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A Distinct Nuclear Import Pathway Used by Ribosomal Proteins

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Summary

Protein transport into the nucleus is governed by the interaction of soluble transport factors with their import substrates and nuclear pore complexes. Here, we identify a major distinct nuclear import pathway, mediated by a previously uncharacterized yeast β karyopherin Kap123p. The predominant substrates for this pathway are ribosomal proteins, which must be imported into the nucleus prior to assembly into preribosomes. Kap123p binds directly to its transport substrates, repeat motif–containing nucleoporins, and Ran-GTP. We show that the related protein Pse1p is also a karyopherin and can functionally substitute for Kap123p; both are capable of specifically directing a ribosomal nuclear localization signal reporter to the nucleus in vivo.

Introduction

Nuclear pore complexes (NPCs) function as the only known mediator of nucleocytoplasmic exchange, and as such, they define the contents of the nucleus. Ions and small molecules can passively diffuse through ~9 nm diameter aqueous channels. In contrast, specific macromolecules are actively transported across the NPC; these include imported proteins and snRNPs, exported RNPs, and "shuttling" (repeatedly imported and exported) proteins. Transport is saturable, energy-dependent, regulated, and highly selective (for reviews, see Rout and Wente, 1994; Görlich and Mattaj, 1996a).

Nuclear protein import begins in the cytoplasm with the recognition of nuclear localization sequences (NLSs) on substrate proteins by soluble transport factors. Typically, proteins bearing a classical NLS of the SV40 large T antigen (SV40 LT) or bipartite type (Dingwall and Laskey, 1991; Makkerh et al., 1996) are bound by karyopherin α (importin α), which then docks as a heterodimeric complex with karyopherin β (importin β) to particular repeat-containing nucleoporins at the cytoplasmic face of the NPC. A subsequent active translocation step across the NPC requires other soluble factors, including the small GTPase Ran (which provides a potential source of energy) and its cofactor p10 (Melchior et al., 1993; Moore and Blobel, 1993, 1994; Adam and Adam, 1994; Görlich et al., 1995; Imamoto et al., 1995a, 1995b; Moroianu et al., 1995; Paschal and Gerace, 1995; Radu et al., 1995; Weis et al., 1995). It has long been established that there exist separate, saturable, noncompeting nuclear import pathways, presumed to be mediated by alternative karyopherins recognizing different types of NLSs (Fischer et al., 1991; Garcia-Bustos et al., 1991; Goldfarb and Michaud, 1991; Michaud and Goldfarb, 1991, 1992). Recently, one such pathway was discovered. The karyopherin β homolog Kap104p/transportin imports a particular set of mRNA-binding proteins. Although it also docks to repeat motif–containing nucleoporins, it is unlike karyopherin β in that it binds directly to its substrate (Aitchison et al., 1996; Pollard et al., 1996; Bonifaci et al., 1997).

Here, we demonstrate that Kap123p is a Saccharomyces karyopherin β, mediating a major alternative transport pathway.

Results

Kap123p Is a β Karyopherin Homolog

A detailed analysis of the composition of a highly enriched NPC fraction from the yeast Saccharomyces has led to the identification of not only numerous nucleoporins, but also transport factors presumably caught during the NPC-associated phase of their cycle. Two known karyopherins were identified: Srp1p/Kap60p and Kap95p (Yano et al., 1992; Enenkel et al., 1995). Sequence data from another protein in the fraction identified an ORF in the genome database (YER110c), predicted to encode a polypeptide of 123 kDa. Because this protein proved functionally homologous to Kap95p (below), we renamed it Kap123p, following standard Saccharomyces nomenclature (Enenkel et al., 1995; Iovine et al., 1995). Comparisons of the yeast database reveal significant similarities between Kap95p, Kap123p, and two other yeast ORFs: Pse1p (YMR308c), originally characterized as a gene affecting protein secretion (Chow et al., 1992), and another recently characterized karyopherin β homolog, Kap104p (YBR017c) (Aitchison et al., 1996). Sequence alignment and structural prediction algorithms revealed a number of features shared between Kap95p, Kap123p, and Pse1p (Figure 1). Each protein is approximately 20% identical and 50% similar to the others along its entire length by pairwise FASTA analysis, though Kap123p most resembles Pse1p. Pse1p is also a karyopherin (below). All carry a redundant WPEL motif between amino acids 120 and 140, and a stretch of glutamic and aspartic acids around amino acid 330 that is conserved in vertebrate karyopherin β and has been reported to form part of the overlapping Ran-GTP and karyopherin α binding domains (Enenkel et al., 1996; Moroianu et al., 1996). Extensive stretches of antiparallel α-helical motif "HEAT" repeats (proposed to be protein binding sites) are predicted in all the karyopherin β homologs, karyopherin α, and certain other proteins involved mainly in transport processes (Andrade and Bork, 1995).

KAP123, like *KAP104*, is not essential; unlike *KAP104*, deletion of the gene does not result in a temperature-sensitive phenotype but does cause a growth defect on rich media (<75% normal growth rate) (Smith et al., 1996). This was strikingly demonstrated when the deletion strain and its wild-type counterpart were grown

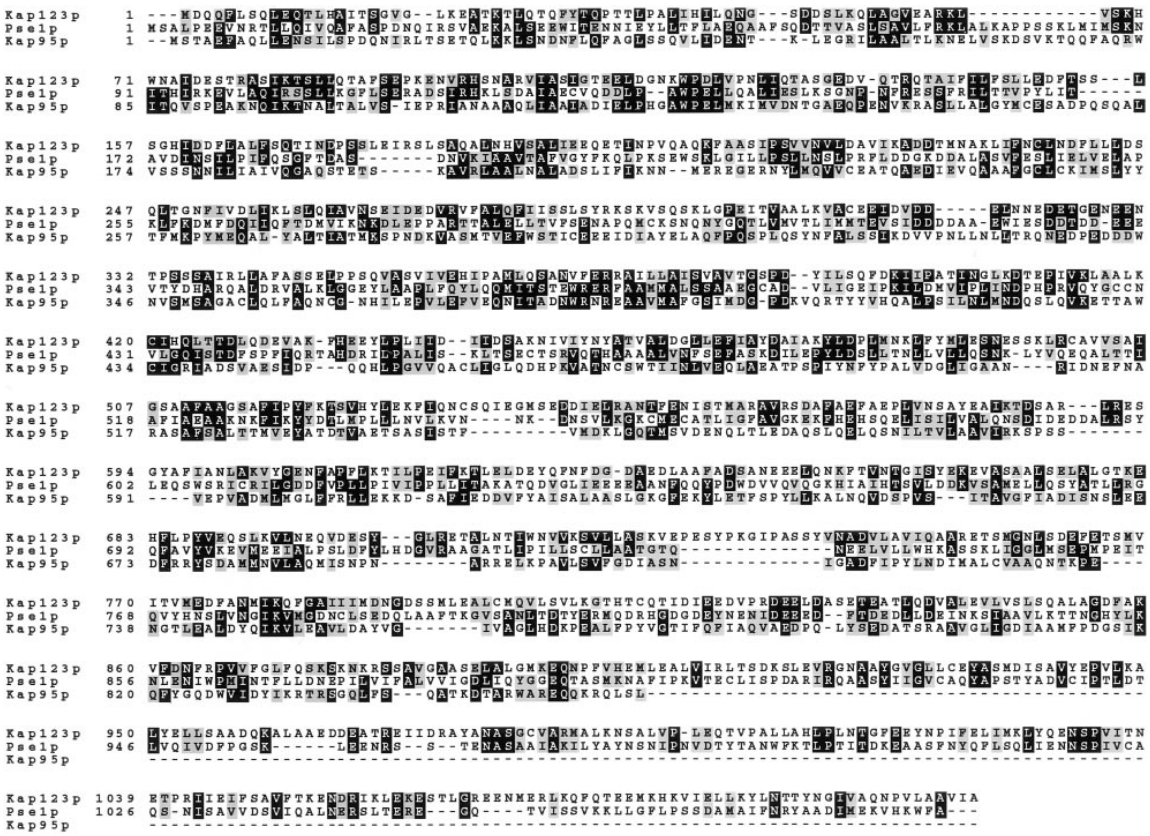

Figure 1. Alignment of Kap95p, Kap123p, and Pse1p Sequences

Amino acid identities are indicated by black boxes, whereas similarities are indicated by gray ones. Sequences were aligned using CLUSTALW v.1.6 and analyzed with BOXSHADE.

together competitively. The deletion strain lost ground rapidly in a comparatively short time, even though it was initially added in 2-fold excess (Figure 2A). Previously, we characterized a novel β karyopherin by comparing its behavior against that of the classical β karyopherin, Kap95p (Aitchison et al., 1996), and a similar approach was used here. To achieve this, strains were created in which the only copy of the karyopherin in question carried a Protein A (PrA) tag at its carboxy terminus (Aitchison et al., 1995a). The Kap123p-tagged strain showed no growth defects when grown competitively against the wild-type strain (Figure 2A), demonstrating that the Kap123p-PrA fusion protein, like Kap95p-PrA and Kap104p-PrA (Aitchison et al., 1996), is fully functional. The presence of the same tag on each of the different β karyopherins allowed direct and semiquantitative comparisons; immunoblotting of cell lysates from these strains suggested that Kap123p is the most abundant of all the homologs (Figure 2B; data not shown). Subcellular fractionation (Figure 2B) indicated that both Kap95p-PrA and Kap123p-PrA were mainly cytosolic, though a significant fraction of each remained associated with nuclei and NEs (nuclear envelopes). An identical fractionation pattern was observed using wild-type cells and an antibody specific for Kap123p (data not shown). Under these conditions, foreign reporter proteins containing an NLS cofractionated with the nuclei, suggesting that loss of nuclear-associated proteins during this procedure is minimal (Figures 5B and 5C). Immunofluorescence microscopy confirmed this mainly cytoplasmic localization, though again some nuclear and NE-associated signal was also observed. Under short fixation conditions, a strong signal coincident with the localization pattern for NPC markers could be detected (Figure 2C), showing that both proteins associate with NPCs.

It has previously been shown that Kap95p, either in isolation or as a complex with Kap60p, binds strongly to the repeat motif regions within a particular class of nucleoporins, reflecting its docking to the NPC (Iovine et al., 1995; Kraemer et al., 1995; Rexach and Blobel, 1995; Aitchison et al., 1996). To compare the pattern of binding of Kap95p with Kap123p in vitro, we probed a 2D separation of total proteins from enriched yeast NPCs (Rout and Blobel, 1993) with cytosols containing either tagged Kap95p or Kap123p (Figure 3A). Cytosols (rather than purified expression constructs) were chosen for most of these experiments, as they provided both the accessory factors and competitors potentially found in vivo; for example, cytosolic Kap95p is found mainly as a heterodimer with Kap60p, the probable form in which we detected it in all the assays shown here (Enenkel et al., 1995) (Figure 5). Both Kap95p and Kap123p bound to nucleoporins containing repeat motif regions

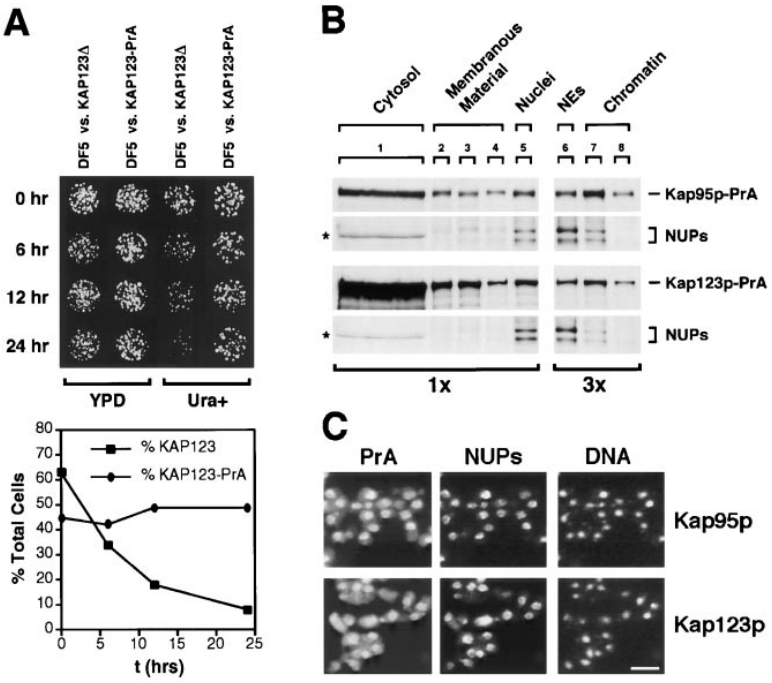

Figure 2. Initial Characterization of Kap123p

(A) Top: Cultures in which the parent strain (DF5) was grown with cells containing either a deletion of *KAP123* (KAP123Δ) or a *KAP123*-PrA fusion (KAP123-PrA) were sampled at the indicated time points (0, 6, 12, and 24 hr). Aliquots containing ~100 logarithmically growing cells were plated on either YPD (representing the total number of cells) or Ura$^-$ (representing the number of deletion mutant or tagged cells in the aliquot) medium. Initially, 123Δ-14-1 and DF5a cells were mixed 2:1 to a final concentration of 3×10^5 cells/ml, and KAP123-A and DF5a cells were mixed 1:1 to a final concentration of 2.4×10^5 cells/ml. Cell cultures were diluted at each time point to the original concentration, and 10 μl (neat and 10-fold dilutions) of the culture was plated. Bottom: Graphical representation of the percentages of the deletion or tagged strains in the cultures at the measured time points.

(B) Behavior of Kap95p-PrA and Kap123p-PrA during subcellular fractionation of KAP95-A and KAP123-A cells. Equal numbers of yeast cells from both strains were fractionated, and the proteins from each fraction analyzed by SDS–PAGE and immunoblotting. Lanes 1–5 were loaded at 1 cell equivalent (1×) and lanes 6–8 at 3 cell equivalents (3×), and the major contents of the fractions are noted; lane 1 is wider for loading purposes. The karyopherins were detected via their PrA tags, and the nuclear envelopes (NEs) were followed with nucleoporin markers (NUPs). The asterisk marks a cross-reacting cytoplasmic band, and the two nucleoporins shown are Nup57p (upper band) and Nup49p (lower band). Semiquantitative analysis indicated that approximately 75% of both karyopherins were found in the cytosolic fraction, 5%–10% in the nuclei fraction, and 1%–3% in the NE fraction.

(C) Immunofluorescence microscopy of KAP95-A and KAP123-A cells. Both karyopherins (detected via their PrA tags) gave some cytoplasmic labeling plus a punctate peripheral nuclear signal (PrA) coincident with the nucleoporin marker (NUPs), indicating an association of Kap95p and Kap123p with NPCs. The position of the nuclei was visualized with DAPI (DNA). Bar, 7 μm.

(though apparently not all members of that class were recognized) and shared most of their binding specificities; however, when compared with Kap95p, Kap123p bound with generally lesser intensities. It is not known whether any of these differences were due to Kap60p's association with Kap95p. Furthermore, when the karyopherins were presented with either a long portion of the repeat-containing region of recombinant Nup1p (characterized by redundant XFXFG repeats), or a short portion of the repeat region of recombinant Nup159p (PSFG repeats) (Davis and Fink, 1990; Kraemer et al., 1995; Rexach and Blobel, 1995), both preferred the former. Kap123p expressed in E. coli bound similarly, indicating that the interaction between Kap123p and the nucleoporin fragment was direct (Figure 3B). These results imply that the NPC docking sites for Kap123p are the repeat motif–containing nucleoporins and that it, like Kap95p, binds directly to at least the repeat motif–containing regions themselves, perhaps by a similar mechanism.

Ran (Gsp1p) has been shown to be an essential cofactor in the import of classical NLS substrates and to bind to Kap95p (Rexach and Blobel, 1995). Similarly, Kap123p directly bound Ran-GTP in an overlay assay (Figure 3C). It should be noted that under these conditions, Ran-GTP bound with a markedly lower affinity to Kap123p than to Kap95p (data not shown). The similarities between Kap95p and Kap123p in sequence, abundance, subcellular distribution, nucleoporin affinities, and binding to Ran-GTP indicate that Kap123p is indeed a karyopherin.

Kap123p Imports Ribosomal Proteins into the Nucleus

The results presented above suggested that the overlay assay might also be effective in determining transport substrate specificities. Comparison of the binding of Kap95p and Kap123p to proteins in a yeast nuclear lysate (many of which by definition carry NLSs) showed that while Kap95p/Kap60p recognized proteins mainly above 50 kDa, Kap123p bound strongly to a series of proteins between 15 and 50 kDa (Figure 4A). Abundant nuclear proteins in this molecular weight range include those associated with chromatin, hnRNPs, and nucleolar preribosomal assemblages. Ribosomal protein import into the nucleus has been shown to be active and NLS-mediated (Moreland et al., 1985; Underwood and Fried, 1990; Schaap et al., 1991; Schmidt et al., 1995). Therefore, ribosomal proteins were directly tested in the overlay assay. In fact, of the four homologs, only Kap123p bound with high affinity to purified yeast ribosomal proteins (Figure 4B). None of the four homologs recognized E. coli ribosomal proteins (data not shown), which carry no NLSs, making it unlikely that the interaction of Kap123p was based simply on the generally basic pIs of ribosomal proteins, and suggesting that the interaction might be via the yeast ribosomal proteins' NLSs. Many, but not all, of the ribosome-associated proteins

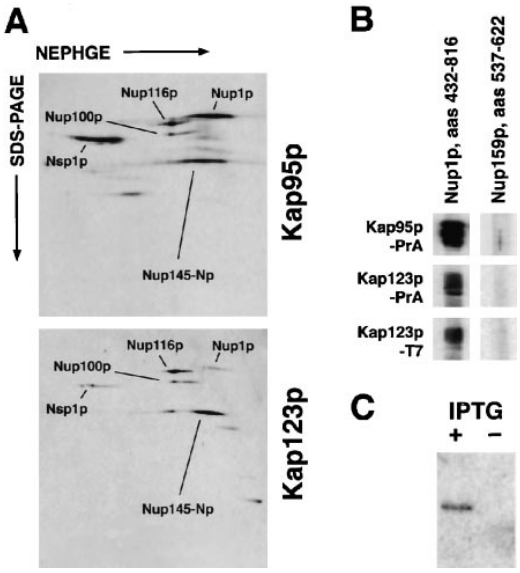

Figure 3. Kap123p Binds to Repeat Motif–Containing Nucleoporins and Ran

(A) Overlay assay of a 2D electrophoretic separation of enriched NPC proteins probed with cytosols from either KAP95-A (Kap95p) or KAP123-A (Kap123p) cells. The binding of either Kap95p-PrA or Kap123p-PrA to the indicated repeat-containing nucleoporins was detected via their PrA tags. The identity of the other spots, though believed to be other known repeat motif–containing nucleoporins, has not been confirmed.

(B) Overlay assay showing that cytosolic Kap95p and Kap123p and bacterially synthesized Kap123p bind to the repeat motif regions themselves, strongly preferring one type of repeat motif region. E. coli lysates containing fragments of repeat motif regions from two different nucleoporins fused to GST (Nup1p, aa 432–816 or Nup159p, aa 537–622) were probed with cytosols from either KAP95-A (Kap95p-PrA) or KAP123-A (Kap123p-PrA) cells, or lysate from E. coli expressing T7-tagged Kap123p (Kap123p-T7).

(C) Ran-GTP binds to Kap123p. E. coli lysates in which expression of Kap123p was induced (+) or uninduced (−) by IPTG were probed with Ran-γ^{32}P-GTP in an overlay assay.

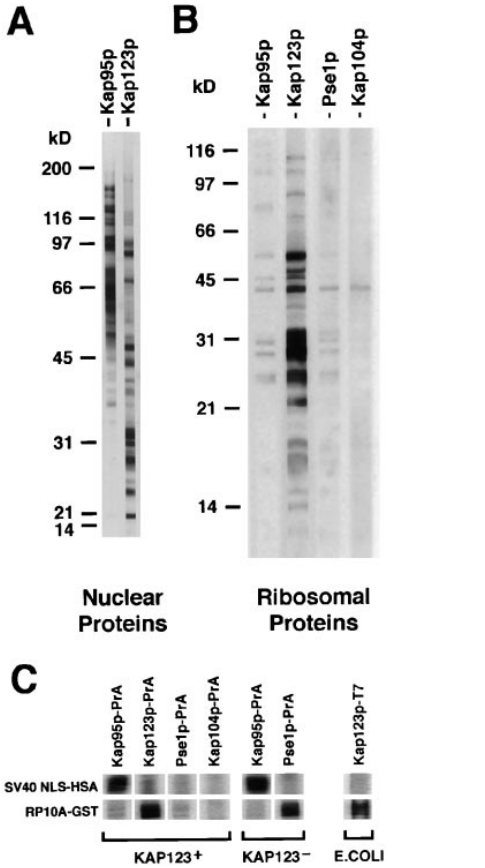

Figure 4. Kap123p Binds a Subset of Ribosomal Proteins in Overlay Assays

(A) Yeast nuclear proteins separated by SDS–PAGE and probed with cytosol from either KAP95-A (Kap95p) or KAP123-A (Kap123p) cells, showing Kap123p recognizes a group of low molecular weight proteins.

(B) Yeast ribosomal proteins separated by SDS–PAGE and probed with cytosol from either KAP95-A (Kap95p), KAP123-A (Kap123p), PSE1-A (Pse1p), or KAP104-A (Kap104p) cells, showing that Kap123p specifically recognizes numerous ribosomal proteins.

(C) Partiality of various karyopherins for NLS-bearing proteins. SV40 NLS-HSAp (Moore and Blobel, 1992) or GST-RP10Ap were probed with cytosols prepared from cells carrying (KAP123$^+$) or lacking (KAP123$^-$) a functional copy of *KAP123*, in which the indicated karyopherin was PrA-tagged. In cells expressing normal levels of Kap123p, only Kap95p/Kap60p strongly recognized the SV40 LT NLS, and only Kap123p the ribosomal NLS. Bacterially expressed T7-tagged Kap123p (E. COLI [T7]) showed a similar specificity. However, in the absence of any Kap123p, Pse1p bound effectively to RP10Ap, though the partiality of Kap95p/Kap60p remained unchanged.

bound strongly in the overlay assay. The denaturation and partial refolding of ribosomal proteins during their preparation for the overlay assay may inactivate many NLSs. Also, the ribosomes used for this assay contain translation accessory factors and proteins added upon final cytoplasmic maturation of the ribosome, none of which need NLSs (Zinker and Warner, 1976; Warner, 1989).

We then compared the affinities of the tagged cytosolic β karyopherins for the yeast ribosomal protein RP10Ap (which we identified as one of the strongest binding proteins in the overlay assay; data not shown) or the classical SV40 LT NLS, which also functions in yeast (Nelson and Silver, 1989). While the SV40 LT NLS bound only Kap95p/Kap60p, the RP10Ap construct was most strongly recognized by Kap123p (Figure 4C). These results raise the possibility of an alternative NLS, carried by ribosomal proteins and preferred by Kap123p. Notably, E. coli–expressed Kap123p can also bind specifically to the RP10Ap construct, indicating that Kap123p requires no α karyopherin–like cofactor to bind ribosomal NLSs (Figure 4C).

We also affinity purified the tagged Kap123p from cytosol, as an alternative to the overlay assay in determining the interactions of the karyopherin with soluble proteins. Kap95p was similarly purified, from the same number of cells, for comparison. Both proteins were recovered in high yield, at greater than 60% of their cellular totals (Figures 2B and 5A), indicating that any interactions were likely representative. Semiquantitative immunoblotting and quantitative amino acid analysis showed that there was approximately three times as much Kap123p as Kap95p in the cells, representing

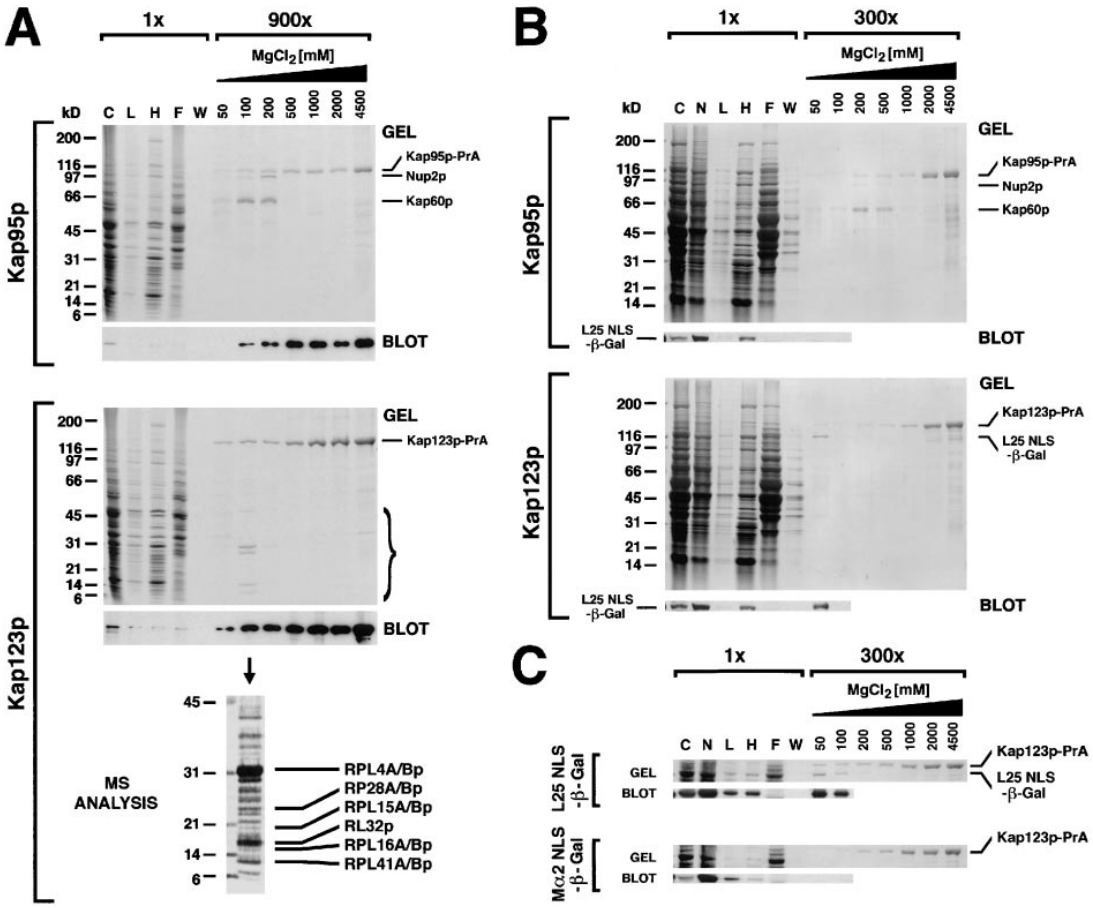

Figure 5. Immunoisolation of Protein A–Tagged Karyopherins from Yeast Cytosol

Cytosols (C) from KAP95-A or KAP123-A cells were centrifuged to pellet particulates ([L] and [H], for pellets from the low and high speed spins) and incubated with IgG Sepharose. The resin was washed to remove unbound material ([F] and [W], for flowthrough and wash fractions) (1 cell equivalent loaded [1×]). Karyopherin complexes were eluted in a $MgCl_2$ gradient ($MgCl_2$ [mM]; molarity of elution step is indicated) and loaded at either 300 cell equivalents (300×) or 900 cell equivalents (900×). Gel samples from the fractions were loaded for Coomassie blue–stained gels (GEL) or immunoblots of the tagged protein (BLOT).

(A) Immunoisolation of Kap95p-PrA, Kap123p-PrA, and their associated proteins. Top: the positions of Kap95p-PrA and the coisolating Kap60p and Nup2p are indicated. Middle: the position of Kap123p-PrA is indicated, and the bracket shows the molecular weight range of numerous bands coisolating in apparently submolar amounts with Kap123p-PrA. Bottom: higher protein loadings of the 100 mM $MgCl_2$ eluate allowed the mass spectrometric analysis of the bands and the tentative identification of six proteins in the bands (indicated with lines), all of which were ribosomal proteins: RPL4A/Bp (YHL033c/YLL045c), RP28A/Bp (YOL120c/YNL301c), RPL15A/Bp (YDR418w/YEL054c), RL32p (YBL092w), RPL16A/Bp (YPR102c/YGR085c), and RPL41A/Bp (YNL162w/YHR141c).

(B and C) Specific coimmunoisolation of a ribosomal NLS-bearing reporter protein with Kap123p-PrA. In (B), KAP95-A or KAP123-A cells were transformed with a plasmid expressing an L25 NLS-β-galactosidase fusion protein (L25 NLS-β-gal). Fewer cell equivalents of the eluate fractions were loaded than in Figure 6A, so the coisolating ribosomal proteins are not apparent. The L25 NLS-β-galactosidase chimera coimmunoisolated with the Kap123p-PrA in high enough amounts to be visible as a Coomassie-stained band (50 mM lane of the $MgCl_2$ elution). Immunoblotting (BLOT) confirmed that this was the L25 NLS-β-galactosidase reporter, that the reporter was expressed at approximately equal levels and was mainly localized to the nucleus in both strains, and that not even trace amounts coisolated with Kap95p-PrA. In (C), *KAP123-A* cells were transformed with plasmids expressing either a Matα2 NLS- or L25 NLS-β-galactosidase fusion protein (Mα2 NLS-β-gal or L25 NLS-β-gal). Only the L25 NLS chimera coimmunoisolated with the Kap123p-PrA (50 mM lane of the $MgCl_2$ elution). Immunoblotting (BLOT) confirmed that both reporters were expressed at approximately equal levels and localized mainly to the nucleus. There was more cytoplasmic reporter in the case of the L25 NLS, but most of this was associated with particulates removed prior to immunoisolation (lanes L and H).

some 1/15,000 and 1/50,000, respectively, of the total protein mass in a cell. Kap95p copurified with a roughly equimolar amount of Kap60p and a little Nup2p, as previously shown (Enenkel et al., 1995; Aitchison et al., 1996). Several minor bands copurified with Kap123p. The identification of these copurifying bands by mass spectrometry was limited by the low amounts of each band to analyze, the potential of multiple proteins in each band, and contamination with proteolytic breakdown products of the far more abundant Kap123p-PrA. However, all those that could be identified proved to be ribosomal proteins (Figure 5A). These bands were unique to the Kap123p-PrA purification, as ribosomal proteins were not found to copurify in similar experiments with Kap95p-PrA, Kap104p-PrA (Aitchison et al., 1996), or Pse1p-PrA (data not shown). Kap123-PrA did

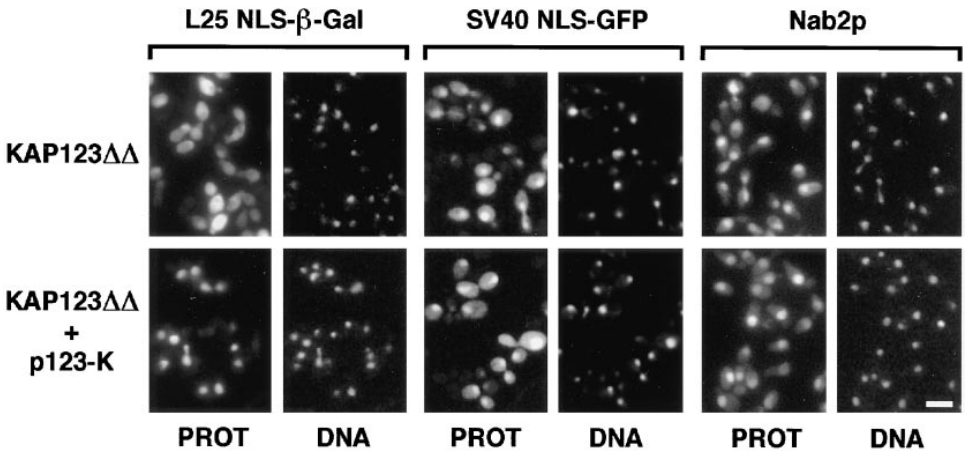

Figure 6. Efficient Nuclear Localization of a Ribosomal NLS Reporter Protein Depends upon the Presence of Kap123p

The localization of various nuclear transport markers (PROT) was determined by fluorescence microscopy of yeast cells. When no Kap123p was present (KAP123ΔΔ), the ribosomal protein NLS reporter (L25 NLS-β-gal) was distributed throughout the cytoplasm and nucleoplasm of these cells (with occasional slight nuclear accumulation); however, when Kap123p was present (KAP123ΔΔ + p123-K), the same reporter was localized mainly to the nucleus. The nuclear signal of markers known to be imported by either Kap95p/Kap60p (SV40 NLS-GFP) or Kap104p (Nab2p) remained unaffected by the absence or presence of Kap123p. Some signal heterogeneity in this figure and in Figure 7 was due to loss of plasmids during growth on the nonselective media. The position of the nuclei was visualized with DAPI (DNA). Bar, 5 μm.

not pellet with the ribosomes (lane H), indicating that it did not associate with mature ribosomes. To test if Kap123p bound a known ribosomal NLS, Kap95p-PrA or Kap123p-PrA were next immunoisolated from KAP95-A or KAP123-A cells expressing a reporter protein with the NLS-containing region of the ribosomal protein L25 (RPL25 [YOL127w]) amino-terminally fused to the full length of β-galactosidase, a construct previously shown to be efficiently imported into the nucleus (Schaap et al., 1991; Mutvei et al., 1992; Nehrbass et al., 1993). The reporter coisolated only with Kap123p-PrA (Figure 5B). The absence of a coisolating band of similar abundance to the reporter protein indicates again that no cofactor is required to maintain the interaction between Kap123p and a ribosomal NLS, but, rather, the binding is direct. Kap123p-PrA was also immunoisolated from equal numbers of *KAP123-A* cells expressing either the homeodomain NLS of Matα2 (Hall et al., 1990) or the L25 NLS amino-terminally fused to β-galactosidase (Figure 5C). Only the L25 NLS-β-galactosidase reporter coisolated with Kap123p-PrA. Thus, the interaction of Kap123p with the reporter is specific to the NLS carried and independent of the β-galactosidase. Together, the immunoisolation experiments and overlay assays provide independent evidence that Kap123p binds specifically to ribosomal proteins, probably in all cases (as with the L25 NLS above) via their NLS-bearing regions.

The specific recognition of the L25 NLS-β-galactosidase reporter by Kap123p suggested that it would also be a suitable reporter for the function of Kap123p in vivo. Therefore, this reporter was expressed in cells either carrying or lacking *KAP123*. As shown (Figure 6), the efficient nuclear localization of the reporter depended upon the presence of Kap123p in these cells. This was due to its nuclear import rather than selective cytoplasmic degradation, because the total cellular amounts of the reporter remained unchanged (data not shown; see also Figure 7). The comparatively large size of this reporter may slow its transport, enhancing its cytoplasmic accumulation. The subcellular distribution of other reporter proteins specifically recognized by other karyopherins was similar in both strains (Figure 6), indicating that Kap123p had a negligible effect on their nucleocytoplasmic transport. Taken together, these results indicate that Kap123p is a functional β karyopherin with a major role in the nuclear import of ribosomal proteins.

A Potential Secondary Ribosomal Protein Import System

A potential problem has been raised by the results presented above. Deletion of *KAP123* is not lethal, whereas ribosomal protein transport is an essential process, so how can yeast survive in its absence? Kap104p showed no affinity for ribosomal proteins (Figure 4B) (Aitchison et al., 1996). Therefore, two other possible candidates, Kap95p and Pse1p, were PrA-tagged in cells lacking Kap123p for use in an overlay assay on the RP10Ap or SV40 LT NLS constructs. Our initial characterization indicated that deletion of *PSE1* was lethal, but that cells carrying PrA-tagged Pse1p grew normally (data not shown). The affinities of the tagged β karyopherins in the cytosols from these cells were compared with the affinities of the same karyopherins in cytosols from cells carrying normal amounts of Kap123p. Although the binding pattern of Kap95p remained unchanged, the affinity of Pse1p for the RP10Ap construct (and other ribosomal proteins; data not shown) increased markedly in the absence of Kap123p (Figure 4C). It appears that, normally, Kap123p out-competes Pse1p, but in its absence, Pse1p may supplant it. To test this hypothesis in vivo, the distribution of the L25 NLS-β-galactosidase reporter was studied by immunofluorescence microscopy in the cells lacking *KAP123* and carrying an inducible plasmid-borne copy of *PSE1*. Although the presence of the plasmid when not expressing *PSE1* did not alter

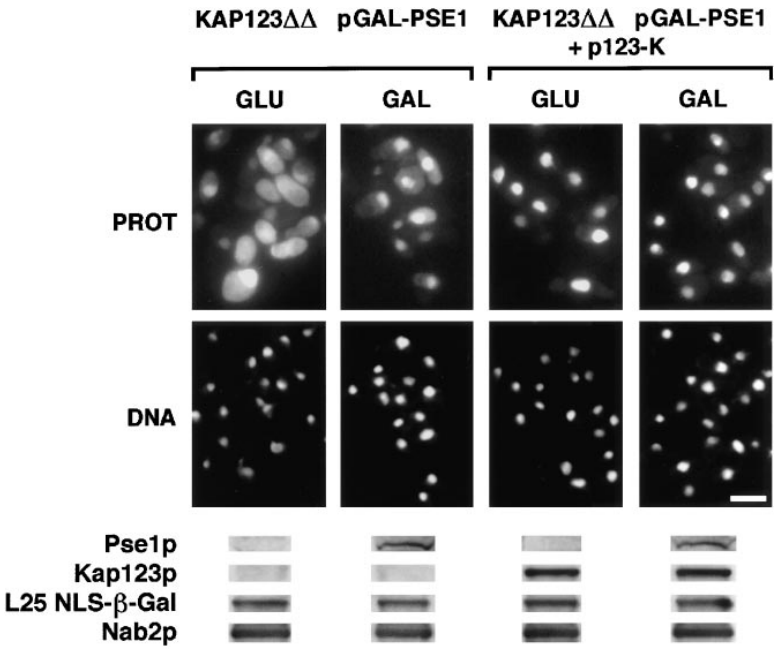

Figure 7. Pse1p Can Replace the Function of Kap123p as a Ribosomal Protein Karyopherin

GAL-10-driven overexpression of Pse1p in cells either lacking *KAP123* (KAP123ΔΔ pGAL-PSE1) or expressing *KAP123* (KAP123ΔΔ pGAL-PSE1 + p123-K). The localization of the ribosomal protein NLS reporter L25 NLS-β-galactosidase (PROT) was determined by immunofluorescence microscopy of yeast cells. With normal levels of Pse1p (GLU) and no Kap123p, the reporter was mainly cytoplasmic, but overexpression of Pse1p in the same cell line (GAL) could effectively drive much of the reporter into the nucleus. However, the localization of the reporter was even more strongly nuclear in the presence of Kap123p, regardless of the expression level of Pse1p. The position of the nuclei was visualized with DAPI (DNA). Bar, 5 μm. Beneath each immunofluorescence panel are shown the relevant portions of a lane from an immunoblot of protein from a whole cell lysate, demonstrating the levels of expression of the two karyopherins (Pse1p, detected by its HA tag, and Kap123p), the reporter protein (L25 NLS β-gal), and a control protein (Nab2p) in cells of the same strain grown under the same conditions; each lane contained the same number of cell equivalents (as shown by the control protein).

the mainly cytoplasmic distribution of the L25 NLS reporter, overexpression of *PSE1* in this strain effectively drove much of the reporter into the nucleus, although some cytoplasmic signal remained. Interestingly, upon readdition of Kap123p, the localization of the reporter was even more strongly nuclear and became independent of the expression level of Pse1p, affirming the strong preference of at least this ribosomal NLS for Kap123p over Pse1p. Again, the nucleocytoplasmic distribution of a control reporter protein (Nab2p; Figure 6, data not shown) was unaffected by these manipulations, and immunoblotting confirmed that the changing distribution of the reporter was not due to its selective degradation (Figure 7). Because ribosomal assembly is metabolically regulated (see Discussion), we were aware of the possibility that a variation in carbon source alone could result in a redistribution of the L25 NLS-β-galactosidase reporter. However the reporter's mainly cytoplasmic localization was almost unaffected by growth on either carbon source in the KAP123ΔΔ strain lacking the *GAL-PSE1* plasmid (data not shown). Though we have tested only one ribosomal NLS, these experiments are consistent with the idea that Pse1p is a functional karyopherin that is able to substitute for Kap123p in vivo.

Discussion

A Separate Pathway for Ribosomal Protein Import into the Nucleus

All ribosomal proteins are potentially small enough to passively diffuse into the nucleus. However, as ribosomal proteins concentrate 50-fold in the nucleolus within 5 minutes of their synthesis, and free cytoplasmic ribosomal proteins are degraded with a half-life of 2–3 minutes or less (Warner et al., 1985; Warner, 1989), they would seem to require active nuclear import. Here, we identify a major alternative nuclear import pathway for ribosomal proteins and show that it is primarily mediated by the previously uncharacterized β karyopherin Kap123p.

Kap123p was identified by its association with isolated NPCs. This, and its significant sequence similarity to known β karyopherins, suggested that it may have a role in nuclear transport. Like the other β karyopherins, it is mainly cytosolic but also interacts with the NPC through, at least, repeat motif–containing nucleoporins. It specifically binds to numerous ribosomal proteins as well as a defined ribosomal protein NLS. Unlike Kap95p, Kap123p requires no partner but interacts directly with its transport substrates, as well as with the NPC and Ran-GTP. Both Kap123p and another related β karyopherin, Pse1p, are able to mediate the import of a ribosomal NLS-bearing substrate into the nucleus in vivo. Given this similarity in sequence and function, it seems likely that the interactions of Pse1p with the NPC and NLSs are also direct. We propose that Pse1p be provided with the supplementary name Kap121p, reflecting at least one of its functions and in accordance with the systematic nomenclature established in yeast for this family of proteins (Enenkel et al., 1995; Aitchison et al., 1996).

The NLSs for three yeast ribosomal proteins have been defined (Moreland et al., 1985; Underwood and Fried, 1990; Schaap et al., 1991). None of these NLSs fit the minimal tetrapeptide or bipartite consensus for NLSs of substrates transported by the classical karyopherin β/α system (Chelsky et al., 1989; Dingwall and Laskey, 1991; Makkerh et al., 1996). However, the three NLSs of human S6 do fit the tetrapeptide consensus

(Schmidt et al., 1995), supporting the idea that the nuclear import of ribosomal proteins may also occur by this classical pathway. Indeed, although the existence of alternative nuclear import pathways has been known for some time (Fischer et al., 1991; Garcia-Bustos et al., 1991; Goldfarb and Michaud, 1991), ribosomal protein import was not generally considered among them. Interestingly, it had been shown that while the depletion of the repeat motif–containing nucleoporin Nsp1p in yeast prevented the nuclear accumulation of a β-galactosidase reporter fused to classical NLSs, the reporter remained nuclear when fused to a ribosomal NLS (Mutvei et al., 1992). Similar results were obtained for a temperature-sensitive strain of Nsp1p (Nehrbass et al., 1993). This could be interpreted as evidence of a separate import pathway recognizing the ribosomal NLS (Mutvei et al., 1992), and may be explained by the much weaker binding of Kap123p for Nsp1p than Kap95p/Kap60p (Figure 3A).

Although Kap123p bound numerous cytosolic ribosomal proteins, it did not associate with mature ribosomes. It is likely that in the mature ribosome the NLSs are masked, as suggested by the fact that antibodies recognizing ribosomal NLSs do not bind to mature ribosomes (Goldfarb and Michaud, 1991). In RPL25p, the NLS-containing region adds some 50% to the mass of the protein compared with its prokaryotic counterpart (L23), raising the possibility that the addition of such sequences may account in part for the larger mass of the eukaryotic ribosome (Schaap et al., 1991). Other ribosomal proteins may not have an NLS at all, being imported as subcomplexes (which may explain why not all ribosomal proteins were recognized in the overlay assays). However, the short half-life of free ribosomal proteins in the cytoplasm would argue against this. Furthermore, though most ribosomal proteins may be imported by the Kap123p and Pse1p (Kap121p) pathway, some may also utilize other import pathways (see below).

Possible Functions of an Alternative Import Pathway for Ribosomal Proteins

One likely reason for the existence of multiple separate import pathways converging at the NPC is to reduce competition for import between major classes of import substrates. Therefore, to maintain an efficient balance, several alternative pathways may have evolved to separate the import processes of major classes of import substrates. The members of the substrate classes determined so far seem loosely related in function. Thus, Kap104p imports mRNA binding proteins, while Kap123p imports ribosomal proteins. However, we also show that some ribosomal NLSs (and by inference, perhaps the NLSs of other substrate classes) may be recognized by several karyopherins, though strongly preferred by just one, potentially providing a multiply redundant transport system. Such strong preferences would prevent interference between karyopherins, but minor cross affinities would allow the karyopherins to back each other up, as with Kap123p and Pse1p (Kap121p). It seems likely that the essential Pse1p (Kap121p) transports a separate and necessary substrate class not efficiently recognized by other karyopherins. It also seems likely that Kap123p recognizes NLSs carried by proteins other than ribosomal proteins, particularly as it recognizes proteins on an overlay assay of nuclear lysate that do not coincide with the position of ribosomal proteins or repeat motif–containing nucleoporins (Figure 4A). Furthermore, as with the other karyopherins, a role for either Kap123p or Pse1p (Kap121p) in protein or RNP export (for example, in the export of ribosomal subunits) cannot be excluded at present.

It is not clear how these different pathways avoid mutual interference when they converge at the NPC. Data presented here and elsewhere (Aitchison et al., 1996; Pollard et al., 1996; Bonifaci et al., 1997) show that the karyopherins mediating different pathways recognize different classes of NLS on different classes of substrate. The karyopherins seem to have differing but overlapping affinities for the repeat motif–containing nucleoporins, as shown here for Kap95p and Kap123p, and in Aitchison et al. (1996) for Kap95p and Kap104p. Again, like the NLSs, each type of repeat motif docking site on the NPC might be recognized by several karyopherins but strongly preferred by just one, reducing the competitive interference between karyopherins at the docking step but maintaining maximum use of the available docking sites for the highest efficiency. This may partially explain the large number of different repeat motif–containing nucleoporins in the NPC. That the alternative pathways can use separate docking sites is demonstrated by the results of Mutvei et al. (1992) and Nehrbass et al. (1993), as discussed above. These differences may be further exaggerated by differences in the kinetics of the karyopherins. Thus, the karyopherins may act in dynamic competition, by maintaining separate pathways that can nonetheless partially cross-talk at the levels of both NLS recognition and docking sites preferences, and hence maximize the use of the available transport resources, whatever the transport burden.

Yeast cells grow at a wide variety of rates, dependent upon factors such as carbon source and nutrient availability, and under ideal growth conditions, they are among the fastest dividing eukaryotic cells. Therefore, their maximal rate of de novo protein synthesis is also correspondingly high. To achieve this, the rate of ribosomal protein synthesis and nuclear import may vary over a 20- to 50-fold range, reaching a maximum of some 15 ribosomal proteins/NPC/sec. The enormous yeast nucleoli, occupying nearly half the total nuclear volume, then assemble the requisite large numbers of ribosomes (Boehlke and Friesen, 1975; Warner, 1989). Correspondingly, Kap123p is by far the most abundant of the four β karyopherins. We propose that Kap123p and Pse1p act in concert as a partially redundant system, providing the capacity and flexibility necessary to coordinate the efficient nuclear import of ribosomal proteins under a variety of conditions, and with the more abundant Kap123p prevailing at the higher rates of synthesis. Strong evidence for this is provided by the competitive growth experiments, which indicate that Kap123p is required under conditions of rapid cell growth providing cells with a competitive edge. As is graphically demonstrated in Figure 2A, the loss of Kap123p would be lethal outside the specialized environment of the laboratory.

Evolution of the Karyopherins

Searches of the Saccharomyces genomic database with the known yeast karyopherins reveal no other close potential karyopherin homologs, though it should be noted that sequence similarities do exist between the four β karyopherins and other proteins in the yeast database, which may also play a role in nucleocytoplasmic transport. Included in these is Crm1p, the human homolog of which has been implicated as a nuclear transport factor (Fornerod et al., 1997). Given the important functions of Kap123p and Pse1p (Kap121p), one might expect potential homologs to be present in other kingdoms and phyla. Surprisingly, our comprehensive searches revealed more sequences closely related to Pse1p (Kap121p) than Kap123p. Perhaps the apparent Pse1p (Kap121p) homologs functionally resemble Kap123p more closely in these organisms. However, the conservation of the Kap123p/Pse1p (Kap121p) karyopherin type over a wide variety of phyla argues that this alternative import pathway is conserved in all eukaryotes. Evidence for this is provided by the functionality of the yeast L25 ribosomal NLS, specifically recognized by Kap123p and Pse1p (Kap121p) (above), in vertebrate cells (Schaap et al., 1991).

Interestingly, Kap60p carries an NLS-like sequence, by which it binds to Kap95p (Görlich et al., 1996b; Moroianu et al., 1996; Weis et al., 1996). Kap60p originally could have been a transport substrate itself, distantly related to β karyopherins and with its own NLS. It then replaced the NLS-binding function of Kap95p with a more efficient system of its own, making Kap95p/Kap60p a later evolving system (Görlich and Mattaj, 1996a). Kap95p might therefore still be able to bind certain NLSs directly. Kap104p recognizes NLSs apparently unlike any other, again suggesting a later arrival. If Kap123p is a more specialized karyopherin, then perhaps Pse1p (Kap121p) is closest to the ancestral state for karyopherins.

Experimental Procedures

Protein Analysis

Proteins of the highly enriched NPC fraction were analyzed as described (Rout and Blobel, 1993; Aitchison et al., 1995b). Three protein bands of ~60 kDa, 90 kDa, and 120 kDa were isolated for peptide sequence analysis (Fernandez et al., 1994). The resulting peptide sequence data from the three bands uniquely matched amino acids 505–530 of the ORF YNL189w (Srp1p / Kap60p), amino acids 78–89 of the ORF YLR347c (Kap95p), and amino acids 73–78, 487–497, 588–600, 684–693, and 1087–1092 of the ORF YER110c (termed Kap123p), respectively. Individual ribosomal proteins were identified by MALDI–TOF mass spectrometry as described (Gharahdaghi et al., 1996). Protein amino acid analysis was performed as described (Gharahdaghi et al., 1992).

Yeast Strains

The *KAP123* gene was replaced in DF5 cells by direct integration of a PCR product containing the *URA3* gene (Rothstein, 1991) and nucleotides −60–3 and 3289–3234 of *KAP123* as described (Aitchison et al., 1995a). Ura$^+$ haploids were confirmed to contain the deletion by the PCR and immunoblotting. Sister spores were mated to generate the diploid KAP123ΔΔ. Genes were tagged with the IgG binding domains of PrA using the following regions for homologous recombination: *KAP123*, 3276–3338 and 3506–3447; *KAP95*, 2523–2582 and 2648–2586; *PSE1*, 3201–3276 and 3348–3290 (Aitchison et al., 1995a). Haploids yielding the functional chimeras were isolated and designated KAP95-A, KAP123-A, and PSE1-A. pGAL-PSE1 was made by fusing the *GAL1–10* promoter upstream of *PSE1* containing an amino-terminal Hemagglutinin (HA) epitope (Wozniak et al., 1994) in pRS314 and transformed into KAP123ΔΔ cells in combination with pL25/β-gal (YEp13 containing L25-NLS [amino acids 1–49 of RPL25]) fused to LacZ, pMα2/β-gal (YEp13 containing the Matα2 homeodomain NLS fused to LacZ) (Mutvei et al., 1992), and pKAP123-K (pRS317 containing the *KAP123* gene). Cells carrying plasmids with a selectable marker were grown for 24 hr in the relevant selection medium containing 2% raffinose as the carbon source, then transferred to YPD or YPGal and maintained at mid log phase for 8 hr before harvesting.

Expression Constructs

The ORFs encoding full-length *KAP123* or amino acids 1–400 were amplified from total S. cerevisiae DNA and subcloned into pET21a (Novagen) for expression in E. coli BL21(DE3) as a fusion with an amino-terminal T7 epitope tag and a carboxy-terminal 6× HIS tag. For the generation of antibodies, amino acids 1–400 were isolated as suggested (QIAGEN) and injected into Balb-c mice.

The RP10A ORF was ligated into the pGEX 2TK expression vector (Pharmacia) to generate an amino-terminal fusion of glutathione S-transferase to the full-length ORF of RP10A.

Immunoblotting and Immunofluorescence Microscopy

The PrA tags were detected with rabbit IgG, nucleoporins with mAb414 (Davis and Fink, 1990; Rout and Blobel, 1993), β-galactosidase fusions with monoclonal anti-β-galactosidase (Boehringer Mannheim), the HA epitope with MAb 16B12 (Berkeley Antibody Co.), and Nab2p with a polyclonal mouse antiserum (Aitchison et al., 1996). Antibodies were visualized with HRP-conjugated secondary antibodies and ECL (Amersham Corp.).

For immunofluorescence microscopy, cells were fixed as described (Kilmartin and Adams, 1984; Wente et al., 1992). PrA-tags, Nab2p, nucleoporins, and the β-galactosidase reporter were detected as above, followed by either Cy3 or DTAF-conjugated donkey anti-rabbit IgG or anti-mouse IgG (Jackson ImmunoResearch Laboratories), as appropriate. Cells containing SV40 NLS-GFP (detected by autofluorescence [Chalfie et al., 1994]) were fixed as above and mounted directly. Controls confirmed that there was no cross-contamination during double labeling.

Subcellular Fractionation and Immunopurification

Yeast nuclei and NEs were prepared essentially as described (Rout and Kilmartin, 1990; Rout and Kilmartin, 1994; Strambio-de-Castillia et al., 1995), except that the 2.30 M sucrose-PVP and 1.50 M sucrose-bt fractions were omitted, as they contained no protein. Ribosomes were prepared as per Zinker and Warner (1976). PrA-tagged karyopherins and their associated proteins were immunoisolated as described (Aitchison et al., 1996).

Overlay Assays

For the overlay assays, proteins to be probed were first separated by SDS–PAGE and electrophoretically transferred to nitrocellulose. The proteins of the NPC fraction were separated by NEPHGE/SDS–PAGE on a mini 2D gel apparatus (BioRad) as suggested by the manufacturer. All overlay incubations were in 5% dried skimmed milk, 20 mM HEPES, 110 mM KOAc, 2 mM $MgCl_2$, 0.1% Tween 20 (pH 7.5) (Radu et al., 1995) plus 0.001 volumes Solution P (Rout and Kilmartin, 1990). For the primary reactions, equal protein loadings were used for comparative samples and for yeast cytosols or E. coli lysates and incubated for 18 hr at 4°C. Bound proteins were detected with rabbit IgG (Cappel) or anti-T7 monoclonal antibody (Novagen), followed by HRP-conjugated secondary antibodies and ECL (Amersham corp.). Ran-GTP (Gsp1p; YLR293c) overlay assays were performed as described (Coutavas et al., 1993).

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