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Kap104p: A Karyopherin Involved in the Nuclear Transport of Messenger RNA Binding Proteins

John D. Aitchison, Günter Blobel,* Michael P. Rout

A cytosolic yeast karyopherin, Kap104p, was isolated and shown to function in the nuclear import of a specific class of proteins. The protein bound directly to repeat-containing nucleoporins and to a cytosolic pool of two nuclear messenger RNA (mRNA) binding proteins, Nab2p and Nab4p. Depletion of Kap104p resulted in a rapid shift of Nab2p from the nucleus to the cytoplasm without affecting the localization of other nuclear proteins tested. This finding suggests that the major function of Kap104p lies in returning mRNA binding proteins to the nucleus after mRNA export.

Transport across the nuclear envelope occurs through nuclear pore complexes (NPCs) and is governed by the interaction of soluble transport proteins (karyopherins) with the transport substrate and the NPC (1–12). Most of our understanding of the mechanism of translocation comes from studying protein import in senipermeabilized cells (1) of model karyophilic proteins that carry a nuclear localization signal (NLS) from either the SV40 large T antigen or nucleoplasm (2). These classical NLSs are recognized by karyopherin α in a dimeric cytosolic complex with karyopherin β (3–8). The complex docks at the NPC through its interaction with nucleoporins that contain characteristic repeated peptide motifs (6–11). The small guanosine triphosphatase, Ran, and p10 are required for the subsequent translocation of the substrate (and karyopherin α) through the NPC (11, 12).

Distinct saturable and noncompeting pathways for the import of different karyophiles have been uncovered through the use of microinjection studies in oocytes (13–15). Similarly, saturable noncompeting pathways exist for the export of macromolecules from the nucleus (14, 16, 17). The signals that mediate many of these processes are different from classical NLSs (14, 15, 17–19) and thus may use recognition factors other than karyopherin α and karyopherin β for nuclear transport. Here we characterize the first such factor, which we term Kap104p and which is required for the import of at least two yeast nuclear mRNA binding proteins.

The Saccharomyces cerevisiae proteins Kap60p and Kap95p are homologs of mammalian karyopherin α and karyopherin β (20). Sequence comparisons of Kap95p with the complete yeast genome database uncovered three additional proteins that are structurally similar to Kap95p; two of these, which we term Kap123p and Kap104p, have not been previously characterized (21), and the third, Pse1p, was identified as a multicopy enhancer of protein secretion (22). The sequence alignment of Kap104p with Kap95p is shown (Fig. 1A). The proteins bear substantial similarity over their entire lengths, and secondary structural predictions suggest that Kap95p and Kap104p share the same overall domain structure of HEAT motifs (23).

Deletion of KAP104 resulted in a severe growth defect and temperature sensitivity (24). Immunofluorescence microscopy (25) with antibodies specific for Kap104p (in wild-type cells) showed that Kap104p was mainly cytosolic and was apparently absent from the nucleus (Fig. 1B). However, in nag120Δ cells, which cluster their NPCs to a region of the nuclear envelope opposite the nucleolus (26), Kap104p colocalized with the nucleoporin Nup1p (27) (Fig. 1C). The ability to detect coincident staining of the nucleoporins and Kap104p under these conditions likely was due to an interaction of Kap104p with NPCs.

Subcellular fractionation (28) was consistent with the distribution of Kap104p detected by immunofluorescence. Kap104p was present mainly in the cytosolic fraction.
but cofractionated partly with the nucleus (29). Further fractionation of the nucleus caused Kap104p to be released into a soluble fraction, and so it did not copurify with NPCs (28, 29). This result is in contrast to that for Kap95p and Kap60p, which were found in the highly enriched NPC fraction (see below) (30). Thus, the interaction of Kap104p with the NPC was weaker than that of the Kap95p–Kap60p complex.

We used an overlay blot assay to investigate which (if any) NPC proteins are bound by Kap104p. Both Kap95p and Kap104p were tagged with a functional in-frame COOH-terminal fusion of protein A (31). Cytosolic extracts (28) containing the chimeras were incubated with NPC proteins immobilized on nitrocellulose, and bound karyophorins were detected by means of the tag (Fig. 2A) (10). For reference, the positions of repeat-containing nucleoporins identified through the use of well-characterized monoclonal antibodies are shown (28). Kap104–protein A bound most strongly to proteins in the NPC fraction that comigrated with Nup116p, Nup100p, Nup145p (65-kD fragment), and Nup57p. Although Kap95–protein A (likely complexed with Kap60p) (8) bound to these nucleoporins, it also bound strongly to proteins comigrating with Nap1p, Nsp1p, and Nup49p. Kap104p expressed as a glutathione-S-transferase (GST) fusion and purified from Escherichia coli bound to the same set of nucleoporins as the cytosolic extract containing tagged Kap104p. Thus, whereas Kap95p has been reported to bind to nucleoporins in a complex with Kap60p, Kap104p bound directly to these proteins and required no additional factors from yeast cytosol. The strong signal, with a relative molecular mass of ∼60,000, observed in NPCs with Kap95–protein A was identified by immunoblotting as Kap60p (29), confirming that Kap95p binds Kap60p. In contrast, Kap104–protein A did not bind Kap60p. Similarly, purified Kap104p did not bind to immobilized Kap60p, whereas purified Kap95p did (29). This result strongly suggests that whereas Kap60p binds Kap95p, it does not bind Kap104p.

Because of the comigration of Kap104p binding proteins with the repeat-containing nucleoporins, we expressed GLFG, XPFPG, and PSFG repeat-containing nucleoporins in E. coli and compared the ability of Kap104– and Kap95–protein A to bind to each (Fig. 2B). Kap104p bound to Nup116p, Nup100p, and Nup145p, whereas Kap95p bound to Nup1p, Nsp1p, and Nup49p. Kap104p binding was not as robust as Kap95p binding; however, Kap104p binding was still observed in E. coli, and total lysates from each strain or from uninduced cells (U1) were assayed for binding to Kap95–protein A and Kap104–protein A as described in (A).
Nup100p, and to the XPF/FG repeat-containing region of Nup1p. Kap95p bound most strongly to the XPF/FG repeat-containing region of Nup1p. Neither Kap95p nor Kap104p bound to PS9 repeats from Nup159p or to any E. coli proteins present in lysates lacking an expressed nucleoporin. It is difficult to assess the relative binding strength of each Kap to the nucleoporins in this assay because each protein may have reassociated on the blot to a different extent. It is clear, however, that Kap104p bound to repeat-containing nucleoporins in this assay, and it is likely that Kap95p and Kap104p bound with relatively different affinities to different nucleoporins.

To study the interaction of Kap104p with soluble proteins, we purified the Kap104–protein A fusion from the post-nuclear cytosolic extract of KAP104-A cells using immunoglobulin G (IgG)–Sepharose (32). The same procedure was used to isolate proteins bound to Kap95p (30). Two major proteins (p68 and p70) specifically bound to Kap104–protein A (Fig. 3). The behavior of these proteins in this isolation procedure matched that of Kap66p bound to Kap95–protein A, suggesting that the strength and nature of the interaction of p68 and p70 with Kap104p was similar to that of Kap66p with Kap95p. Quantitation of the recovery of Kap104–protein A by immunoblotting showed that under these conditions, 60 to 70% of the total cellular Kap104p was recovered. Also, the Coomassie-blue staining intensity of p68 and p70 compared with that of Kap104p suggests that they were present in approximately equimolar amounts. Thus, most of Kap104p in the cytosol was bound to p68 and p70, although it remains to be determined whether p68 and p70 bind to Kap104p separately or as a complex. By partial protein A sequencing, p68 and p70 were identified as Nab2p and Nab4p (also called Hrp1p) (33, 34). These proteins have been shown to be nuclear mRNA binding proteins, both with similarity to a class of heterogeneous nuclear ribonucleoproteins (hnRNPs) that shuttle between the nucleus and cytoplasm of mammalian cells. Nab4p is the closest structural homolog of hnRNP A1 in yeast (33). Although neither Nab2p nor Nab4p contains the M9 sequence shown to be important for shuttling of hnRNP A1 (19), both have glycine-rich regions similar to the M9 sequence of hnRNP A1. Furthermore, searches of the yeast database with the M9 sequence revealed no direct homologs in yeast, indicating that this part of the "shuttling" sequence may not be highly conserved between yeast and mammals.

Because Nab2p and Nab4p are nuclear in the steady state (33), and the cytosolic fraction used for immunosolation of Kap104–protein A was prepared under conditions that keep the majority of nuclei intact (28), the cytosolic fraction we recovered bound to Kap104p cannot represent the major fraction of these proteins. The similarity of Nab4p and Nab2p to the shuttling hnRNP suggests that the cytosolic fraction of each protein bound to Kap104p may represent a cytoplasmic pool of these mRNA binding proteins.

Temperature-sensitive mutants of Kap104p were constructed by plasmid shuffling and assayed for their effect on Nab2p (35). One of these mutants (k104-16) was of particular interest, because after the temperature shift to 37°C, Kap104p was rapidly turned over, presumably degraded after misfolding of the protein. Immunoblotting of total cell lysates demonstrated that 1 hour after the temperature shift, less than 10% of Kap104p remained (Fig. 4A), and it was barely detectable after 3 hours. Immunofluorescent localization of Nab2p demonstrated that depletion of Kap104p was coincident with redistribution of Nab2p from the nucleus to the cytoplasm (Fig. 4B). Other nuclear marker proteins, including Nsr1p, Nop1p, Npl3p (36), and the SV40 large T NLS fused to the green fluorescent protein were not mislocalized under these conditions (29). Thus, depletion of Kap104p after the temperature shift resulted in the specific depletion of Nab2p from the nucleus in these cells, which suggests that the accumulation of Nab2p in the cytosol was a direct result of the inability to reimport Nab2p after it was exported from the nucleus. Similar results were obtained when Kap104p was depleted from a strain containing KAP104 under the glucose-repressible GAL1 promoter (29). Electron microscopy of these cells showed no apparent morphological defects (29). In addition, different alleles of k104ts mutants mislocalized Nab2p to a varying extent. Thus, Kap104p appeared to play a role in the import of Nab2p, but not its export. This result is also consistent with the cytosolic location of Kap104p.

We examined the distribution of polyadenylated [poly(A)+] mRNA by in situ hybridization of oligo(dT) in the k104-16 strain. Poly(A)+ RNA accumulated within the nucleus after the temperature shift, but in only a subpopulation of cells after 3 hours (29), considerably slower than the rapid mislocalization of Nab2p and loss of detectable Kap104p, suggesting that Kap104p depletion did not directly affect mRNA exons.
port. The most straightforward interpretation of our results [and by analogy to hnRNA1A (19)] is that Nab2p and Nab4p exit the nucleus as a major component of an hnRNP complex and upon release are reimported into the nucleus. The late-onset mRNA transport block we observed after the temperature shift in kap104-16 cells may thus have been caused by depletion of the nuclear pool of essential mRNA binding proteins like Nab2p.

Thus, Kap104p represents a karyopherin that participates in a nuclear import pathway independent of the classical NLS-mediated pathway of Kap95p and Kap60p. It interacted directly both with its substrate and nucleoporins, bypassing the requirement of an NLS binding adapter like Kap60p. We suggest an import-export cycle in which Nab2p and Nab4p are imported into nuclei via Kap104p, are assembled with mRNA, and are exported to the cytoplasm as major components of hnRNPs, where they dissociate from the mRNA and upon rebinding of Kap104p, begin another cycle. A putative mammalian Kap104p homolog has recently been cloned (37), suggesting an evolutionary conservation of this transport system.

REFERENCES AND NOTES

3. Nomenclature: Karyopherin α: importin α60, NLS receptor, SP1P, hSRP14, karyopherin β: importin β9/7, p97, p10, NTF2.
5. 2.4. deletion and disruption of the Kap104 gene was accomplished by replacement of the Kap104 open reading frame, selectable marker and the diploid strain DFS (38) by integrative transformation [J. Rothstein, Methods Enzymol. 194, 281 (1991) with modifications as described (38)]. Hygromycin-resistant diploid and a dysplastic copy of Kap104 were spored, and totals were dissected on YP plates at 23°C. Although all four spores could be recovered, haploid cells containing a copy of Kap104 failed to germinate at 30°C, were severely impaired in their growth at 23°C, and died when transferred to 30°C.
6. Indirect immunofluorescence was performed after 8-hour incubation of yeast spheinoplasts in 3.7% formaldehyde as described (25), with mouse antisera to purified Kap104 or Nab2p, monoclonal antibodies to Nsr1p and Nop1p, or rabbit antisera to Nop1p. Double immunofluorescence labeling was done with donkey DTAF (dichlorotriazinylaminomethylation-conjugated antiserum to rabbit IgG and donkey Cy3-conjugated antisera to mouse IgG). Composite images were collected as described (25).

31. The protein A gene was integrated into the genomic copy of each Kap gene, yielding functional chimeras as the only copy of each gene as described (38) (these strains are referred to as Kap104-A and Kap104-A1). No growth defects were observed as a result of the presence of either tagged protein, and both subcellular fractionation and immunofluorescence microscopy of each of these constructs reflected the distribution observed with the wild-type proteins.
32. A postnuclear supernatant fraction containing cytosol was isolated as described (29). Cytosol ( 2 ml corresponding to ~30 mg of protein) was dialyzed with 12.0 ml of 20 mM Hepes, 110 mM KOAc, 2 mM MgOCl, 1 mM NaClO, 1 mM CaClO, 1 mM dithiothreitol, 0.1% (w/v) N-womenol (20 ml) plus 50 μl of a protease inhibitor cocktail (28). This mixture was centrifuged at 2000 g for 20 min (low-speed spin) at 4°C, and the supernatant was centrifuged at 260,000 g for high-speed spin (10 min). The resulting supernatant was loaded onto 10 μl of prewashed, packed rabbit IgG-Sepharose (Cappel, Durham, NC) and incubated with gentle agitation at 4°C for at least 1 h. After washing, the bound proteins were eluted with a step gradient of 25 mM, 50 mM, 100 mM, 200 mM, 500 mM, 1 M, 2 M, and 4 M MgOCl in 20 mM Hepes, 0.05% Tween-20 (pH 7.0) in 70% ethanol (11,000 dilution) and precipitated with trichloroacetic acid for analysis by SDS-polyacrylamide gel electrophoresis (PAGE).
34. The protein sequence of p68 and p70 yielded amino acids 396 to 512 of Nab2p and amino acids 245 to 257 of Nab4p or HIP1p (33). Nab2p YPD has the accession number YGL122c; HIP1p is also Nab4p (YPD accession number, YOL234).
35. The Kap104p gene was cloned into PRS14, PRS316, and PRS317 to yield p104-TIP, p104-URA4, and p104-LYS. p104-URA4 was transformed into diploid DFS cells (38), carrying a deletion or disruption of one copy of Kap104 (kap104-ura3-HIS3). These cells were spored, tetrads dissected, and kap104-ura3-HIS3, p104-URA4 haploids were selected. These cells were then transformed with p104-TIP that had been passage through the murine monoclonal antibody 7.1.1.1 Red Transfectants were transferred to 5-fluorocytosine acid to select against p104-TIP at 23°C and then replica-plated at 30 and 37°C to identify temperature-sensitive strains. That means that strain means that either temperature sensitivity or YPD, and were rescued when retransformed with p104-LYS, were selected.
37. N. Bonifac and G. Blobel, personal communication.
40. We thank J. Fernandez and The Rockefeller University-Howard Hughes Medical Institute Biotpolymer Facility for protein sequence analysis; S. Wente for Nup100p and Nup116p expression constructs; J. Kilmartin for antisera to Nup1p and J. Aris for antisera to Nup1p; M. Rixach for many reagents and critically reading the manuscript; U. Nehrbass, F. Kessler, M. Rixach, the other members of the Blobel lab, and W. Rizzoni for helpful discussions; and A. Antinucci de Mauelo and E. Ellison for technical help. J.D.A. was supported by a postdoctoral fellowship from the Medical Research Council of Canada during a portion of this work. 7 August 1996; accepted 24 September 1996.
7 Identification of a Protein Complex that is Required for Nuclear Protein Import and Mediates Docking of Import Substrate to Distinct Nucleoporins
Aurelian Radu; Gunter Blobel; Mary Shannon Moore
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11 Role of the Nuclear Transport Factor p10 in Nuclear Import
Ulf Nehrbass; Günter Blobel
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12 Purification of a Ran-Interacting Protein that is Required for Protein Import into the Nucleus
Mary Shannon Moore; Gunter Blobel
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20 Yeast Srp1p has Homology to Armadillo/Plakoglobin/β-Catenin and Participates in Apparently Multiple Nuclear Functions Including the Maintenance of the Nucleolar Structure
Ryoji Yano; Melanie L. Oakes; Michelle M. Tabb; Masayasu Nomura
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