The EMBO Journal vol.15 no.8 pp.1810–1817, 1996

A 41 amino acid motif in importin-α confers binding to importin-β and hence transit into the nucleus

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The complex of importin-α and -β is essential for nuclear protein import. It binds the import substrate in the cytosol, and the resulting trimeric complex moves through the nuclear pores, probably as a single entity. Importin-α provides the nuclear localization signal binding site, importin-β the site of initial docking to the pore. Here we show that the conserved, basic N-terminus of importin-α is sufficient for importin-β binding and essential for protein import. The fusion product of this 41 amino acid domain to a heterologous protein is transported into the nucleus in the same way as full-length importin-α itself. Transport is dependent on importin-β but competed by importin-α. As no additional part of importin-α is needed for translocation, the movement which drives the import substrate complex into the nucleus appears to be generated between importin-β and structures of the nuclear pore. The domain that binds to importin-β appears to confer import only, but not re-export out of the nucleus, suggesting that the return of importin-α into the cytoplasm is not a simple reversal of its entry.

Keywords: IBB domain/importin/nuclear pore/nuclear transport/Ran

Introduction

Protein import into the nucleus proceeds through the nuclear pore complex (Feldherr *et al.*, 1984), requires energy (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988) and is triggered by nuclear localization signals (NLSs) (Dingwall *et al.*, 1982; Hall *et al.*, 1984; Kalderon *et al.*, 1984; Lanford and Butel, 1984). It has been studied using digitonin-permeabilized mammalian cells as an *in vitro* system (Adam *et al.*, 1990). A fluorescent labelled import substrate can be introduced through the leaky plasma membrane into the cells, and its uptake by the nuclei can be visualized by fluorescence microscopy. The permeabilization also results in the loss of the soluble cell contents. Active uptake of an NLS-containing import substrate depends on the re-addition of cytosol or cytosolic fractions (Adam *et al.*, 1990; Moore and Blobel, 1992). Based on this observation, four soluble factors have been purified and implicated in nuclear protein import: importin-α (Adam and Gerace, 1991; Görlich *et al.*, 1994, 1995a,b; Imamoto *et al.*, 1995b,c; Weis *et al.*, 1995), importin-β (Adam and Adam, 1994; Görlich *et al.*, 1995a; Chi *et al.*, 1995; Imamoto *et al.*, 1995a; Radu *et al.*, 1995), the GTPase Ran/TC4 (Melchior *et al.*, 1993; Moore and Blobel, 1993) and pp15 (Moore and Blobel, 1994; Paschal and Gerace, 1995). Importin has also been called karyopherin.

Three stages of nuclear protein import can be distinguished easily. First the import substrate binds to the importin-αβ heterodimer in the cytoplasm (Imamoto *et al.*, 1995c; Görlich *et al.*, 1995a). This NLS recognition complex docks to the nuclear pore complex (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988) via importin-β (Görlich *et al.*, 1995b; Moroianu *et al.*, 1995b) and subsequently is translocated through the pore by an energy-dependent, Ran-dependent mechanism (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988; Moore and Blobel, 1993). The constituents of the trimeric complex become separated as a result of this process. Import substrate and importin-α reach the nucleoplasm, whereas importin-β accumulates at the nuclear envelope, but not in the nucleoplasm (Görlich *et al.*, 1995b; Moroianu *et al.*, 1995b). Immunoelectron microscopy detects importin-β at both sides of the nuclear pore complex (Görlich *et al.*, 1995b), suggesting that it does not remain at its initial docking site, but moves with importin-α and the import substrate through the pore.

Here we show the importin-beta binding domain (IBB domain) is located near the N-terminus of importin-α. This 41 amino acid domain is sufficient for importin-β binding and essential for nuclear protein import. A fusion between the IBB domain and a heterologous protein is imported into the nucleus extremely efficiently, bypassing the requirement for importin-α and entering in the same way as importin-α itself. As no other part of importin-α is needed for the actual translocation, it appears that the movement which drives importin-α into the nucleus with the import substrate is generated between importin-β and structures of the nuclear pore. The IBB fusion to a heterologous protein accumulates to completion in the nuclei, in contrast to wild-type importin-α which does not, as it is returned rapidly to the cytoplasm. Thus it appears that the IBB fusion lacks the domain responsible for rapid re-export. These different requirements for entry and exit suggest that the return of importin-α to the cytoplasm is not just the reversal of its import.

Results

The complex of import substrate with importin-α and -β appears to move as a single entity through the pore

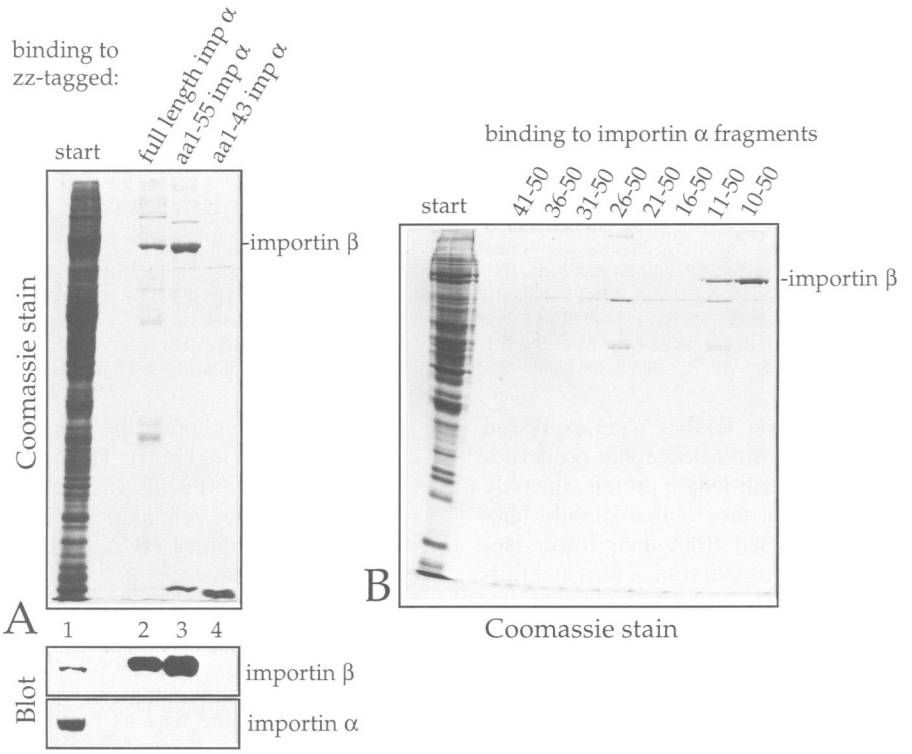

Fig. 1. Definition of an importin-α domain sufficient for high affinity binding to importin-β. Different importin-α fragments were tested for their ability to bind importin-β from a *Xenopus* egg extract (high speed supernatant, HSS). Numbers refer to the *Xenopus* importin-α amino acid sequence. (**A**) Full-length importin-α (impα), or residues 1–55 or 1–43 from importin-α were each fused to the zz-domain (IgG binding domain) from protein A and expressed in *E.coli*. Ten μg of each fusion were pre-bound to IgG–Sepharose and used to bind importin-β from 1 ml of HSS. The load of the bound fractions corresponds to 20 times the starting material; analysis was by SDS–PAGE followed by Coomassie staining or immunodetection of importin-α or -β. (**B**) Binding was as in (A), except that immmobilized synthetic peptides were used as affinity matrix and SDS was used for elution. Analysis was by PAGE followed by Coomassie staining.

(Görlich *et al.*, 1995b), and importin-β is responsible for at least the initial contact with the pore. Therefore, the question arises of whether importin-α contributes directly to the generation of movement through the pore, in addition to its role in NLS binding. Alternatively, is it only the contact of importin-β with the nuclear pore complex that matters? These questions can be addressed by monitoring accumulation in the nucleus of importin-α or its derivatives, rather than nuclear accumulation of an import substrate. Fusion of importin-α domains to a heterologous protein should assay which domain of importin-α is necessary for its transit through the pore and, specifically, for its interaction with importin-β.

Amino acids 10–50 of importin-α are sufficient for high affinity binding to importin-β

Binding to importin-β is essential in order for importin-α to get into the nucleus. Therefore, we determined which part of importin-α binds to -β. We observed previously that binding of an antibody to importin-α's N-terminus causes its quantitative dissociation from importin-β (Görlich *et al.*, 1995a). The simplest explanation would be that the importin-β binding site is near the N-terminus. To test this assumption, full-length importin-α, or its first 55 or first 43 amino acids were each tagged with the IgG binding zz-domain of protein A and expressed in *Escherichia coli*. The tag does not affect function as zz–importin-α is active in an import assay (not shown). To test the capacity of the fusions to bind importin-β, they were pre-absorbed to IgG–Sepharose and incubated with *Xenopus* egg extract. The bound fractions were released from the fusions after thorough washing, and were analysed by SDS–PAGE followed by Coomassie staining and Western blotting. As seen from Figure 1A, the first 55 amino acids of importin-α are at least as efficient as the full-length protein in binding to importin-β. Deletion of a further 12 amino acids, however, abolishes binding completely and thus defines a C-terminal border of the binding site. The alignment of the N-terminal region of *Xenopus* importin-α with other family members reveals striking conservation in amino acid positions 10–50 (Figure 2). This region is dominated by basic residues, not dissimilar to NLSs. Therefore, it should be noted that the NLS receptor, importin-α, does not bind *in trans* to its own basic clusters (Figure 1A).

To find out which of the conserved residues are crucial, a series of peptides of increasing length was synthesized, immobilized and tested for their capacity to bind importin-β from a *Xenopus* egg extract (Figure 1B). The shortest peptide that could bind covered residues 11–50. The inclusion of the conserved arginine in position 10 improved binding further.

The importin-β binding domain is essential for importin-α function

To test whether the binding domain is essential for importin-α function, a series of N-terminal importin-α truncations was expressed in *E.coli*. They seemed properly

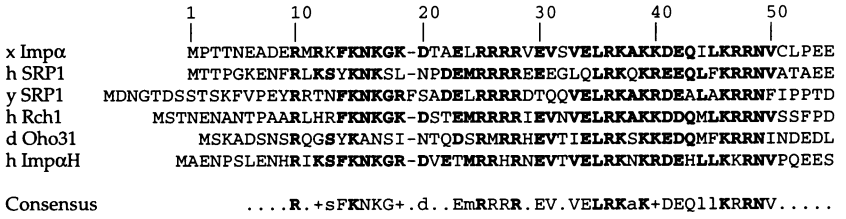

Fig. 2. Evolutionary conservation of the importin-β binding site in importin-α. The N-termini of the following members of the SRP1p/importin-α protein family were aligned: 'xImpα' is *Xenopus* importin-α form 1b (Görlich *et al.*, 1994); 'hSRP1' is human SRP1p (Cortes *et al.*, 1994); 'ySRP1' is from *Saccharomyces cerevisiae* (Yano *et al.*, 1992); 'hRch1' is human Rch1p (Cuomo *et al.*, 1994); 'dOho31' is *Drosophila melanogaster* Oho31p (Török *et al.*, 1995); 'hImpαH' is a human importin-α homologue found as a sequence tag in the database (accession no. D61288). Bold letters in the alignment indicate residues conforming to the consensus of the IBB domain. The extent of conservation is indicated in the consensus as follows: bold 6/6, upper case 4/6–5/6, lower case 3/6. '+' stands for lysine or arginine. Glutamic and aspartic acid were considered as equivalent.

folded as judged by three criteria: (i) they were expressed as soluble proteins; (ii) their chromatographic properties resembled closely those of the full-length protein, the only detectable difference being that they eluted slightly later from MonoQ, which is expected from their lower isoelectric point; (iii) the amino-terminal truncations and full-length protein bind equally well to a bovine serum albumin (BSA)–NLS conjugate but not to a conjugate with reversed NLS peptides (not shown). These truncated forms were compared with full-length importin-α for their capacity to restore import activity of an egg extract that had been depleted of importin-α (Görlich *et al.*, 1994). Fluorescein-labelled nucleoplasmin was used as a substrate. As can be seen from Figure 3, only the deletion of the first nine amino acids is tolerated without loss of activity. Deletion of the conserved arginine in position 10 roughly halves the import activity. Deletion of the next four amino acids results in a further 3-fold decrease. Hardly any import activity is detectable in the truncation lacking the first 22 amino acids; import activity is completely abolished if the first 29 or 40 amino acids have been deleted. The import activity of the N-terminal deletions is in good agreement with their ability to bind zz-importin-β (not shown). The comparison between the experiments shown in Figures 1B and 3 indicates that, in the context of the whole molecule, the importin-β binding domain is slightly more tolerant towards a partial deletion than when it is isolated.

The importin-β binding domain confers transit into the nucleus

Having identified the part of importin-α which is necessary and sufficient to bind importin-β, we wanted to know how a heterologous protein to which this domain is fused behaves in an import assay. One should expect it at least to bind to the nuclear envelope in an importin-β-dependent fashion. If any additional part of importin-α is essential for the transit through the pore, then the fusion protein should not be able to reach the nucleoplasm. If no other part is essential, the fusion should accumulate inside the nucleus.

Wild-type nucleoplasmin and the nucleoplasmin core domain alone were used as controls for nuclear accumulation and exclusion from the nuclei, respectively. A fusion between the nucleoplasmin core domain and residues 16–55 of importin-α is excluded from the nuclei (Figure 4). The fusion with residues 11–55, however, showed a low level of accumulation in the nucleus. If the conserved arginine in position 10 was included as well (amino acids 10–55 imp α npl-core), the uptake was complete and extremely rapid (Figure 4). The addition of the first nine amino acids (amino acids 1–55-imp α npl-core) did not result in a further stimulation. The comparison of the experiments in Figures 1B and 4 indicates that the ability of the tested sequences to bind importin-β correlates well with the nuclear accumulation of the corresponding fusions, even though the arginine in position 10 has a more dramatic effect on import of the fusion proteins.

If, as these results suggest, the IBB fusion enters the nucleus in the same way as importin-α, then one would predict two things. First, the IBB–core should need Ran and importin-β, but not importin-α, for nuclear accumulation, and, second, the fusion should compete with importin-α for uptake. This is confirmed by the experiment shown in Figure 5. The docking of the fusion to the nuclear envelope depends on importin-β but is competed efficiently by importin-α (see left panels 'minus Ran'). All incubations in the right hand panels were in the presence of Ran. The low level of accumulation in the absence of any extra addition is probably due to endogenous importin-β still being present in the permeablized cells (Görlich *et al.*, 1995b). Importin-α does not stimulate, but severely competes with the fusion for uptake. As expected, import is enhanced greatly by exogenous importin-β. Thus, the fusion of the IBB domain to core is taken up into the nuclei in the same way as wild-type importin-α. Not only does the IBB domain allow transit from the cytoplasmic side to the nucleoplasmic side of the pore, but the dissociation from importin-β is also triggered at the right point, resulting in the release of the fusion protein into the nucleoplasm.

The importin-β binding domain confers rapid import only but not re-export

Importin-α would be expected to shuttle, entering the nucleus with the import substrate, returning into the cytoplasm unbound, and then participating in the next round of import. Although this still needs to be demonstrated directly, a rough estimation shows that rapid recycling of the import receptor must occur to explain the import efficiency observed in a *Xenopus* egg extract. The extract, in which the cytoplasmic and nuclear compartments are still mixed, contains nucleoplasmin pentamers at a 6 μM concentration (Mills *et al.*, 1980). The same concentration of additional fluorescein-labelled nucleoplasmin is also easily taken up by the nuclei during a 20 min incubation. Therefore, although importin-α concentration is ~3 μM (Görlich *et al.*, 1994), it has to

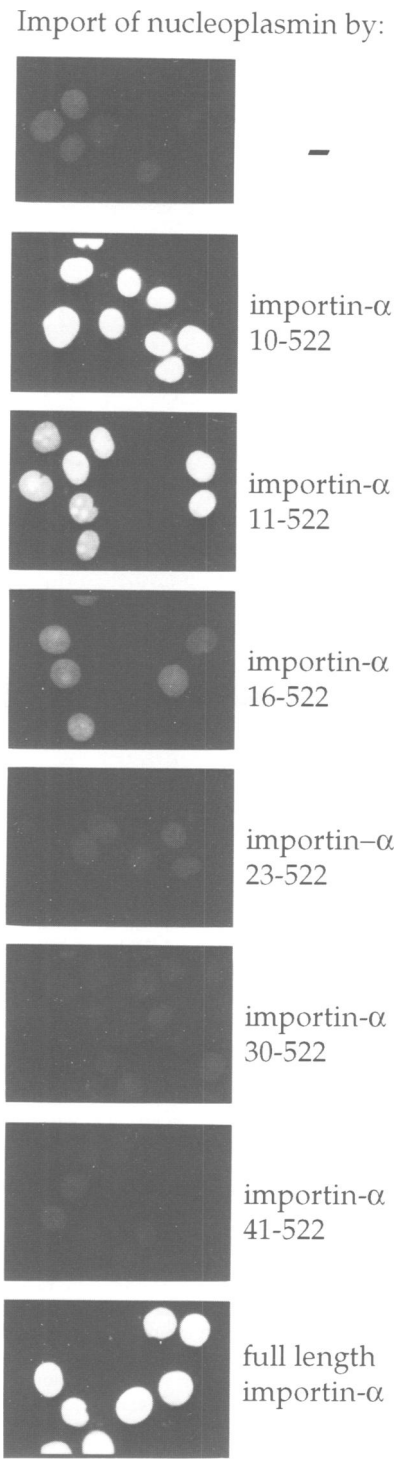

Fig. 3. Deletion of the IBB domain abolishes importin-α function. The import activities of truncated forms of importin-α were compared with the full-length protein. The importin derivatives were added to 2 μM; the substrate, fluorescein-labelled nucleoplasmin, was added to 2 μM (pentamers); all other import factors were supplied by an egg extract (high speed supernatant at 4 mg/ml protein concentration) that had been depleted of importin-α. Import was for 30 min at 23°C. Panels show confocal sections through the fixed nuclei.

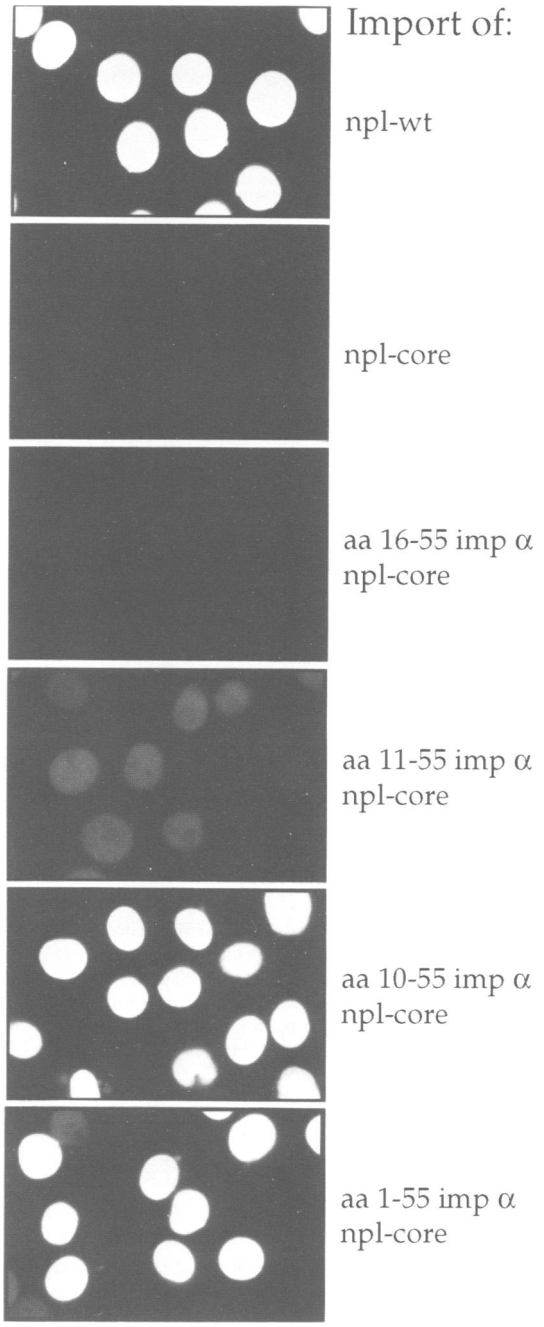

Fig. 4. The IBB domain confers transit through the pore into the nucleus. The indicated importin-α residues were fused in front of the nucleoplasmin core domain. The fusions were expressed in *E.coli*, fluorescein labelled and tested for nuclear accumulation. Full-length nucleoplasmin and the core domain are controls. Nuclei were pre-incubated for 10 min in a complete *Xenopus* egg low speed supernatant (LSS) supplemented with an energy-regenerating system. Import was started by addition of 0.4 μM import substrate (pentamers) and continued for 20 min. Panels show confocal sections through the fixed nuclei.

import 12 μM nucleoplasmin. Even assuming that one nucleoplasmin pentamer is taken in by a single importin-α molecule and that only nucleoplasmin is imported, this still means that each importin-α molecule has participited in four import cycles on average, and it gives an upper limit of 5 min for its cycling time. As nucleoplasmin accounts for only ~10% of the total nuclear protein (Mills *et al.*, 1980), in reality the cycling time for importin-α might be <1 min.

Perhaps because the return into the cytoplasm is so

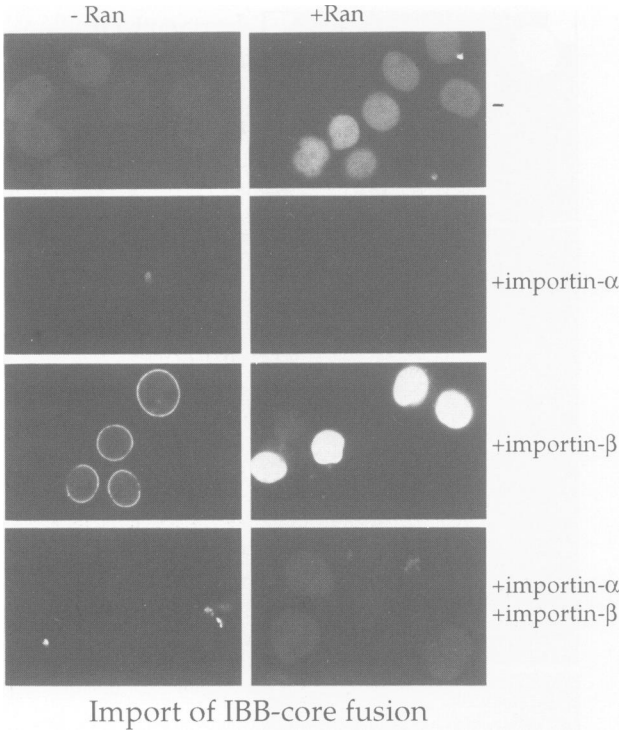

Fig. 5. The IBB–core fusion enters into the nucleus the same way as importin-α. Import of the fluorescein-labelled IBB–core fusion (residues 1–55 of importin-α) into nuclei of permeabilized HeLa cells was followed for 20 min at 23°C. Additions were as indicated: 3 μM Ran; 0.1 μM importin-β, 6 μM *Xenopus* importin-α. The order of addition was such that importin-α and -β were mixed before addition of the import substrate. Panels show confocal sections through the fixed nuclei. All reactions contained 0.3 μM IBB–core pentamers, 1 mM ATP, 200 μM GTP, 10 mM creatine phosphate, 50 μg/ml creatine kinase, 2 mM magnesium acetate, 100 mM potassium acetate, 10 mg/ml BSA and 2 mg/ml unlabelled nucleoplasmin core (to prevent unspecific binding). The pp15 homodimer (1.5 μg/ml) was also present in all reactions, even though it had no effect with this batch of nuclei (not shown).

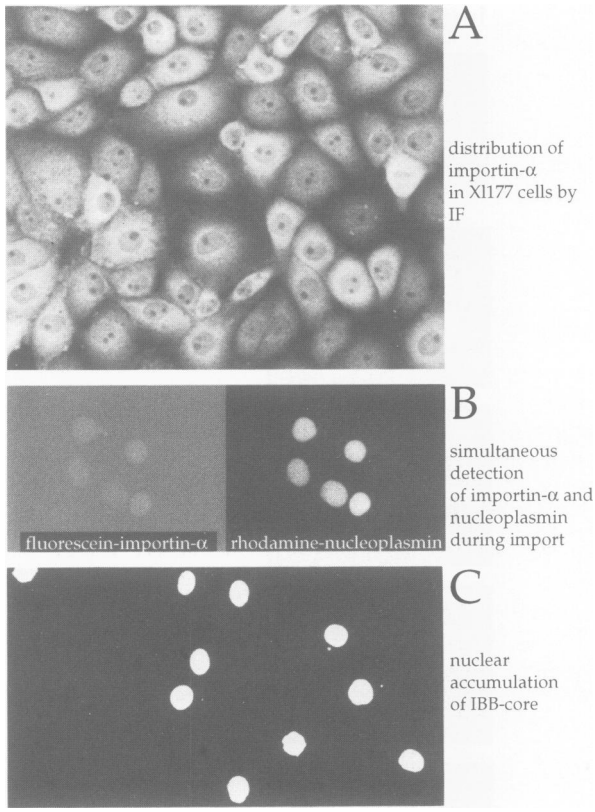

Fig. 6. The IBB domain confers nuclear entry only, but not re-export. (**A**) *Xenopus* Xl177 cells were grown on coverslips, fixed and stained as described (Görlich *et al.*, 1995b) with affinity-purified antibodies raised against recombinant *Xenopus* importin-α followed by FITC-labelled donkey anti-rabbit secondary antibody. A confocal section through the equator of the nuclei is shown. (**B**) HeLa nuclei were pre-incubated for 15 min in an undiluted *Xenopus* egg extract (low speed supernatant) which was supplemented with an energy-regenerating system. Fluorescein–importin-α and rhodamine X–nucleoplasmin (1 μM each) were added and import was allowed to proceed for 1 h at 23°C. Panels shows double channel recording of a confocal section through the unmanipulated sample, thus allowing direct comparison between cytoplasmic and nuclear concentrations of the fluorescent probes. There was no spill-over between the two channels (not shown). Note that the fluorescence intensity for importin-α remained high in the cytoplasm (the ratio of nuclear:cytoplasmic importin-α concentration was 1.2), whereas the uptake of nucleoplasmin was essentially complete. (**C**) Import reaction and analysis was essentially as in (B), but the import substrate was an IBB–core fusion (residues 10–50 of importin-α). It was detected subsequently in the fluorescein channel. A confocal section through the unmanipulated sample is shown, thus comparing directly the cytoplasmic and nuclear concentration of the fluorescent import substrate. Fluorescence intensities were ≥255 inside the nuclei (on a 0–255 scale) and <1 in the cytoplasm.

rapid, most cells in a random population of Xl177 cells show a lower nuclear than cytoplasmic importin-α concentration by immunofluorescence (Figure 6A). Similarly, fluorescein-labelled importin-α is not taken up completely into the nuclei when incubated in complete egg extract. By confocal scanning of unfixed samples, we estimate a ratio of nuclear to cytoplasmic importin-α concentration close to 1:1 (Figure 6B). In contrast, the IBB–core fusion is taken up to completion with a nuclear/cytoplasmic ratio >250 (Figure 6C). The failure of the IBB–core fusion to exit the nuclei again is not due to intranuclear binding, because it is only retained if the nuclear envelope is intact, and >98% is released if the nuclear membrane is permeabilized by 0.1% digitonin (not shown). The difference between wild-type importin-α and the fusion clearly indicates that entry into the nucleus and return into the cytoplasm have distinct requirements. We conclude that the domain responsible for rapid re-export of importin-α is missing from the IBB–core fusion and therefore that re-export involves interactions different from those needed for import.

Discussion

We have identified the domain in importin-α that binds it to importin-β. The N-terminal 55 amino acids fused to the zz-domain bind importin-β specifically and efficiently. The binding to importin-β via this domain is sufficient for a heterologous protein to transit through the nuclear pore into the nucleus in the absence of full-length importin-α. *Xenopus* and human importin-β function with different members of the importin-α protein family such as *Xenopus* importin-α, human Rch1p and *Drosophila* Oho31p (D.G., T.Török, and B.M.Mechler, unpublished).

This implies that the crucial residues for importin-β binding must be conserved between the different members of the importin-α family.

The alignment in Figure 2 shows several clusters of conserved residues between positions 10 and 50. The deletion of the preceding nine amino acids has no effect on either import activity of importin-α itself or the efficiency of import of the IBB–core fusion. Deletion of the conserved Arg10 decreases binding of the peptide to importin-β and the transport activity of importin-α to ~50%. The effect on the import of the IBB–core fusion is far more severe, thus the extent of the contribution of this residue shows some context dependence. The peptide lacking a further five amino acids shows no detectable importin-β binding and the corresponding fusion to the core domain is excluded from the nuclei. The corresponding truncated importin-α, however, retained ~15% activity as compared with full-length protein. The first 29 amino acids need to be deleted to abolish importin-α activity entirely. In conclusion, the IBB domain is absolutely required for function; furthermore, the complete domain is needed for full activity. The extent to which a partial deletion of the domain reduces activity depends on the assay applied.

A parallel study by Weis and colleagues (1996, accompanying paper) also provides unequivocal evidence that the N-terminus of the import receptor (a human importin-α homologue) binds to importin-β and is absolutely required for function. In the light of this corroborating evidence, the question arises of how Moroianu *et al.* (1995a) could have obtained data showing that the human importin-α homologue, hSRP1p, functions in nuclear import when their constructs lacked the entire IBB domain (the first 49 amino acids) and therefore should neither bind importin-β nor promote transport. This question needs to be answered.

The middle domain of importin-α consists of eight hydrophobic repeats called arm motifs (Peifer *et al.*, 1994). This domain constitutes the NLS binding site (Cortes *et al.*, 1994). Interestingly, importin-β has similar repeats; depending on the algorithm used, up to 11 internal repeats can be found (Bork and Andrade, 1995; Görlich *et al.*, 1995a), two of which are particularly closely related to two of the arm motifs found in the α-subunit (Görlich *et al.*, 1995a). As the IBB domain and a NLS have clusters of basic amino acids in common, we speculate that their recognition site might be similar as well. Therefore, it does not seem unlikely that the arm motifs in importin-β might be involved in importin-α binding.

This IBB domain can be considered as an archetypal nuclear targeting sequence in both a structural sense (basic clusters) and a functional sense (targeting of the import receptor to the nucleoplasm). Why do nuclear proteins use NLSs and not the IBB domain to enter the nucleus? The 41 residue IBB domain is considerably larger than even a bipartite NLS. An additional constraint appears to be that the sequence preceding the IBB domain needs to be quite flexible. For example, the IBB domain works well when fused in front of the nucleoplasmin core domain, but not when fused straight to the C-terminus; there the spacer length had to be increased to 28 residues to make it function at all (data not shown). A classical NLS is certainly easier to accommodate within a given protein structure. This might be one reason why importin-α is needed as an adapter between the structurally constrained 'primary nuclear targeting signal' (namely the IBB domain) and an NLS which can better be integrated into the structure of a nuclear protein. The presence in higher eukaryotes of different SRP1p/importin-α family members probably increases the range of substrate specificity, i.e. the range of NLS that can function.

How can a shuttling NLS receptor achieve a net import? For example, how can nucleoplasmin from an egg extract be completely taken up into nuclei, but nuclear importin-α hardly exceeds the cytoplasmic concentration at any time? How can importin-α return to the cytosol without the import substrate? The most likely explanation is that the return of importin-α out of the nucleus is not the simple reversal of its entry. Importin-α and -β enter the nucleus together as a complex, i.e. at the same rate. However, only importin-α accumulates in the nucleoplasm, importin-β does not. Thus the two subunits are returned to the cytoplasm at different rates and possibly by separate routes. The observation that the IBB domain apparently confers rapid import only, but not export, is a further indication that some other factor and not importin-β mediates the return of importin-α to the cytoplasm, or that importin-α makes a direct contact with the pore on its way back. In either case, the export-competent conformation of importin-α must be the low affinity form for NLS binding, so that importin-α can leave the nucleus without the cargo that it has carried in.

Materials and methods

cDNA constructs and recombinant expression

The expression of *Xenopus* importin-α (C-terminally His-tagged), human importin-β, Ran/TC4 and nucleoplasmin were described before (Görlich *et al.*, 1994, 1995a,b). Nucleoplasmin core was expressed in this study from pQE9 (Qiagen) with an N-terminal His tag.

Appropriate restriction sites for the construction of new constructs were introduced by PCR with Pwo proofreading DNA polymerase (Boehringer); those constructs were controlled by DNA sequencing.

For zz tagging, we modified pQE70 (Qiagen) as follows: the cDNA for the IgG binding zz-domain (without signal sequence) was amplified from pezz18 (Pharmacia) generating an *Nla*III site at the 5′ end (after cutting gives compatible ends to *Sph*I) and a *Sph*I site at the 3′ end. This fragment was cloned into the *Sph*I site of pQE70 and selected for the correct orientation. The resulting pzz70 vector is used for expressing proteins with an N-terminal zz tag and a C-terminal His tag.

zz full-length importin-α was generated by direct re-cloning from pQE70 into pzz70. To generate zz–amino acids 1–55 importin-α and zz–amino acids 1–43 importin-α, a *Bam*HI site was introduced after the codons for the 55th or 43rd amino acids, respectively, and the resulting fragments were cloned into the *Sph*I–*Bam*HI sites of pzz70.

For the construction of the N-terminal truncations of importin-α, a *Sph*I site was introduced before the indicated amino acid, the fragments were cloned into the *Sph*I–*Bam*HI sites of pQE70 and expressed with a C-terminal His tag. The cloning procedure resulted in the following amino acids preceding the actual coding sequence (one letter code, numbers refer to residues in importin-α): MQ 10–522; none 11–522; MQ 15–522; MN 23–522; ML 30–522; MQ 41–522.

The starting point for the fusions of the N-terminal importin-α sequences to nucleoplasmin core was a pQE9 vector (Qiagen) containing in its *Bam*HI–*Hin*dIII sites the nucleoplasmin core domain (residues 1–149 of nucleoplasmin). The indicated segments were cloned as *Bam*HI–*Bam*HI fragments between the N-terminal His tag and the core domain. Thus the actual importin-α sequence was preceded by GS.

The protein A and nucleoplasmin core fusions were expressed in *E.coli* at 37°C and purified on nickel–agarose; importin-α and its N-terminal truncations were expressed at 28°C and purified on nickel–agarose followed by MonoQ.

Peptide synthesis

Peptides were synthesized on an ABI433A Peptide Synthesizer using FastMoc chemistry essentially according to a standard protocol (Atherton and Sheppard, 1989). Peptides were purified by HPLC on a 300×40 mm VYDAC TPB 1520 300A C18 reverse phase column and checked by mass spectroscopy. Peptides were synthesized with an acetylated N-terminus and a C-terminal cysteine. The peptide corresponding to residues 10–50 had two additional non-conserved residues (QE) at its N-terminus. The peptides were coupled to sulfo-Link (Pierce) aiming at a concentration of 1 mg/ml resin. Unreacted sites were quenched with mercaptoethanol.

Binding to importin-β

A low speed supernatant from activated *Xenopus* eggs was diluted 5-fold in binding buffer (50 mM Tris–HCl, 500 mM NaCl, 5 mM magnesium acetate, 10 mM mercaptoethanol). The high speed supernatant (HSS) was prepared by ultracentrifugation aiming to sediment particles >30S. Importin-β was bound for 5 h from 1 ml of HSS to 20 µl of IgG–Sepharose (Pharmacia) with ~10 µg of the zz fusions pre-bound, or to 20 µl of the immobilized peptides. The resins were washed five times with 1 ml of binding buffer, including an overnight wash. Bound fractions were released with SDS (Figure 1B) or with 1 M magnesium chloride followed by precipitation with 90% isopropanol (Figure 1A).

Fluorescence labelling

Labelling was performed in Tris-buffered saline (TBS). Fluorescein 5-maleimide (Pierce) or rhodamine X iodoacetamide (Molecular Probes), dissolved in dimethylformamide were added to the protein in a 1:1 molar ratio (referring to monomers for the nucleoplasmin derivatives). Reactions were allowed to proceed for 30 min at 37°C (nucleoplasmin derivatives) or 2 h on ice (importin-α). Reactions were quenched by 50 mM mercaptoethanol, and free label was removed by subsequent chromotographies on G25 and Superdex 200 (Pharmacia) equilibrated in TBS. Labelling was nearly quantitative for nucleoplasmin derivatives and ~70% for importin-α.

Import assays

The basic method has been described before (Görlich *et al.*, 1994, 1995a,b). Importin-α was depleted in two rounds from the *Xenopus* egg HSS (in 20 mM HEPES–KOH pH 7.5, 80 mM potassium acetate, 3 mM magnesium acetate, 10 mM mercaptoethanol) by an immobilized antibody raised against importin-α's N-terminus (Görlich *et al.*, 1994). Additional details are mentioned in the figure legends.

Acknowledgements

We thank A.D.Mills for performing the immunofluorescence. Iain W.Mattaj for stimulating discussion and K.Weis *et al.* for communicating results prior to publication. This work was supported by the Cancer Research Campaign, the Deutsche Forschungsgemeinschaft and an HFSPO long term fellowship (to D.G.).

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Received on November 15, 1995; revised on December 18, 1995