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Nuclear Import of TFIIB Is Mediated by Kap114p, a Karyopherin with Multiple Cargo-binding Domains[D]

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Nuclear import and export is mediated by an evolutionarily conserved family of soluble transport factors, the karyopherins (referred to as importins and exportins). The yeast karyopherin Kap114p has previously been shown to import histones H2A and H2B, Nap1p, and a component of the preinitiation complex (PIC), TBP. Using a proteomic approach, we have identified several potentially new cargoes for Kap114p. These cargoes include another PIC component, the general transcription factor IIB or Sua7p, which interacted directly with Kap114p. Consistent with its role as a Sua7p import factor, deletion of *KAP114* led to specific mislocalization of Sua7p to the cytoplasm. An interaction between Sua7p and TBP was also detected in cytosol, raising the possibility that both Sua7p and TBP can be coimported by Kap114p. We have also shown that Kap114p possesses multiple overlapping binding sites for its partners, Sua7p, Nap1p, and H2A and H2B, as well as RanGTP and nucleoporins. In addition, we have assembled an in vitro complex containing Sua7p, Nap1p, and histones H2A and H2B, suggesting that this Kap may import several proteins simultaneously. The import of more than one cargo at a time would increase the efficiency of each import cycle and may allow the regulation of coimported cargoes.

INTRODUCTION

In eukaryotic cells the nucleocytoplasmic transport of most proteins and some RNAs is mediated by an evolutionarily conserved family of soluble transport factors, the karyopherins (also referred to as importins and exportins; reviewed in Weis, 2003; Harel and Forbes, 2004; Mosammaparast and Pemberton, 2004). After synthesis in the cytoplasm, most nuclear protein cargoes are bound by a member of the karyopherin family, through direct interaction with a nuclear localization sequence contained in the cargo protein. Transport through the nuclear pore complex occurs via transient interactions of the karyopherin with the NPC. Once in the nucleus, the karyopherin encounters a high concentration of RanGTP, which acts as a regulator of transport. Interaction of the karyopherin with RanGTP leads to dissociation of the karyopherin from its nuclear cargo (Weis, 2003; Harel and Forbes, 2004; Mosammaparast and Pemberton, 2004). In some circumstances, nuclear-binding partners of the cargo appear to also play a role in stimulating the dissociation of Kap and cargo (Senger *et al.*, 1998; Lee and Aitchison, 1999; Pemberton *et al.*, 1999). In yeast, there are 14 members of the karyopherin family, with >20 members in mammalian cells (Mosammaparast and Pemberton, 2004). Karyopherins appear to function in either nuclear import or nuclear export, with only two examples of a Kap that works in both directions (Weis, 2003; Harel and Forbes, 2004; Mosammaparast and Pemberton, 2004). In yeast, 11 members of the karyopherin family must import at least 1500 nuclear proteins, suggesting that each receptor must have many cargoes. To date specific transport receptor-cargo pairs have only been shown for ~30 cargoes; in addition the NLS sequences recognized by most Kaps is not known, nor have the distinct binding sites for those NLSs been elucidated (Weis, 2003; Harel and Forbes, 2004; Mosammaparast and Pemberton, 2004). Several cargoes have been shown to be imported as protein complexes and are thought to interact with each other directly after synthesis, before Kap binding (Titov and Blobel, 1999; Mosammaparast *et al.*, 2001; Yoshida and Blobel, 2001; Mosammaparast *et al.*, 2002b; Leslie *et al.*, 2004). It is also possible that Kaps can bind to several distinct cargoes at a time, suggesting that these cargoes can be cotransported by the same Kap. Apart from being a more efficient mode of import, this could have significant implications for the function of these cargoes. Coregulation of import could ensure that similar amounts of specific proteins are imported at the same time or delivered to the same place in the nucleus. To add to the complexity of determining which Kap imports a particular cargo, it has been shown that several proteins can be imported into the nucleus by more than one Kap (Rout *et al.*, 1997; Jakel and Gorlich, 1998; Pemberton *et al.*, 1999; Mosammaparast *et al.*, 2001, 2002b; Muhlhausser *et al.*, 2001). Indeed many essential yeast nuclear proteins appear to have more than one route into the nucleus, with the preferred route being mediated by a nonessential karyopherin (Rout *et al.*, 1997; Pemberton *et al.*, 1999; Mosammaparast *et al.*, 2001, 2002b).

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It has been previously shown that the TATA-binding protein is imported by the karyopherin Kap114p (Morehouse *et al.*, 1999; Pemberton *et al.*, 1999). However in the absence of Kap114p, other karyopherins appear to be able to take its place (Pemberton *et al.*, 1999). Transcription of genes by RNA polymerase II (RNAP II) involves many steps, some of the earliest being the recruitment of general transcription factors and RNAP II to form the preinitiation complex (PIC; Hampsey, 1998; Hahn, 2004). TBP binds to the TATA box at many promoters and nucleates the formation of the PIC. After TBP-DNA binding a second PIC component, TFIIB, binds to TBP and DNA and stabilizes their association. The rest of the PIC including TFIID components, TFIIF, TFIIE, and TFIIH, and RNAP II are assembled onto this platform, allowing the RNA polymerase to be brought to genes to be transcribed (Hampsey, 1998; Hahn, 2004). TFIIB plays a central role in PIC formation through interactions with DNA, TBP, TFIIF, and RNAP II (Hampsey, 1998; Hahn, 2004). In addition TFIIB is thought to function in start site selection (Hampsey, 1998; Hahn, 2004). High-resolution crystal structures have elucidated the TFIIB-TBP-DNA and RNAP II complexes, but little is known about TFIIB before it is incorporated into this complex (Hampsey, 1998; Hahn, 2004). It is thought that with each round of transcription TFIIB does not remain bound to the promoter, suggesting that the study of the import of new TFIIB may be relevant to transcription (Yudkovsky *et al.*, 2000). Here we identify the import pathway of Sua7p, the yeast version of TFIIB. Surprisingly, Sua7p uses the same import receptor as TBP (Morehouse *et al.*, 1999; Pemberton *et al.*, 1999). This import receptor, Kap114p, is also utilized by histones H2A and H2B and their chaperone Nap1p (Mosammaparast *et al.*, 2001, 2002a).

MATERIALS AND METHODS

Yeast Strains

The yeast strains in this study were derived from DF5 (Finley *et al.*, 1987), except yeast strain Sua7-TAP (a kind gift from Dr. Tilman Borggrefe), which is described in Borggrefe *et al.* (2001). Yeast strains from DF5 were manipulated as previously described (Sherman *et al.*, 1986). The Δ*kap114* and Δ*kap123* strains have been described previously (Rout *et al.*, 1997; Pemberton *et al.*, 1999). *KAP114* was deleted in the haploid Sua7-TAP strain by integrative transformation of NAT^R. The Kap114-Myc/Sua7-TAP haploid strain was constructed by integrative transformation of 13 Myc epitopes into the C-terminus of *KAP114* immediately upstream of the stop codon (using a cassette kindly provided by John Aitchison, Institute for Systems Biology).

Plasmids

For overexpression of MBP-tagged proteins, the open reading frames (ORFs) corresponding to Sua7p, TBP, and Kap114p were cloned into pMAL-c2X (NEB, Beverley, MA). MBP-lacZα was overexpressed from the parental plasmid, pMAL-c2X. All GST-fusions were expressed by cloning the relevant sequences into pGEX4T1 (Stratagene, La Jolla, CA). pQE32-RanQ69L was kindly provided by Ian Macara (University of Virginia). GFP reporter constructs were based on $pGFP_2$-C-FUS as described previously, which result in the fusion of tandem GFP moieties to the carboxy terminus of Sua7p (Mosammaparast *et al.*, 2001).

Cytosol Preparation and Western Blotting

Postribosomal cytosol was prepared from 1 l of Kap114-PrA yeast, and 4 l of each indicated Sua7-TAP strain as previously described (Aitchison *et al.*, 1996). Kap114-PrA or Sua7-TAP and their associated proteins were isolated using IgG-Sepharose as described (Aitchison *et al.*, 1996; Pemberton *et al.*, 1997). Western blotting was performed by transferring the proteins onto polyvinylidene difluoride membrane and probing with antibodies as noted. The Sua7p antibody used in Western blots (Pardee *et al.*, 1998) was a kind gift from Dr. Alfred Ponticelli (University of Buffalo, NY). Kap114-Myc was probed with monoclonal antic-myc antibody (Sigma, St. Louis, MO). Rabbit polyclonal antibody was used to detect TBP. The TBP and yeast Sua7 antibodies also interact with PrA and TAP, allowing simultaneous detection of Kap114-PrA or Sua7-TAP. All antibody interactions were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

Purification of Recombinant Proteins and In Vitro Binding Assays

All MBP-tagged proteins and GST-tagged proteins were purified as previously described (Mosammaparast *et al.*, 2001, 2002b). Tag-free Nap1p and the $H2A_{1-46}$ NLS peptide were purified initially as GST-tagged fusions, and the GST tag was removed by thrombin (Sigma). His6-tagged RanQ69L, His6-tagged Ran T24N, and His6-tagged Gsp1 Q71L was purified on Ni-NTA agarose (Qiagen, Chatsworth, CA). On purification, all recombinant proteins were dialyzed against 1× TB-T20/15% glycerol. In vitro binding assays were performed as previously described (Mosammaparast *et al.*, 2002b), except for Figure 7A, where 0.5% Tween20 was added to the buffer (replacing 0.1% in the other assays). Beads were blocked in 20% bovine serum albumin (BSA)/tris-buffered saline/0.1% Tween 20 before use, resulting in visible "bovine serum albumin" band in many binding assays. For competition assays, indicated proteins were preincubated together for 30 min at 4°C before adding immobilized protein and beads. Purified His6-tagged TBP was a kind gift of David Auble (University of Virginia). The purified recombinant untagged histone H2A-H2B dimer was a kind gift of Robert Dutnall (UCSD).

Cell Culture and Microscopy

Strains containing the reporter constructs based on $pGFP_2$-C-FUS were grown and induced as described previously (Mosammaparast *et al.*, 2001). All microscopy and image manipulation were performed as described previously (Mosammaparast *et al.*, 2001) with a Nikon Microphot-SA microscope (Melville, NY) and OpenLab software (Improvision, Lexington, MA) using a 100× objective . For each figure, the GFP images were acquired using identical exposure settings and manipulated identically using Adobe Photoshop (San Jose, CA). After fixation in 3.7% formaldehyde for 20 min, immunofluorescence microscopy on yeast spheroplasts was performed as previously described (Pemberton *et al.*, 1997). Anti-Sua7 rabbit polyclonal antibodies were used to detect Sua7p (Liu *et al.*, 2001; a gift from David Auble, University of Virginia), followed by Cy3-conjugated donkey anti-rabbit IgG (Jackson Laboratories, ImmunoResearch Laboratories, West Grove, PA). Cells were mounted in Hoechst stain solution (bisbenzimide). All immunofluorescence images were taken with the Texas Red filter and an exposure time of 200 ms.

Nano-HPLC Microelctrospray Ionization Mass Spectrometry Analysis and Database Searching

For analysis of the entire interacting fraction, the sepharose and associated proteins were washed extensively in TB and 50 mM $MgCl_2$. The associated proteins were then eluted together with 1 M $MgCl_2$ (in one experiment the interacting fractions were eluted in 100 mM $MgCl_2$, followed by 1 M $MgCl_2$ and the derived peptide data were subsequently pooled). This mixed protein containing fraction was in most cases digested with modified trypsin and analyzed by nano-HPLC microelectrospray ionization (μESI) mass spectrometry (MS) as previously described (Mosammaparast *et al.*, 2001). In one case chymotrypsin digestion and peptide analysis was also used. Only peptides with cross correlation scores of >2 were considered good. As a negative control a nontagged wild-type strain was incubated with IgG sepharose and interacting proteins were analyzed as above. Proteins identified from the control were subtracted from the specific Kap114-PrA interacting protein data set.

RESULTS

Identification of Kap114-PrA Interacting Proteins

The karyopherin Kap114p is one of 11 import receptors in yeast (Mosammaparast and Pemberton, 2004). To identify the cargo repertoire of Kap114p, proteins that interacted with a PrA-tagged version of Kap114p were isolated from yeast cytosol using IgG sepharose. Proteins were eluted with 1 M $MgCl_2$, digested with proteases, and analyzed by mass spectrometry. In six separate experiments multiple peptides of known Kap114p partners (histone H2A and H2B, Nap1p and Spt15p/TBP) were identified, validating our approach (Supplementary Data Table 1). The data set also included some known proteins not previously shown to interact with Kap114p and some uncharacterized ORFs that represented potential Kap114p cargoes. The previously characterized proteins included the general transcription factor Sua7p or TFIIB (Pinto *et al.*, 1992), Sok2p, a transcription factor that regulates pseudohyphal development (Ward and Garrett, 1994), and Ddr48p, a protein involved in DNA damage

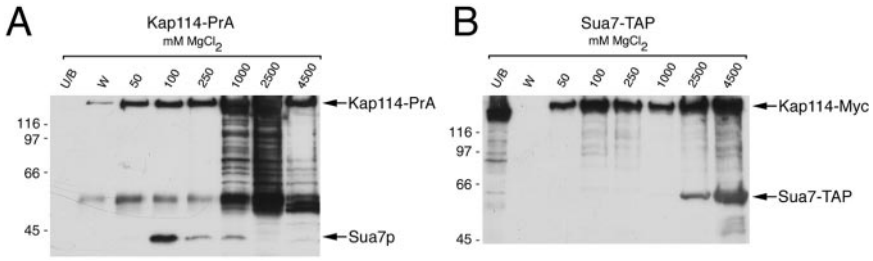

Figure 1. Kap114p interacts with Sua7p in yeast cytosol. (A) Kap114-PrA and associated proteins were isolated from cytosol by IgG-Sepharose, eluted with a $MgCl_2$ gradient, separated by SDS-PAGE, and visualized by Western blotting with an anti-Sua7p antibody. U/B, unbound fraction; W, final wash. (B) Sua7-TAP and associated proteins were isolated as described as described above from a strain also expressing Kap114-Myc. Kap114-Myc and Sua7-TAP were visualized by Western blotting with an anti-Myc antibody. Positions of molecular mass standards in kDa are shown.

response (McClanahan and McEntee, 1986). We also observed many peptides from RpL6B, suggesting this interaction may be specific to Kap114p. Kap114p itself was identified and most likely was released from the column. Some proteins were also identified from a control experiment using cytosol from an untagged yeast strain, and these were subtracted from the dataset. In addition, some ribosomal proteins, heat shock factors and translation elongation factors have been identified routinely with unrelated PrA tagged-proteins, suggesting that they may also represent nonspecific interactions (LFP unpublished data; Supplementary Data Table 1). Other proteins from which at least three separate peptides were identified, included several components of the regulatory or cap particle of the proteasome; Rpn3, Rpn5, Rpn7, Rpn9, Rpn11, and Rpn12 (Finley *et al.*, 1998; Glickman *et al.*, 1999), as well as Kap95p and Gsp1p (Supplementary Data Table 1). A further 51 proteins were represented by only 1 or 2 peptides (Supplementary Data Table 1). We chose to focus on the further characterization of Sua7p, because this potential cargo was interesting because it functions in the nucleus with another Kap114p cargo, TBP.

Kap114p Interacts with Sua7p in Yeast Cytosol

We set out to validate our mass spectrometry data and determine whether Sua7p was indeed a cargo for Kap114p. Kap114-PrA–interacting proteins were again isolated from cytosol and eluted with a $MgCl_2$ gradient. After separation by SDS-PAGE, the proteins were Western-blotted with an antibody to Sua7p. Sua7p was clearly visible, suggesting that it did indeed interact with Kap114p (Figure 1A). To further confirm this interaction, a TAP-tagged version of Sua7p was isolated from a strain expressing Myc-tagged Kap114p. Western blotting determined that Kap114p interacted with Sua7p in cytosol (Figure 1B).

Kap114p Imports Sua7p In Vivo

To determine whether Kap114p plays an important role in Sua7p import in vivo, we observed the localization of a Sua7-GFP fusion in wild-type yeast and in strains bearing a *kap114* deletion. As expected at steady state Sua7-GFP was mostly nuclear in wild-type cells (Figure 2A). Surprisingly in the Δ*kap114* strain, Sua7-GFP was completely mislocalized with no apparent nuclear accumulation (Figure 2A). Because Sua7p has an essential nuclear function, whereas Kap114p is not encoded by an essential gene, we concluded that other Kaps must play a role in Sua7p import. Sua7-GFP was observed in strains deleted for *KAP123*, *KAP104*, *MTR10*, *SXM1*, *NMD5*, and strains with temperature sensitive alleles of *KAP95* and *KAP121*: No obvious cytoplasmic accumulation of Sua7-GFP was noted in these strains (Figure 2A and unpublished data).

The degree of mislocalization observed with Sua7-GFP in the Δ*kap114* strain was greater than seen with other Kap114p-cargoes such as TBP or H2A and H2B (Pemberton *et al.*, 1999; Mosammaparast *et al.*, 2001). To verify that the endogenous Sua7p behaved similarly to Sua7-GFP, we carried out indirect immunofluorescence of fixed cells using an anti-Sua7p polyclonal antibody. In these experiments Sua7p was nuclear in wild-type cells, whereas a large proportion of Sua7p was mislocalized to the cytoplasm in the Δ*kap114* strain. These results suggest that Kap114p plays a major role in the import of Sua7p in vivo.

We have previously shown that Kap114p imports TBP, raising the possibility that Kap114p functioned as a specific import Kap for components of the PIC, or the RNAP II machinery (Pemberton *et al.*, 1999). We tagged Rpb3p (RNAP II core subunit), Taf12p (TFIID subunit), Tfa1p (TFIIE subunit), and Tfb1p (TFIIH subunit) with GFP and analyzed their localization in strains lacking Kap114p. We found that in these strains the localization of these proteins appeared nuclear and indistinguishable from their localization in wild-type cells, suggesting that Kap114p does not play a major role in their import (unpublished data).

The NLS of Sua7p Is Contained in the "Core" Region

Structural analyses of TFIIB have defined distinct domains of the protein; an amino terminal domain that forms a zinc ribbon and interacts with RNAP II and TFIIF (Zhu *et al.*, 1996; Chen and Hahn, 2003), a conserved B finger domain that also interacts with RNAP II (Bushnell *et al.*, 2004) followed by a linker, and the core or central/carboxy terminal domain (Figure 3). The core domain consists of two alpha helical repeats that interact with TBP and promoter DNA (Bagby *et al.*, 1995; Nikolov *et al.*, 1995; Lagrange *et al.*, 1998). We attempted to map the NLS of Sua7p by creating GFP fusions of different domains of Sua7p and determining which domain was sufficient to direct nuclear localization of the fusion. As expected unfused GFP was localized to both the nucleus and cytoplasm. Fusion of amino acids 101–345 to GFP (which approximately corresponds to the core domain) showed tight nuclear accumulation (Figure 3), which was abrogated in the absence of Kap114p (unpublished data). Fusion of amino acids 1–100 (zinc ribbon and B finger), 1–220 (zinc ribbon and B finger and first repeat of core), and 100–220 (first repeat of core) did not show any significant nuclear accumulation in wild-type cells (Figure 3). A further fusion of 220–345 was not expressed, suggesting the fusion was misfolded (unpublished data). These results suggested

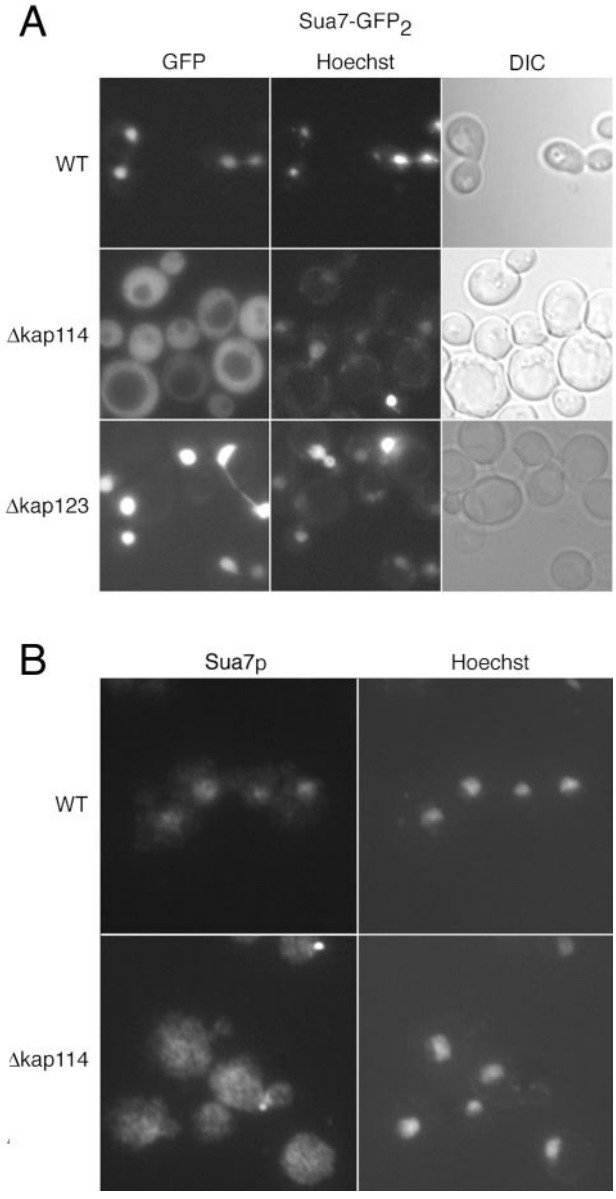

Figure 2. Kap114p imports Sua7p in vivo. (A) Wild-type (WT) and Δ*kap114* and Δ*kap123* mutant strains (as indicated) were transformed with plasmids expressing Sua7p fused to tandem copies of GFP under the control of the inducible *MET25* promoter as indicated. The GFP moiety detected by fluorescent imaging. The coincident Hoechst staining and DIC image is shown. (B) Indirect immunofluorescence was carried out on fixed yeast spheroplasts from WT and Δ*kap114* strains using an antibody to Sua7p. Antibody binding was visualized with Cy3-conjugated secondary antibodies; the coincident Hoechst staining is shown.

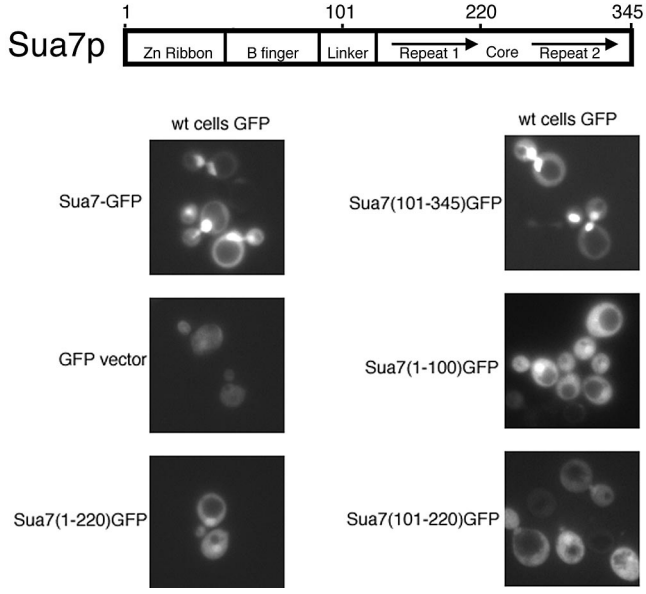

Figure 3. The core domain of Sua7p is necessary for import. Different fragments of Sua7p (as indicated by amino acid number; see schematic above) were expressed as GFP fusions in wild-type cells. Cells were transformed with plasmids expressing fragments of Sua7p fused to tandem copies of GFP under the control of the inducible *MET25* promoter. In addition, cells also expressed GFP alone as indicated (GFP vector), and the GFP moiety detected by fluorescent imaging.

that the NLS for Sua7p overlapped with the core domain of Sua7p, which also mediates interaction with TBP and DNA.

TBP and Sua7 Interact in Cytosol

Because both Sua7p and TBP were imported by Kap114p, this raised the possibility that they were coimported into the nucleus. This would serve a possible mechanism to coregulate their concentrations. As the domain of Sua7p necessary for nuclear import was the core or TBP-binding domain, it was also possible that Sua7p was imported via interaction with TBP. Both proteins coprecipitated with Kap114p from cytosol, and we tested whether a cocomplex of Kap114p, TBP, Sua7p could be formed. As before, we isolated Sua7-TAP from the cytosol of yeast strains bearing Myc-tagged Kap114p. Interacting proteins were eluted with a $MgCl_2$ gradient and analyzed by SDS-PAGE and Western blotting. We observed that both TBP and Kap114p were found in a complex with cytosolic Sua7-TAP, suggesting the presence of a trimeric complex in cytosol (Figure 4A).

To determine whether Sua7p was interacting with TBP through Kap114p, we repeated the experiment using cytosol from a Sua7-TAPΔ*kap114* strain. In the absence of Kap114p, TBP was also isolated with Sua7p (Figure 4B). This suggested that TBP and Sua7p interact directly in cytosol, or possibly through an additional protein such as another Kap, that is still present in the Sua7-TAPΔ*kap114* strain. These results suggested that TBP and Sua7p interact in cytosol, suggesting they could be coimported into the nucleus.

Sua7p Binds Kap114p and TBP Directly

We then wanted to determine whether the interaction between Sua7p and Kap114p was direct. Recombinant GST-Sua7 and MBP-Kap114 were expressed and purified from bacteria. MBP-Kap114 was shown to interact with immobilized GST-Sua7 but not with GST alone, and a control protein MBP-lacZ did not interact with Sua7p (Figure 5A). This suggested that Kap114p could bind and import Sua7p directly. We also tested whether TBP and Sua7p interact directly as suggested from the cytosol experiments. We could show using either immobilized GST-TBP or immobilized GST-Sua7 that these proteins interact directly with each other, and with Kap114p (Figure 5A). Addition of DNase I to the binding reaction confirmed that this interaction was not

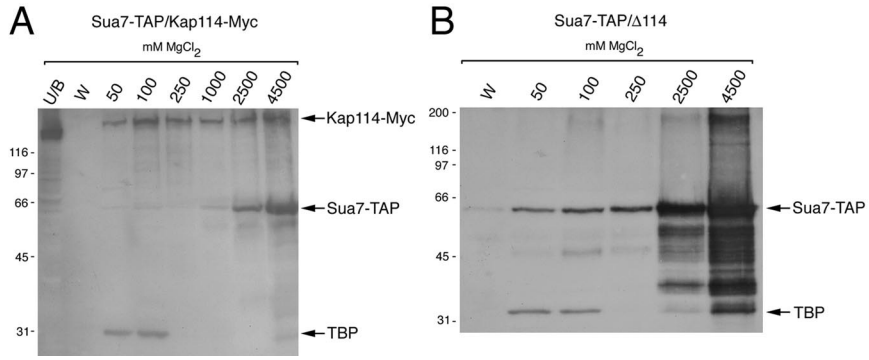

Figure 4. TBP and Sua7 interact in cytosol. Sua7-TAP and associated proteins were isolated as described above from (A) a Sua7-TAP/Kap114-Myc strain and (B) a Sua7-TAP/Δ*kap114* strain. Sua7-TAP and TBP and Kap114-Myc were visualized by Western blotting with an anti-TBP and anti-Myc antibodies. Positions of molecular mass standards in kDa are shown.

dependent on DNA (unpublished data). These results suggested that TBP and Sua7p interact in cytosol.

Kap-cargo interactions are usually sensitive to the addition of RanGTP (Weis, 2003; Mosammaparast and Pemberton, 2004). To test whether this was the case with Kap114p and Sua7p, MBP-Kap114 was preincubated with RanQ69L (a mutant version of human Ran that cannot hydrolyze GTP and is therefore constitutively GTP-bound). The Kap114p-RanGTP complex was then incubated with GST-Sua7 or GST-H2A NLS (another Kap114p cargo; Mosammaparast *et al.*, 2001). As expected RanGTP inhibited interaction of Kap114p with the both Sua7p and H2A (Figure 5B). The inhibition of Sua7p binding was less than observed with H2A, even at high RanGTP concentrations, raising the possibility that another factor is needed (Mosammaparast *et al.*, 2002a). In a parallel experiment MBP-Kap114p was preincubated with Ran T24N, a mutant version of human Ran that is defective in nucleotide loading, and with the yeast Ran (Gsp1-Q71L) that cannot hydrolyze GTP and is therefore constitutively GTP-bound. As before the yeast RanGTP partially inhibited interaction of Kap114p with GST-Sua7p, whereas the Ran T24N control had no effect. Taken together these results described above suggest that Sua7p is a new cargo of Kap114p and further suggests that Sua7p and TBP could be coimported into the nucleus together or be imported separately via direct interaction with Kap114p.

Kap114p Has Multiple Binding Sites for Its Different Binding Partners

As well as Sua7p, we have previously showed that Kap114p imports TBP, histones H2A and H2B and Nap1p (Pemberton *et al.*, 1999; Mosammaparast *et al.*, 2001, 2002a). How does Kap114p interact with these different cargoes that have no obvious homology to each other? In addition, integral to its function as a transport factor, Kap114p likely interacts directly with RanGTP and components of the NPC. We wanted to determine whether Kap114p could bind its cargoes simultaneously or whether this Kap contained only one cargo binding site. Structural information is not available for this Kap, however, structures of other members of the Kap family bound to Ran, Nups and cargoes have been solved (Chook and Blobel, 2001). These suggest that RanGTP and distinct nucleoporins bind to the amino terminal half of the

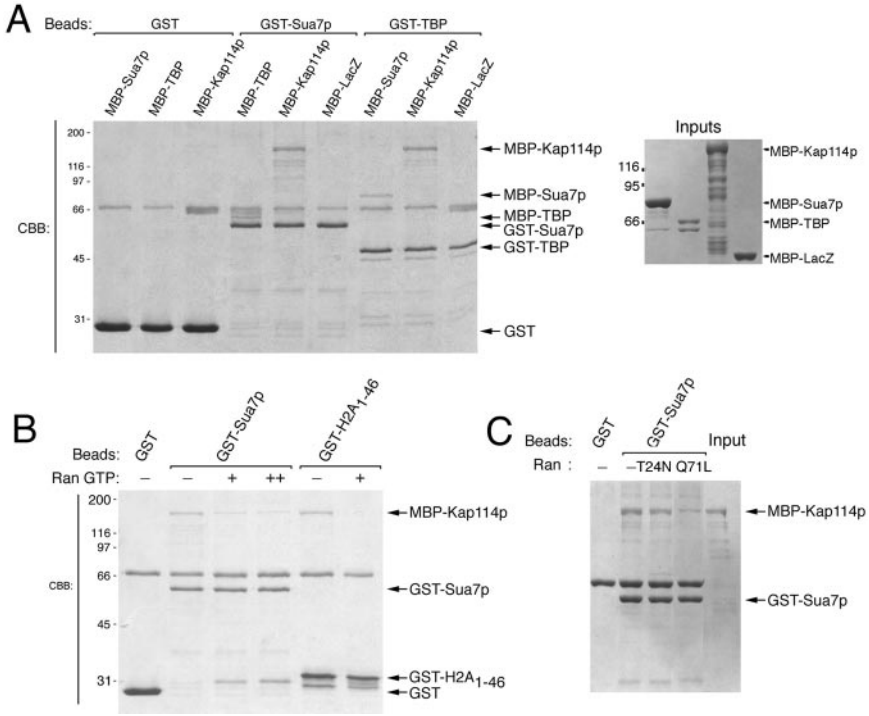

Figure 5. Sua7p binds Kap114p and TBP directly. (A) Immobilized GST (2 μM), GST-Sua7 or GST-TBP (0.5 μM of each) was incubated with recombinant MBP-Sua7p, MBP-TBP, MBP-Kap114p, or MBP-LacZ as indicated (all 1 μM). Bound fractions were separated by SDS-PAGE and visualized by Coomassie blue staining (CBB). Inputs gel (right) shows 10% of input protein in reaction for comparison; MBP-Sua7p (100 nM), MBP-TBP (100 nM), MBP-Kap114 (100 nM), or MBP-LacZ (100 nM) was visualized with CBB. (B) MBP-Kap114p (200 nM) was preincubated without (−) or with 10 μM (+) or 20 μM (++) human Ran Q69L, and binding was then tested with immobilized GST (200 nM), GST-Sua7p (200 nM) or GST-H2A$_{1-46}$ (2 μM). Bound fractions were analyzed as above. (C) MBP-Kap114p (200 nM) was preincubated without (−) or with 10 μM human Ran T24N or 10 μM yeast Ran Q71L, and binding was then tested with immobilized GST (200 nM), GST-Sua7p (200 nM). Bound fractions were analyzed as above. Band migrating at 66 kDa in all lanes is BSA. Input, 10% of the MBP-Kap114 (20 nM), in the reaction in B and C, is shown for comparison.

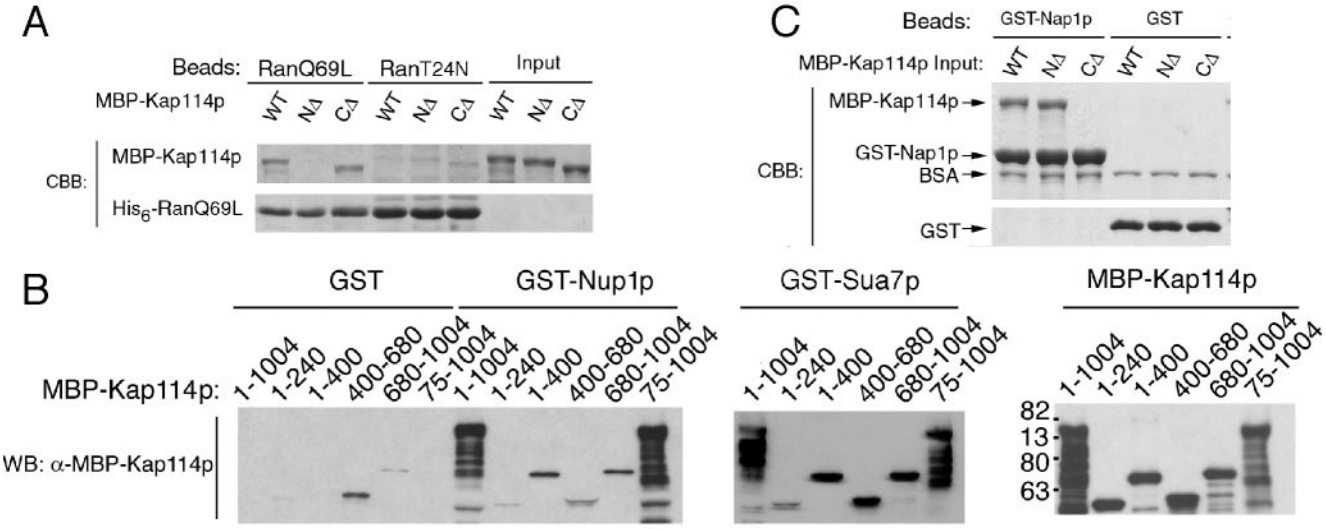

Figure 6. Kap114p interacts directly with Ran, Nup1p, Nap1p and Sua7p via different sites. (A) Immobilized His6-RanQ69L or His6-RanT24N (both 10 μM) were incubated with recombinant MBP-Kap114p (WT) or MBP-Kap114p truncations; NΔ, corresponding to amino acids 75–1004 or CΔ (1–897) as indicated (all 500 nM). Bound fractions were separated by SDS-PAGE and visualized Coomassie blue (CBB) staining. Input shows 10% (50 nM) of each MBP-Kap114 protein in the reaction for comparison. (B) Immobilized GST, GST-Nup1, or GST-Sua7 (1 μM of each) was incubated with recombinant MBP-Kap114p truncation mutants (numbering shows amino acids present; all 0.2 μM). Bound fractions were separated by SDS-PAGE and visualized by Western blotting (WB) with the anti-MBP antibody. Input gel (right) shows 10% of MBP-Kap114 proteins (20 nM) in reaction for comparison. (C) Immobilized GST (2 μM) or GST-Nap1 (400 nM) was incubated with recombinant MBP-Kap114p, NΔ (75–1004), or CΔ (1–897) truncation mutants as indicated (all 300 nM). Bound fractions were separated by SDS-PAGE and visualized by Coomassie blue staining (CBB) and Western blotting (WB) with the anti-MBP antibody.

Kap, whereas the localization of cargo binding sites is less clear, and cargoes may contact extended interaction interfaces within the Kap (Chook and Blobel, 2001; Mosammaparast and Pemberton, 2004).

We tried to determine where in Kap114p the binding sites for its various partners were located. Different fragments of Kap114p were expressed as MBP fusion proteins. In vitro binding assays were carried out with the Kap114p fragments and immobilized RanQ69L-His, RanT24N-His, GST-Nup1p, GST-Sua7p, and GST-Nap1. As expected RanQ69L bound to the amino terminal half of Kap114p, but no longer interacted when the first 75 amino acids were removed, suggesting that the Ran binding site is in the N-terminal half of Kap114p. RanT24N did not bind specifically to Kap114p (Figure 6A). The nucleoporin Nup1p appeared to interact with several domains of Kap114p. Fragments of Kap114p consisting of amino acids 1–400 and 680-1004 both interacted strongly with GST-Nup1. The Kap114p aa400–680 fragment interacted with Nup1p but also interacted with GST alone, suggesting the interaction may not be specific. Confirming that the amino terminus is not necessary for binding, a fragment consisting of 75–1004 also interacted strongly, whereas binding of the fragment consisting of Kap114p 1–240 was much weaker (Figure 6B). The same fragments were tested for binding to GST-Sua7p. Sua7p interacted strongly with all fragments except 1–240, suggesting its Kap114p binding site may be complex and not part of a discrete domain of Kap114p (Figure 6B).

Nap1p binding required amino acids 897-1004 of Kap114p, suggesting that the binding site was contained in this domain (Figure 6C). We have previously shown that histone H2A binds to at least three domains within Kap114p, in the amino terminal, central, and carboxy terminal domains of the protein (Mosammaparast *et al.*, 2005). This binding site analysis suggested that Kap114p uses extensive nonlinear binding sites for its interacting partners and raised the possibility that several partners could bind simultaneously.

Kap114p Has Overlapping Binding Sites for Sua7p and Nap1p

To determine whether Sua7p, H2A and Nap1p could simultaneously interact with Kap114p, in vitro binding assays were carried out. As shown before, immobilized GST-H2A bound MBP-Kap114p, and addition of excess histone H2A/H2B dimer competed with the immobilized H2A for Kap114p, resulting in a loss of Kap114p binding (Mosammaparast *et al.*, 2002a; Figure 7A). Excess recombinant Sua7p also competed with the immobilized GST-H2A for Kap114p, resulting in a loss of bound Kap114p (Figure 7A). This suggested that histones H2A/H2B and Sua7p share an overlapping binding site on Kap114p. To verify this result we carried out a similar assay with immobilized GST-Sua7 and again observed competition between Sua7p and H2A/H2B for Kap114p (Figure 7A).

We next determined by a similar assay if Sua7p and Nap1p could compete with each other for Kap114p binding. MBP-Kap114p bound to GST-Sua7p, and as expected addition of excess MBP-Sua7p competed with GST-Sua7p, resulting in a loss of Kap114p bound to the beads. However, addition of excess Nap1p did not appear to compete with GST-Sua7p for Kap114p (Figure 7B). This result suggests that Sua7p and Nap1p bind to different sites on Kap114p and raises the possibility that these proteins could be coimported by Kap114p into the nucleus.

Kap114p Can Form a Complex with Sua7p, Nap1p, and Histones H2A and H2B

To confirm that Sua7p and Nap1p can simultaneously interact with Kap114p, we attempted to assemble a cocomplex of

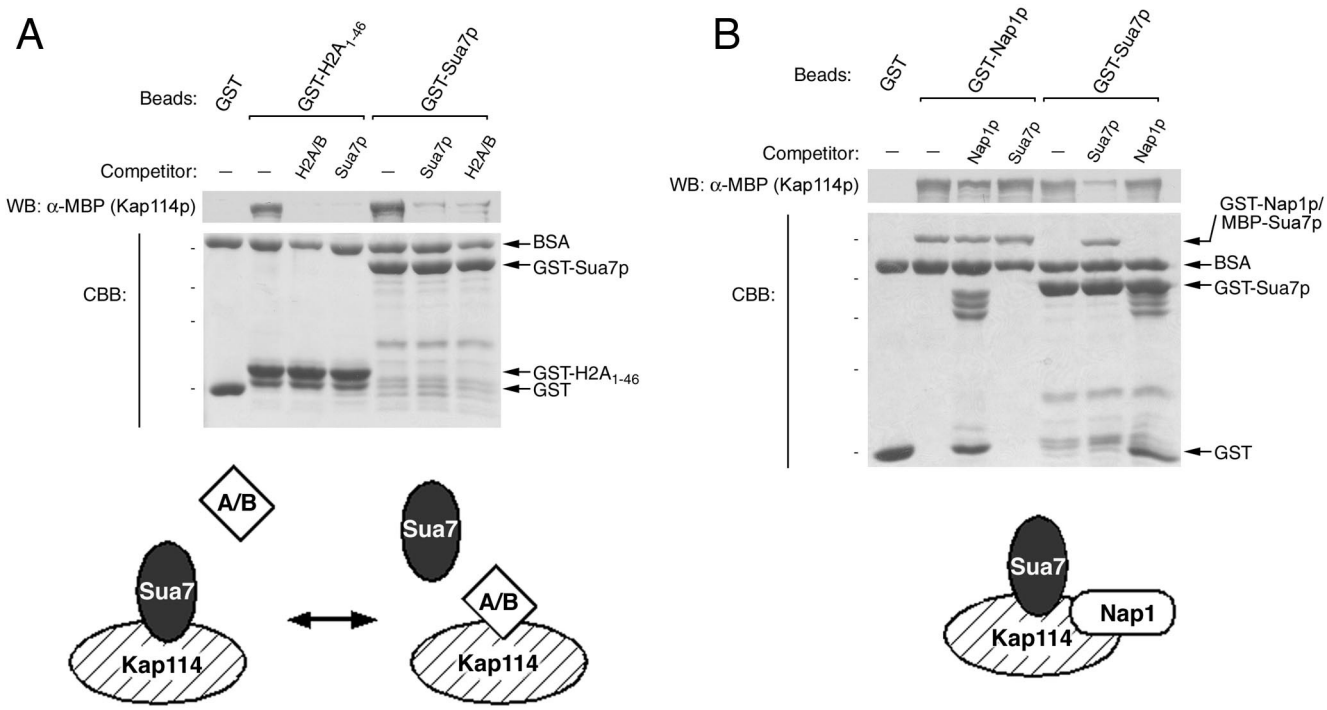

Figure 7. Sua7p competes with histone H2A and H2B for Kap114p binding. (A) MBP-Kap114p (200 nM) was preincubated without (−) or with competitor protein as indicated (MBP-Sua7p, 5 μM; or H2A/H2B, 3 μM) and binding was then tested with immobilized GST (2 μM), GST-Sua7p (100 nM), or GST-$H2A_{1-46}$ (200 nM). Bound fractions were separated by SDS-PAGE and visualized by Coomassie blue staining (CBB) and Western blotting (WB) with the anti-MBP antibody. Schematic model of Kap114 binding is shown below. (B) MBP-Kap114p (200 nM) was preincubated without (−) or with competitor protein (MBP-Sua7p, 5 μM; or Nap1p, 10 μM as indicated) and binding was then tested with immobilized GST-Sua7p (1 μM). Bound fractions were analyzed as above. Schematic model of Kap114 binding is shown below.

Kap114p, Sua7p, and Nap1p. As a control we first confirmed that Sua7p and Nap1p do not interact directly, whereas Nap1p does interact directly with the H2A NLS (Figure 8A). We then tested whether recruitment of Sua7p to immobilized Nap1p was dependent on the presence of Kap114p. We could show that MBP-Sua7p was only recruited to GST-Nap1p when MBP-Kap114p was also added to the reaction, suggesting that we had formed a Nap1p-Kap114p-Sua7p cocomplex. As GST-Nap1p and MBP-Sua7p have the same mobility, recruitment of Sua7p was visualized by western blotting (Figure 8B). As further evidence that these proteins are in a cocomplex, we also tested whether we could recruit histones H2A and H2B to the Nap1p-Kap114p-Sua7p complex in a Nap1p dependent manner to form a Sua7-Kap114p-Nap1p-H2A/B cocomplex. GST-Kap114p was immobilized, and binding of Sua7p, histone H2A and H2B dimer, and Nap1p could be observed in independent binding assays (Figure 8C). These proteins also did not interact with immobilized GST protein. Binding of the H2A and H2B dimer to GST-Kap114p was observed in the presence of an excess of Nap1p, but as before, was not observed in the presence of an excess of Sua7p. Therefore both Sua7p and Nap1p bound to GST-Kap114p independently, and when added together. When all four proteins (H2A and H2B dimer, Sua7p, and Nap1p) were added together, binding of the H2A and H2B dimer to Kap114p was now observed, suggesting that, these proteins can interact with GST-Kap114p via Nap1p, in the presence of Sua7p (see model in Figure 8C). These results suggested that Kap114p could simultaneously import different cargoes into the nucleus.

DISCUSSION

We have identified several potentially new cargoes for the karyopherin, Kap114p, and further characterized one of these cargoes, the general transcription factor IIB, or Sua7p. Sua7p can bind directly to Kap114p, likely via the Sua7p core domain, which is necessary and sufficient for import. The core domain of Sua7p is also necessary for interaction with TBP and with DNA at the promoter of RNAP II encoded genes (Bagby *et al.*, 1995; Nikolov *et al.*, 1995; Lagrange *et al.*, 1998). Because Sua7p can interact with TBP in cytosol and TBP is itself a cargo for Kap114p, this raised the possibility that both Sua7p and TBP can be imported together as a complex by Kap114p. In addition to Sua7p and TBP, integral to its function in nuclear transport, Kap114p also interacts with several other proteins, including RanGTP, nucleoporins, and its other cargoes, histones H2A and H2B and Nap1p (Morehouse *et al.*, 1999; Pemberton *et al.*, 1999; Mosammaparast *et al.*, 2001, 2002a). We have shown that Kap114p possesses multiple overlapping binding sites for these partners, which suggests that this Kap may import several proteins simultaneously.

We took a proteomic approach in order to identify the cargo repertoire of different karyopherins. Here we present data for Kap114p. The proteins that were identified included all the known binding partners of Kap114p including TBP, histones H2A and H2B and Nap1p, which validated our approach (Morehouse *et al.*, 1999; Pemberton *et al.*, 1999; Mosammaparast *et al.*, 2001, 2002a). We chose Sua7p for further analysis and showed it was indeed a Kap114p cargo. We presently do not know how many more cargoes this

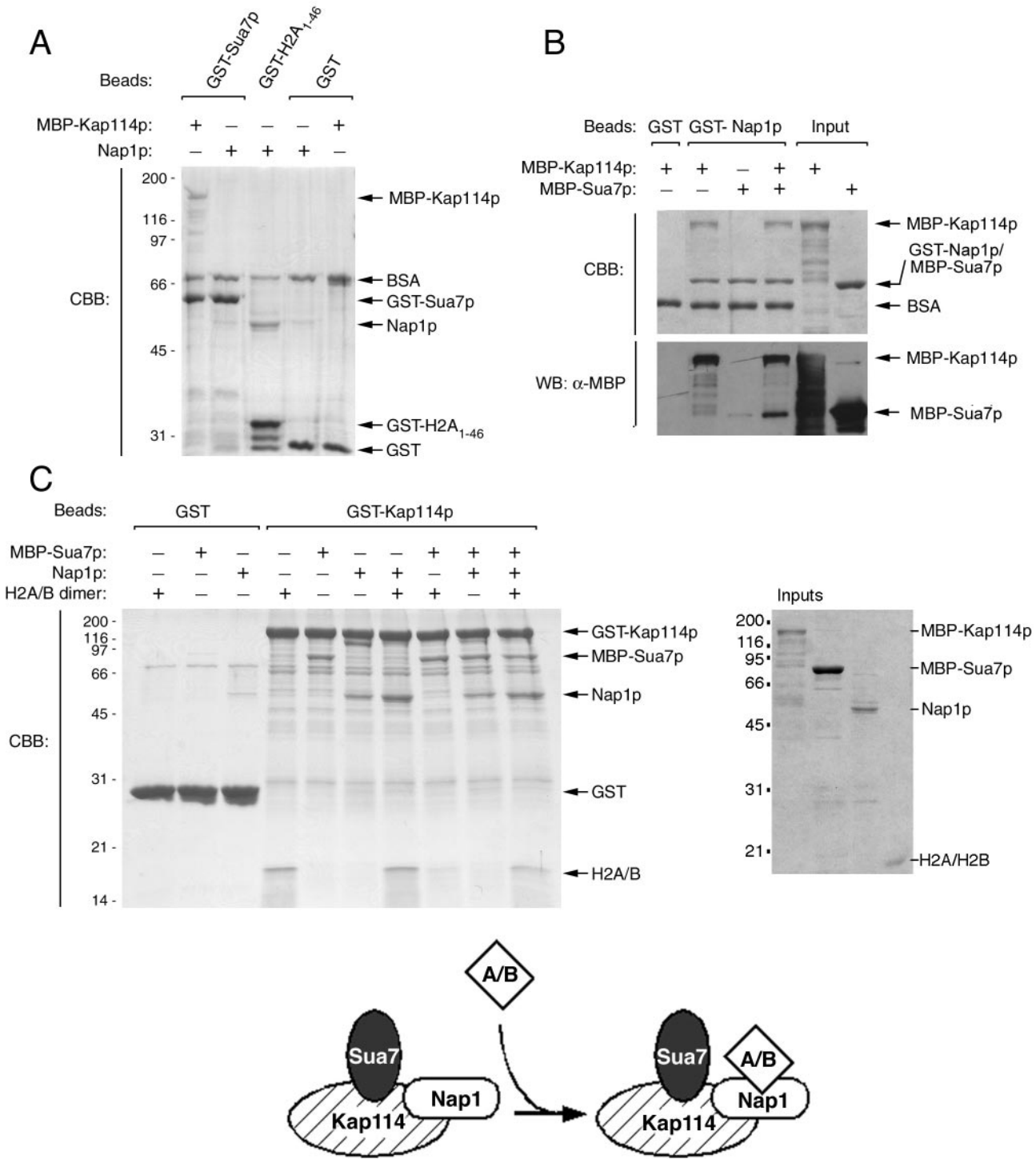

Figure 8. Kap114p can form a cocomplex with Sua7p, Nap1p, and histones H2A and H2B. (A) Immobilized GST, GST-Sua7p, or GST-H2A$_{1-46}$ (all 500 nM) was incubated with recombinant MBP-Kap114p or Nap1p (both 500 nM) as indicated. Bound fractions were analyzed as above. (B) Immobilized GST (2 μM) or GST-Nap1 (40 nM) was incubated with MBP-Kap114 (400 nM) and/or MBP-Sua7 (400 nM) as indicated. Bound fractions were separated by SDS-PAGE and visualized by Coomassie blue staining (CBB) and Western blotting (WB) with the anti-MBP antibody. Input shows 10% of protein in reaction (MBP-Sua7p, MBP-Kap114p, both 40 nM). (C) Immobilized GST (2 μM) or GST-Kap114 (200 nM) was incubated with recombinant MBP-Sua7 (2.5 μM), Nap1p (500 nM), and H2A/H2B dimer (500 nM) as indicated. Bound fractions were separated by SDS-PAGE and visualized by Coomassie blue staining (CBB). Input gel with the following input proteins is shown for comparison; MBP-Kap114 (20 nM), MBP-Sua7p (50 nM), Nap1p (50 nM), and H2A-H2B (50 nM). Schematic model of Kap114 binding in B and C is shown below.

karyopherin has; we identified multiple peptides from Sok2p, Ddr48p, and Rpl6B. Sok2p and RpL6B are found in the nucleus, and further experiments will determine whether they too utilize Kap114p for import (Lehmann *et al.*, 2002; Huh *et al.*, 2003; Wendler *et al.*, 2004). The experiments presented here, and others published for Kap114p, Kap121p, and Kap123p suggest that each karyopherin has multiple cargoes, although little is currently known about the distinct cargo repertoires for each karyopherin (Leslie *et al.*, 2004; Mosammaparast and Pemberton, 2004). We obtained three or more peptides from a further 13 proteins, and additional experiments are required to determine whether they indeed interact with Kap114p in vivo. Ten of these proteins have been shown to be nuclear and included several components of the regulatory cap of the proteasome, and some ribosomal proteins, suggesting they may also represent specific Kap114p-cargoes. Surprisingly we also identified Gsp1p and the karyopherin Kap95p. Gsp1p is a Kap114p binding partner, but in its cytosolic form (Gsp1-GDP) would not be expected to interact with Kap114p. We do not know the significance of the interaction between Kap114p and Kap95p (and Srp1p, which was also identified) or whether it is physiologically relevant. We obtained a limited number of peptides (1–2) from 50 additional proteins, approximately half are predicted to be nuclear and further experiments will determine whether any represent cognate cargoes for Kap114p.

It is interesting that the experimentally verified Kap114p cargoes seem so far to be involved in RNAP II transcription or chromatin assembly, although we could not show that Kap114p played a role in the import of other PIC components. (Morehouse *et al.*, 1999; Pemberton *et al.*, 1999; Mosammaparast *et al.*, 2001, 2002a). This raises the possibility that distinct Kaps may import groups of cargoes that are functionally related, and interestingly Kap123p has been shown to important for the import of several cargoes involved in ribosome biogenesis and nucleolar functions (Rout *et al.*, 1997; Sydorskyy *et al.*, 2003).

Using GFP-tagged fusions and antibodies to the endogenous protein, we observed that a significant fraction of Sua7p was mislocalized from the nucleus to the cytoplasm in the absence of Kap114p. In light of this mislocalization it is surprising that there is no obvious growth defect in the *kap114* deletion strain and suggests that a fraction of Sua7p must be gaining access to the nucleus by another pathway. We previously observed a similar result for TBP, although the TBP mislocalization was not as pronounced (Pemberton *et al.*, 1999). We do not know how Sua7p enters the nucleus in the absence of Kap114p. However, we have shown that Kap123p and Kap121p functionally overlap with Kap114p in the import of TBP and histones, suggesting that they may also play a secondary role in Sua7p import (Pemberton *et al.*, 1999; Mosammaparast *et al.*, 2001).

Several crystal structures of TFIIB in complex with other PIC components have been solved (Bagby *et al.*, 1995; Nikolov *et al.*, 1995; Zhu *et al.*, 1996; Bushnell *et al.*, 2004). In addition recent findings have determined how TFIIB plays a central role in the PIC in transcription initiation and mediates interactions with TBP, DNA, RNAP II components, and TFIIF (Hampsey, 1998; Chen and Hampsey, 2004; Chen and Hahn, 2004; Hahn, 2004). We wanted to determine which domain of TFIIB was important for nuclear import and tested domains of Sua7p for their ability to mediate the nuclear accumulation of GFP. We determined that the core domain was responsible for this activity. We were not able to further refine this mapping as smaller deletions did not appear to form functional fusion proteins. As the core domain is responsible for interaction with TBP, this raised the possibility that Sua7p was entering the nucleus via interaction with TBP (Bagby *et al.*, 1995; Nikolov *et al.*, 1995). Interestingly, in support of this model we have been able to show that Sua7p and TBP interact in cytosol, in the absence of DNA. However, TBP and Sua7p can both also bind directly to Kap114p, indicating that there is more than one possible configuration for a cocomplex, that are not necessarily exclusive: Each cargo may bind directly to Kap114p, or else one cargo binds Kap114p and the second cargo can piggyback off the first. Similar configurations have recently been shown for the Kap121p-mediated import of Nop1p and Sof1p (Leslie *et al.*, 2004). We have attempted to carry out gel filtration experiments to determine whether a trimeric complex exists; however, because of the differences in MW of the proteins, and their ability to bind each other, it has not proved possible to resolve the Kap-cargo dimers from Kap and cargo trimers.

If TBP and Sua7p import is mediated by the same Kap, this may allow coregulation of their nuclear concentrations. We have previously shown that the RanGTP-mediated release of TBP from Kap114p appears to be stimulated by TATA-containing DNA and proposed that the Kap114p might target TBP to its final nuclear destination at the TATA box (Pemberton *et al.*, 1999). In vitro, the Kap114p-Sua7p interaction appears to be more sensitive to RanGTP than the Kap114p-TBP complex and less sensitive than Kap114p-H2A complex, suggesting that they may all interact differently with Kap114p. Future experiments will determine whether other factors are required for the RanGTP-mediated dissociation of Kap114p-Sua7p and whether Sua7p and TBP are dissociated at similar locations in the nucleus.

Little is known about the specific binding sites of Kap-interacting proteins at the atomic level, although it has been shown that RanGTP and nucleoporins bind to the amino terminal half of the protein and that at least one cargo binds to the carboxy terminal half (reviewed in Chook and Blobel, 2001; Mosammaparast and Pemberton, 2004). We attempted to understand how Kap114p could import five proteins that show little sequence or structural homology (H2A, H2B, Nap1p, TBP, and Sua7p), and we analyzed the different binding sites for Kap114p partners. As expected, RanGTP required the amino terminus, whereas Nup1p interacted with two nonoverlapping domains, the N terminal domain, and also a nonoverlapping C terminal fragment. Interestingly, previous studies on mammalian karyopherin β1 have demonstrated the presence of an additional C terminal nucleoporin-binding domain (Bednenko *et al.*, 2003). The identification of the cargo-binding sites has proved more difficult. We have previously shown that H2A interacts with at least three distinct, nonoverlapping sites within Kap114p and that H2A and H2B bind to overlapping sites on Kap114p (Mosammaparast *et al*, 2005). Here we show that Nap1p requires the carboxy terminal part of the protein. We have been unable to map a distinct domain for Sua7p, because this protein interacted to some extent with all fragments of Kap114p tested except 1–240. Future structural studies should be able to provide a clearer picture of how Kaps interact with their cargoes and determine how the three-dimensional structure of the protein contributes to the various binding sites, but the data presented here suggests that the binding sites within the Kaps may often be complex and nonlinear. Alternative approaches, however, using competition studies have allowed us to demonstrate that the Sua7p-binding site overlaps with that of histones H2A and H2B, but not with that of Nap1p. Interestingly we could recruit histones to Kap114p, in the presence of excess Sua7p,

if Nap1p was also present, presumably because histones can also interact indirectly with the Kap via Nap1p. We could also form a complex with Kap114p, Sua7p, and Nap1p. The formation of these cocomplexes leads us to propose a model whereby Kap114p can coimport more than one of its cargoes at a time. In the case of TBP and Sua7p, if these two cargoes are coimported it may serve to regulate their nuclear accumulation, leading to the observed similar concentrations of these factors in the nucleus (Lee and Young, 1998). As discussed above, coimport by Kap114p may possibly play a role in ensuring their release at the same distinct nuclear location. In the case of Sua7p, Nap1p, and histones it is possible that dependence on Kap114p for import serves to coregulate a common function. It should be noted that during RNAP II transcription, histones H2A and H2B are removed from nucleosomes at the transcribed gene and then must be replaced, in a reaction mediated by the FACT complex and possibly Nap1p (reviewed in Belotserkovskaya and Reinberg, 2004). It is also possible that during S phase, when there is a burst of histone synthesis, Sua7p can be outcompeted by the histones, which could temporarily attenuate its import into the nucleus. In other phases of the cell cycle, histones would be out-competed by Sua7p, but any remaining cytoplasmic histone that still needed to access the nucleus could bind Kap114p via Nap1p. In this way competition for the Kap could serve to negatively regulate cargo import.

In summary we have identified a new cargo for Kap114p and shown that two components of the PIC can be imported by the same Kap. Because of the ability of some Kap114p cargoes to interact in the cytoplasm before import and the fact that Kap114p can simultaneously bind more than one cargo, we suggest that Kap114p and other Kaps may be able to import more than one cargo at a time. Coimport of cargoes would increase the efficiency of nuclear import and could serve as an important mechanism whereby the nuclear concentrations of cargoes could be coregulated.

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