Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope

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Background: Selective protein import into the cell nucleus occurs in two steps: binding to the nuclear envelope, followed by energy-dependent transit through the nuclear pore complex. A 60 kD protein, importin, is essential for the first nuclear import step, and the small G protein Ran/TC4 is essential for the second. We have previously purified the 60 kD importin protein (importin 60) as a single polypeptide.

Results: We have identified importin 90, a 90 kD second subunit that dissociates from importin 60 during affinity chromatography on nickel (II)–nitrolotriacetic acid–Sepharose, a technique that was originally used to purify importin 60. Partial amino-acid sequencing of *Xenopus* importin 90 allowed us to clone and sequence its human homologue; the amino-acid sequence of importin 90 is strikingly conserved between the two species. We have also identified a homologous budding yeast sequence from a database entry. Importin 90 potentiates the effects of importin 60 on nuclear protein import, indicating that the importin complex is the physiological unit responsible for import. To assess whether nuclear localization sequences are recognized by cytosolic receptor proteins, a biotin-tagged conjugate of nuclear localization signals linked to bovine serum albumin was allowed to form complexes with cytosolic proteins in *Xenopus* egg extracts; the complexes were then retrieved with streptavidin–agarose. The pattern of bound proteins was surprisingly simple and showed only two predominant bands: those of the importin complex. We also expressed the human homologue of importin 60, Rch1p, and found that it was able to replace its *Xenopus* counterpart in a functional assay. We discuss the relationship of importin 60 and importin 90 to other nuclear import factors.

Conclusions: Importin consists of a 60 and a 90 kD subunit. Together, they constitute a cytosolic receptor for nuclear localization signals that enables import substrates to bind to the nuclear envelope.

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Background

Protein import into the nucleus is an active process that requires both energy, in the form of nucleoside triphosphates [1,2], and a signal, in the form of nuclear localization sequences (NLSs) [3–5]. The transport process proceeds in at least two steps: binding of a karyophilic protein to the nuclear envelope and its subsequent energy-dependent translocation through the nuclear pore complex [1,2].

NLSs are characterized by clusters of basic amino acids. Traditionally, they are classified into two major types. The first, as exemplified by the simian virus 40 (SV40) large T antigen [6,7], contains a single cluster of basic residues within one polypeptide chain. Signals of the second class are more complex, and the bipartite NLS found in nucleoplasmin, consisting of two basic clusters separated by a 10 amino-acid spacer is an example of this class [8].

Protein import into the nuclei of permeabilized cultured cells is dependent on cytosolic factors [9]. Binding to the nuclear pore and subsequent translocation through it require different cytosolic fractions [10,11]. These fractions were called A and B, respectively, by Moore and Blobel [10]. The G protein Ran/TC4 [12,13] and an interacting partner, pp15 [14], were found to be active components of the B fraction that is implicated in the second, translocation, step.

We have previously isolated a 60 kD protein, which we call importin 60, that is essential for the first step of nuclear protein import, namely the signal-dependent binding of karyophilic substrates to the nuclear envelope [15]. Importin 60 is homologous to SRP1p, a protein associated with the nuclear pore complex in *Saccharomyces cerevisiae*, which is the product of an essential gene [16–18]. Importin 60 is also homologous to mammalian proteins Rch1p [19] and mSRP1p [20], and to the *Drosophila* protein, Pendulin.

Previously, we observed a basal level of nuclear protein import using recombinant importin 60 and Ran/TC4 alone [15]. However, transport efficiency could be greatly

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enhanced by other factors present in the cytosol. Here, we identify one of these factors as a second, 90 kD subunit of importin (importin 90). We have cloned the importin 90 gene from a human cDNA library, and its deduced amino-acid sequence closely matches the partial peptide sequence of *Xenopus* importin 90. Furthermore an importin 90 homologue from *S. cerevisiae* is 34 % identical at the sequence level to the human importin 90, indicating a high degree of evolutionary conservation. The 60 and 90 kD importin subunits cooperate in the first nuclear import step.

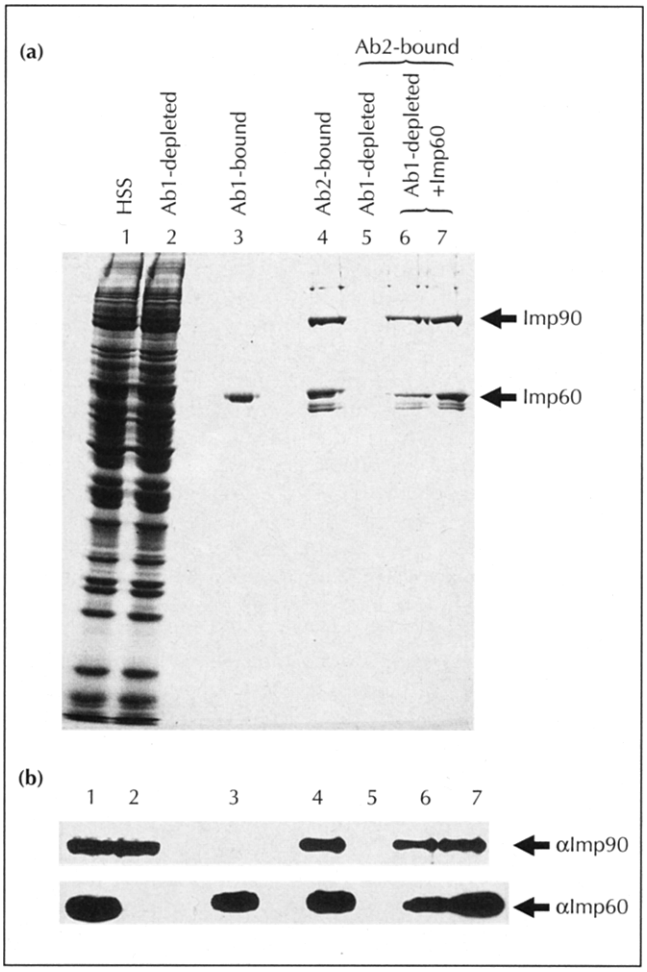

Fig. 1. Importin 60 associates with a second subunit of 90 kD. **(a)** Immunoprecipitation of importin 60 using the Ab1 or Ab2 antibodies, followed by SDS–PAGE and Coomassie staining. The starting material (lane 1) was *Xenopus* egg high-speed supernatant (HSS). Ab1 was used to deplete the HSS specifically of importin 60, yielding an 'Ab1-depleted' extract (lane 2), and an 'Ab1-bound' fraction that contains importin 60 (lane 3). Lanes 4–7 are fractions that were bound to Ab2. The starting material for binding to 25 μl Ab2–Sepharose was 300 μl of either HSS (yielding the bound fraction shown in lane 4), Ab1-depleted extract (lane 5), or Ab1-depleted HSS to which 20 or 40 μg ml^{-1} recombinantly expressed importin 60 had been re-added (lanes 6 and 7, respectively). Proteins bound to Ab2 were released using 4 % SDS. The doublet below the importin 60 band seems to be due to non-specific binding, as it originates from proteins that are far more abundant than importin and that vary in intensity between different experiments (see also Fig. 2a). 'Imp90' indicates the co-precipitating 90 kD (importin 90) band. The 'bound' fractions correspond to 30 times the starting material for staining. **(b)** Immunoprecipitation of importin 60 using the Ab1 or Ab2 antibodies, followed by western immunoblotting. Equivalent amounts of samples from (a) were subjected to SDS–PAGE and immunoblots were probed either with a mixture of Ab1 and Ab2 anti-importin 60 antibodies (αimp60) or with anti-importin 90 antibodies (αimp90).

These two subunits of importin resemble in size two proteins of 54/56 kD and 97.5 kD that were purified from bovine erythrocytes, and that bind nuclear import substrates to the nuclear envelope [21,22]. We have previously pointed out that the 60 kD importin molecule exhibits different biochemical properties from those described by Adam and co-workers for their 54/56 kD protein [21,22]. These differing properties include its native size and sensitivity to N-ethylmaleimide and ammonium sulphate, as well as the sensitivity of the import system to GTP analogues. Nevertheless, the sequence of the 54/56 kD protein from bovine erythrocytes is homologous to that of importin 60 (S.A. Adam, personal communication).

The 54/56 kD protein from bovine erythrocytes was initially purified by virtue of its binding to a radioactive large T antigen NLS peptide, suggesting that it constitutes a cytosolic NLS receptor [21]. In support of this conclusion, Adam and Gerace [21] showed that NLS binding was competed by wild-type, functional, NLS peptide more efficiently than by a non-functional mutant or reverse-sequence peptide. However, several other more questionable candidates for NLS receptors have undermined confidence in such assignments (reviewed in [23]).

Here, we use an affinity-chromatographic approach to test directly which proteins bind to a functional import substrate, but not to a nonfunctional one. When a biotinylated conjugate protein made up of bovine serum albumin (BSA) linked to NLS peptides was incubated with a *Xenopus* egg extract and subsequently recovered with streptavidin–agarose, two predominant proteins were bound — the two importin subunits. This observation supports the conclusion of Adam and Gerace [21] that a protein of around 55 kD recognizes nuclear localization signals. Our observations also directly demonstrate that the two subunits of importin together constitute a cytosolic receptor that binds nuclear localization sequences to the nuclear envelope. In addition, we tested whether human Rch1p is a functional homologue of *Xenopus* importin 60. Recombinantly expressed Rch1p can substitute fully for importin 60 in an import reaction *in vitro*.

Results and discussion

Importin has a second subunit of 90 kD

When subjecting a high-speed supernatant made from activated *Xenopus* eggs to gel filtration on Superdex 200, we previously observed that the 60 kD importin polypeptide is part of a large, heterogeneous complex of 300–1 000 kD molecular weight [15]. However, in the course of purification, the apparent size of the complex

containing importin 60 decreased to approximately 120 kD, suggesting that interacting partner proteins had been lost during the procedure.

To assess whether interacting partners had indeed been lost, we raised two different antibodies against importin 60. The first (Ab1) was raised against an amino-terminal peptide, and the second (Ab2) was raised against the entire recombinantly expressed importin 60. On western immunoblots, both antibodies were found to be monospecific for importin 60, and both antibodies immunoprecipitated importin 60 from a high-speed supernatant of egg extract (Fig. 1; compare lanes 1, 3 and 4). The identity of importin 60 was confirmed by immunoblotting (Fig. 1b; 'αimp 60'). The Coomassie-stained protein gel revealed that a 90 kD polypeptide is precipitated by Ab2 (lane 4) but not Ab1 (lane 3). This observation was confirmed by probing a blot with an antibody raised against an internal peptide of the 90 kD protein (Fig. 1b; 'αimp 90').

There are two alternative possible explanations for why the 90 kD protein is found in the eluate from Ab2 immunoprecipitates. The first is that the 90 kD protein is a subunit of a complex that includes importin 60 and therefore co-immunoprecipitates with anti-importin 60 antibodies. The second is that the Ab2 antibody cross-reacts with the 90 kD protein in solution. If the 60 and 90 kD bands represent two subunits of the same protein, antibody binding of the 90 kD polypeptide should depend on the presence of importin 60. If, however, the 90 kD protein is a cross-reacting antigen, the 90 kD protein would be predicted to bind to the Ab2 column even in the absence of importin 60; in fact, the two proteins should compete with one another for binding to Ab2.

To distinguish between these possibilities, binding to Ab2 was performed either using unfractionated egg high-speed supernatant, containing both importin 60 and the 90 kD protein, or with a high-speed supernatant that had been specifically depleted of importin 60 by Ab1, without affecting the 90 kD protein content (Fig. 1; compare lanes 1 and 2). As seen from a comparison of lanes 4 and 5 in Figure 1, binding of the 90 kD band was completely abolished by prior depletion of importin 60, and binding was restored by the re-addition of recombinantly expressed importin 60. Thus, the 90 kD protein forms a genuine complex with importin 60. Consistent with this view, we found that the two polypeptides co-purified on ion-exchange chromatography, gel filtration (data not shown) and, most strikingly, during NLS-affinity chromatography (see below). Therefore, the 90 kD protein behaves as a second subunit of an importin 60-containing complex, and we refer to it henceforth as importin 90.

Binding of importin 60 to Ab1 or to nickel (II)–nitrolotriacetic acid (NTA)–Sepharose interferes with the interaction between the two importin subunits and leads to the displacement of importin 90. This interaction is also sensitive to very high ionic strength, a property that we exploited in purifying importin 90. Purification was achieved by binding the importin complex to an affinity column made up of antibody against recombinant importin 60 (Ab2), washing the column extensively and dissociating the two subunits from each other with a magnesium chloride gradient (Fig. 2). The 90 kD subunit eluted at about 0.4 M $MgCl_2$, whereas the 60 kD protein remained bound to the antibody at this salt concentration. Substoichiometric amounts of a 32 kD protein eluting slightly earlier on the salt gradient were observed in some experiments. Importin 90 was further purified from the magnesium chloride eluate by gel filtration on Superdex 200. Most contaminants eluted close to the void volume, whereas nearly homogeneous importin 90 eluted at a position expected for its size.

Molecular cloning of importin 90

We first obtained a partial amino-acid sequence from *Xenopus* importin 90. Based on this information, we cloned importin 90 from a HeLa cDNA library. The

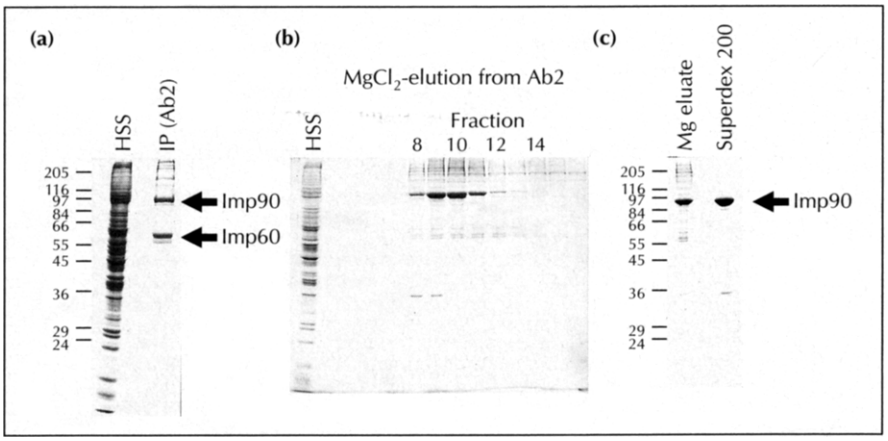

Fig. 2. Purification of importin 90. **(a)** A high-speed supernatant from activated *Xenopus* eggs (HSS) was subjected to immunoaffinity chromatography using affinity-purified Ab2 antibodies raised against recombinant importin 60. The protein patterns of the starting material 'HSS' and the pH 2.5 eluate from the antibody 'IP (Ab2)' are visualized by SDS–PAGE and Coomassie staining. 'Imp 90' indicates the 90 kD protein that co-precipitates and co-purifies with importin 60 (see Figs 1 and 6). **(b)** The importin complex from an egg high-speed supernatant was bound to the antibody column as in (a). The subunits were dissociated from one another by a magnesium chloride gradient. The 90 kD subunit was released from the column at about 0.4 M $MgCl_2$ (peak at fractions 9 and 10), whereas the 60 kD subunit, against which the antibodies were raised, remained bound. **(c)** The peak fractions from the magnesium chloride elution were pooled and subjected to gel filtration on Superdex 200; the resulting Coomassie-stained protein patterns are seen in the lanes labelled 'Mg eluate' and 'Superdex 200', respectively.

molecular weight of the human protein as calculated from the deduced amino-acid sequence is 97 kD, slightly higher than the 90 kD estimated for the *Xenopus* protein by SDS gel electrophoresis. The peptides obtained from the *Xenopus* protein match almost perfectly (93 % identity in 188 amino acids) the sequence of the cloned human protein (see Fig. 3). This is particularly remarkable as Rch1p, the closest known mammalian homologue of *Xenopus* importin 60, shares only 61 % identical amino acids with the *Xenopus* importin.

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Imp90,  Xenopus      **V*******-****RX*    +***Q*********V****K*L***A******
Imp90,  human      MELITILEKTV-SPDRLELEAAQKFLERAAVENLPTFLVELSRVVANPGNSQVARVA    56
                     E:  :LE::: SPD:    :::. L.: : :N:  F    S:V: : ...  :R:
Imp90H, yeast      MSTAEFAQLLENSILSPDQNIRLTSETQLKKLSNDNFLQFAGLSSQVLIDENTKLEGRIL    60

Imp90,  Xenopus                  +******XX*-*X***P
Imp90,  human      AGLQIKNSLTSKGPDIKAQYQQRWLA-IDANARREVKNYVLQTL-GTETYRPSSASQCVA   114
                   A:L :KN.L.SK::  ..Q:.QRW:: :.::A:.::K. .L :L :.E.  :::A:Q :A
Imp90H, yeast      AALTLKNELVSKDSVKTQQFAQRWITQVSPEAKNQIKTNALTALVSIEPRIANAAAQLIA   120

Imp90,  Xenopus                                  ***X****D**X***
Imp90,  human      GIACAEIPVNQWPELIPQLVANVTNPNSTEHMKESTLEAIGYICQDIDP--EQLQDKSNE   172
                   :IA  E:P ..WPEL:  :V.N T.:: .E::K ::L A:GY:C:. DP  :.L ..SN:
Imp90H, yeast      AIADIELPHGAWPELMKIMVDN-TGAEQPENVKRASLLALGYMCESADPQSQALVSSSNN   179

Imp90,  Xenopus
Imp90,  human      ILTAIIQGMRKEEPSNNVKLAATNALLNSLEFTKANFDKESERHFIMQVVCEATQCPDTR   232
                   IL.AI:QG :..E.S:.V:LAA NAL :SL F.K.N.::E:ER:::MQVVCEATQ  D.
Imp90H, yeast      ILIAIVQGAQSTETSKAVRLAALNALADSLIFIKNNMERGERNYLMQVVCEATQAEDIE   239

Imp90,  Xenopus                                                          *
Imp90,  human      VRVAALQNLVKIMSLYYQYMETYMGPALFAITIEAMKSDIDEVALQGIEFWSNVCDEEMD   292
                   V:.AA:  L KIMSLYY :M..YM..AL:A:TI.::MKS  D.VA  .:EFWS.:C:EE:D
Imp90H, yeast      VQAAAFGCLCKIMSLYYTFMKPYMEQALYALTIATMKSPNDKVASMTVEFWSTICEEEID   299

Imp90,  Xenopus    ************X***X* +**E*
Imp90,  human      LAIEASEAAEQGRPPEHTSKFYAKGALQYLVPILTQITLTKQDENDDDDDWNPCKAAGVCL   352
                   :A E :: :   ::P :: :F A :::: :VP L : LT:Q:E: :DDDWN ..:AG.CL
Imp90H, yeast      IAYELAQFP---QSPLQSYNF-ALSSIKDVVPNLLNLLTRQNEDPEDDDWNVSMSAGACL   355

Imp90,  Xenopus                      +****      +Y***
Imp90,  human      MLLATCCEDDILPHVLPFIKEHIKNPDWRYRDAAVMAFGCILEGPEPSQLKPLVIQAMPT   412
                    L:A  C.::IL .VL F:.::I.. :WR R:AAVMAFG.I::GP:  Q .  V QA:P:
Imp90H, yeast      QLFAQNCGNHILEPVLEFVEQNITADNWRNREAAVMAFGSIMDGPDKVQRTYYVHQALPS   415

Imp90,  Xenopus       ******
Imp90,  human      LIELMKDPSVVVRDTAAWTVGRICELLPEAAINDVYLAPLLQ-CLIEGLSAEPRVASNVC   471
                   :::LM:D.S: V::T:AW :GRI : ::E:   : .L: ::Q CLI GL .:P:VA:N .
Imp90H, yeast      ILNLMNDQSLQVKETTAWCIGRIADSVAESIDPQQHLPGVVQACLI-GLQDHPKVATNCS   474

Imp90,  Xenopus                                      +***************
Imp90,  human      WAFSSLAEAAYEAADVADDQEEPATYCLSSSFELIVQKLLETTDRPDGHQNNLRSSAYES   531
                   W:: :L.E      ::A:.  .P .Y   : :  :V: L:.:::R D :: N R:SA:.:
Imp90H, yeast      WTIINLVE------QLAEATPSP-IY---NFYPALVDGLIGAANRID-NEFNARASAFSA   523

Imp90,  Xenopus
Imp90,  human      LMEIVKNSAKDCYPAVQKTTLVIMERLQQVLQMESHIQSTSDRIQFNDLQSLLCATLQNV   591
                   L .:V. ::..   : ...:  :M::L Q.: ::.:  : .D   :::LQS : :.L..V
Imp90H, yeast      LTTMVEYATDIVAETSASISTFVMDKLGQTMSVDENQLTLEDAQSLQELQSNILTVLAAV   583

Imp90,  Xenopus                ********XM**X*******
Imp90,  human      LRKVQHQDALQISDVVMASLLRLFQSTAGSGGVQEDALMAVSTLVEVLGGEFLKYMEAFK   651
                   :RK .   . .::D::M: ::RL::.. :S: :::D.: A:S:L.. LG .F KY:E:F.
Imp90H, yeast      IRKSPSSVE-PVADMLMGLFFRLLEKK-DSAFIEDDVFYAISALAASLGKGFEKYLETFS   641

Imp90,  Xenopus                                          *F********X*
Imp90,  human      PFLGIGLKNYAEYQVCLAAVGLVGDLCRALQSNIIPFCDEVMQLLLENLGNENVHRSVKP   711
                   P:L  :L:: .: .V.::AVG:::D:..:L:.:: .:.D.:M::L : ::N N.:R.:KP
Imp90H, yeast      PYLLKALNQ-VDSPVSITAVGFIADISNSLEEDFRRYSDAMMNVLAQMISNPNARRELKP   700

Imp90,  Xenopus            *******XX*              +G***
Imp90,  human      QILSVFGDIALAIGGEFKKYLEVVLNTLHEASQAQVDKSDYDMVDYLNELRESCLEAYTG   771
                   .:LSVFGDIA .IG::F  YL: ::.   .A ::: :::. : :DY  .: E: L:AY.G
Imp90H, yeast      AVLSVFGDIASNIGADFIPYLNDIMALCVAAQNTKPENGTLEALDYQIKVLEAVLDAYVG   760

Imp90,  Xenopus       +*********  X******R*X**X*
Imp90,  human      IVQGLKGDQENVHPDVMLVQPRVEFILSFIDHIAGDED--HTDGVVACAAGLIGDLCTAF   829
                   IV.GL     : .P:.::  P V. I: FI.::A.D :   .D:.   A.GLIGD: : F
Imp90H, yeast      IVAGL-----HDKPEALF--PYVGTIFQFIAQVAEDPQLYSEDATSRAAVGLIGDIAAMF   813

Imp90,  Xenopus       +******  ********          +****X****DV
Imp90,  human      GKDVLKLVEARPMIHELLTEGRRSK--TNKAKTLATWATKELRKLKNQA              876
                    .: :K   ::  : : :. .R.::  :: :K. A WA .: ::  :
Imp90H, yeast      PDGSIKQFYGQDWVIDYIKRTRSGQLFSQATKDTARWAREQQKRQLSL               861
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Fig. 3. Sequence of human importin 90 and homologies with yeast and *Xenopus* proteins. A 188 amino-acid partial protein sequence was obtained from 19 internal *Xenopus* importin 90 peptides. Based on this information, importin 90 was cloned from a HeLa cDNA library. The figure shows the deduced amino-acid sequences (single-letter code) of human importin 90, the *Xenopus* importin 90 partial sequences, and the deduced amino-acid sequence of an open reading frame in *S. cerevisiae* 'Imp 90H, yeast' (accession number U19028) that encodes a homologous protein. *Xenopus* and human importin 90 sequences are 93 % identical in the overlapping region, whereas human importin 90 and the yeast importin 90 homologue share 34 % identical amino acids. Asterisks indicate amino acids identical in *Xenopus* and human importin 90s. Amino acids deviating between the *Xenopus* and human proteins are denoted by single-letter code; '+' precedes the amino terminus of a tryptic peptide and stands for either lysine or arginine; 'X' indicates an unreadable amino acid. The human and yeast sequences were aligned using AALIGN of the DNASTAR programme; in the line between these sequences, a single letter indicates an identical amino acid; a colon indicates a positively-related amino acid; a dot indicates a neutrally-related amino acid; and a space indicates a negatively-related amino acid.

We next searched the GenBank Database for homologues using the human importin 90 sequence. As well as a number of identical human expressed-sequence tags (accession numbers: T07554, T09278 and T05917), homologous sequences from rice (accession numbers: D22848 and D40074), *Caenorhabditis elegans* (D37275) and *Plasmodium falciparum* (T18034) emerged. However, the most striking outcome of the search was the identification of a 95 kD homologous protein in *S. cerevisiae* that shares 34 % identical amino acids with the human importin 90 (Fig. 3). It remains to be seen whether this yeast protein is a functional homologue of importin 90 — whether it acts with SRP1p to facilitate nuclear protein import. Furthermore, weak similarities were found to the yeast protein Pse1 and some uncharacterized open reading frames (SCPSE1 and SCYBR017c; accession numbers Z11538 and Z35886, respectively).

Importin 90 also contains a 42 amino-acid region (positions 400–442) that shows significant similarity to one of the 'arm' motifs present in the SRP1p/importin 60 protein family (corresponding to positions 151–193 of importin 60). The flanking sequences of this 42 amino-acid stretch might constitute three more arm-like motifs, although the similarity here is less clear.

The 60 and 90 kD importin subunits cooperate in mediating the first step of nuclear protein import

We have previously shown that importin 60 is essential for the first step of nuclear import — NLS-dependent binding of import substrates to the nuclear envelope. The effects of the second subunit were first tested in the absence of Ran/TC4 (thus assaying only the first step of transport). As shown in Figure 4, although the presence of importin 60 alone allowed binding of a fluorescein-labelled BSA–NLS conjugate to the nuclear envelope, importin 90 did not allow envelope binding. But stimulation by importin 60 of the conjugate's binding to the envelope could be greatly enhanced by the simultaneous presence of importin 90. This enhancement by importin 90 was particularly significant for recombinant importin 60 or for low concentrations of native importin 60.

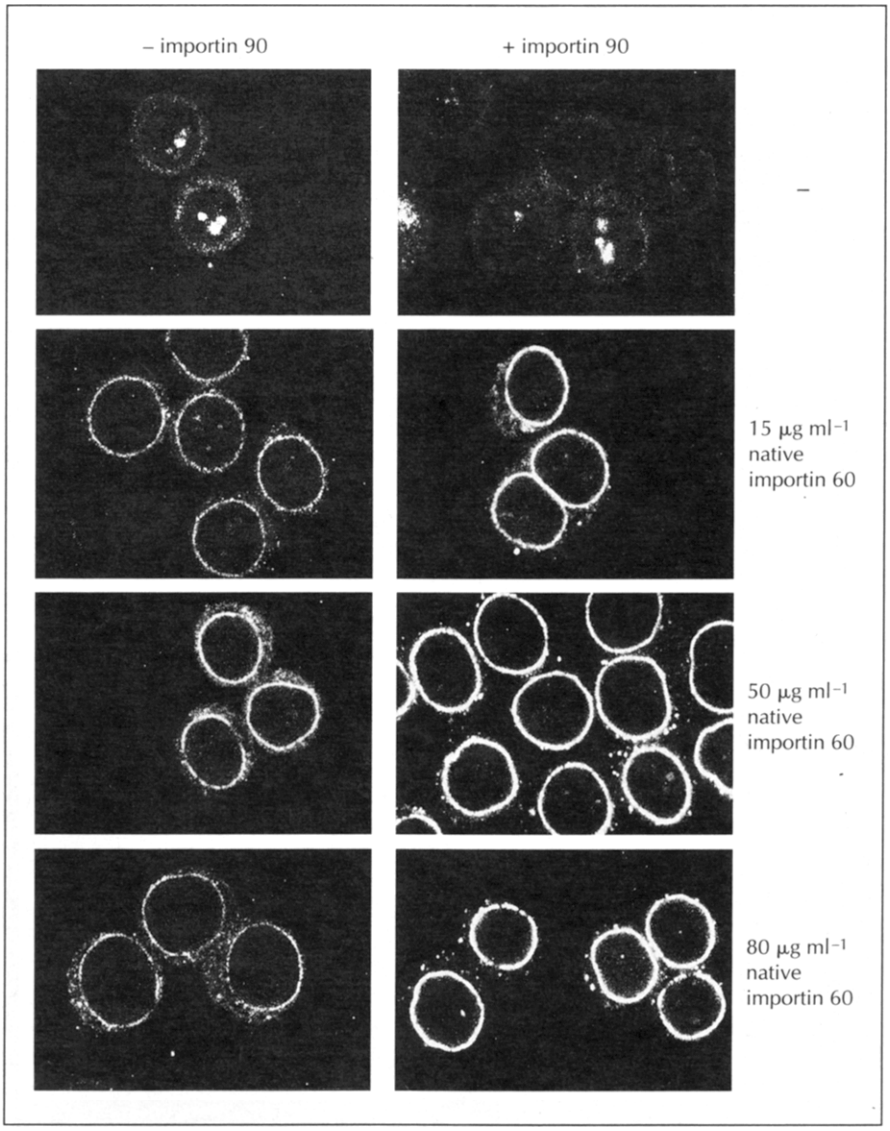

Fig. 4. Importin 60 and Importin 90 cooperate in binding an import substrate to the nuclear envelope. Panels show confocal sections through nuclei of permeabilized HeLa cells after import reactions with fluorescein-labelled BSA–NLS conjugate; '– importin 90' or '+ importin 90' indicates omission or addition of 10 μg ml^{-1} of the 90 kD importin subunit. Native importin 60 was purified from *Xenopus* eggs. Recombinant importin 60 is importin form 1 expressed in *E. coli* [15]. All panels were scanned and photographed under identical conditions.

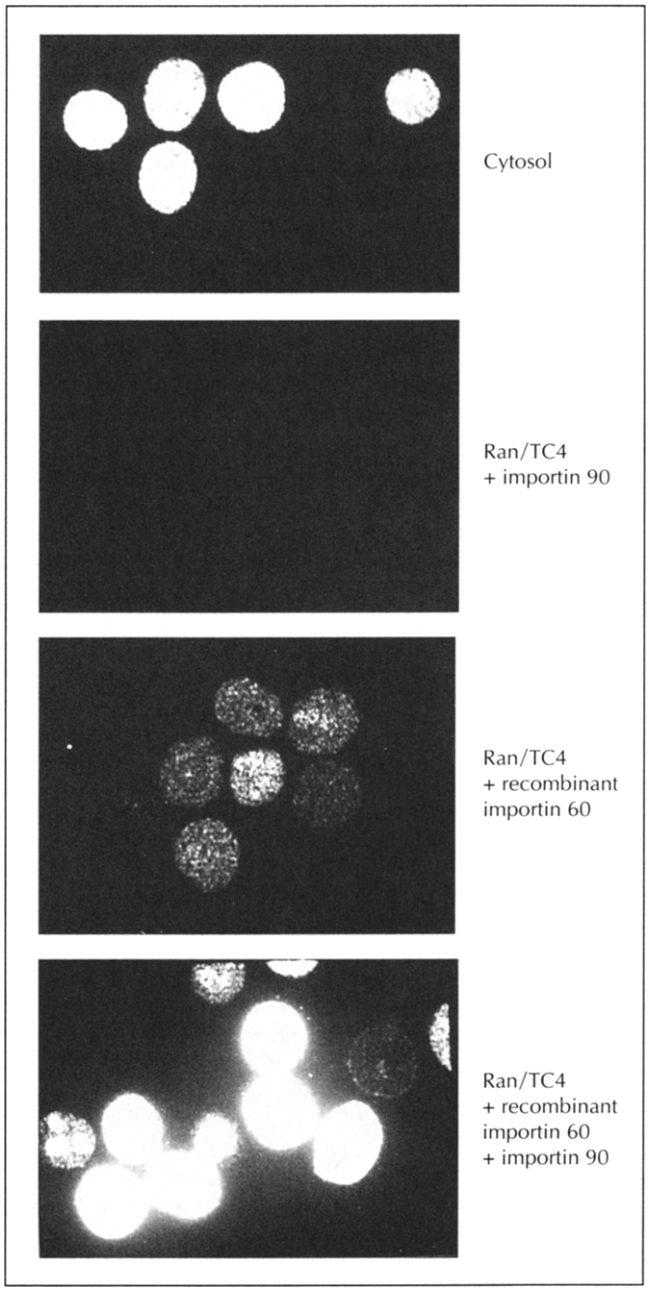

Fig. 5. Importins 60 and 90 cooperate in complete transport of an import substrate into the nucleus. The figure shows confocal sections through nuclei of permeabilized cells after import reactions with fluorescein-labelled nucleoplasmin. The reactions were performed in the presence of ATP, GTP and an energy-regenerating system. Additions were as indicated: 'cytosol' denotes *Xenopus* egg high-speed supernatant at a final protein concentration of 8 mg ml^{-1}; Ran/TC4 was at 100 μg ml^{-1}; importin 90 was at 10 μg ml^{-1}; and recombinant importin 60 was at 100 μg ml^{-1}. All photographs were obtained and printed under identical settings. The fluorescence of the nuclei in the panel 'Ran/TC4 + importin 90' is below the detection limit, whereas most nuclei in the panel 'Ran + Importin 90 + recombinant Importin 60' are above the linear range; the fuzzy appearance of the latter is therefore due to overexposure.

To prove that the effect on envelope binding relates to faithful nuclear transport, a similar experiment was performed with addition of an energy-regenerating system and Ran/TC4 (Fig. 5). In the presence of Ran/TC4 alone (not shown) or Ran/TC4 in combination with importin 90, no import was observed. The combination of Ran/TC4 and recombinant importin 60 gave basal-level transport, as reported previously [15]. However, if all three components were present simultaneously, highly efficient import was observed. When unfixed samples were viewed, import with this combination of proteins was so efficient that nucleoplasmin appeared to be taken up completely. It should, however, be noted that the fluorescence intensity of these nuclei appeared heterogeneous (Fig. 5). About 10 % were extremely bright, the nuclear fluorescence being more than five-fold higher than the whole-cytosol control. These nuclei appeared decondensed, perhaps due to the excessive uptake of nucleoplasmin [24]. About 40 % of the nuclei had fluorescence intensities between one and five times the signal obtained from the cytosol control. The rest appeared pale, or remained at the stage of envelope binding, indicating that additional factors needed for translocation through the nuclear pore were limiting.

One protein factor that might be responsible for this variability is pp15; this factor was recently identified as a second active component of the fraction B that is required for the second step of nuclear import [14]. Alternatively, the heterogeneity among the population of nuclei might be due to heterogeneity in cell-cycle stage [25]. Of course, the most interesting possibility is that these two explanations overlap — meaning that pp15 might be a factor whose activity varies within the cell cycle. The fact that Moore and Blobel [14] use confluent (and thus perhaps G0 stage, quiescent) buffalo rat liver cells, whereas we use exponentially growing HeLa cells as a source of nuclei, might then explain why we have not seen a strict requirement for exogenously added pp15 for nuclear import.

The complex of importin 60 and 90 constitutes a cytosolic NLS receptor

To look for cytosolic factors that bind NLSs specifically, we allowed an NLS–BSA conjugate tagged with biotin to form complexes with putative receptors in the cytosol. In order to compete out cytosolic proteins interacting non-specifically with the BSA–NLS conjugate, a 50-fold excess of untagged conjugate made up of BSA with reversed-sequence NLS-peptide was also added; the reverse-sequence peptide made up of the same amino acids is unable to function as an NLS but has an identical electrostatic charge [26]. Biotinylated complexes were then retrieved with streptavidin–agarose. After extensive washing, the proteins bound to the NLS conjugate were released by 1 M $MgCl_2$ (note that this does not affect the streptavidin–biotin interaction). Immunoblotting showed (Fig. 6a) that 30–50 % of importins 60 and 90 were recovered in the NLS-bound fraction. No importin complex was bound if the non-functional BSA–reversed-NLS conjugate was used as a probe.

The Coomassie-stained protein pattern of the NLS-bound fraction consisted of only two major bands, of

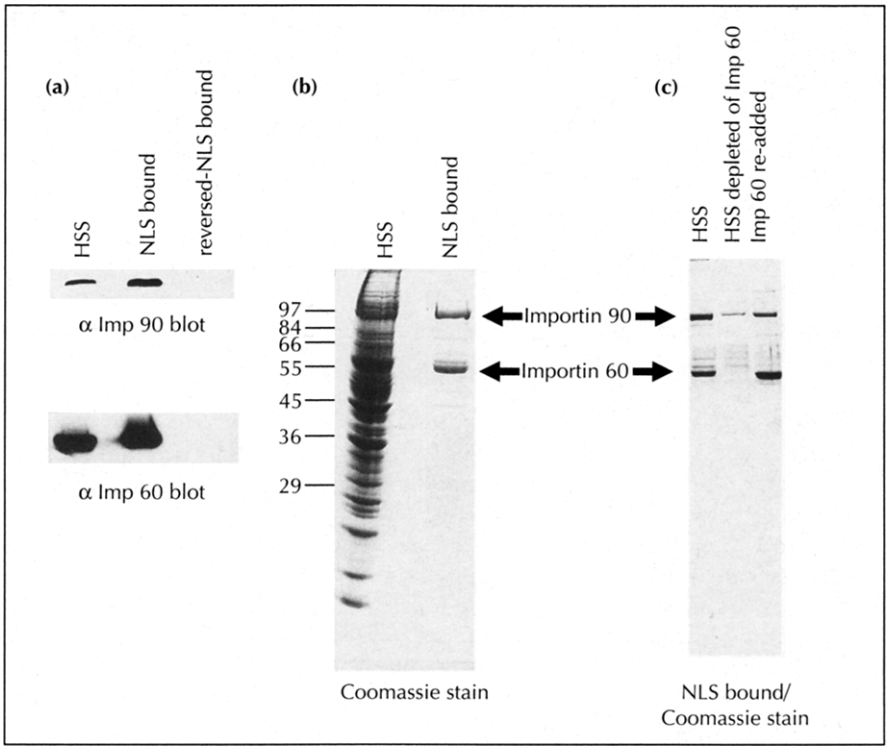

Fig. 6. The importin complex constitutes the cytosolic NLS receptor. A high-speed supernatant from activated *Xenopus* eggs was incubated with 40 μg ml^{-1} biotinylated BSA–NLS conjugate (efficient nuclear-import substrate) in the presence of a 50-fold excess of non-biotinylated BSA–sequence-reversed-NLS conjugate as non-specific competitor, which provides a negative control as an inactive import substrate. The reverse experiment was also performed — using biotinylated BSA–sequence-reversed-NLS conjugate and the BSA–NLS conjugate as a competitor. Aggregates were removed by ultracentrifugation and the biotinylated conjugates recovered with streptavidin–agarose. Proteins bound to the biotinylated probes were released with 1 M $MgCl_2$, precipitated with 90 % ethanol and analyzed by SDS–PAGE. **(a)** Samples were analyzed by immunoblotting with antibodies raised against recombinant importin 60 or with an antibody raised against an internal peptide of importin 90. 'HSS' corresponds to the starting material. The load in lanes labelled 'NLS bound' and 'reversed-NLS bound' is four times more than the starting material. **(b)** Samples were analyzed by Coomassie staining. The load in the 'NLS bound' lane corresponds to 15 times the starting material. **(c)** The NLS-binding assay was performed using either unfractionated high-speed supernatant, supernatant depleted of the 60 kD subunit of importin with the antibody Ab1, or the same Ab1-treated high-speed supernatant to which 50 μg ml^{-1} recombinant importin 60 had been re-added. The immunodepletion lowered the content of importin 60 to the detection limit of western immunoblotting. Note that binding to the amino-terminal antibody dissociates the importin complex, thus leaving importin 90 undepleted.

60 and 90 kD, which strikingly resemble the immunoprecipitate with Ab2 anti-importin antibodies (compare Figs 2a and 6b). Not only does the 90 kD NLS-bound protein comigrate with importin 90 and blot with anti-importin 90 antibodies, it also has the same amino-acid composition. The 60 kD band was not detectable in the NLS-bound fraction if importin 60 had been depleted using antibodies raised against its amino terminus. The band reappeared if recombinant importin 60 was re-added, thus proving that the 60 kD NLS-bound protein is importin 60. Specific depletion of the 60 kD importin subunit diminished, but did not completely abolish, the binding of the 90 kD subunit to the import substrate (Fig. 6c). Thus, importin 90 alone might also have some NLS-binding activity. However, binding of the 90 kD protein to the NLS–BSA conjugate was clearly reinforced by the re-addition of recombinant importin 60. Recombinantly expressed importin 60 presented in the form of a bacterial lysate also binds to the import substrate in the absence of the 90 kD protein (data not shown), indicating that importin 60 on its own has NLS-binding activity. Thus, it appears that both importin subunits contribute to NLS binding, and NLS recognition seems to be the result of the cooperation of the two subunits.

The mammalian homologues of importin 60, Rch1p and hSRP1p, have been found in two-hybrid screens to be interaction partners of the recombination activating protein, Rag1p [19,20]. This observation probably reflects an interaction between the Rag1p NLS and the Rch1p and human/mouse SRP1p NLS receptors. Mapping has revealed [20] that four out of the eight arm motifs [27] of hSRP1p are needed for its interaction with Rag1p, indicating that these motifs constitute the NLS-binding site. Similarly, the presence of arm-like motifs in importin 90 could explain its NLS-binding activity. The importin complex might have multiple binding sites for basic clusters, as both subunits appear to bind NLSs. The cooperative recognition of different parts of an import substrate might then contribute crucially to the fidelity of nuclear import.

As mentioned earlier, we found importin 60 in an egg extract in complexes of 300–1 000 kD molecular weight. It is not clear at present whether association with the 90 kD subunit and the binding of NLS-containing proteins can entirely explain the size of these complexes. The question of whether there are further interaction partners of importin is currently under investigation.

Evolutionary conservation of importin 60 function

On the basis of sequence homology, importin 60 is a member of a growing family of similar proteins that, so far, includes the yeast nuclear pore protein SRP1p [16], the mammalian proteins Rch1p and m/hSRP1p [19,20], and the *Drosophila* protein Pendulin (accession number: U12269). The degree of amino-acid sequence identity between the different members of the family is only

41–61 %. This degree of sequence divergence cannot be due solely to species differences between homologous proteins, because Rch1p and mammalian SRP1p, which are 47 % identical, have been found to coexist in a single species (as shown for mouse and humans). To ask whether the members of this protein family are functional homologues, we cloned human Rch1p and yeast SRP1p on the basis of their published sequences, expressed them as recombinant proteins in *E. coli* and assessed their ability to substitute for importin 60 in the import of nucleoplasmin into the nuclei of permeabilized HeLa cells (Fig. 7a).

A *Xenopus* egg extract depleted of importin 60 shows very little import activity (Fig. 7a), but it can be rescued by addition of recombinant importin 60 (Fig. 7b), as reported previously [15]. Addition of Rch1p has the same effect (Fig. 7c) thus demonstrating Rch1p and importin 60 are functional homologues. In contrast, yeast SRP1p cannot substitute for importin 60 (Fig. 7d). The evolutionary distance between yeast and vertebrates is probably too great to provide complete compatibility of components of the nuclear-import apparatus. This observation is consistent with the finding that yeast cytosol is unable to stimulate protein import into mammalian nuclei [9]. However, we have found that SRP1p binds specifically to functional nuclear import substrates but not to non-functional ones, indicating that SRP1p functions as an NLS receptor in yeast (D.G. and E.H., unpublished observations).

Relationship of importin to other nuclear import factors: emergence of a consensus opinion?

As explained in the Background section, there have been many claims of receptors for NLSs (reviewed in [23]). Amongst these, one of the most promising is the protein doublet of 54/56 kD purified from bovine erythrocytes [21,22]. After our NLS-binding experiments were completed, we heard that the sequence of this bovine protein is homologous to importin 60, SRP1p, hSRP1p and Rch1p (S. Adam personal communication). Therefore, our results and those of Adam and colleagues [21,22] fully agree on the assignment of the function of NLS-binding to importin 60. It remains to be seen whether the different biochemical properties described for the bovine and *Xenopus* proteins are due to differences between species, the isolation of different family members, or another reason.

In addition, our results agree with the observation by Adam and Adam [22] that a 97 kD protein is also involved in the first stage of nuclear import. Here, we present the sequence of a functional protein of this size, together with the following observations. First, it is a second subunit of importin 60. Second, it cooperates with importin 60 in selective nuclear-envelope binding, as well as in complete transport. And third, the human and *Xenopus* sequences of importin 90 are highly similar. We have also identified a good candidate for a yeast homologue of importin 90. Furthermore, we have demonstrated a functional conservation between human Rch1p and *Xenopus* importin 60. This work has integrated nuclear-import studies based on the *Xenopus* system [10–12,15] with those in bovine erythrocytes [21,22]. In addition, Imamoto and colleagues have now informed us that similar proteins of approximately 60 and 90 kD constitute an NLS receptor in mouse ascites cells [28].

Conclusions

A consensus is now emerging that, in a wide range of species, a similar pair of proteins is required for the first step of nuclear protein import. The importin complex,

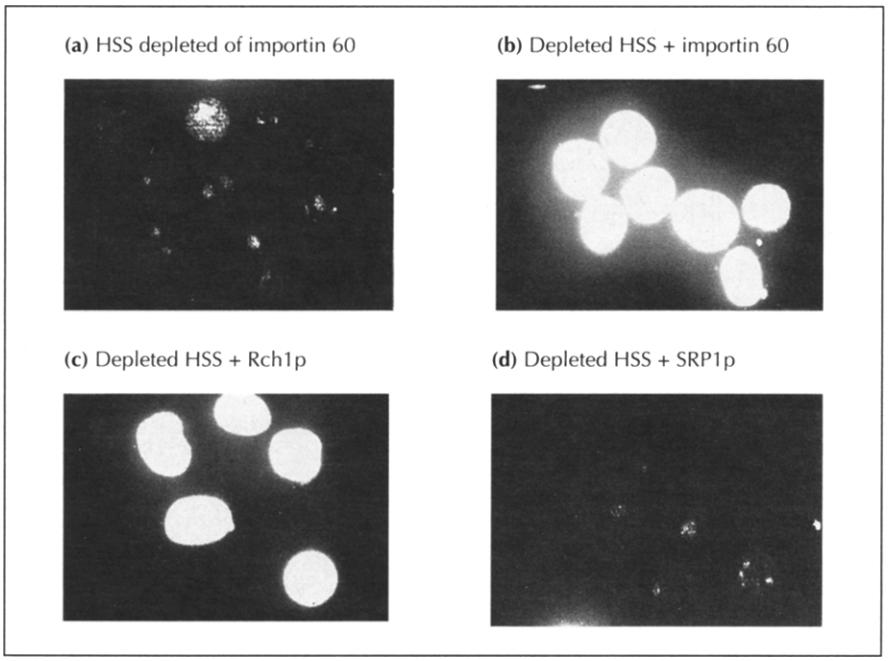

Fig. 7. Human Rch1p mimics importin 60 in nuclear import. **(a–d)** *Xenopus* importin 60, human Rch1p and yeast SRP1p were expressed in *E. coli* and tested for the ability to restore protein import activity of a high-speed supernatant depleted of importin 60. Proteins were added to a final concentration of 50 $\mu g\ ml^{-1}$. Panels show confocal sections through the nuclei of permeabilized cells after import reactions utilising fluorescein-labelled nucleoplasmin. All panels were scanned and photographed under identical settings.

which consists of two subunits of 60 and 90 kD, is essential for this first step. The human Rch1p protein is able to substitute fully for *Xenopus* importin 60 in an import reaction *in vitro*, and, furthermore, the human sequence of importin 90 shows striking conservation with both the *Xenopus* protein and a protein encoded by an open reading frame in *S. cerevisiae*. The 60 and 90 kD subunits of importin cooperate to form an import receptor which distinguishes functional nuclear localization signals from non-functional ones and selectively binds import substrates to the nuclear envelope.

Materials and methods

Nuclear protein import assay and recombinant expression

Nuclear import reactions, preparation of nuclear import substrates, purification of importin 60 from *Xenopus* eggs and the expression and purification of Ran/TC4 were all described previously [15]. In the experiment shown in Figure 4, the nucleoplasmin core domain was included in the assay to compete out non-specific background binding. The expression and purification of recombinant importin 60 was modified as follows: the protein was expressed without a histidine tag and purified from the bacterial lysate in essentially the same way as from a *Xenopus* egg extract. Rch1 and SRP1 were cloned on the basis of their published sequences [16,19] from human and *S. cerevisiae* libraries, respectively. Rch1p was expressed from pQE70 (Qiagen) without a histidine tag, and was purified from the bacterial lysate essentially as importin 60, except that the binding stringency to Ni–NTA Sepharose was lowered by replacing Tris by 50 mM Hepes–KOH, pH 7.5. SRP1p was expressed from pQE32 (Qiagen) with an amino-terminal histidine tag, and was purified on Ni-NTA agarose. Experiments with importin 60 have shown that an amino-terminal histidine tag does not interfere with function (D.G., unpublished observations).

Antibodies

All antibodies were affinity-purified on sulfoLink Gel (Pierce) to which the appropriate antigens had been coupled. Antibodies were immobilized as described [29] and were prewashed with a pH 2.5 buffer before equilibration and use. Antibodies raised against the amino terminus of *Xenopus* importin form 1 were described previously [15]. The antigenicity of 60 kD importin expressed in *E. coli* [15] was enhanced by coupling it to sulfo-SMCC-activated BSA prior to injection into rabbits. Antibodies against importin 90 were raised against an internal peptide (cDLAIEASEAAEQG; 'c' at the start of this sequence denotes an additional cysteine that was used as a coupling group).

Immunoaffinity chromatography and purification of the 90 kD importin subunit

A low-speed supernatant (LSS) was prepared from activated *Xenopus* eggs as described [30]. It was diluted three-fold in column buffer (50 mM Tris–HCl pH 7.4, 100 mM NaCl, 3 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10 μg ml^{-1} leupeptin, 5 μg ml^{-1} chymostatin) and ultracentrifuged to sediment particles larger than 25 S, yielding a high speed supernatant (HSS).

For selective depletion of importin 60, 10 ml of HSS was passed at 2 ml h^{-1} through a 2 ml immobilized-Ab1 (anti-amino-terminal importin 60) column. Fractions of 0.5 ml were collected and aliquots were analyzed by immunoblotting. Protein-containing fractions that were completely depleted of importin 60 were pooled and frozen in small aliquots. In experiments in which depleted and non-depleted extracts were directly compared, an aliquot of starting HSS was diluted with column buffer to the same optical density (at 280 nm) as the depleted extract. Proteins bound to Ab1 were released after extensive washing with 1 mg ml^{-1} antigenic peptide in 1 M NaCl at room temperature (flow rate 2 ml h^{-1}).

In experiments in which importin 60 was re-added to a depleted extract, all samples were supplemented with an energy-regenerating system and were pre-incubated for 20 min at room temperature after the addition of recombinant importin 60 to allow, for example, phosphorylation to occur and complexes to reform.

To purify importin 90, 50 ml HSS was applied at a flow rate of 10 ml h^{-1} to a 5 ml column of antibodies (about 5 mg) raised against recombinant importin 60 (Ab2), equilibrated in column buffer and washed with 100 ml column buffer. A 20 ml gradient from column buffer to buffer B (50 mM Tris–HCl pH 7.4, 1 M $MgCl_2$) was applied. The 90 kD protein was released from the column at about 40 % buffer B. The peak fractions (2 ml) were pooled, dialyzed against column buffer, cleared by ultracentrifugation and finally purified on Superdex 200 (Pharmacia) equilibrated in column buffer. The peak fractions were then pooled, adjusted to 250 mM sucrose and frozen in small aliquots.

Sequence analysis and molecular cloning of importin 90

Internal peptides were generated in one of three ways: (i) from SDS–PAGE blotted proteins by *in situ* tryptic cleavage and separation by HPLC; (ii) from ethanol-precipitated proteins by cyanogen bromide cleavage and PAGE; (iii) by Asp-N cleavage and HPLC separation. An automated protein sequencer system (model 477A/120A; Applied Biosystems) was used for sequencing. Two of the peptides matched almost perfectly a human sequence tag (accession number T07554 [31]) from which three non-degenerate oligonucleotides were chosen in order to screen a randomly primed HeLa cDNA library. Two overlapping clones coding for amino acids 1–781 and 8–876 of human importin 90 were sequenced on both strands. Sequences were compared with the GenBank (database release 85+) using the tblastn program from the NCBI e-mail server [32].

NLS-binding assay

SV40 NLS peptides and the reversed-sequence peptides were conjugated to BSA as described [15]. Biotin was coupled to the conjugates in a 4:1 molar ratio using Biotin-X-NHS,WS (number 203189, Calbiochem).

A typical NLS-binding assay was performed with 100 μl HSS in 50 mM Tris–HCl, pH 7.5, 80 mM NaCl (about the physiological ionic strength in *Xenopus* eggs) and 3 mM magnesium acetate. Each step was carried out on ice or in the cold room. The first addition of 2 mg ml^{-1} BSA–reversed-NLS conjugate was followed by the addition of 40 μg ml^{-1} biotinylated BSA–NLS conjugate. After 1 h incubation, aggregates that had formed were removed by a 10 min 100 000 × *g* spin. The supernatant was then bound to 50 μl streptavidin agarose (Sigma) for 1 h. The beads were washed six times with 1 ml 20 mM Tris–HCl, pH 7.5, 80 mM NaCl, 2 mM magnesium chloride, and bound proteins were eluted twice with 100 μl 1 M MgCl. Elution could also be achieved by low (2.5) or high (11.5) pH. Proteins were precipitated with 90 % ethanol and analyzed by SDS–PAGE on a 10 % polyacrylamide gel, followed by either

staining with Coomassie brilliant blue G250 or immunoblotting. The parallel binding to the reversed-NLS conjugate was performed identically, except that the untagged competitor was the NLS–BSA conjugate and the biotinylated probe contained the reversed-NLS peptide sequence.

GenBank Accession number

The accession number for the human importin 90 sequence reported in this paper is L38951.

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