Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 2008–2011, March 1995
Cell Biology

Previously identified protein of uncertain function is karyopherin α and together with karyopherin β docks import substrate at nuclear pore complexes

(human NPI-1/SRP-1/recombinant human karyopherin α/recombinant rat karyopherin β/ligand blot assay)

JUNONA MOROIANU, GÜNTER BLOBEL*, AND AURELIAN RADU

Laboratory of Cell Biology, The Rockefeller University, Howard Hughes Medical Institute, 1230 York Avenue, New York, NY 10021

Contributed by Günter Blobel, December 1, 1994

ABSTRACT **Previously, we had purified a cytosolic protein complex, termed karyopherin, that functions in docking import substrate at the nuclear envelope in digitonin-permeabilized cells and also had molecularly cloned and sequenced its 97-kDa β subunit. We now report that the karyopherin α subunit is the previously identified protein NPI-1/SRP-1 of hitherto uncertain function. Using purified recombinant karyopherin α or β subunit, we showed that neither karyopherin α nor karyopherin β alone was sufficient for docking of import substrate at the nuclear envelope. Docking occurred only when both subunits were present. Moreover, docking of import substrate by the two recombinant karyopherin subunits was productive, as it led to nuclear internalization of the docked substrate in the presence of additional, previously characterized cytosolic factors. In a binding assay using immobilized karyopherin α and β subunits and import substrate as a ligand, we found that only karyopherin α bound ligand. We suggest that karyopherin β functions as an adaptor that binds both to karyopherin α and to any of a large number of docking sites that are represented by a repetitive peptide motif containing nucleoporins on both the cytoplasmic and nucleoplasmic side of the nuclear pore complex (NPC), bidirectionally ferrying a complex of karyopherin α–substrate across the NPC.**

In an *in vitro* nuclear import system that utilizes a nuclear localization sequence (NLS)-containing import substrate and digitonin-permeabilized cells (retaining intact import-competent nuclei but being largely depleted of cytosolic proteins), import is dependent on exogenous cytosol (1, 2). The required cytosolic factors appear to be highly conserved because an amphibian (*Xenopus* ovary) cytosol can compensate for leaked out cytosol from digitonin-permeabilized mammalian cells (2). Biochemical subfractionation of the *Xenopus* ovary cytosol yielded two fractions (A and B) with distinct activities (2). Fraction A functions in the recognition of the NLS-containing substrate and its docking at the nuclear envelope, whereas fraction B is required for subsequent nuclear internalization of the docked substrate (2). The active components of both *Xenopus* cytosolic subfractions have been purified. The small GTP-binding protein designated "Ran" (3) for *ras*-related nuclear protein and a Ran-interactive protein, p10 (4), represent fraction B's activity, whereas a 9-S complex of three proteins of 54, 56, and 97 kDa represents fraction A's activity (5). Because of its function in docking the import substrate prior to nuclear internalization, the 9-S complex has been named karyopherin, with the 54- and 56-kDa proteins termed its α subunit and the 97-kDa protein named its β subunit (5).

Import factors have also been identified in cytosol fractions of mammalian cells. Sieving of a HeLa cell cytosol to remove proteins of low molecular mass resulted in depletion of the sieved cytosol's import activity, which could be restored by the addition of recombinant human Ran (ref. 6; see also ref. 3). Using bovine erythrocyte cytosol and based on crosslinking by an NLS-containing peptide, two proteins of 54 and 56 kDa were purified and termed the NLS receptor(s) (7). Subsequent purification of a third bovine erythrocyte protein of 97 kDa (p97) showed that both p97 and p54/56 were required for docking of import substrate at the nuclear envelope of digitonin-permeabilized cells (8). Because of their similar functions and molecular mass, it is likely that these three bovine erythrocyte proteins are karyopherin homologs, although this remains to be proven.

A karyopherin homolog was also isolated from a rat liver cytosolic subfraction A by a single-step affinity procedure using the recombinant immobilized nuclear pore complex (NPC) protein called nucleoporin Nup98 (5). One of the eluted proteins of 97-kDa was molecularly cloned and sequenced (5). Partial protein sequencing of the *Xenopus* karyopherin β subunit showed it to be the homolog of the molecularly cloned and sequenced rat p97 (5).

In this paper we report the characterization of the α subunit of mammalian karyopherin. We show that it is a previously identified mammalian protein of hitherto uncertain function termed human SRP-1 (9) or NPI-1 (10). Because of its near 50% identity with yeast SRP-1 (11), human NPI-1/SRP-1 was proposed (9, 10) to be the mammalian homolog of yeast SRP-1. Using recombinant human karyopherin α and recombinant rat karyopherin β, we show that both karyopherin subunits are required for efficient docking of import substrate at the nuclear envelope of digitonin-permeabilized cells and for its subsequent *Xenopus* fraction B-mediated nuclear internalization.

MATERIALS AND METHODS

The nuclear import assay using digitonin-permeabilized BRL (buffalo rat liver) cells was as described (2, 3, 5). Cells were permeabilized with digitonin under two conditions as specified in the figure legends: either as previously described (2, 3) with 35 μg of digitonin per ml on ice or with 50 μg of digitonin per ml at room temperature (8) for 5 min in each case.

The preparation of rat liver cytosolic subfraction A was as described (5).

A HeLa cell cytosol was prepared as described (6).

Purified recombinant human SRP-1 lacking 49 N-terminal residues and being His_6-tagged at its C terminus was a gift of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: NLS, nuclear localization sequence; HSA, human serum albumin; NPC, nuclear pore complex; Ran, *ras*-related nuclear protein; RAG-1, recombination-activating gene 1.

*To whom reprint requests should be addressed.

Patricia Cortes (Rockefeller University). Although this protein is a derivative of a full-length karyopherin α, it is referred to as karyopherin α.

Purification of Recombinant Karyopherin β. A DNA segment coding for rat karyopherin β (minus 12 amino acid residues from the N-terminal end) (5) was obtained by PCR with a λgt10 clone as template and primers containing *Sac* I and *Xho* I restriction sites. The PCR product was digested and ligated in the His_6-tag vector pET-21b(+) (Novagen). The expression of protein was induced with 0.1 mM isopropyl β-D-thiogalactoside for 3 hr in the *Escherichia coli* BL21(DE3) strain. The cells were harvested and resuspended in buffer A (20 mM Hepes, pH 7.3/110 mM KOAc/2 mM $Mg(OAc)_2$/1 mM EGTA) supplemented with 2 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg each of pepstatin, leupeptin, and aprotinin (Boehringer Mannheim) per ml. The cells were lysed at 1500 psi (1 psi = 6.89 kPa) in a French pressure cell (SLM Aminco, Urbana, IL), and the insoluble material was pelleted by centrifugation at 128,000 × *g* for 20 min at 4°C. The supernatant was supplemented with 0.3 M NaCl, 5 mM ATP, 10 mM 2-mercaptoethanol, 5 mM imidazole, and 20% (vol/vol) glycerol and was applied on a Ni-nitrilo-triacetic acid column (Qiagen, Chatsworth, CA) equilibrated with buffer A containing the same ingredients. After 1 hr of incubation at room temperature, the column was washed with the same buffer containing 40 mM imidazole and eluted with 1 M imidazole in the same buffer (pH 7.3); the eluate was dialyzed at 4°C against buffer A containing 2 mM dithiothreitol. The supernatant of a final centrifugation at 350,000 × *g* for 15 min at 4°C was used for the import assays.

Immunodepletion. Two-hundred and fifty microliters of protein A-conjugated agarose (Sigma) was incubated with 500 μl of anti-NPI-1 antiserum (10) for 1 hr at 4°C. After extensive washing with buffer A, the retained IgG was crosslinked to protein A-agarose with dimethylpimelimidate as described by Harlow and Lane (12). After washing first with 0.1 M glycine hydrochloride (pH 3.0) and then with buffer A, we incubated 50 μl of IgG-protein A-agarose for 1 hr at 4°C with either 100 μl of HeLa cell cytosol or 100 μl of rat liver cytosolic subfraction A to yield immunodepleted fractions.

RESULTS

Human NPI-1/SRP-1 Is the Karyopherin α Subunit. SRP-1 has first been identified in yeast as a suppressor of a temperature-sensitive mutation in RNA polymerase I (11). As immunofluorescence microscopy yielded punctate staining in the nuclear periphery typical for nucleoporins (a collective term for NPC proteins) it was suggested that SRP is a nucleoporin (11). This conclusion was further supported by the demonstration of genetic and physical interactions between yeast SRP-1 and the yeast nucleoporins Nup1 and Nup2 (13). Nevertheless, even if it were a structural nucleoporin (or a nucleoporin-associated protein), the function of SRP-1 remained unclear, in particular its relationship to RNA polymerase I. However, besides a possible interaction with RNA polymerase I, recent analysis of various temperature-sensitive mutant alleles of *srp1* has revealed pleiotropic effects on several nuclear processes, such as RNA transcription, nuclear division, and nucleolar structure (14). To explain these pleiotropic effects of *srp1* mutations, it was suggested that SRP-1 may serve some general karyoskeletal function or may effect transport across the NPC (14). However, no defect in protein import could be detected with the *srp1* allele, arguing against the idea that the primary function of SRP-1 is in nuclear protein import (13).

The putative mammalian homologs of yeast SRP-1 were all isolated by the use of the yeast two-hybrid protein interaction system with various nuclear proteins as baits (9, 10). Again, it was suggested that the mammalian SRP-1 homologs serve some karyoskeletal function (9, 10) or a nucleocytoplasmic transport function (10), or both.

To determine whether a mammalian SRP-1 homolog is one of the cytosolic factors required for import of a model substrate into digitonin-permeabilized cells, we used an immunodepletion approach. Antibodies against the human SRP homolog NPI-1 (10) were immobilized and incubated with HeLa cell cytosol or with a karyopherin-enriched rat liver cytosol subfraction A. This immunodepletion resulted in a dramatic loss of import activity from either HeLa cell cytosol (Fig. 1*A*) or from rat liver cytosolic subfraction A (Fig. 1*B*). That the immunodepletion of the human SRP homolog NPI-1 from either HeLa cell cytosol (not shown) or rat liver cytosolic subfraction A was indeed complete was shown by Western blot analysis (Fig. 1*C*).

As the rat liver cytosolic subfraction A's function in protein import is the karyopherin-mediated docking of import substrate at the nuclear envelope (5) and as depletion of NPI-1 resulted in loss of this activity (Fig. 1 *B* and *C*), it was likely that NPI-1 was either one of the karyopherin subunits itself (from the similarity of molecular mass, it would have to be karyopherin α) or a karyopherin-associated protein.

To test whether NPI-1 is indeed the karyopherin α subunit, we took advantage of the fact that cDNAs for human NPI-1/SRP-1 (9, 10)—i.e., the putative karyopherin α, and karyopherin β (5)—are available. Hence, after expression of appropriate cDNA constructs in *E. coli*, either subunit should be obtainable in sufficient amounts and purity and, if active, should allow testing for function separately or together. Fig. 2 shows a purified recombinant His_6-tagged human SRP-1 (containing all but 49 N-terminal residues (see *Materials and Methods*) (lane 1) and a purified recombinant His_6-tagged rat

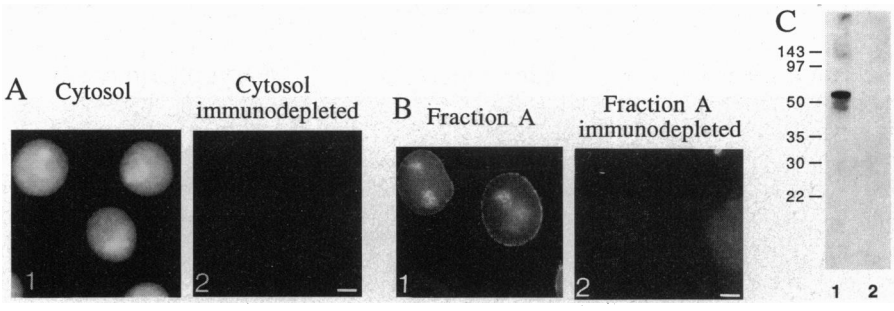

FIG. 1. Immunodepletion of nuclear import activity present in cytosol or a cytosolic subfraction A by immobilized anti-NPI-1 antibodies. (*A* and *B*) Digitonin-permeabilized cells (5 min on ice) were incubated with NLS-conjugated human serum albumin (HSA) in the presence either of HeLa cell cytosol (5 mg of protein per ml) before (*A1*) or after (*A2*) immunodepletion or of a rat liver cytosolic subfraction A (1 mg of protein per ml) before (*B1*) or after (*B2*) immunodepletion. (Bar = 5 μm.) (*C*) Proteins of the rat liver cytosolic subfraction A before (lane 1) and after (lane 2) immunodepletion were separated by SDS/PAGE, transferred to nitrocellulose, and probed with anti-NPI-1 antibodies. Detection was by ^{125}I-labeled protein A and subsequent autoradiography.

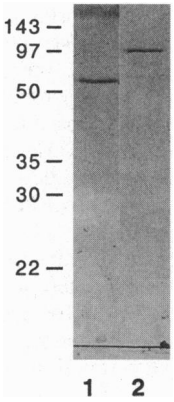

FIG. 2. Analysis of purified recombinant karyopherin α (lane 1) and β (lane 2) subunits by SDS/PAGE and subsequent staining with Coomassie blue.

karyopherin β subunit (lane 2) after analysis by SDS/PAGE and staining with Coomassie blue.

Recombinant α and β Karyopherin Function in Docking and Import. To test for docking of import substrate at the nuclear envelope of digitonin-permeabilized cells, the import reaction was carried out at near 0°C (Fig. 3). When rat liver cytosol subfraction A was present in the reaction, there was docking at the nuclear envelope (Fig. 3*D*). There was no docking of import substrate in the presence of either recombinant proteins alone (Fig. 3 *A* and *B*, respectively), but there was docking when both recombinant proteins were present (Fig. 3*C*). These data indicated that the two recombinant proteins are indeed active. More importantly, the data showed that neither karyopherin α nor karyopherin β were by themselves sufficient to obtain docking at the nuclear envelope. Docking occurred only in the presence of both subunits. Adam and Adam (8) have previously reported similar results with

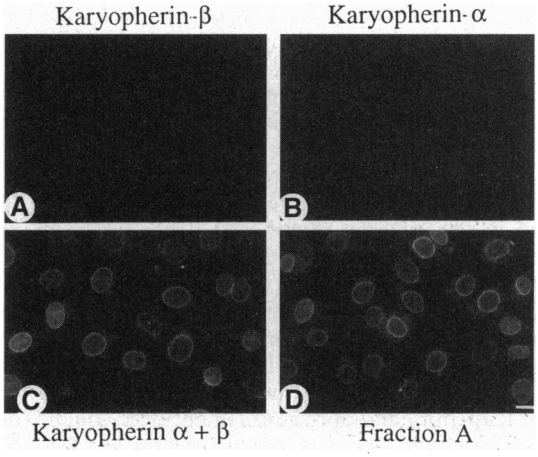

FIG. 3. Docking of import substrate at the nuclear envelope requires the presence of both karyopherin α and β subunits. Digitonin-permeabilized cells (5 min at room temperature) were incubated for 30 min on ice with import substrate in the presence of 50 nM of karyopherin β (*A*); or of 50 nM of karyopherin α (*B*); or of both karyopherins, each at 50 nM (*C*); or of rat liver cytosolic subfraction A (*D*). (Bar = 10 μm.)

their purified bovine erythrocyte proteins: there was docking only when both the p54/p56 and p97 were present.

To test whether the recombinant karyopherin α and β subunits would also allow nuclear internalization of import substrate, the import reaction was carried out at room temperature and at saturating concentrations of *Xenopus* fraction B (Fig. 4). As expected, there was little import when either karyopherin α or β alone was present in the reaction mixture (Fig. 4 *A*, *D1*, and *D2*). However, at a constant concentration of karyopherin β, there was increasing import with increasing concentration of karyopherin α (Fig. 4 *B* and *D3*). These data indicate that karyopherin-mediated docking at the nuclear

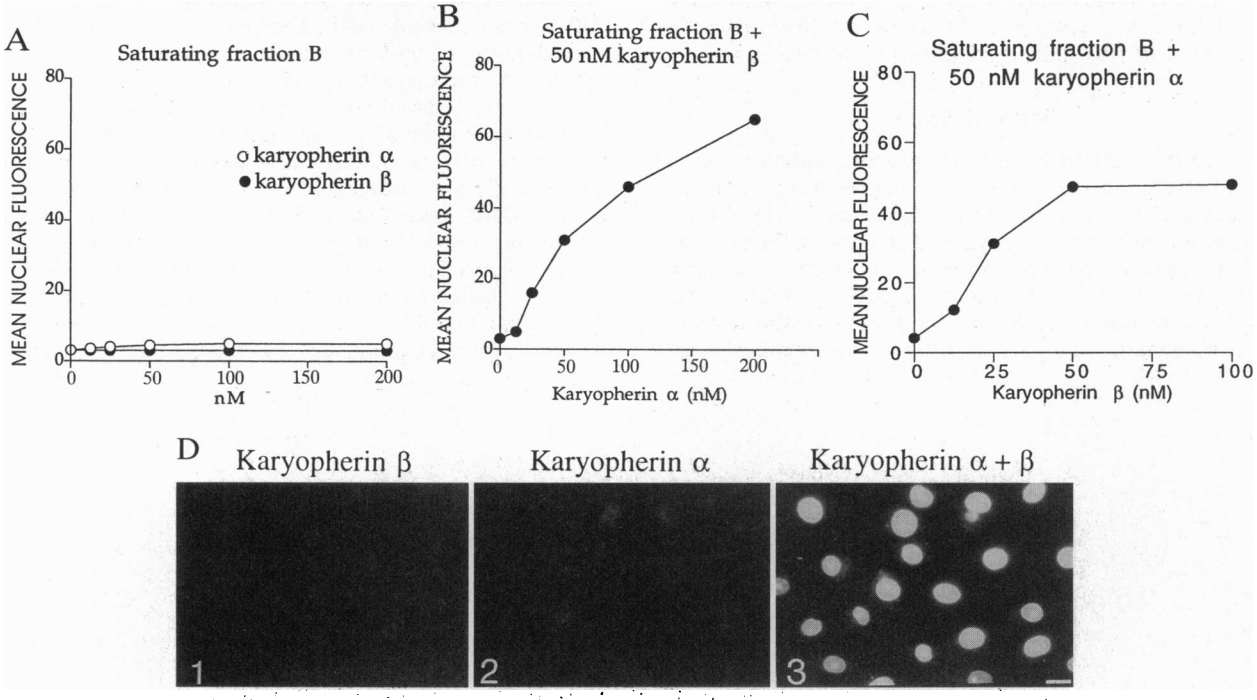

FIG. 4. Nuclear import requires the presence of both karyopherin α and β subunits. (*A–C*) Digitonin-permeabilized cells (5 min at room temperature) were incubated for 30 min at room temperature in the presence of saturating amounts of *Xenopus* fraction B (4, 5) and either karyopherin α or β alone (*A*), or of 50 nM karyopherin β and various concentrations of karyopherin α (*B*), or of 50 nM karyopherin α and various concentrations of karyopherin β (*C*). An average of 30 nuclei were scanned per point. (*D*) Corresponding immunofluorescence images at some end points are shown with 50 nM karyopherin β (*D1*; compare with *A*), with 200 nM karyopherin α (*D2*; compare with *A*), and with 50 nM karyopherin β and 200 nM karyopherin α (*D3*; compare with *B*). (Bar = 10 μm.)

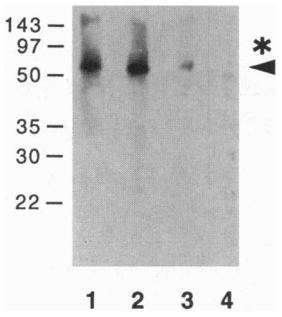

FIG. 5. Probing of immobilized karyopherin α (lanes 1–3) and karyopherin β (lane 4) with import substrate (NLS-HSA). Karyopherin α (100 ng) or β (200 ng) was subjected to SDS/PAGE and transferred to nitrocellulose. The strips were then incubated with NLS-HSA alone (lanes 1 and 4) or in the presence of a 100 M excess of wild-type NLS (2) (lane 3) or mutant NLS (2) (lane 2). Ligand binding was detected by incubation with anti-HSA antibodies (Sigma), ^{125}I-labeled protein A, and autoradiography (5).

envelope is productive for subsequent fraction B-mediated nuclear internalization. Interestingly, there was still no saturation of import activity with a 4-fold molar excess of karyopherin α relative to karyopherin β (Fig. 4*B*; see *Discussion*). We also carried out a titration of karyopherin β subunits at a constant concentration of karyopherin α (Fig. 4*C*). In this case, saturation of import activity was reached with an equimolar concentration of karyopherin β (Fig. 4*C*; see *Discussion*).

Immobilized Recombinant Karyopherin α but Not β Binds Import Substrate. Recombinant karyopherin α or β was immobilized on nitrocellulose and probed with import substrate. Bound substrate was detected with antibodies and ^{125}I-labeled staphylococcal protein A (Fig. 5). Only karyopherin α (lane 1) but not β (lane 4) bound NLS-HSA. Binding to karyopherin α appeared to be specific as it could be blocked by competition with wild-type NLS peptide (lane 3) but not with mutant NLS peptide (lane 2). These data suggest that karyopherin α but not β functions in recognition of the NLS. However, it remains possible that karyopherin β was not sufficiently renatured to bind NLS or that it binds NLS only in combination with karyopherin α.

DISCUSSION

Our data here demonstrate that the human protein NPI-1/SRP-1, previously identified via a yeast two-hybrid system using unrelated nuclear proteins as bait, but of hitherto uncertain function (9, 10), is the α subunit of karyopherin. We show here that karyopherin α, together with the previously identified karyopherin β (5), functions in docking an NLS-containing substrate at the nuclear envelope of digitonin-permeabilized cells. Docking was obtained with recombinant human karyopherin α and recombinant rat karyopherin β and was productive because the docked substrate could be imported into the nucleus in the presence of previously characterized factors (3, 4) present in the *Xenopus* cytosolic subfraction B. There was no docking (or import) when one of the karyopherin subunits was omitted.

The α and β subunits of karyopherin are likely to carry out different tasks. In blotting experiments using immobilized karyopherin α and β subunits and an NLS-containing substrate as ligand, we found that only α but not β bound ligand. This suggests that karyopherin α functions as a NLS receptor. This would explain why two entirely different nuclear proteins, the influenza virus nucleoprotein NP and the recombinase RAG-1 (for recombination-activating gene), both containing NLS, interact with karyopherin α in the yeast two-hybrid system. In the case of RAG-1, the karyopherin α-interactive domain has, in fact, been mapped to the N-terminal region of RAG-1 (9) that contains a putative NLS.

There is at least one other human protein, Rch-1, that is 44% identical to karyopherin α and that has also been isolated via a yeast two-hybrid system using RAG-1 as a bait (15). Thus, it is possible that there is a family of karyopherin α subunits with overlapping specificities for a spectrum of degenerate NLSs. The *Xenopus* (5) and bovine erythrocyte (7) p54 and p56 could represent related α subunits. Each of the related karyopherin α subunits may combine with one common karyopherin β subunit in a stoichiometric complex.

The function of karyopherin β remains to be determined. It could function as an adaptor that binds both to nucleoporins containing repetitive peptide motifs (5) and to an import substrate–karyopherin α complex. Preliminary data indicate that karyopherin α but not β enters the nucleus (J.M. and G.B., unpublished data). This suggests a cycle of α- and β-subunit association and dissociation. Consistent with only karyopherin α entering the nucleus and karyopherin β not doing so is our finding that, at a constant concentration of karyopherin β, import of NLS-containing substrate into the nucleus is not saturated at a 4-fold molar excess of karyopherin α (Fig. 4*B*), whereas at a constant concentration of karyopherin α, nuclear import is saturated by equimolar concentrations of karyopherin β (Fig. 4*C*).

Our finding here that recombinant karyopherin α and β subunits are both functionally active should now greatly facilitate a detailed analysis of their respective roles in nuclear import.

Note Added in Proof. A *Xenopus* homolog of yeast SRP-1 has recently been reported to be essential for protein import into nuclei (16).

We are greatly indebted to Robert E. O'Neill and Peter Palese for their generous gift of antibodies to NPI-1 and to Patricia Cortes for her generous gift of purified recombinant human SRP-1. We thank Mary Moore and Philip Bernstein for numerous discussions and suggestions.

1. Adam, S. A., Sterne-Marr, R. & Gerace, L. (1990) *J. Cell Biol.* **111,** 807–816.
2. Moore, M. S. & Blobel, G. (1992) *Cell* **69,** 939–950.
3. Moore, M. S. & Blobel, G. (1993) *Nature (London)* **365,** 661–663.
4. Moore, M. S. & Blobel, G. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 10212–10216.
5. Radu, A., Blobel, G. & Moore, M. S. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 1769–1773.
6. Melchior, F. B., Paschal, J., Evans, J. & Gerace, L. (1993) *J. Cell Biol.* **123,** 1649–1659.
7. Adam, S. A. & Gerace, L. (1991) *Cell* **66,** 837–847.
8. Adam, E. J. H. & Adam, S. A. (1994) *J. Cell Biol.* **125,** 547–555.
9. Cortes, P., Ye, Z. S. & Baltimore, D. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 7633–7637.
10. O'Neill, R. E. & Palese, P. (1994) *Virology* **206,** 116–125.
11. Yano, R., Oakes, M., Yamagishi, M., Dodd, J. A. & Nomura, M. (1992) *Mol. Cell. Biol.* **12,** 5640–5651.
12. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), p. 725.
13. Belanger, K. D., Kenna, M. A., Wei, S. & Davis, L. I. (1994) *J. Cell Biol.* **126,** 619–630.
14. Yano, R., Oakes, M. L., Tabb, M. M. & Nomura, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6880–6884.
15. Cuomo, C. A., Kirch, S. A., Gyuris, J., Brent, R. & Oettinger, M. A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6156–6560.
16. Görlich, D., Prehn, S., Laskey, R. A. & Hartmann, E. (1994) *Cell* **79,** 767–778.