the nucleus. EMBO J. (Eur. Mol. Biol. Organ.) 16:6237–6249.

Seedofor, M., and P.A. Silverman. 1999. A RanGTP import/karyopherin protein family members required for mRNA export from the nucleus. Proc. Natl. Acad. Sci. USA. 94:8590–8595.

Senger, B., G. Simos, F.R. Bischoff, A. Podtelejnikov, M. Mann, and E. Hurt. 1998. Mtr10p functions as a nuclear import receptor for the binding.
protein Npl3p. EMBO J. 17:2196–2207.
Sherman, F., G.R. Fink, and J.B. Hicks. 1986. Methods in Yeast Genetics. Cold Spring Harbor Press, Cold Spring Harbor, NY. 186 pp.
Tan, S., Y. Hunziker, D.F. Sargent, and T.J. Richmond. 1996. Crystal structure of a yeast TFIIA/TBP/DNA complex. Nature 381:127–151.
Truant, R., and B.R. Cullen. 1999. The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin beta-dependent nuclear localization signals. Mol. Cell Biol. 19:1210-1217.
Vetter, I.R., A. Arndt, U. Kutay, D. Gorlich, and A. Wittinghofer. 1999. Structural view of the Ran-Importin beta interaction at 2.3 Å resolution. Cell. 97:635–646.
Wente, S.R., M.P. Rout, and G. Blobel. 1992. A new family of yeast nuclear pore complex proteins. J. Cell Biol. 119:705–723.
Wilson, S.M., K.V. Datar, M.R. Paddy, J.R. Swellday, and M.S. Swanson. 1994. Characterization of nuclear polyadenylated RNA-binding proteins in Saccharomyces cerevisiae. J. Cell Biol. 127:1173–1184.
Wozniak, R.W., M.P. Rout, and J.D. Aitchison. 1998. Karyopherins and kissing cousins. Trends Cell Biol. 8:184–188.
Yang, Q., M.P. Rout, and C.W. Akey. 1998. Three-dimensional architecture of the isolated yeast nuclear pore complex: functional and evolutionary implications. Mol. Cell. 1:225–234.