The Karyopherin Kap142p/Msn5p Mediates Nuclear Import and Nuclear Export of Different Cargo Proteins

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Abstract. We have identified a novel pathway for protein import into the nucleus. Although the product of Saccharomyces cerevisiae gene MSN5 was previously shown to function as a karyopherin (Kap) for nuclear export of various proteins, we discovered a nuclear import pathway mediated by Msn5p (also referred to as Kap142p). We have purified from yeast cytosol a complex containing Kap142p and the trimeric replication protein A (RPA), which is required for multiple aspects of DNA metabolism, including DNA replication, DNA repair, and recombination. In wild-type cells, RPA was localized primarily to the nucleus but, in a KAP142 deletion strain, RPA was mislocalized to the cytoplasm and the strain was highly sensitive to bleomycin (BLM). BLM causes DNA double-strand breaks and, in S. cerevisiae, the DNA damage is repaired predominantly by RPA-dependent homologous recombination. Therefore, our results indicate that in wild-type cells a critical portion of RPA was imported into the nucleus by Kap142p. Like several other import-related Kap–substrate complexes, the endogenous RPA–Kap142p complex was dissociated by RanGTP, but not by RanGDP. All three RPA genes are essential for viability, whereas KAPI42 is not. Perhaps explaining this disparity, we observed an interaction between RPA and Kap95p in a strain lacking Kap142p. This interaction could provide a mechanism for import of RPA into the nucleus and cell viability in the absence of Kap142p. Together with published results (Kaffman, A., N.M. Rank, E.M. O’Neill, L.S. Huang, and E.K. O’Shea. 1998. Nature. 396:482–486; Blondel, M., P.M. Alepuz, L.S. Huang, S. Shaham, G. Ammerer, and M. Peter. 1999. Genes Dev. 13:2284–2300; Devit, M.J., and M. Johnston. 1999. Curr. Biol. 9:1231–1241; Mahanty, S.K., Y. Wang, F.W. Farley, and E.A. Elion. 1999. Cell. 98:501–512) our data indicate that the karyopherin Kap142p is able to mediate nuclear import of one set of proteins and nuclear export of a different set of proteins.

Key words: Saccharomyces cerevisiae • karyopherin • nuclear transport • single-stranded DNA binding protein • bleomycin

Introduction

Eukaryotic cells are characterized by the existence of a double-membrane nuclear envelope, which separates the nucleus and the cytoplasm. Transport of molecules between these compartments occurs through nuclear pore complexes (NPCs) that penetrate the nuclear envelope. NPCs form aqueous channels through which small molecules can pass by free diffusion. Macromolecules that are unable to pass efficiently through the NPC by free diffusion are transported into or out of the nucleus in an active manner via nuclear localization signals (NLSs) and nuclear export signals (NESs), respectively (for reviews see Pemberton et al., 1998; Wozniak et al., 1998; Görlich and Kutay, 1999; Nakielny and Dreyfuss, 1999). Signal-dependent transport pathways are mediated by recognition of these signals by soluble nuclear transport receptors known as karyopherins (Kaps; also called importins [Görlich et al., 1994], nuclear pore targeting complexes [PTACs; Imamoto et al., 1995], p97 [Chi et al., 1995], transportins [Pollard et al., 1996], and exportins [Stade et al., 1997]). Comparative sequence analysis revealed that in Saccharomyces cerevisiae, the Kap family is composed of 1 Kapα and 14 structurally related Kapβ proteins. Kapβ-related proteins carry cargo molecules into or out of the nucleus by binding to their substrates, the NPC and RanGTP. Kaps, Kap60p in yeast, functions as an adapter protein able to bind substrate and Kapβ1 (Kap95p in yeast) (Conti et al., 1998; Kobe, 1999). To date, it has been shown in yeast that nine Kaps function as import receptors and four Kaps function as export receptors (Enenkel et al., 1995; Aitchison et al., 1996; Pemberton et
otic cells and in yeast is composed of three subunits of (ssDNA). This complex is highly conserved in all eukaryotic complexes that have a high affinity for single-stranded DNA, allowing export cargo, Kap, and RanGDP to be liberated in RanBP1 (Yaseen and Blobel, 1999). This nucleotide conversion is hydrolyzed to RanGDP by the action of RanGAP and docks to FG nups on the cytoplasmic side, where RanGTP is thought to act analogously to Kap. XRIPα orthologues are found in humans and in Drosophila, but not in yeast. The absence of an XRIPα in yeast suggests that yeast and Xenopus use divergent pathways to import RPA.

MSN5/KAP142 was originally isolated as a multiplicity suppressor in yeast mutants defective in the Smf1p protein kinase and subsequently as a gene required for the calcineurin-dependent induction of PMC1 (Estruch and Carlson, 1990, 1993; Matheos et al., 1997). Recent studies have shown multiple functions of MSN5, including regulation of carbohydrate metabolism, cell stress response, mating response, signal transduction control, cell cycle control, and pseudohyphal differentiation of diploid cells (Akada et al., 1996; Lorenz and Heitman, 1998; Alepuz et al., 1999). By using mutant strains that are missing individual Kaps, it was shown recently that Msn5p mediates several cellular responses through functioning as a nuclear export receptor of various proteins, including transcription factors, Pho4p and Mig1p; inhibitor of G1 cyclin–Cdk complex, Far1p; as well as a scaffold protein of a mitogen-activated protein kinase cascade, Ste5p (Kaffman et al., 1999; Mahanty et al., 1999). Each of these substrates is exported only in certain conditions, including high phosphate or low glucose in the growth medium, or mating response. It is not known whether Kap142p has any activities independent of environmental stimulation. (To reflect the primary function of Msn5p, we hereafter refer to it as Kap142p. For nomenclature issues and open reading frame details, see http://genome-www4.stanford.edu/cgi-bin/SGD/locus.pl?locus=s0002743.)

Here we show that Kap142p is a Kap that mediates not only nuclear export, but also nuclear import. We have identified the ssDNA-binding protein complex RPA as an import substrate for Kap142p. KAP142 is not essential for viability, whereas all three RPA genes are essential. This led us to identify Kap95p as a Kap likely to be involved in a secondary import pathway for RPA.

**Materials and Methods**

**Yeast Strains**

All strains were derived from *S. cerevisiae* DF5 (Finley et al., 1987). All yeast manipulations were performed according to described protocols (Atchison et al., 1995). KAP142 and YBR137W were deleted by integrative transformation of HIS5 (Schizosaccharomyces pombe). The HIS5 re-
placement cassette was generated by PCR amplification with primers that contained 60 nucleotides flanking the KAP142 or YBR137W open reading frames. Heterozygous diploids were sporulated and tetrads were dissected to generate KAP142 deletion (Δ) or YBR137W haploid strains. Deletion of genes was confirmed by PCR on genomic DNA with internal and external 3’ noncoding primers. The KAP120Δ strain (Chaves and Blobel, personal communication) was also generated using the integrative transformation of HIS3. The HIS5 replacement cassette was generated by PCR amplification with primers that contained 60 nucleotides flanking the KAP120 open reading frames. Heterozygous diploids were sporulated and tetrads dissected to generate KAP120Δ strain. Deletion of genes was confirmed by PCR on genomic DNA with internal and external 3’ noncoding primers. COOH-terminal genomic KAP142-PrA or YBR137W-PrA was constructed by integrative transformation of PCR-amplified cassette containing four and a half IgG-binding domains of protein A (PrA; Staphylococcus aureus) immediately after 60 nucleotides of 3’ sequence of KAP142 or YBR137W and including a HIS5 selection marker (these templates were kindly donated by Dr. R. Beckmann, Institut für Biochemie, Charité Berlin-Mitte, Berlin, Germany). Heterozygous diploids were sporulated and tetrads were dissected to generate KAP142-PrA and YBR137W-PrA haploid strains. RPA1-PrA and RPA2-PrA strains were created by direct integration of a PCR product into the DF5a haploid strain. RPA1-PrA and RPA2-PrA/KAP142Δ strains were constructed by direct replacement of KAP142 with URA3 in RPA1-PrA and RPA2-PrA strains. RPA2-PrA/YBR137W strain was constructed similarly to the RPA2-PrA/KAP142Δ strain. Correct integration was verified by PCR and immunoblotting.

Green fluorescent protein (GFP)-tagged fusion proteins were expressed by transforming plasmid pXY242-GFP in which human La, YL1, or PHO4 was cloned into wild-type or KAP142 deletion haploid strains. pYX242-GFP, pXY8sLa-GFP, and pXYYla1p-GFP were kindly provided by Dr. J.S. Rosenblum (Rockefeller University).

Results

Immunofluorescence Microscopy

PrA-tagged cells were fixed in 3.7% formaldehyde for 10 min or as indicated. Immunofluorescence microscopy on yeast spheroplasts was done as described previously (Wente et al., 1992). PrA tags were visualized using rabbit anti-mouse IgG (preadsorbed against formaldehyde-fixed wild-type yeast cells) followed by Cy3-conjugated donkey anti–rabbit IgG (Jackson ImmunoResearch Laboratories). Nuclei were visualized with DAPI staining. Direct fluorescence was used to visualize live, unfixed cells transformed with a plasmid-encoding GFP fusion protein. All images were viewed under the 63X objective on a Zeiss Axioptih microscope; images were collected in a video imaging system and manipulated in the computer program Adobe Photoshop®.

Cell Fractionation and Immunosolation

Fractionation and immunosolation of PrA fusion proteins were performed as described previously (Aitchison et al., 1996). In brief, 300 ml of postnuclear postribosomal cytosol was prepared from 10 liters of the KAP142-PrA strain and 50 ml of cytosol was prepared from 2 liters of RPA2-PrA, RPA2-PrA/KAP142Δ, and YBR137W-PrA strains. All strains were grown to an OD600 of 1.6. KAP142-PrA, Rpa2p-PrA, or Ybr137wp-PrA and associated proteins were immunosolated by overnight incubation of cytosol at 4°C with 25 μl of rabbit IgG–Sepharose (made from coupling CNBr-activated Sepharose 4B [Amersham Pharmacia Biotech] to rabbit IgG [Cappel]) per 50 ml cytosol. The IgG-Sepharose was isolated and washed extensively by TB (20 mM Heps/Cl, pH 7.5, 110 mM KOAc, 2 mM MgCl2, 1 mM DTT, and 0.1% Tween 20) with protease inhibitors (1 mM 4-[2-aminoethyl]-benzenesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A) before elution with a step gradient of 50-4,500 mM MgCl2 or with SDS sample buffer. Proteins eluted with MgCl2 were precipitated with methanol and chloroform before separation by SDS-PAGE on a 4–20% gradient acrylamide gel (Invitrogen). Coomasie staining bands were excised and protein masses were identified by mass spectrometry and triton-assisted laser desorption/ionization time-of-flight mass spectrometry (Fernandez et al., 1994; Gharahdaghi et al., 1996). Peptide masses were compared with several databases (http://prospector.ucsf.edu/ucsfhtml3/3a/msfit.htm or http://prowl.rockefeller.edu/cgi-bin/ProFound). Anti-Kap60p antibody was a generous gift of Dr. M. Rexach (Stanford University, Stanford, CA).

Dissociation Experiments

Yeast Ran (Gsp1p) was expressed in bacteria, purified, and loaded with GTP as described previously (Floer and Blobel, 1996). RanGTP was incubated with the yeast RanGap (RnA1p) overnight at room temperature to convert RanGTP to RanGDP as described previously (Albertini et al., 1998). Kap142p-PrA and bound proteins were immunosolated by IgG-Sepharose as described above. After extensive washing, the IgG-Sepharose was divided into three siliconized tubes and incubated with buffer alone, RanGDP, or RanGTP (final concentration 5 μM) in a volume of 100 μl TB with protease inhibitors for 20 min at room temperature. After incubation, dissociated proteins were collected in the supernatant. Proteins bound to the Sepharose were eluted with 50 and 250 mM MgCl2. All fractions were precipitated and separated by SDS-PAGE, followed by immunoblotting with rabbit polyclonal antibody against Rpa1p. Antibodies against Rpa1p and Rpa2p were kindly provided by Dr. B. Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Drug Sensitivity Assay

Stock solutions of bleomycin (BLM; Sigma-Aldrich) were prepared with sterile water. Assays measuring BLM sensitivity were carried out with YED medium containing 0.5% yeast extract and 1% dextrose. BLM concentrations were varied between 0.5 and 2 μg/ml. For the BLM sensitivity assay, wild-type strain and Kap mutants including KAP104Δ (Aitchison et al., 1996), KAP110Δ (Rosenblum et al., 1997), KAP111Δ (Pemberton et al., 1997), KAP114Δ (Pemberton et al., 1999), KAP119Δ (Rout et al., 1997), xpo1-1 (Stade et al., 1997), KAP142Δ (this study), KAP120Δ (Chaves and Blobel, personal communication), and YBR137WΔ (this study) were grown to an OD600 of 0.5–0.8 with YED medium, diluted, and 7 μl of cell cultures was dropped onto YED medium solidified by 2% agar (YEDA). Cells grown in the absence of various concentrations of BLM were incubated at 30°C for 1–4 d. For survival tests, cells grown in YED medium at 30°C to an OD600 of 0.5–0.8 were diluted and 100 μl of cell cultures were spread on YEDA in the presence and the absence of BLM. Cells were incubated at 30°C for 1–4 d and colonies were counted. 4-nitroquinoline N-oxide (4NQO) (Sigma-Aldrich) was prepared in 50% DMSO. Assays measuring 4NQO sensitivity were carried out with YEDA containing 1 μM 4NQO. Drop test was performed analogously to the BLM sensitivity assay. Cells in the presence of 4NQO were incubated at 30°C for 4 d.

Results

Immunolocalization of Kap142p-PrA

To determine the subcellular localization of Kap142p, we generated a strain expressing Kap142p fused to four and a half IgG-binding repeats of PrA. The haploid strain (KAP142-PrA), which expresses Kap142p-PrA from the endogenous promoter of Kap142p, did not show any changes in growth rate relative to wild-type cells at 30°C. The resulting fusion protein was used to determine the localization of Kap142p by indirect immunofluorescence.

Figure 1. Kap142p-PrA localizes both to the cytoplasm and the nucleus. Haploid cells whose endogenous Kap142p was tagged with PrA were examined by indirect immunofluorescence of the PrA tag (left), DNA was stained with DAPI (middle), and whole cells were visualized by Nomarski optics (right).
Figure 2. (a) Immunoisolation of cytosolic proteins interacting with Kas142p-PrA. The cytosol from a haploid KAP142-PrA strain was incubated with IgG-Sepharose. The last wash fraction and fractions eluted with a stepwise MgCl₂ gradient were separated by SDS-PAGE and stained with Coomassie blue. Kas142p-PrA eluted between 1,000 and 4,500 mM MgCl₂. Major bands migrating at ~70, 37, and 14 kD were identified by mass spectrometry as Rpa1p, Rpa2p, and Rpa3p, respectively. The band migrating to ~20 kD was identified as Ybr137wp. Relative molecular mass standards are indicated on the left. (b) Kas142p-PrA is insoluble at 4.5 M MgCl₂ but soluble in SDS-PAGE sample buffer. The cytosol of a haploid KAP142-PrA strain was incubated with IgG-Sepharose. The proteins bound to the IgG-Sepharose were eluted by buffer (lane 1) and eluted with SDS sample buffer (lane 2). The proteins were separated by SDS-PAGE and then stained with Coomassie blue. Kas142p-PrA appeared as doublet bands (double arrows), h.c., heavy chain; l.c., light chain.

Fixation of the cells for 2.5 min revealed Kas142p-PrA to be localized to both the nucleus and the cytoplasm (Fig. 1). In addition, some of the cells had strong immunofluorescent intensity in the nucleus. The result that Kas142p-PrA localized to both the nucleus and the cytoplasm was consistent with the localization of other PrA-tagged Kas.

Identification of Proteins Interacting with Kas142p
Kas that function in protein import form stable complexes with their substrates in the cytoplasm where the RanGTP concentration is low. To isolate proteins that interact with Kas142p, a postnuclear, postribosomal cytosol fraction from our Kas142p strain was prepared and incubated with IgG-Sepharose.

Bound proteins were eluted with a stepwise gradient of 50–4,500 mM MgCl₂. Eluted proteins were precipitated and separated by SDS-PAGE followed by staining with Coomassie blue. SDS-PAGE analysis revealed the existence of four major bands eluting between 100 and 1,000 mM MgCl₂ (Fig. 2a). The elution properties of these proteins were similar to those of previously described nuclear import substrates bound to their cognate Kas. This was in contrast to the Kas-PrA, which forms a very stable complex with the IgG and is only eluted at very high MgCl₂ concentrations. Kas142p-PrA was eluted above 1,000 mM MgCl₂ but at high MgCl₂ concentrations was mostly insoluble (see below and Fig. 2b).

To identify the potential import substrates, the bands were excised and the proteins were digested with trypsin and analyzed by mass spectrometry. The proteins migrating at ~70, 37, and 14 kD corresponded to the three subunits of RPA, Rpa1p, Rpa2p, and Rpa3p, respectively. 17 matching peptides derived from the 70-kD band covered 31% of Rpa1p, 8 matching peptides from the 37-kD band covered 49% of Rpa2p, and 5 matching peptides from the 14-kD band covered 63% of Rpa3p. The proteins that were eluted by 250 mM MgCl₂ were additionally immunoblotted with antibodies that specifically recognize Rpa1p or Rpa2p, confirming the identification of these two proteins (data not shown). None of these proteins have been identified in similar experiments with other Kas-PrA fusions. The RPA protein complex binds ssDNA and is required for eukaryotic DNA metabolism, such as DNA replication, DNA repair, and recombination. The protein migrating at ~20 kD corresponded to Ybr137wp, a protein of unknown function (Kucharczyk et al., 1999). Since RPA and Ybr137wp formed a complex with Kas142p in cytosol, these proteins might be import substrates for Kas142p.

The amount of Kas142p-PrA seemed substoichiometric relative to the amount of eluted RPA (Fig. 2a). It is possible that when Kas142p-PrA was eluted with high concentrations of MgCl₂ and precipitated with chloroform and methanol, it remained mostly insoluble in SDS sample buffer and therefore only a small amount entered the gel. To test this possibility, the amount of Kas142p-PrA bound to IgG-Sepharose was examined. Proteins that bound to the resin after incubation with cytosol from KAP142-PrA strain were eluted by adding SDS sample buffer to the resin directly and proteins were analyzed by SDS-PAGE. By Coomassie blue staining, doublet bands that corresponded to Kas142p-PrA by mass spectrometry appeared as well as RPA, Ybr137wp protein, and IgG heavy and light chains (Fig. 2b). This result may explain the apparent substoichiometry of Kas142p-PrA in Fig. 2a. The significance of the migration of Kas142p-PrA as a doublet remains to be investigated.

Rpa2p-PrA Forms a Cytosolic Complex with Rpa1p and Rpa3p and This Complex Binds to Kas142p and Ybr137wp
From the results shown above in Fig. 2, a and b, RPA subunits formed a complex with Kas142p-PrA in cytosol. To examine whether RPA exists as a trimeric complex in the yeast cytoplasm, proteins bound to Rpa2p-PrA were immunoisolated from cytosol of an RPA2-PrA strain by IgG-Sepharose affinity chromatography (Fig. 3). After extensive washing (lane 1), proteins bound to IgG-Sepharose were eluted with SDS sample buffer, boiled, and separated by SDS-PAGE followed by staining with Coomassie blue (lane 2). There were several bands in addition to IgG heavy and light chains. The band migrating at ~70 kD was immunoreactive with an anti-Rpa1p antibody, and was therefore Rpa1p. From its stoichiometry to Rpa1p and its migration, the 14-kD band was most likely Rpa3p. The band migrating...
at ~55 kD was thought to be a mixture of Rpa2p-PrA and IgG heavy chain because the 57-kD Rpa2p-PrA most likely was not resolved from the IgG heavy chain. RPA subunits were pulled out simultaneously from the cytosol and were partially disassembled only at high MgCl$_2$ concentrations (see also Fig. 9 a), suggesting that its subunits existed in the cytosol as a stable trimeric complex.

An additional band that migrated in the mass range expected for Kaps was seen to bind to cytoplasmic Rpa2p-PrA. The band migrating at ~150 kD (Fig. 3, single asterisk) was excised and the eluted protein was analyzed by mass spectrometry. 12 peptides matched Kap142p, covering 16% of the protein, confirming that the band contained Kap142p. The band migrating at ~20 kD (Fig. 3, double asterisk) was also excised and analyzed by mass spectrometry. Seven peptides derived from this band covered 37% of Ybr137wp. From these results, we conclude that Rpa2p-PrA existed in the cytoplasm as a complex with Rpa1p and Rpa3p and bound to Kap142p and Ybr137wp.

**Defective Nuclear Import of RPA in KAP142Δ Strain**

As RPA is nuclear at steady state, RPA that was isolated as a cytoplasmic complex with Kap142p could have been intercepted en route to the nucleus. To examine whether RPA is indeed an import substrate for Kap142p, the localization of Rpa1p and Rpa2p was examined in both wild-type and KAP142Δ strains. Like a similar strain reported previously (Alepu et al., 1999), our KAP142Δ haploid strain grew slowly at 35°C in rich medium compared with a wild-type haploid strain. To determine whether deletion of KAP142 leads to temperature-sensitive phenotype and mislocalization of Rpa1p-PrA and Rpa2p-PrA, Strain as indicated, were streaked on yeast extract/petri plate and incubated at 30°C (left) and at 35°C (right). (b) Wild-type (WT) or KAP142Δ strain whose endogenous Rpa1p or Rpa2p was tagged with PrA was grown at 30°C and examined by indirect immunofluorescence of the PrA tag (left). DNA was stained with DAPI (middle) and whole cells were visualized by Nomarski optics (right).

**Other Nucleocytoplasmic Pathways Are Not Affected by Deletion of KAP142**

To determine whether deletion of KAP142 would affect the localization of other transport substrates, several proteins whose transport pathways have been characterized previously were examined in both wild-type and KAP142Δ strains. For this experiment, GFP-tagged human and yeast La proteins, which are imported into the nucleus in yeast cells by Kap60p/Kap95p and Kap108p, respectively, were used as import substrates (Rosenblum et al., 1998). In both wild-type and KAP142Δ deletion strains, these substrates showed nuclear localization, indicating that deletion of Kap142p did not affect at least two of the
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The sensitivity of KAP142Δ to BLM provided additional independent evidence that the repair of DSBs is carried out predominantly by mechanisms that promote homologous recombination and require RPA (for review see Pâques and Haber, 1999). To investigate the physiological role of Kap142p in RPA-mediated DNA metabolism in vivo, we tested for defects in DNA DSB repair using BLM in cells lacking, or with mutated, Kaps.

We tested for BLM sensitivity with wild-type and the following strains: KAP104Δ, KAP108Δ, KAP111Δ, KAP114Δ, KAP119Δ, pse1-1, KAP122Δ, KAP123Δ, xpo1-1, KAP142Δ, and KAP120Δ. Although KAP111Δ, KAP119Δ, pse1-1, xpo1-1, and KAP120Δ strains grew more slowly than the wild-type strain at 30°C, they did not show any additional growth phenotype in the presence of BLM (data not shown). We found that KAP104Δ, KAP122Δ, and KAP142Δ strains were sensitive to BLM. The sensitivity of KAP104Δ to BLM became apparent only at high BLM concentration. In contrast, the KAP122Δ and KAP142Δ strains showed high sensitivity to 1 μg/ml BLM (Fig. 7 a). Strikingly, at 2 μg/ml BLM, KAP142Δ cells survived at <1% of the rate of KAP122Δ cells, which themselves survived at a rate of only 1.7% of wild-type cells (Fig. 7 b). High sensitivity of the KAP142Δ to BLM provided additional independent evidence that the import of RPA, which plays crucial roles in the repair of DNA DSB, was disturbed in the absence of Kap142p.

To study the physiological role of Kap142p on RPA-mediated DNA metabolism further, we tested for 4NQO sensitivity with wild-type and KAP142Δ strains. 4NQO causes DNA damage that is primarily repaired by way of nucleotide excision repair (NER) where RPA plays a role with other repair proteins. In contrast to the effect of BLM on KAP142Δ strain, 1 μM 4NQO did not affect the growth of KAP142Δ compared with the growth of the wild-type strain (Fig. 7 c). Although the direct reason for insensitivity of KAP142Δ strain to 4NQO is unknown, NER might require less nuclear RPA than DNA DSB repair (see also Discussion).
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Nuclear Import of RPA by Kap142p

Function of Ybr137w Protein Remains Unclear

Since Ybr137wp was isolated in a complex with Kap142p-PrA, Ybr137wp may be an import substrate of Kap142p or an adapter protein between Kap142p and RPA. To examine these possibilities, YBR137W was genomically tagged with PrA and the resultant YBR137W-PrA strain was examined for the localization of Ybr137wp. Unlike Kap142p and RPA, Ybr137wp was localized diffusely throughout the cell and in a cytoplasmic focus both in wild-type (Fig. 8a) and KAP142Δ strains (data not shown), making it unlikely that Ybr137wp was an import substrate for Kap142p. If Ybr137wp were an adapter protein between Kap142p and RPA, the localization of RPA would require the presence of Ybr137wp. To examine the effect of Ybr137wp on nuclear import of RPA, the localization of Rpa2p-PrA, the localization of Rpa2p-PrA was examined in wild-type and YBR137WΔ deletion (YBR137WΔ) strains. YBR137WΔ was not encoded by an essential gene and the YBR137WΔ strain did not show any growth phenotype in rich media at any temperature. Immunofluorescence microscopy showed that there was no apparent difference in localization of Rpa2p-PrA between an isogenic wild-type strain (Fig. 8b, top) and YBR137WΔ (Fig. 8b, bottom). To further investigate a possible role of Ybr137wp in import of RPA in the nucleus, we examined the sensitivity of YBR137WΔ to BLM. We found that deletion of YBR137WΔ did not lead to a decrease in DNA DSB repair efficiency (Fig. 7, a and b), whereas deletion of KAPI42 lead to a marked decrease, suggesting that Ybr137wp was not required for import of RPA into the nucleus. From these observations, Ybr137wp seemed to be neither an import substrate of Kap142p nor an adapter protein between Kap142p and RPA. The function of Ybr137wp remains unclear.

RPA Is Imported into the Nucleus by Overlapping Pathways

Unlike each of three RPA genes, KAPI42 is not essential for viability. Further, some nuclear accumulation of RPA was seen in some of the KAPI42Δ cells (Fig. 4b). RPA that localizes to the nucleus must be imported via active transport, as its mass exceeds 110 kD. Taken together, these observations indicate that in the absence of Kap142p, RPA is imported into the nucleus via alternative Kap-dependent pathways. To explore this issue, we purified Rpa2p-PrA from cytosol deleted for KAPI42 (Fig. 9a). As expected from their high affinity for Rpa2p, Rpa1p and Rpa3p were eluted from Rpa2p-PrA only at very high MgCl2 concentrations. In addition to RPA subunits, a prominent band in the molecular mass range for Kaps was eluted between 250 and 1,000 mM MgCl2 (Fig. 9a, single asterisk), which is a typical range of MgCl2 concentrations to dissociate Kap–substrate complexes (Fig. 2a). Mass spectrometry revealed that this band was Kap95p. 13 matching peptides covered 14% of the protein. This result suggests that in the absence of Kap142p, RPA may be imported into the nucleus in a Kap95p-dependent manner.
To examine whether import of RPA into the nucleus in the absence of Kap142p is mediated by the Kap60p–Kap95p complex, we immunoblotted the Rpa2p-PrA–associated proteins with an antibody that specifically recognizes Kap60p (Fig. 9 b). We found no specific signal that corresponded to Kap60p, indicating that RPA was imported into the nucleus in the absence of Kap142p by a Kap95p-mediated pathway that was independent of Kap60p. The z20-kD band (Fig. 9 a, double asterisk) eluting between 100 and 1,000 mM MgCl₂ was identified as Ybr137wp by mass spectrometry. The five matched peptides covered 25% of the protein. Since Ybr137wp was purified in complex with Rpa2p-PrA from a strain lacking Kap142p, Ybr137wp appeared to specifically bind RPA.

**Discussion**

It has been shown previously that Kap142p is the export receptor for several proteins. Here, we show that Kap142p also serves as the import receptor for RPA. This is the first demonstration, to our knowledge, of a Kap that imports one set of proteins and exports a different set of proteins.

Although KAPI42 was first identified in a genetic screen as a multicopy suppressor of mutants defective in the SNF1 protein kinase, based on its sequence similarity, the product of KAPI42 of S. cerevisiae was recently suggested to be a member of the Kap transport factor family (Görlich et al., 1997). The members of the Kap family have so far been characterized only as import or export receptors. Kap142p was shown to be an export Kap for various proteins, including the transcription factors, Pho4p, Mig1p, inhibitor of G1 cyclin–Cdk complex, Far1p, and a mitogen-activated protein kinase cascade scaffold protein, Ste5p (Kaffman et al., 1998a; Blondel et al., 1999; DeVit and Johnston, 1999; Mahanty et al., 1999). In this paper, we report that the product of the KAPI42 gene functions not only as an export Kap, but also as an import Kap for RPA.

We found that Kap142p was localized both to the cytoplasm and the nucleus when tagged at its COOH terminus with PrA. However, it has been reported that Kap142p localizes predominantly to the nucleus when it is tagged in different ways (Alepuz et al., 1999; Blondel et al., 1999). The reason that there is a discrepancy of localization of Kap142p remains unclear, although it has precedent in other Kaps, but this may be due to the orientation of the tag (NH₂ terminus or COOH terminus), what kind of moiety is attached (PrA, Myc, or GFP), how it is studied (in living or fixed cells), or how it is expressed (under its own promoter or overexpressed). The sensitivity of nucleocytoplasmic localization of Kap142p to tags or expression is consistent with its function as a Kap shuttling between the nucleus and the cytoplasm.

We found that cytosolic Kap142p exists as a complex with RPA. Previous findings showed that Kaps that function in nuclear import, in general, form stable complexes with their cognate import substrate in the cytoplasm where RanGTP is low. After translocation into the nucleus where RanGTP is high, this complex is dissociated via a mechanism that includes binding of RanGTP to the Kap. Therefore, formation of the Kap142p–RPA complex in the cytoplasm (Fig. 2 a) and dissociation of the complex by RanGTP (Fig. 6) suggested that Kap142p might function as an import Kap for RPA. Our ability to show partial mislocalization of RPA subunits at 30°C in a KAPI42Δ strain (Fig. 4 b) confirmed that at least some portion of...
RPA was imported into the nucleus by Kap142p. The *KAP142* gene is not essential for viability in yeast, whereas each *RPA* gene is essential. Including all of the identified transport substrates that are mediated by Kap142p, RPA is the only essential one characterized so far. Our cells lacking *KAPI42* showed slow growth at 35°C (Fig. 4 a) like a similar strain reported previously (Alepuz et al., 1999). The direct cause of growth inhibition in the *KAPI42Δ* strain might be that the essential RPA is not imported into the nucleus efficiently in the absence of Kap142p at 35°C and therefore DNA replication would be impaired. Nevertheless, we cannot exclude the effect of nuclear export substrates on cell growth.

If RPA is imported into the nucleus mainly by Kap142p, cells lacking *KAPI42* should have defects in RPA-mediated DNA metabolism. To look for such defects, we examined the sensitivity of Kap mutants to BLM. BLM induces DNA DSB in cells and the damage is repaired predominantly by a mechanism that promotes recombination, wherein RPA plays a crucial role (for reviews see Pâques and Haber, 1999; Flores-Rozas and Kolodner, 2000; Haber, 2000). Once DNA DSB occurs, the ends of the DSB are restricted by a 5′ to 3′ exonuclease or by a helicase coupled to an endonuclease to produce long, frequently >1 kb, 3′-ended ssDNA tails that can invade a homologous template. RPA binds to the ssDNA tails that are created by the nuclease and is suggested to have a role in sensing DNA damage (Lee et al., 1998). The repair of DSB by homologous recombination is dependent on a Rad52p-mediated mechanism in which Rad52p stimulates DNA strand exchange through specific protein–protein interaction between Rad52p, Rad51p, and RPA (New et al., 1998). Among the Kap mutants we examined, the *KAPI42Δ* strain was most sensitive to BLM (Fig. 7, a and b). This can be explained, taken together with the direct binding of Kap142p with RPA in vitro (Fig. 2 a), partial mislocalization of RPA in *KAPI42Δ* strain in vivo (Fig. 4 b) and the crucial roles RPA plays in DNA DSB repair, by a defect in RPA-mediated DNA repair in the *KAPI42Δ* strain due to insufficient import of RPA into the nucleus in the absence of Kap142p.

To examine RPA-mediated DNA metabolism further, we studied the sensitivity of the *KAPI42Δ* strain to 4NQO. 4NQO forms bulky adducts with nucleobases and this DNA damage is primarily targeted for NER. In the process of NER, DNA lesions are excised in the form of a DNA fragment ~25–30 nucleotides long and leaves ssDNA where RPA binds with other NER factors (for reviews see Araujo and Wood, 1999; Prakash and Prakash, 2000). The occluded binding sites for yeast RPA have been shown to be ~20–40 nucleotides (Sugiyama et al., 1997; Sibenaller et al., 1998). We found that 1 μM 4NQO did not affect the growth of the *KAPI42Δ* strain (Fig. 7 c). Although the direct cause of insensitivity of the *KAPI42Δ* strain to 4NQO remains to be elucidated, it could be that ssDNA generated in the process of NER is short enough that the amount of RPA imported into the nucleus in the absence Kap142p is sufficient, whereas ssDNA generated in the presence of DNA DSB repair requires more RPA than is imported into the nucleus in the absence of Kap142p. Therefore, this differential drug sensitivity could arise because ssDNA generated in DNA DSB is >1,000 nucleotides long at each end of the break, whereas that generated in NER is 25–30 nucleotides long. As such, DNA DSB repair could require >80-fold more nuclear RPA than NER per lesion.

As we purified Ybr137wp bound to Kap142p-PrA, Ybr137wp could be either an import substrate for Kap142p or an adapter protein between Kap142p and RPA. However, Ybr137wp localized to the cytoplasm diffusively and the localization of Rpa2p-PrA was unchanged in the absence of Ybr137wp. A *YBR137WΔ* strain was not sensitive to BLM in contrast to *KAPI42Δ*, suggesting that import of RPA into the nucleus was independent of Ybr137wp. In addition, Ybr137wp was purified as a binding protein to RPA from cytosol lacking Kap142p. From these results, we conclude that Ybr137wp binds to RPA specifically. The function of Ybr137wp remains to be elucidated.

Pho4p has been shown to be a nuclear export substrate of Kap142p under phosphate-rich conditions (Kaffman et al., 1998a). We also showed that Pho4p-GFP was localized to the cytoplasm when cells were grown in phosphate-rich medium and that it was mislocalized to the nucleus in the absence of Kap142p even if the cells were grown in phosphate-rich medium. However, proteins that bound to Kap142p-PrA in the cytoplasm did not include Pho4p by Coomassie blue staining. This might be explained as follows. Phosphorylated Pho4p forms a complex with Kap142p and RanGTP in the nucleus but as soon as this complex is exported into the cytoplasm, RanGTP is converted to RanGDP or phosphorylated Pho4p is dephosphorylated by phosphatase and this modification leads to dissociation of the complex faster than we are able to purify it with our method.

In common with the Kap114p-mediated import pathway for the TATA box-binding protein, Kap122p-mediated import pathway for transcription factor IIA, Kap123p-mediated import of ribosomal proteins, and Los1p-mediated export of tRNA, this Kap142p-mediated import pathway for RPA represents another example of a nonessential Kap importing an essential substrate (Rout et al., 1997; Hellmuth et al., 1998; Pemberton et al., 1999; Titov and Blobel, 1999). RPA does not pass through the NPC by free diffusion, because RPA forms a complex in the cytoplasm whose mass exceeds 110 kD. This led us to search for alternative pathways into the nucleus for RPA. We could identify Kap95p as a protein that forms a complex with Rpa2p-PrA in a strain lacking Kap142p. This case was similar to those for TATA box-binding protein, which binds Kap123p and/or Kap121p in the absence of Kap114p, and for ribosomal proteins, which bind Kap121p in the absence of Kap123p (Rout et al., 1997; Pemberton et al., 1999).

In addition to basic NLS (bNLS)-containing proteins, which interact with Kap6, vertebrate Kapβ1 can contact and import several proteins either via other adapters or directly (Palacios et al., 1997; Jäkel et al., 1999; Moore et al., 1999; Palmeri and Malim, 1999; Truant and Cullen, 1999). Kap95p, the yeast orthologue of Kapβ1, has so far only been shown to interact with bNLSs via Kap60p (Rexach and Blobel, 1995). Recently, RPA from *Xenopus* egg extract was demonstrated to be imported into the nucleus by Kapβ1 in combination with XRIPα as an adapter protein. However, XRIPα orthologues were found only in humans.
and in Drosophila, but not in yeast (Jullien et al., 1999). The absence of XRIPα in yeast suggested that the import pathway of yeast RPA might be different from that of Xenopus RPA. The interaction of Kap95p with RPA in the absence of Kap142p may be indicative of a cryptic role for Kap95p in RPA import and may be evolutionarily relevant to involvement of orthologues of Kap95 in import of RPA orthologues. In this fashion, the divergence in nuclear import of RPA is similar to that seen in the La proteins (Rosenblum et al., 1998). Yla1p was shown to be imported into the nucleus by Kap108p in yeast cells, whereas the human orthologue of Yla1p is imported into the nucleus by the Kapo–Kapβ1 complex in human cells. The nuclear import of RPA, a highly conserved complex, appears to be the second example of an evolutionary divergence in the import of orthologous proteins.

In summary, we have identified a new pathway for protein import into the nucleus, mediated by the Kap, Kap142p. Although Kap142p has been previously established as a nuclear export receptor for various proteins, we have now shown that it is also able to import RPA into the nucleus. We also present data suggesting that Kap95p is involved in the inefficient nuclear import of RPA that takes place in the absence of Kap142p. Interestingly, the mechanism of nuclear import RPA has diverged between yeast and Xenopus. The import of RPA into the nucleus by Kap142p is the first example for one transport receptor functioning as both an import and an export receptor for completely different proteins. It remains to be seen if this is a general feature of Kaps.

We thank Bruce Stillman for antibodies against Rpa1p and Rpa2p; Michael Rexach for antibody against Kap60p; Roland Beckmann for pFlu3xHis5 and pBXAHis5; Farzin Gharahdaghi and Denise Ann Meagher (Rockefeller University, Protein/DNA Technology Center) for mass spectral analysis; Monique Floer for purified Ran and RanGAP; Jonathan S. Rosenblum for GFP fusion protein–expressing vectors; Chi, N.C., E.J. Adam, and S.A. Adam, 1995. Sequence and characterization of cytoplasmic nuclear protein import factor p97. J. Cell Biol. 130:265–274.

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