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Isolation of a Protein That Is Essential for the First Step of Nuclear Protein Import

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Summary

We have purified a cytosolic protein from Xenopus eggs that is essential for selective protein import into the cell nucleus. The purified protein, named importin, promotes signal-dependent binding of karyophilic proteins to the nuclear envelope. We have cloned, sequenced, and expressed a corresponding cDNA. Importin shows 44% sequence identity with SRP1p, a protein associated with the yeast nuclear pore complex. Complete, signal-dependent import into HeLa nuclei can be reconstituted by combining importin purified from Xenopus eggs or expressed in E. coli with Ran/TC4. Evidence for additional stimulatory factors is provided.

Introduction

Selective import of proteins into the cell nucleus occurs in two steps, both of which require the presence of a nuclear localization sequence (NLS; Newmeyer and Forbes, 1988; Richardson et al., 1988; Akey and Goldfarb, 1989). The first step is binding to the cytoplasmic surface of the nuclear pore complex. It does not require ATP or GTP. The second step is the energy-dependent translocation through the nuclear pore complex. Protein import into the nucleus has been reviewed extensively (Garcia-Bustos et al., 1991; Silver, 1991; Yamasaki and Lanford, 1992; Fabre and Hurt, 1994; Moore and Blobel, 1994).

Permeabilized cells (Adam et al., 1990) have been powerful tools for investigating nuclear import. The plasma membrane of cultured mammalian cells is selectively solubilized with a low concentration of digitonin, releasing the soluble contents of the cell, but leaving internal membranes such as the nuclear envelope intact. A fluorescent import probe can readily enter the leaky plasma membrane, and its uptake into the nucleus can be followed by fluorescence microscopy. The active import of karyophilic proteins was found to be absolutely dependent on the readdition of cytosol (Adam et al., 1990).

The biochemical approaches applied so far to identify factors involved in nuclear protein import can be classified into two main lines: first, the search for NLS-binding proteins by means of peptide cross-linking, affinity chromatography, or ligand-blotting techniques (for review see Yamasaki and Lanford, 1992), and second, fractionation of cytosol in respect to import activity (Newmeyer and Forbes, 1990; Moore and Blobel, 1992, 1993; Melchior et al., 1993; Adam and Adam, 1994).

The two steps of nuclear import, envelope binding and the subsequent energy-dependent translocation, were found to require different cytosolic fractions, called A and B, respectively (Moore and Blobel, 1992). Only one essential cytosolic component has been identified so far (Moore and Blobel, 1993; Melchior et al., 1993). It is the small GTP-binding protein Ran/TC4 from fraction B (Drivas et al., 1990; Bischoff and Pongstingl, 1991; Ren et al., 1993; Moore and Blobel, 1993; Melchior et al., 1993).

Here, we report the purification from Xenopus eggs, cDNA cloning, and sequencing of a cytosolic factor that is essential for the first step of nuclear protein import. It is a single polypeptide with an apparent molecular mass of 60 kDa on SDS gels. In its chromatographic properties and its effects on nuclear transport, it resembles the crude fraction A described by Moore and Blobel (1992). It mediates binding of karyophilic proteins to the nuclear envelope. Complete transport can be achieved with the purified protein and Ran/TC4 alone, although we present evidence for an additional stimulatory factor(s).

The recombinant 60 kDa protein expressed in bacteria is as active as the frog protein in restoring import activity of cytosol that has been depleted of the 60 kDa protein.

The 60 kDa protein is 64% identical with human Rch1 (Rag cohort 1; Cuomo et al., 1994) and 44% identical with SRP1p (suppressor of temperature-sensitive mutations of RNA polymerase I) in Saccharomyces cerevisiae (Yano et al., 1992). The SRP1 protein has previously been shown to be encoded by an essential gene (Yano et al., 1992, 1994) and to be a constituent of the yeast nuclear pore complex (Yano et al., 1992; Belanger et al., 1994).

Given the fact that the 60 kDa protein is essential for protein import into the cell nucleus, we suggest naming it importin.

Results

Purification of a 60 kDa Protein That Is Essential for Nuclear Protein Import

To identify further cytosolic factors required for nuclear import, we used a nuclear import system based on permeabilized cultured cells (Adam et al., 1990) and nucleoplasmin that had been labeled with fluorescein as a natural import probe.

On starting the fractionation of cytosol, the number of

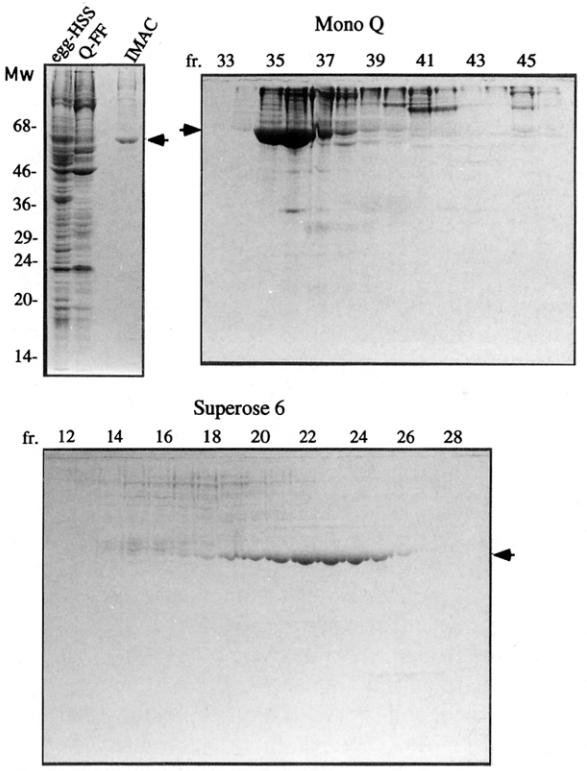

Figure 1. Purification of a 60 kDa Protein, Importin

Protein patterns of fractions at different stages of purification, visualized by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie staining. A high speed supernatant of activated Xenopus eggs (egg-HSS) was applied to Q–Sepharose fast flow at 0.2 M NaCl. The protein fraction eluted with 1 M NaCl (Q-FF) was subjected to immobilized metal ion affinity chromatography (IMAC) on nitrilotriacetic acid–Sepharose charged with Ni^{2+}. IMAC represents the pool of active fractions eluting between 40–100 mM imidazole. Further purification was achieved on Mono Q (200–520 mM NaCl gradient). High activity was found in fractions 35 and 36, while less but still significant activity was found in fraction 37. Mono Q fractions 35–37 were applied to Superose 6. Significant activity was detected in fractions 18–26, and high activity was detected in fractions 21–24. Fraction 24 was used for the experiment shown in Figure 2, and fraction 23 was used for protein sequencing.

essential factors was unknown. To follow the purification of one component, we had to simplify the system. Therefore, bacterially expressed Ran/TC4 (100 μg/ml) was added to all assays, since it is known to be essential (Moore and Blobel, 1993; Melchior et al., 1993). Using Q–Sepharose fast flow (Pharmacia) as the initial fractionation step (see Figure 1), we first identified the most stringent binding conditions that still allowed depletion of an essential component, hoping that under these conditions only one, the most acidic, would bind (see legend to Figure 1). To ask which protein(s) has to be added back to restore activity, we used the Q–flowthrough (supplemented with Ran/TC4) as a depleted cytosol.

The eluate from the Q–Sepharose fast flow column was used for the further purification (see Figure 1). It was subjected to immobilized metal ion affinity chromatography (IMAC) on nickel(II)–nitrilotriacetic acid–Sepharose (Ni–NTA–Sepharose; Qiagen). This turned out to be the crucial purification step, allowing some 100-fold purification of the activity. After further ion exchange chromatography on Mono Q (Pharmacia) and gel filtration on Superose 6 (Pharmacia), a 60 kDa protein was purified to near homogeneity (Figure 1). Trace impurities were only evident in overloaded gels (as in Figure 1), and their pattern was variable in different active fractions and in different variants of the purification protocol. In contrast, we always observed a perfect correlation between the stimulation of import by the fractions and the presence of the 60 kDa band.

After the last column step, approximately 1 mg of purified 60 kDa protein was obtained from 1 g of protein in the starting material, corresponding to an approximately 15% yield as estimated by immunoblotting with antibodies raised against the 60 kDa protein (see below). The yield in terms of activity was in the same range (see Table 1). The abundance of the 60 kDa protein is approximately 0.7% of total protein in the postribosomal supernatant of Xenopus egg extract (this compares with some 1% Ran/TC4 for Xenopus oocyte extract; Moore and Blobel, 1993). The cytosolic concentration of the 60 kDa protein in Xenopus eggs is about 200 μg/ml (3 μM). Its maximum effect on import was reached within the same concentration range of 100–200 μg/ml (see below).

The determined N-terminal sequence of the 60 kDa protein did not match with any known protein in the data base. Given the fact that the 60 kDa protein is necessary and sufficient to restore the protein-import activity of Ni–NTA–Sepharose-depleted cytosol, we suggest naming it importin.

Importin elutes earlier from Superose 6 and Superdex 200 than one would expect for a 60 kDa protein. The apparent molecular mass of purified importin by means of gel filtration is close to 120 kDa, indicating either a very extended conformation or dimerization of this protein. In contrast, in crude cytosol, importin is exclusively found in higher molecular mass forms, ranging from 300 kDa to perhaps 1000 kDa (exclusion limit of Superdex 200; data not shown).

Importin, the 60 kDa Protein, Is Required for the First Step of Nuclear Protein Import

Having a purified protein in hand, we investigated its role in nuclear import (Figure 2). Unfractionated cytosol (at 4 mg/ml protein concentration) gave efficient transport of fluorescein-labeled bovine serum albumin (BSA)–NLS conjugate in the presence of an energy-regenerating system. In the absence of ATP (depleted by apyrase), transport was completely abolished. Instead, accumulation at the nuclear envelope was observed as reported previously (Newmeyer and Forbes, 1988; Richardson et al., 1988; Moore and Blobel, 1992).

Ran/TC4 alone had no effect whether or not an energy-regenerating system was present.

When both importin and Ran/TC4 were present at saturating concentrations (100 μg/ml each), the effects resembled those with unfractionated cytosol. In the presence of ATP, import occurred at a rate comparable with the cytosol

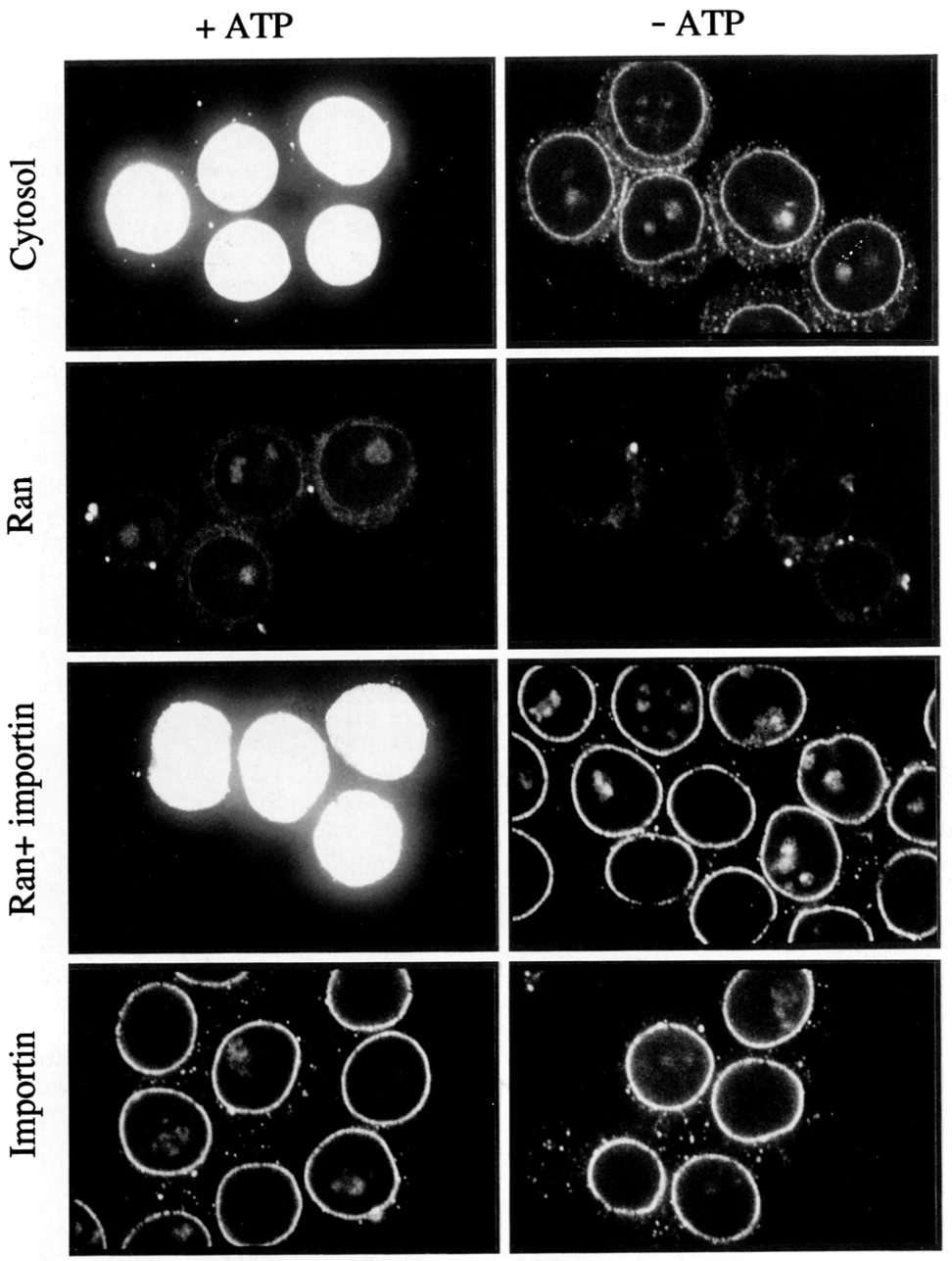

Figure 2. Effects of Importin on Nuclear Import

Confocal images after import reactions of fluorescein-labeled BSA–NLS conjugates into nuclei of permeabilized HeLa cells for 1 hr at 23°C.

Plus ATP indicates the presence of an energy-regenerating system (ATP plus GTP plus creatine phosphate and creatine kinase); minus ATP indicates the absence of the energy-regenerating system and inclusion of 50 U/ml apyrase; cytosol indicates the presence of a postribosomal supernatant of an activated Xenopus egg extract at a protein concentration of 4 mg/ml. Importin (Superose 6 fraction 24; see Figure 1) and recombinant Ran/TC4 were added at 100 μg/ml when indicated.

All panels were scanned and photographed under identical conditions. Therefore, the panels plus ATP/cytosol and plus ATP/Ran plus importin are overexposed.

control. In the absence of ATP, intense staining of the nuclear envelope was observed. When importin was the only cytosolic protein present, nuclear envelope binding was observed, but no import, whether or not the energy-regenerating system was present (Figure 2).

Envelope Binding Mediated by Importin Is Specific for NLSs

Next, we tested whether the envelope binding mediated by importin is specific. As seen in Figure 3, this phenomenon is crucially dependent on the addition of importin and

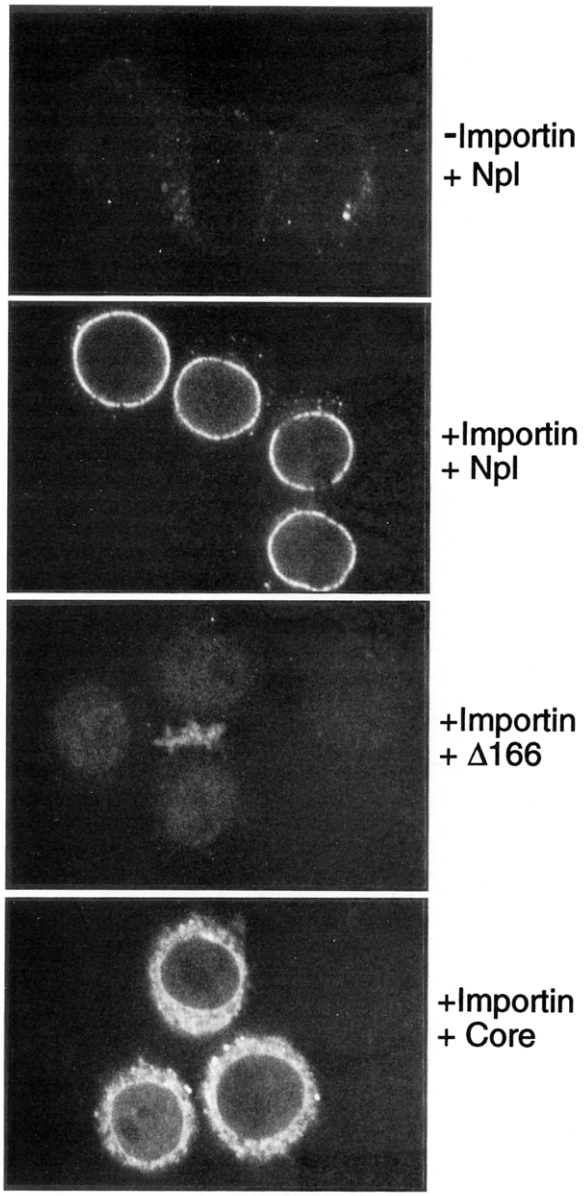

Figure 3. Envelope Binding of Fluorescein-Labeled Nucleoplasmin Depends Both on Importin and on an Intact NLS

Confocal scanning images after standard incubations with nuclei of permeabilized HeLa cells as indicated.

Minus or plus importin indicates the absence or presence of 100 μg/ml importin. Npl indicates 100 μg/ml fluorescent-labeled full-length nucleoplasmin. Δ166 is a fluorescent-labeled nucleoplasmin truncation consisting of the first 166 amino acid, thus lacking the downstream basic cluster of the NLS. Core indicates fluorescent-labeled nucleoplasmin core (first 149 amino acids), lacking the entire NLS. Note that the nucleoplasmin core stains the cytoplasmic remnants intensely.

All panels were scanned and photographed under identical conditions.

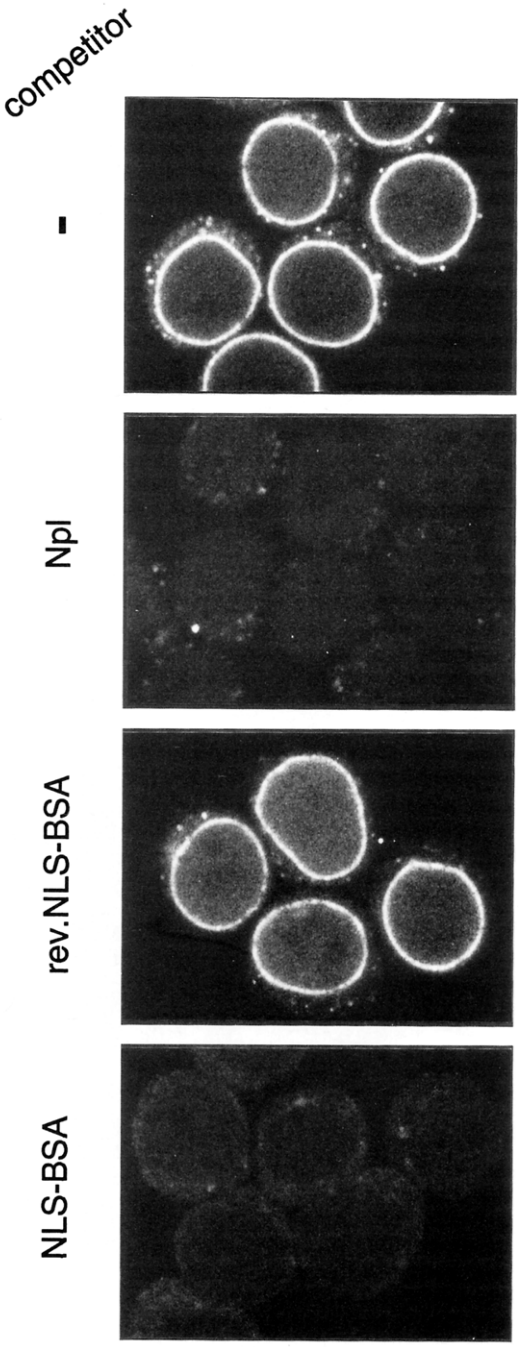

Figure 4. Functional NLSs Compete with Nucleoplasmin for Importin-Mediated Envelope Binding, Whereas Nonfunctional NLSs Do Not Compete

Confocal scanning images after import reactions using nuclei of permeabilized HeLa cells in the presence of 100 μg/ml importin, 50 μg/ml fluorescein-labeled full-length nucleoplasmin, and a 50-fold excess of unlabeled competitors as indicated. Npl indicates nucleoplasmin; rev.NLS–BSA indicates a (nonfunctional) reversed NLS peptide from SV40 large T antigen coupled to BSA; NLS–BSA indicates functional NLS peptides conjugated to BSA. All images were obtained under identical conditions.

the presence of an NLS within the import substrate. Envelope binding was not observed when truncated nucleoplasmin consisting of the first 166 amino acids (Δ166) was used. This lacks the downstream lysine cluster of the bipartite NLS (Robbins et al., 1991). Similarly, the nucleoplasmin core, lacking the entire NLS, failed to bind to the envelope. Instead, a bright background binding to the cytoplasmic remnants in the permeabilized cells was observed.

Competition between nucleoplasmin and BSA–T antigen–NLS conjugates for import into the nucleus has been

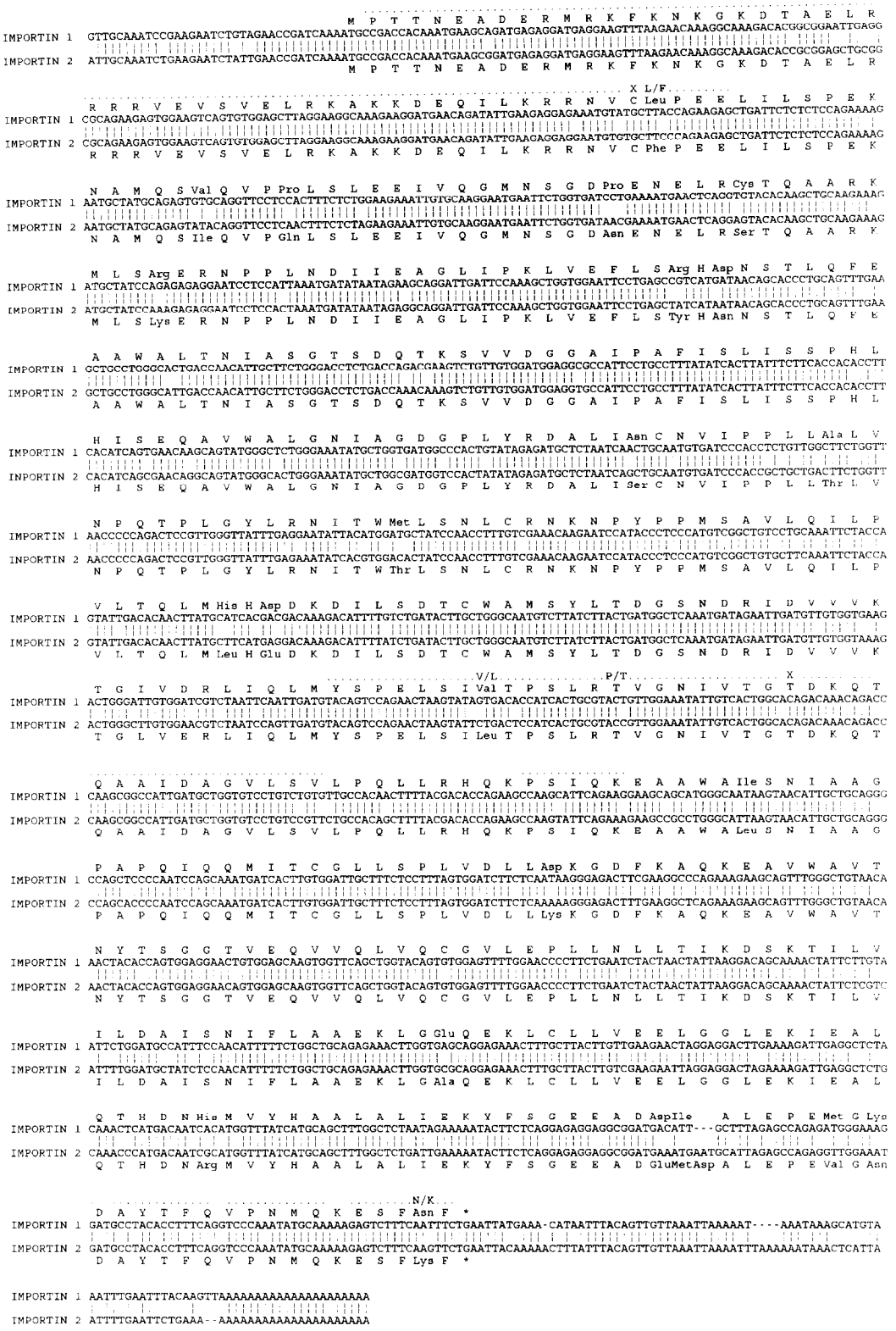

Figure 5. Sequence of Importin from Xenopus

A cDNA library from Xenopus ovary was screened with oligonucleotides corresponding to importin partial protein sequence. Several full-length clones were found, some of them varying in a few amino acid positions. The figure shows the alignment the cDNA sequences and the translated amino acid sequences of forms 1 and 2 of Xenopus importin. Amino acids are written in single letter code; three letter code was used in positions that are not identical in forms 1 and 2. Residues identified by direct peptide sequencing are indicated by a dotted line above; positions differing from the deduced amino acid sequence of either importin forms 1 or 2 are indicated by single letter code in this line.

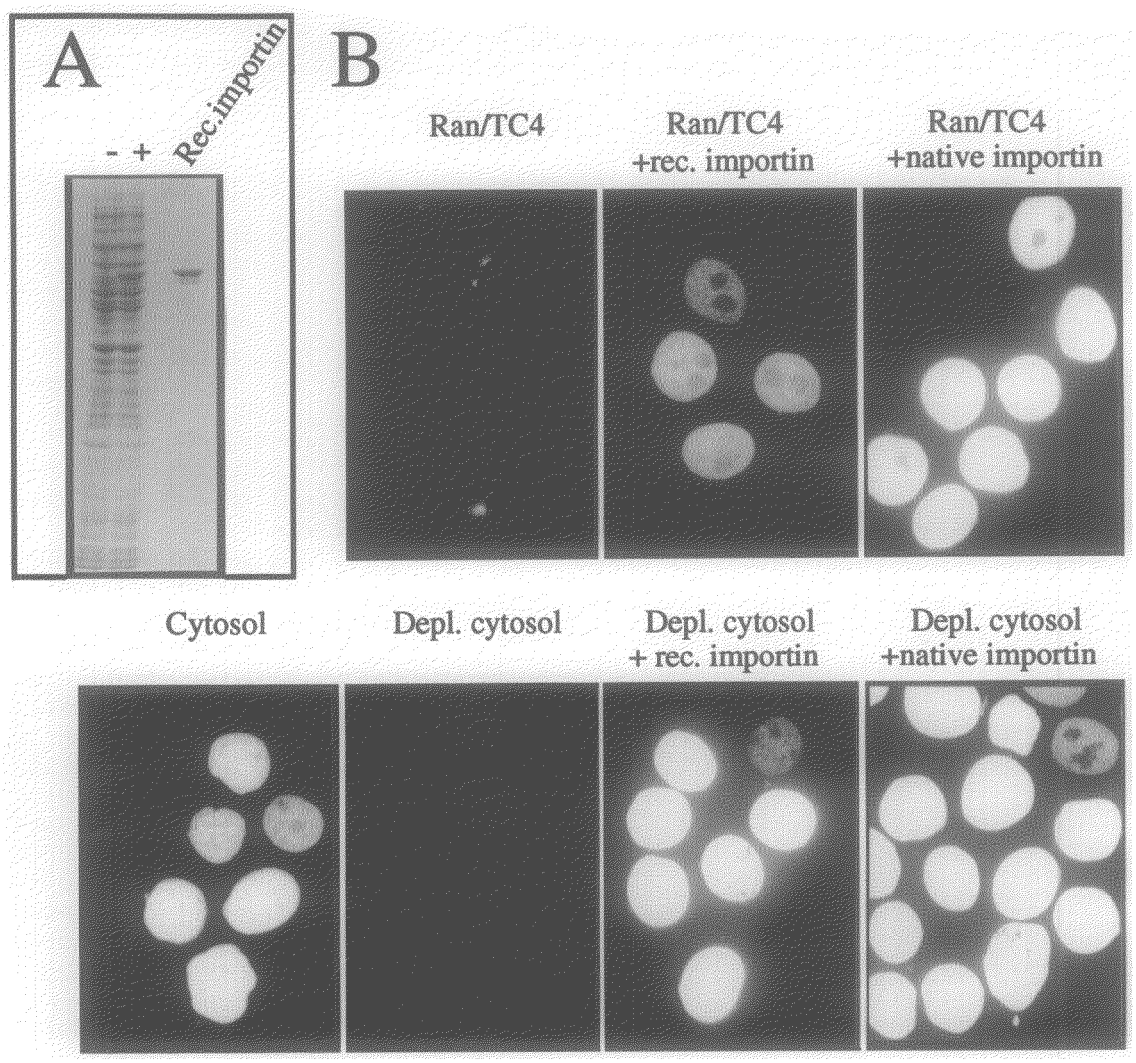

Figure 6. Expressed Recombinant Importin Is Active

(A) Recombinant expression of importin. Minus and plus indicate the total protein pattern after 3 hr induction with IPTG of E. coli strain BL21/Rep4 transformed either with pQE70 vector alone or with pQE70 that contained the cDNA insert coding for importin form 1. Rec.importin indicates the expressed recombinant importin after purification on Ni–NTA–Sepharose.

(B) Confocal sections through nuclei of permeabilized HeLa cells after import reactions with 50 μg/ml fluorescein-labeled nucleoplasmin and additions as indicated. Ran/TC4, 100 μg/ml; recombinant importin, 100 μg/ml; native importin, 100 μg/ml, importin purified from Xenopus eggs; depleted cytosol, Xenopus egg extract depleted of importin by passing it through Ni–NTA–Sepharose and used at a final protein concentration of 4 mg/ml; cytosol, Xenopus egg extract mock depleted by passing it through Sepharose CL-6B instead of Ni–NTA–Sepharose. Importin content of depleted cytosol was approximately 8% of the mock depleted.

All panels were scanned and photographed under identical conditions. Therefore, fluorescence intensity of nuclei in panels labeled depleted cytosol plus importin is above the linear range, whereas that of panel Ran/TC4 is below the detection limit.

reported previously (Michaud and Goldfarb, 1991). Therefore, we tested whether there is a similar competition between import substrates for importin-mediated binding to the nuclear envelope (Figure 4). In the absence of a competitor (actually in the presence of nucleoplasmin core; see Experimental Procedures), fluorescein-labeled nucleoplasmin bound to the nuclear envelope showing bright rings. In the presence of a 50-fold excess of unlabeled nucleoplasmin, envelope binding was completely abolished. Addition of the same excess of a BSA conjugate with reverse sequence NLS peptides from SV40 large T antigen did not diminish envelope binding of nucleoplasmin. As reported previously, the BSA–reverse NLS is not functional in nuclear import (Adam et al., 1989). In contrast, the functional NLS–BSA conjugate competed strongly and abolished envelope binding completely (Figure 4).

Thus, importin is essential for generating an NLS-binding site at the nuclear envelope. This binding site is specific for NLSs, and it is able to recognize both the bipartite signal of nucleoplasmin (Figures 3 and 4) and the targeting information in functional BSA–NLS conjugates (Figure 2; also Figure 4).

Molecular Cloning and Primary Structure of Xenopus Importin

We partially sequenced importin and designed degenerate oligonucleotide probes to screen a Xenopus ovary li-

Table 1. Dependence of Nuclear Import Efficiency on Importin Concentration

Additions	Transport
Ran	BG
Ran plus 30 μg/ml recombinant importin	20
Ran plus 100 μg/ml recombinant importin	53
Ran plus 250 μg/ml recombinant importin	69
Ran plus 30 μg/ml native importin	39
Ran plus 100 μg/ml native importin	96
Cytosol (30 μg/ml importin final)	100
Depleted cytosol (2.5 μg/ml importin final)	12
Depleted cytosol plus 30 μg/ml recominant importin	132
Depleted cytosol plus 100 μg/ml recombinant importin	273
Depleted cytosol plus 250 μg/ml recombinant importin	410
Depleted cytosol plus 30 μg/ml native importin	116
Depleted cytosol plus 100 μg/ml native importin	298

Standard import assays into nuclei of permeabilized cells were performed in the presence of 100 μg/ml flourescein-labeled nucleoplasmin, an energy-regenerating system, and additions as indicated. Ran indicates recombinant Ran/TC4 at 100 μg/ml; recombinant importin indicates importin form 1 expressed in E. coli; native importin indicates importin purified from activated Xenopus eggs; cytosol indicates mock-depleted cytosol in 4 mg/ml protein concentration; depleted cytosol indicates cytosol that had been depleted of importin by passing through Ni–NTA–Sepharose and used at 4 mg/ml protein concentration.

Import efficiency was evaluated from confocal images using the NIH Image software. For each sample, the mean fluorescence intensity of at leaset 50 nuclei was averaged. The value for Ran/TC4 alone was set to background (BG), the value for the cytosol control was set to 100.

brary for clones coding for it. Several full-length clones were found and sequenced. Importin mRNA seems to be abundant in oocytes, as the corresponding cDNA clones were found in the library at least at the frequency of actin-coding clones. To our surprise, the clones fell into different classes, deviating from each other in up to 22 amino acid positions out of 528, as seen for importin forms 1 and 2 in Figure 5. So far, four additional forms have been found. Three are closely related to form 1, deviating from it in at least three amino acid positions; one is closely related to form 2, differing from it in at least one position (data not shown). Whether this minimum of six cDNA forms represents more than two genes, or whether they reflect the existence of different alleles of two genes within the tetraploid species Xenopus laevis is unclear at present. Peptide sequencing confirmed that the purified importin is a mixture of various forms; as in positions that differ between forms 1 and 2, two different amino acids were indeed found (see Figure 5). It also seems that different forms of importin migrate at slightly different rates on high resolution SDS gels and elute differently from Ni–NTA–Sepharose probably owing to the difference in histidine content (data not shown; see Figure 5 and below).

Expressed Recombinant Importin Is Active in Import

To prove conclusively that importin is responsible for the activity, we expressed its cDNA (form 1) in Escherichia coli (see Figure 6A). We chose a prokaryotic host because it does not contain a nuclear import apparatus. Although the recombinant protein contains ten additional amino acids (a C-terminal histidine tag), it runs on SDS gels fractionally faster than the native protein isolated from Xenopus eggs (data not shown). A difference in posttranslational modifications is a possible explanation for this effect.

Figure 6B and Table 1 show that a single form of expressed recombinant importin can substitute for native importin isolated from Xenopus eggs in the import assay. As shown before, Ran/TC4 alone did not mediate any transport (see Figure 6B). Ran/TC4 plus saturating amounts of recombinant importin gave transport comparable to unfractionated cytosol at 4 mg/ml protein concentration, but this was only 30%–50% (in separate experiments) of the activity of native importin when tested under identical conditions (see Figure 6B and Table 1). A difference of activity between the recombinant and the native protein was also observed for envelope binding (data not shown). This quantitative difference in activity could be due to the lack of a modification. In that case, the recombinant protein should be able to rescue cytosol from which importin has been depleted. To test this, cytosol was depleted of importin by passing it through a Ni–NTA column (note that this depletion protocol is independent of the one used originally for assaying importin activity). Importin was depleted by about 92% as estimated from quantitative immunoblotting with peptide-specific anti-importin antibodies, S^{35} secondary antibodies, and a phosphoimager. On the autoradiogram, the cytosolic importin was resolved into two closely migrating bands (data not shown). The upper one (probably representing form 1) was more efficiently depleted than the lower one, thus reflecting differences in the histidine content (see Figure 5). Depletion of 92% of the cytosolic importin correlated well with the decrease in transport efficiency by 87% (see Table 1). Addition of a saturating amount of the recombinant protein resulted in a 40-fold restimulation of import efficiency. Equal amounts of recombinant and native protein yielded comparable rates of transport (Table 1 and Figure 6B). Thus, in crude depleted cytosol, the expressed recombinant importin and importin isolated from eggs have comparable activities.

The existence of additional stimulatory factors in the depleted cytosol is also evident (see Table 1 and Figure 6B) as suggested previously (Moore and Blobel, 1993, 1994). They provide a stimulation of transport for recombinant importin by a factor of 5–6 and for native importin by a factor of 2–3. These numbers are still underestimated, since the depleted cytosol contains a high concentration of nuclear proteins competing for import (e.g., nucleoplasmin, N1, histones). The difference in stimulation suggests that the recombinant protein needs still to be folded properly, posttranslationally modified, or both, by factors present in the egg extract in order to gain full activity. The fact that the rate of import mediated by the native importin and Ran/TC4 can be further stimulated indicates that at least one stimulatory activity plays a more direct role.

Structure and Evolutionary Conservation of Importin

Comparison of importin with sequences in the GenBank data base reveals strong homologies to the human Rch1

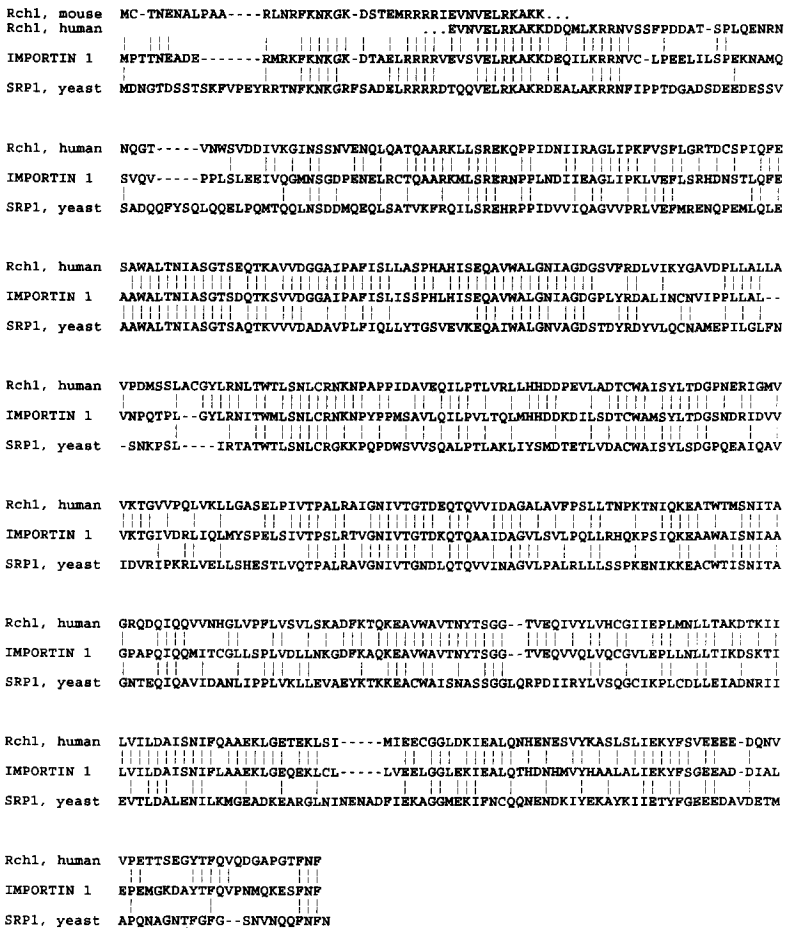

Figure 7. Comparison of Amino Acid Sequences of Xenopus Importin 1, Human and Mouse Rch1, and SRP1p from S. cerevisiae

Multiple alignment of the three proteins was performed with CLUSTALL within the PCGENE program package. The broken vertical lines indicate identical amino acid positions between two sequences. Note that the complete Rch1 sequence had been compiled from two incomplete sequences from mouse and human (Cuomo et al., 1994); however, in the overlapping region, both clones were reported to be identical.

protein (ACC U09559; Cuomo et al., 1994), to the SRP1 protein (ACC M75849) from S. cerevisiae (Yano et al., 1992), and to sequence fragments from rice (ACC D21306, ACC D23592), Arabidopsis thaliana (ACC T14159), maize (ACC T18817), Plasmodium falciparum (ACC T18047), Caenorhabditis elegans (ACC M76111, D28052), mouse (ACC X61876), and human (ACC T08580).

Xenopus importin is 64% identical with human Rch1, a protein found in a yeast two-hybrid system as an interaction partner of Rag1 (Cuomo et al., 1994). Since Rag1 contains an NLS, the interaction found might reflect the recognition of the NLS by Rch1.

SRP1p and importin share 44% identical amino acids. SRP1 was originally found as a suppressor of temperature-sensitive RNA polymerase I mutations (Yano et al., 1992). In contrast with the expected localization in the nucleolus, the protein was found to be a component of the yeast nuclear pore complex (Yano et al., 1992; Belanger et al., 1994). *SRP1* is an essential gene (Yano et al., 1992, 1994).

Xenopus importin, human Rch1, and yeast SRP1p have a characteristic structure in common: the N-terminal and C-terminal domains are very hydrophilic. The N-terminus contains conserved clusters of basic amino acids. These clusters resemble NLS, but preliminary immunofluorescence microscopy data suggest that most importin is found in the cytoplasm (A. D. Mills and D. G., unpublished data).

The middle part of importin/SRP1p consists of an 8-fold repetition of a hydrophobic motif. It was noticed previously that these motifs in SRP1p are similar to repeats (the so-called arm motifs) in other proteins namely in armadillo, β-catenin, plakoglobin, adenomatous polyposis coli (APC), p120, and smgGDS (Peifer et al., 1994; Yano et al., 1994). Interestingly, the GDP/GTP exchange factor smgGDS consists almost exclusively of the arm motif, and it was speculated that the arm domain might mediate G protein interactions in other proteins (Peifer et al., 1994). It remains to be clarified whether importin acts on the GTP cycle of Ran/TC4.

Importin is sensitive to alkylation with N-ethylmaleimide (NEM; data not shown). This is not surprising taking into account that importin form 1 contains eight cysteines; some of them also conserved in SRP1p and Rch1p.

Discussion

We have purified from Xenopus egg extract a 60 kDa protein, importin, that is essential for import of karyophilic proteins into the nucleus. We cloned, sequenced, and expressed this protein in a functional form in E. coli. The transport mediated by importin shows all the characteristic features reported for this process (for reviews see Garcia-Bustos et al., 1991; Silver, 1991; Yamasaki and Lanford,

1992; Fabre and Hurt, 1994; Moore and Blobel, 1994). It is dependent on an intact NLS, an energy-regenerating system, and the small GTP-binding protein Ran/TC4. It can be completely blocked by the lectin wheat germ agglutinin or by the nonhydrolyzable GTP analog GppNp (data not shown). Import mediated by importin occurs in two steps: signal-dependent binding to the nuclear envelope and subsequent translocation. Transport intermediates accumulate at the first stage in the presence of importin and in the absence of either nucleoside triphosphates or Ran/TC4.

Using importin purified from Xenopus eggs and Ran/TC4 as the sole cytosolic proteins, we have reconstituted efficient transport into the nuclei of permeabilized cells (see Figure 2). Furthermore, we have reconstituted transport using bacterially expressed recombinant importin, though it is less efficient than its native counterpart (Figure 6). We suspect that this difference is due to secondary modifications of the protein, but in both cases, transport can be further stimulated by at least one auxiliary factor. This may correspond to factor B2 of Moore and Blobel (1993).

Importin probably represents an active component in the crude fraction A described by Moore and Blobel (1992), as it was purified from a related source; it has similar chromatographic properties and identical effects in the import reaction. Both importin and fraction A are inactivated by ammonium sulfate precipitation or by alkylation with NEM.

In contrast, the relation of importin to nuclear import factor-1 (NIF-1; Newmeyer and Forbes, 1990) is unclear. NIF-1 was defined as the 30%–50% ammonium sulfate cut of Xenopus egg cytosol, a treatment resulting in poor recovery of importin activity. On the other hand, the facts that NIF-1 is sensitive to NEM, it was found in large complexes, and it was implicated in the first step of nuclear import suggest that importin might be an active component of NIF-1.

It is also possible, though not certain, that importin might be related to a 60 kDa protein that was found as the principal cross-linking partner to a wild-type but not to a mutant SV40 NLS peptide in rat liver nuclear envelopes (Adam et al., 1989). However, the authors needed to solubilize the nuclear envelopes with detergent to fully expose the peptide-binding site. Thus, the cross-linking partner might equally be a constituent of the intermembrane space (see Yamasaki and Lanford, 1992) rather than a receptor recognizing proteins coming from the cytosol. In any case, the 55 kDa protein subsequently purified by Adam and Gerace (1991) from bovine red blood cells seems to be different from importin. Thus, the ammonium sulfate precipitation or chromatography on phenyl–Sepharose used in the 55 kDa protein purification would have abolished the activity of importin. Importin is sensitive to alkylation with NEM, whereas p55 is not (Adam and Adam, 1994). Furthermore, the 55 kDa protein of Adam and Gerace (1991) behaves as a monomeric globular protein on sizing columns. In contrast, both purified native and recombinant importin have an apparent molecular mass close to 120 kDa on Superdex 200. Finally, as the nuclear import system of Adam and Gerace (1991) derived from the enucleated mammalian red blood cells is insensitive to GTP analogs (Adam and Adam, 1994), it appears to differ fundamentally from the nuclear import system described here, or by Moore and Blobel (1993) and by Melchior et al. (1993).

Xenopus importin shows 44% amino acid identity to the SRP1 protein from S. cerevisiae. SRP1 is essential for cell viability (Yano et al., 1992, 1994). When SRP1p was depleted from cells by switching off the *Gal7* promotor that controls the only *SRP1* gene in this strain, abolition of transcription, fragmentation of the nucleolus, and defects in both nuclear division and segregation were observed (Yano et al., 1994). These pleiotropic effects of SRP1p depletion as well as the pleiotropic phenotypes of *srp1* and *SRP1^{ts}* mutations (Yano et al., 1994) can be explained as consequences of transport defects.

In the light of 44% amino acid identity between importin and its yeast homolog SRP1p and the reported near identity between mouse and human Rch1 (Cuomo et al., 1994), the 63.7% sequence identity between frog importin and the human Rch1 appears surprisingly low. We found another, shorter human sequence tag with 55% sequence identity with Rch1 (corresponding to positions 234–347) in the data base (ACC T08580). Thus, a family of importin-like proteins might exist in one and the same organism, and the closest mammalian relative to the form of importin in Xenopus eggs might not have been identified yet. The same reservation might apply for yeast. It is tempting to speculate that the different members of the importin family fulfill different functions such as involvement in the import and export of different substrates such as proteins, RNPs, etc. On the other hand, there is the possibility of tissue specificity of different importin forms. The Xenopus egg importin might possibly be a special version to meet the extraordinary demands on nuclear transport during early development.

The actual pore passage of nucleoplasmin-coated gold seems to be preceded by binding to fiber elements extruding at the cytoplasmic face from the nuclear pore complex (Richardson et al., 1988). Importin is essential to generate the NLS-binding site at the nuclear envelope. That suggests that one site of the function of importins is the nuclear pore complex, the associated fibers, or both. In fact, beside its presence in a soluble pool (Yano et al., 1992; Belanger et al., 1994), the yeast homolog SRP1p is physically and functionally associated with the nuclear pore complex in the following ways: it colocalizes with Nup1p immunofluorescence microscopy (Yano et al., 1992), *srp1* genetically interacts with *nup1* (Belanger et al., 1994) and *nup2* (Yano et al., 1994), SRP1p interacts with Nup1p in a two-hybrid system, and SRP1p can be coimmunoprecipitated with Nup1p or Nup2p (Belanger et al., 1994).

Whether importin itself provides the entire NLS-binding site and whether it makes physical contact with the import substrate at all can only be tested by nearest neighbor analysis of import intermediates during nuclear envelope binding. Assays of NLS binding in simplified systems are notoriously error prone, perhaps because of the high density of positive charges in the signal (Yamasaki and Lanford, 1992). At present, it is not certain that the primary NLS recognition step occurs in the cytosol rather than at

the nuclear pore. If NLS recognition does occur in the cytosol, importin is probably the best candidate for this role. However, one does have to consider two more possibilities. First, importin might have to bind first to the nuclear pore before it can bind an NLS. Second, importin might have no NLS-binding site itself, but might be a regulator of an NLS-binding site of the nuclear pore complex.

At least two functional states of the NLS-binding site at the nuclear pore complex must exist: a high affinity state for binding of the import substrate to the pore and a low affinity state to release it either into the nucleoplasm or to downstream components of the import machinery. It remains to be tested whether an import substrate remains bound to the primary NLS recognition site during its entire passage. The affinity of the NLS-binding site has to be regulated (e.g., by Ran/TC4) and might also involve changes in the spatial arrangement of the binding site, e.g., association and dissociation of some part of it. Importin would be an excellent candidate for this loosely bound nuclear pore protein.

Experimental Procedures

Recombinant Expression

The following proteins were expressed in E. coli strain Bl21/Rep4 from cDNA clones using the Qiaexpress system (Qiagen): full-length nucleoplasmin (Dingwall et al., 1987), two nucleoplasmin truncations consisting of the first 166 (Δ166) and the first 149 amino acids (core), Ran/TC4 (Dupree et al., 1992), and "importin" described here. The cDNAs coding for nucleoplasmin, its truncated derivatives, and importin were cloned into the SphI–BamHI sites of pQE70 and were expressed with C-terminal histidine tags. The Ran/TC4 gene was cloned into the SphI–HindIII sites of pQE32, and it was expressed with an N-terminal histidine tag.

Bacteria were grown at 37°C (nucleoplasmin, Δ166, core, Ran/TC4) or at 26°C (importin) in 400 ml 2TY medium to an optical density of 0.9 (600 nm) and were induced with 2 mM IPTG at the same temperature for 6 hr (nucleoplasmin, Δ166, core, Ran/TC4) or 3 hr (importin). PMSF was added to 2 mM, the culture was chilled on ice for 10 min, and cells were pelleted and resuspended in 15 ml of 0.2 M Tris–HCl (pH 8.0), 0.5 M NaCl, 20 μg/ml leupeptin, 10 μg/ml chymostatin, 4 μg/ml elastatinal, 5 mM β-mercaptoethanol. The suspension was subjected to two freeze–thaw cycles, and cells were finally disrupted by ultrasonic sound. The solution was clarified by a 20 min spin at 20,000 × g.

The recombinant proteins were purified from the corresponding homogenates either directly (importin) or from supernatants following ammonium sulfate precipitation (25% saturation for Ran/TC4 or 60% saturation for nucleoplasmin and its derivatives). The protein solutions were adjusted to 10 mM imidazole (pH 7.6) and were loaded onto a 5 ml Ni–NTA–Sepharose column (Qiagen) equilibrated in 500 mM NaCl, 10 mM imidazole (pH 7.0). The column was washed with 30 mM imidazole in the same buffer and finally eluted with a 40 ml gradient of 30–500 mM imidazole. Pooled fractions were adjusted to 20 mM DTT and were dialyzed against several changes of 20 mM HEPES–KOH, 250 mM sucrose, 2 mM DTT. Protein concentration was calculated by UV photometry at 280 and 288 nm using molar extinction coefficients calculated from the tyrosine and tryptophan content (Edelhoch, 1967). Final yield was 40–80 mg/l culture for nucleoplasmin and its derivatives, 25–35 mg/l for Ran/TC4, and 6 mg/l for importin. Since Ran/TC4 tends to aggregate at a high protein concentration, it was diluted to 1 mg/ml in 10% BSA before freezing.

Fluorescein-Labeling of Nucleoplasmin and Its Derivatives

Protein solutions were transferred to 100 mM potassium acetate by gel filtration on a PD10 column (Pharmacia). The concentration of free SH groups was kept in the range of 200–800 μM as determined with Ellmann's reagent (5,5′-dithio-bis(2-nitrobenzoic acid); Sigma). The solution was adjusted to 100 mM MES, 40 mM Tris (pH 7.0), 1.5 M potassium acetate; and fluorescein-5-maleimide (Calbiochem; 10 mg/ml in dimethylformamide) was added in a stochiometric amount to free SH groups. After 30 min, incubation at room temperature the reaction was quenched with 50 mM cysteine (pH 8.0). Free fluorochrome was removed by chromatography on a PD10 column equilibrated in 20 mM HEPES (pH 7.5). The average extent of labeling was usually one fluorescein per nucleoplasmin pentamer as determined from the absorptions at 280 and 490 nm.

BSA–Peptide Conjugates

SMCC-activated BSA was prepared by incubation of BSA at 20 mg/ml in 100 mM HEPES–KOH with a 100-fold molar excess of sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane 1-carboxylate (sulfo-SMCC; Calbiochem) for 1 hr at room temperature. Excess cross-linker was removed by gel filtration on a PD10 column. A 50-fold molar excess of either the SV40 NLS peptide cgggPKKKRKVED or the reverse NLS peptide cgggDEVKRKKKP was added to the SMCC-activated BSA. The pH was readjusted to 7.5, and the reaction was allowed to proceed for 1 hr at 37°C. Noncoupled peptide was separated by gel filtration on a PD10 column equilibrated in 150 mM NaCl. The molar ratio of coupling was 20–30 peptides per BSA molecule as estimated from the electrophoretic mobility.

For fluorescein conjugation, the peptide conjugates were adjusted to 0.2 M $NaHCO_3$, and carboxyfluorescein-N-hydroxysuccinimide ester (Boehringer) dissolved in DMSO was added in a 2:1 molar ratio to BSA. After 30 min incubation at room temperature, the free fluorochrome was removed by gel filtration on a PD10 column and a subsequent precipitation with 60% v/v ethanol. The pellet was then dissolved in 150 mM NaCl.

Preparation of Digitonin Permeabilized HeLa Cells

The method used is based on the one described by Adam et al. (1990) and includes the modification by Leno et al. (1992) to solubilize cells in suspension rather than on coverslips and to freeze permeabilized cells after addition of 5% DMSO.

Digitonin (high purity, Calbiochem) was dissolved to 5% in boiling water. The solution was placed on ice overnight, and the precipitate was removed by centrifugation for 30 min in a microcentrifuge. The supernatant is defined as the 5% stock solution.

HeLa cells were grown in a 500 ml spinner culture. Cells were sedimented at 500 × g for 5 min, and the pellet was resuspended in 100 ml of cell dissociation solution (Sigma C5914) and incubated for 10 min at 37°C. Cells were sedimented and washed three times with cold permeabilization buffer: 50mM HEPES–KOH (pH 7.5), 5 mM magnesium acetate, 2 mM EGTA, 50 mM potassium acetate, 2 mM dithiothreitol, protease inhibitors (10 μg/ml leupeptin, 5 μg/ml chymostatin, 1 μg/ml elastatinal). Cells were finally resuspended in 50 ml of permeabilization buffer. Digitonin was added first to 20 μg/ml from a 100 μg/ml solution made up in permeabilization buffer. The digitonin concentration was then increased by 5 μg/ml increments, and after each addition, the permeabilization of the plasma membrane and the integrity of the nuclear membrane were checked with fluorescein-labeled nucleoplasmin core (inclusion into cells and exclusion from the nucleus). The optimum concentration was usually around 35 μg/ml digitonin. The permeabilization was stopped by addition of BSA to 1% final concentration. The cells were then pelleted, washed three times in permeabilization buffer, and finally resuspended in 10 ml of permeabilization buffer plus 1% BSA plus 5% DMSO and slowly frozen in small aliquots at −80°C.

Import Assay

The method used is similar to that described by Adam et al. (1990). The standard 20 μl assay contained: 20 mM HEPES–KOH, 100 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 250 mM sucrose, 2 mM DTT, 10 mg/ml BSA, energy-regenerating system (unless otherwise indicated) consisting of 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, and 100 μg/ml creatine kinase, 100 μg/ml import probe (fluorescein-labeled nucleoplasmin or its derivatives or BSA–NLS conjugate), and either the fractions or unfractionated cytosol. The reaction was started by addition of some 2×10^4 nuclei (i.e., permeabilized cells) and was usually allowed to proceed for 60 min at room temperature.

As reported before (Moore and Blobel, 1992), there is high nonspecific binding of the fluorescent probe to the cytoplasmic remnants in

the absence of cytosol or at low concentrations of protein fractions. BSA alone does not suppress this nonspecific binding, but the negative control substrates for nuclear import, namely nucleoplasmin core and reverse NLS–BSA conjugate, do suppress it. So routinely 5 mg/ml nucleoplasmin core was added to the assay in experiments with highly purified fractions.

The ionic conditions were found to have only a minor influence on import efficiency, as long as the ionic strength was kept between 70–150 mM and magnesium between 1–5 mM. There is no obvious difference in using HEPES or Tris as a buffer or sodium chloride or potassium acetate as the salt. Therefore, fractions with different ionic compositions were usually not dialyzed before assay, but salt concentration was adjusted to approximately 100 mM final with a compensating buffer. In contrast, imidazole is strongly inhibitory and was removed either by dialysis, gel filtration on G25, or ion exchange chromatography.

To follow the purification of importin, we measured the restimulation of import activity of cytosol that had been depleted of an essential activity by passing it through Q–Sepharose fast flow at 50 mM Tris–HCl (pH 7.4), 200 mM NaCl. To allow a high sample throughout, the import activity was evaluated by fluorescence microscopy of unfixed samples. Import was considered to be significant if the majority of nuclei had accumulated fluorescent nucleoplasmin clearly above the cytoplasmic concentration.

For photographs, import reactions were fixed on ice by dilution to 200 μl with 50 mM HEPES–KOH (pH 7.5), 100 mM potassium acetate, 250 mM sucrose, 1 mM magnesium acetate followed by addition of 200 μl of 8% paraformaldehyde, 100 mM HEPES–KOH (pH 7.5). After 5 min fixation on ice, the nuclei were spun through a 1 ml sucrose cushion (30% w/v in dilution buffer) onto polylysine-coated coverslips (3000 rpm, 6 min). The coverslips were washed briefly in water and mounted on a drop of Vectashield, and the edges of the coverslip were sealed with nail polish. The samples were then scanned with a Bio-Rad MRC600 confocal microsocope using the 60× objective lens under oil. The scanning conditions and exposure times in all subsequent photographic processes were identical for all samples in a given experiment.

To quantitate nuclear import, confocal image files were evaluated with the NIH Image program (version 1.52). For each sample, the mean fluorescence of at least 50 randomly chosen nuclei was averaged.

Purification of Importin

Xenopus eggs were collected, dejellied, and activated as described (Leno and Laskey, 1991). From this point, everything was done on ice or at 4°C. Finally, the eggs were washed in 50 mM HEPES–KOH, 100 mM sucrose, 5 mM DTT, 20 μg/ml leupeptin, 10 μg/ml chymostatin, 2 μg/ml elastatinal and homogenized in 3 vol of the same buffer. The homogenate was spun in a swinging bucket rotor to sediment particles larger than 60S (as calculated from the k-factors for the given rotors). The tubes were punctured to collect the middle layer representing the high speed supernatant (HSS).

Immediately before each column chromatographic step, the material was ultracentrifuged to sediment particles and aggregates larger than 30S.

HSS (260 ml; 1 g of protein) was adjusted to 20 mM Tris–HCl (pH 7.4), 150 mM NaCl and was applied to an 80 ml Q–Sepharose fast flow column (Pharmacia) equilibrated in 50 mM Tris–HCl (pH 7.4), 200 mM NaCl. The column was washed in 120 ml of equilibration buffer, and bound proteins were eluted with 50 mM Tris–HCl (pH 7.4), 1 M NaCl. The protein containing pool (80 ml, 300 mg protein) was adjusted to 5 mM imidazole and loaded onto a 5 ml Ni–NTA–Sepharose column (Qiagen) equilibrated in 50 mM Tris–HCl, 1 M NaCl, 5 mM imidazole. The column was subsequently washed with equilibration buffer and with 50 mM Tris–HCl, 1 M NaCl, 10 mM imidazole until the baseline was reached. Elution was performed with 10 ml steps of 15, 20, 40, 100, and 500 mM imidazole–HCl (pH 7.6) in 50 mM Tris–HCl, 100 mM NaCl, 5% glycerol. Protein containing fractions of each step were pooled and adjusted to 5 mM DTT. Aliquots (100 μl) were dialyzed against 50 mM HEPES–KOH (pH 7.5), 250 mM sucrose, 1 mM DTT and assayed for activity. Highest activity was found in the pools of the 40 and 100 mM imidazole steps.

The 40–100 mM pool (3.7 mg of protein) was diluted with an equal volume of 50 mM Tris–HCl (pH 7.5) and was applied to 1 ml Mono Q column (Pharmacia) equilibrated in 50 mM Tris–HCl (pH 7.4), 200 mM NaCl, 2mM DTT. Proteins were eluted with a 20 ml linear 200–520 mM NaCl gradient in 50 mM Tris–HCl (pH 7.4), 2 mM DTT. Highest activity was found in fractions 35 and 36 (see Figure 1), while less but still significant activity was found in fraction 37 (corresponding to some 300–350 mM NaCl elution position). Fractions 35–37 were pooled (1.5 mg of protein) and loaded onto a Superose 6 column (Pharmacia) equilibrated in 20 mM Tris–HCl (pH 7.4), 100 mM NaCl, 2 mM DTT. Significant activity was found in fractions 18–26 and high activity in fractions 21–24. Final yield was 1 mg of protein corresponding to an overall yield of 15% as estimated from quantitative immunoblotting with anti-importin antibodies. The antibodies were raised in rabbits against the peptide PTTNEADERMc (the N-terminus of importin) coupled to SMCC-activated keyhole limpet hemocyanin.

Size Determination of Importin

A 16/60 Hiload Superdex 200 column in 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 2% glycerol was calibrated (flow rate 1 ml/min) with the following markers: ATP (total volume), cytochrome c (12.5 kDa), ovalbumin (45 kDa), BSA (68 kDa), aldolase from rabbit muscle (158 kDa), catalase from beef liver (240 kDa), ferritin from horse spleen (450 kDa), and plasmid DNA (void volume). The running position of partially purified importin (after IMAC step, see Figure 1) and of expressed recombinant importin was determined by peak detection and was verified by PAGE and Coomassie staining. The running position of importin starting from a crude cytosol was detemined by immunoblotting. Apparent molecular weights were interpolated under the assumption of a semilogarithmic relationship between size and retention time.

Peptide Sequencing and Molecular Cloning

Purified importin was sequenced from its N-terminus and after cleavage with cyanogen bromide or Lys-C protease (Boehringer). Degenerate oligonucleotide probes were designed that correspond to the amino acid sequences MPTTNEA (atg ccn acn acn aa(t/c) ga(a/g) gc) and AKKDEQI (at(t/c) tg(t/c) tc(a/g) tc(t/c) tt(t/c) tt(t/c) ttn gc). Oligonucleotide probes were end-labeled with [γ-^{32}P]ATP and used to screen a Xenopus ovary cDNA library (Stratagene number 937652).

Several full-length clones were picked. The two shown in Figure 5 were sequenced on both stands. The sequences of importin forms 1 and 2 were deposited in the GenBank data base. Beside these clones, others were found that deviate in some positions from both forms 1 and 2. Sequences were analyzed with the following programs: sequence editing and alignments with PCGENE, identification of internal repeats with MCAFEE (Schuler et al., 1991) and tBLASTN algorithm data base screen for sequence homologies with NCBI mailserver (Altschul et al., 1990).

Depletion of Importin from Cytosol

The depletion protocol made use of the unusual property of importin to bind tightly to Ni–NTA–Sepharose. An egg extract was obtained by crushing activated Xenopus eggs in a swinging bucket rotor (as described by Leno and Laskey, 1991) and was respun for 20 min at 100,000 × g. Extract (5 ml) was transferred to depletion buffer (20 mM Tris–HCl [pH 7.4], 5 mM imidazole, 100 mM NaCl, 2 mM $MgCl_2$, 2 mM β-mercaptoethanol) by gel filtration on a 30 ml Sephadex G25 column. The protein containing pool was spun in a TLA100.4 rotor (Beckmann) at 80,000 rpm for 40 min. Half of the supernatant was passed through a 2.2 ml Ni–NTA–Sepharose column at 1 ml/hr (depleted cytosol), the other half was passed through the same volume of Sepharose CL-6B. Protein containing fractions were pooled and transferred into 20 mM HEPES–KOH (pH 7.4), 250 mM sucrose, 0.5 mM magnesium acetate, 2 mM DTT by gel filtration on Sephadex G25, adjusted to the same protein concentration (as estimated from UV absorbtion at 260 and 280 nm), and frozen in liquid nitrogen. Depletion efficiency (comparison between depleted and mock-depleted cytosol) was estimated by quantitative immunoblotting using peptide-specific antibody raised against the ten N-terminal amino acids of importin, ^{35}S-labeled secondary antibodies, a Molecular Dynamics phosphoimager, and recombinant importin of known concentration as a standard.

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GenBank Accession Numbers

The accession numbers for the sequences reported in this paper, importin forms 1 and 2, are L36339 and L36340, respectively.

Note Added in Proof

During the further characterization of stimulatory factors, we found an activity that behaves as a second subunit of importin, but dissociates during the Ni–NTA–Sepharose step. N. Imamoto and Y. Yoneda recently informed us that they also have observed a similar activity that appears to correspond to these two subunits of importin.