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# The conserved amino-terminal domain of hSRP1α is essential for nuclear protein import

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**Nuclear proteins are targeted through the nuclear pore complex (NPC) in an energy-dependent reaction. The import reaction is mediated by nuclear localization sequences (NLS) in the substrate which are recognized by heterodimeric cytoplasmic receptors. hSRP1α is an NLS-binding subunit of the human NLS receptor complex and is complexed *in vivo* with a second subunit of 97 kDa (p97). We show here that a short amino-terminal domain in hSRP1α is necessary and sufficient for its interaction with p97. This domain is conserved in other SRP1-like proteins and its fusion to a cytoplasmic reporter protein is sufficient to promote complete nuclear import, circumventing the usual requirement for an NLS receptor interaction. The same amino-terminal domain inhibits import of NLS-containing proteins when added to an *in vitro* nuclear transport assay. While full-length hSRP1α is able to leave the nucleus, the amino-terminal domain alone is not sufficient to promote exit. We conclude that hSRP1α functions as an adaptor to tether NLS-containing substrates to the protein import machinery.**

*Keywords*: hSRP1α/NLS/nuclear import/nuclear localization signals/protein transport

## Introduction

The transport of molecules between the cytoplasm and the nucleus occurs through a large proteinaceous structure, called the nuclear pore complex (NPC). Whereas small molecules and ions freely diffuse through the NPC, uptake of most karyophilic proteins is energy dependent and is mediated by a specific nuclear localization signal (NLS) in their primary sequence [reviewed in Powers and Forbes (1994) and Melchior and Gerace (1995)]. Experimentally, it is possible to distinguish at least two distinct steps in nuclear protein import. First, there is an energy-independent docking of the import substrate at the nuclear envelope and, second, an energy-requiring translocation step through the NPC (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988).

Recently, an *in vitro* tranport system based on digitonin-permeabilized mammalian cells has been developed (Adam *et al.*, 1990). This has facilitated the identification of cytosolic fractions that are rate limiting for nuclear protein import (Moore and Blobel, 1992). A number of studies using this assay system have reported the characterization and cloning of at least four different protein factors that seem to play an essential role in the uptake of NLS-containing proteins into the nucleus (reviewed in Sweet and Gerace, 1995).

The cytoplasmic recognition of the NLS motif and the subsequent docking of the substrate at the nuclear envelope are mediated by a protein complex which consists of two subunits. One is a 50–60 kDa factor which belongs to a large family of proteins, all homologous to the yeast SRP1p protein (Yano *et al.*, 1992). This subunit has been shown to bind directly to the NLS motif and is therefore believed to be responsible for the primary NLS recognition. Homologues of yeast SRP1p have been isolated and cloned from multiple species including bovine, *Xenopus*, human, mouse and *Drosophila*, and have been called either the NLS receptor (Adam and Gerace, 1991), importin α (Görlich *et al.*, 1994), Rch1/hSRP1α (Cuomo *et al.*, 1994; Weis *et al.*, 1995), hSRP1/NPI-1 (Cortes *et al.*, 1994; O'Neil and Palese, 1995), karyopherin α (Moroianu *et al.*, 1995a), m-importin (Imamoto *et al.*, 1995b) or pendulin/OHO31 (Küssel and Frasch, 1995; Török *et al.*, 1995). The second subunit of the cytoplasmic NLS receptor complex is a 97 kDa protein. Again, homologues have been characterized from different species, including rat, human, mouse and yeast. This factor has been named karyopherin β (Radu *et al.*, 1995a), importin β (Görlich *et al.*, 1995a), p97 (Adam and Adam, 1994; Chi *et al.*, 1995), PTAC 97 (Imamoto *et al.*, 1995a) or Kap95p (Enenkel *et al.*, 1995). The 97 kDa subunit is required for interaction of the substrate–NLS receptor complex with the NPC (Chi *et al.*, 1995; Görlich *et al.*, 1995a,b; Imamoto *et al.*, 1995a; Moroianu *et al.*, 1995b) and direct binding of this factor to nuclear pore proteins has been demonstrated (Radu *et al.*, 1995b). Whereas the 60 kDa subunit enters the nucleus together with its cargo during the import reaction, the 97 kDa subunit stays bound at the nuclear envelope, indicating that the two subunits of this complex dissociate in the course of the import reaction (Görlich *et al.*, 1995b; Moroianu *et al.*, 1995b).

Translocation of the substrate–NLS receptor complex through the NPC *in vitro* is dependent on the GTPase Ran/TC4 (Melchior *et al.*, 1993; Moore and Blobel, 1993), which was originally identified as a small nuclear ras-like protein (Drivas *et al.*, 1990). Apart from its direct role in nuclear protein import, the GDP/GTP cycle of Ran has also been implicated in a large number of other processes, including nuclear RNA export and the regulation of the cell cycle (reviewed by Dasso, 1993; Izaurralde and Mattaj, 1995).

A fourth soluble transport factor, p10, has been shown to enhance the efficiency of nuclear transport *in vitro*. This factor, together with Ran/TC4, was shown to reconstitute the complete translocation step in the permeabilized

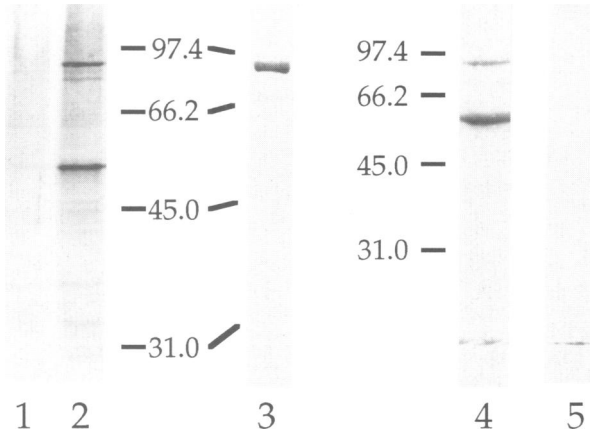


**Fig. 1.** Co-precipitation of native and recombinant p97 with hSRP1α. Immunopurification of hSRP1α from HeLa cell cytosolic extracts with a specific anti-hSRP1α antiserum (Weis *et al.*, 1995) coupled to protein A–Sepharose beads leads to a specific co-precipitation of a protein of ~90 kDa (lane 2). Peptide analysis revealed that the 90 kDa protein is identical to the transport factor p97. Neither hSRP1α nor p97 are recovered with the pre-immune serum coupled to protein A–beads (lane 1). Human cDNA clones for p97 were obtained and used to express the full-length protein in *E.coli*. Recombinant p97 was purified with FPLC on a MonoQ column and an additional hSRP1α affinity column. The purity was analysed by Coomassie staining after separation on SDS–PAGE (lane 3). To test whether the hSRP1α and p97 can also form a complex *in vitro*, 2 μg of untagged recombinant p97 were incubated in 100 μl IPP buffer (see Materials and methods) either in the absence (lane 5) or in the presence of the same amount of recombinant His-tagged hSRP1α (lane 4) and then co-selected with Ni-NTA agarose beads. The selected proteins were analysed by Coomassie staining after 10% SDS–PAGE (lanes 4 and 5).

cell assay (Moore and Blobel, 1994). The same protein was independently isolated by its ability to interact with the nuclear pore protein p62 and again shown to be an essential transport factor *in vitro* (Paschal and Gerace, 1995). In this study, it was called NTF2.

Here, we identify a short amino-terminal domain of the human NLS-binding protein, hSRP1α, that interacts with the protein import factor p97. We demonstrate that this evolutionarily conserved domain in hSRP1α is both necessary and sufficient for the interaction with p97. We identify the role of hSRP1α as an adaptor protein which functions as a link between the NLS-containing substrate and the downstream transport machinery.

## Results

### *Interaction of hSRP1α with p97*

We have recently shown that a human NLS-binding protein, hSRP1α, forms a stable complex *in vivo* with a second protein of ~90 kDa (Weis *et al.*, 1995). Thus, the 90 kDa factor is co-precipitated from cytosolic HeLa cell extracts by an antiserum specific for hSRP1α (Figure 1). This affinity purification allowed the isolation of sufficient amounts of the 90 kDa component to perform direct sequence analysis (see Materials and methods for details). While this work was in progress, the same 97 kDa protein import factor was identified by several other groups, along with homologues from other species (Chi *et al.*, 1995; Enenkel *et al.*, 1995; Görlich *et al.*, 1995a; Imamoto *et al.*, 1995a; Radu *et al.*, 1995a). From these studies, it was established that this conserved protein is essential for protein import into the nucleus and is involved in the docking of the import substrate at the nuclear envelope (Chi *et al.*, 1995; Görlich *et al.*, 1995a; Imamoto *et al.*, 1995a; Radu *et al.*, 1995a).

For *in vitro* studies, we overexpressed the full-length human p97 protein in *Escherichia coli* and purified it as described in Materials and methods (Figure 1). Recombinant human p97 is active in stimulating nuclear protein import in a homologous *in vitro* transport system with pure recombinant human import factors (see below). To test whether the *in vivo* interaction between hSRP1α and p97 can also be mimicked *in vitro*, we performed co-selection experiments on Ni-NTA agarose beads with untagged recombinant human p97 in the presence (Figure 1, lane 4) or absence (Figure 1, lane 5) of $His_6$-tagged recombinant human hSRP1α. This showed that the two proteins can form a stable complex *in vitro*. Furthermore, these data show that the interaction between hSRP1α and p97 is not dependent on the presence of NLS-containing proteins.

### *Amino terminus of hSRP1α mediates interaction with p97*

The SRP1 protein family is characterized by a conserved domain organization comprising eight copies of the arm motif (Pfeifer *et al.*, 1994) flanked by additional short sequences at both the amino and carboxy termini (Figure 2A). Our analysis of the NLS-binding properties of hSRP1α deletion mutants maps the NLS binding site within these conserved arm repeats (K.Weis, in preparation). To identify the site in hSRP1α that interacts with p97, we constructed a further series of deletion mutants of hSRP1α (Figure 2B). These mutants were analysed for their ability to bind *in vitro* both to $^{35}$S-labelled p97 and to an NLS-containing reporter protein (Figure 3). Full-length hSRP1α bound efficiently to $^{35}$S-labelled p97 in this assay system (Figure 3, lane 1). In contrast, deletion of the entire amino-terminal flanking region (amino acids 1–65), leaving intact all eight copies of the arm motifs, completely prevented interaction with p97 (Figure 3, lane 2). However, both this mutant and the full-length hSRP1α could bind the NLS-containing reporter protein (Figure 3, lanes 1 and 2). We therefore conclude that interaction with p97, but not NLS binding, requires the amino-terminal flanking domain of hSRP1α.

We next tested whether this amino-terminal domain was sufficient for binding to p97. For this, the first 65 amino acids of hSRP1α were expressed in *E.coli* fused at the amino terminus to a tag including six histidine residues (see Materials and methods). This truncated domain bound efficiently to p97, but was unable to bind the NLS-containing reporter protein (Figure 3, lane 3). We conclude that the amino-terminal domain of hSRP1α is both necessary and sufficient for interaction with p97, but is not required for NLS binding. This was supported by analysis of a similar amino-terminal fragment of a second human SRP1-homologue, NPI (O'Neil and Palese, 1995). In this case, a fragment of NPI including amino acids 1–71 was also sufficient for binding to p97 (Figure 3, lane 4).

Figure 4 shows a comparison of the amino-terminal domains of five members of the SRP1 protein family

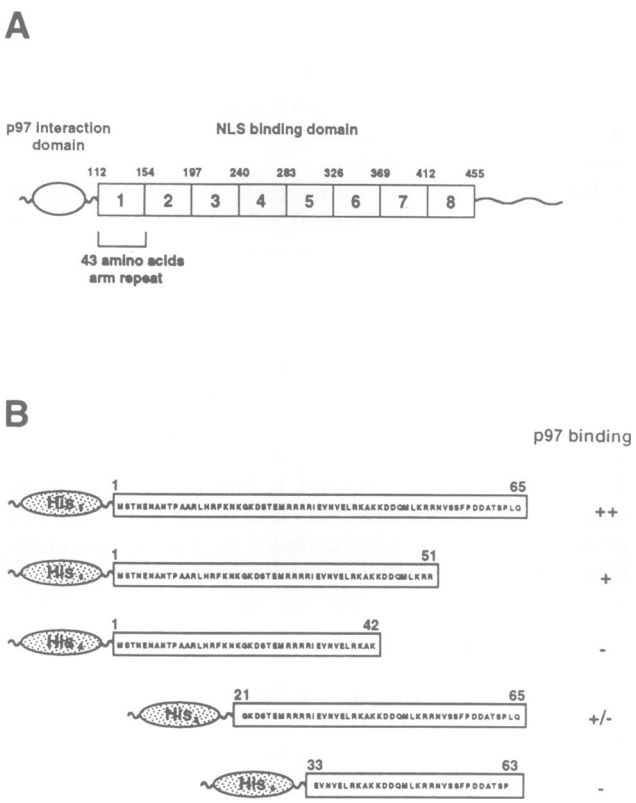


**Fig. 2.** (**A**) Domain organization of hSRP1α. The structure of hSRP1α is characterized by eight repeats of the 43 amino acid long arm motif (Pfeifer *et al.*, 1994) flanked by short sequences both at the amino and carboxy termini. The numbers indicate the amino acid position of the individual arm repeats. (**B**) Deletion mutants of hSRP1α. The figure shows the different deletion mutants of hSRP1α that were used in this study. All mutants were fused to the carboxy terminus of a $His_6$ cassette and expressed in *E.coli*. Numbers indicate the amino acid position in hSRP1α of the different fragments.

from yeast, *Xenopus*, *Drosophila* and human. The amino terminus shows several regions of high conservation and its amino acid composition is strongly basic. For example, the pI of the first 65 amino acids of hSRP1α is 11.3. We note that the extensive stretches of basic residues within this domain show some similarity to a canonical NLS motif. However, we have been unable to detect any interaction between this amino-terminal domain and the arm motifs of hSRP1α (data not shown), suggesting that it does not act as its own substrate.

To better define the region of hSRP1α required for interaction with p97, we analysed additional truncations within the amino-terminal domain (Figure 5). A His-tagged fragment including amino acids 1–51 of hSRP1α still bound to p97 with efficiency similar to full-length hSRP1α (Figure 5, lanes 1 and 3). A shorter fragment including amino acids 1–42 no longer binds p97 (Figure 5, lane 4). A fragment including amino acids 21–65 is still able to bind p97, albeit with clearly reduced affinity compared with either full-length hSRP1α or with the amino-terminal fragment including amino acids 1–65 (Figure 5, compare lane 5 with lanes 1 and 2). A fragment including amino acids 33–63 does not bind p97 (Figure 5, lane 6).

From these mapping data, we conclude that an essential region at the amino terminus between amino acids 21

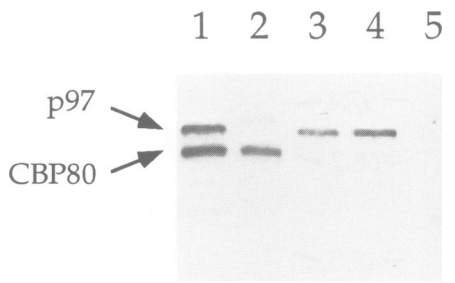


**Fig. 3.** Co-selection of p97 and an NLS reporter by various hSRP1α mutants. Around 10 pmol of either full-length hSRP1α (lane 1), the carboxy terminus of hSRP1α (amino acids 66–529; lane 2), a fragment of hSRP1α containing amino acids 1–65 (lane 3) or the amino-terminal 71 amino acids of NPI (lane 4) were incubated in 100 μl IPP buffer with *in vitro* translated $^{35}S$-labelled p97 and with *in vitro* translated CBP80, a nuclear CAP-binding protein which contains a bipartite NLS (Izaurralde *et al.*, 1994). Lane 5 shows the background binding of $^{35}S$-labelled p97 and CBP80 to Ni-NTA beads in the absence of hSRP1α. Proteins were co-selected with Ni-NTA agarose, separated by 10% SDS–PAGE and analysed with a Phosphorimager.

and 51 is required for binding to p97, while additional sequences between amino acids 1 and 21 can also enhance binding.

### *Amino terminus of hSRP1α is a dominant inhibitor of protein import*

Since the amino-terminal domain of hSRP1α alone can bind to p97, but not to NLS motifs, this predicts that addition of the amino-terminal domain to an *in vitro* transport assay should inhibit nuclear import of NLS-containing proteins. To test this prediction, increasing amounts of the recombinant fragment containing amino acids 1–65 of hSRP1α were added to *in vitro* transport assays performed with a *Xenopus* egg extract (Figure 6A–C). This shows a concentration-dependent inhibition of nuclear transport of a bovine serum albumin (BSA)–NLS reporter protein by the amino-terminal hSRP1α fragment. Quantitation of these data shows that inhibition is >95% with a 20 μM concentration of the amino-terminal fragment, while 50% inhibition is obtained beween 2 and 3 μM (Table I). In contrast, addition of equivalent amounts of the hSRP1α fragment containing amino acids 1–42, that was unable to bind p97 (cf. Figure 5), did not inhibit nuclear transport (Figure 6D). These data support the view that the inhibition arises through a simple competition mechanism, since the concentration of SRP1-like proteins in *Xenopus* egg extracts was estimated to be around 3 μM (Görlich *et al.*, 1994).

### *Fusion of the amino terminus of hSRP1α to a reporter protein directs nuclear import*

Since separate domains of hSRP1α are involved in the binding to p97 and the NLS motif, hSRP1α appears to function as an adaptor that attaches NLS-containing proteins to the downstream transport machinery. If this is the case, fusion of the amino-terminal domain of hSRP1α to a cytoplasmic reporter protein should promote receptor-independent nuclear transport of the reporter by allowing its direct interaction with p97. To test this hypothesis, we fused the amino-terminal domain of hSRP1α to β-galactosidase, which does not enter the nucleus by itself, and analysed whether the fusion protein would migrate to

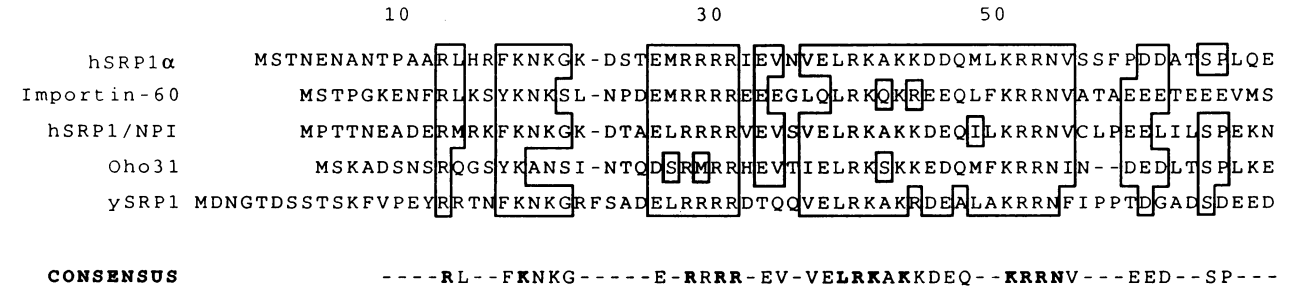


**Fig. 4.** Alignment of the conserved amino termini of different SRP1p homologues. The amino termini of human hSRP1α (Weis *et al.*, 1995), *Xenopus* importin-60 (Görlich *et al.*, 1994), human hSRP1/NPI (Cortes *et al.*, 1994; O'Neil and Palese, 1995), *Drosophila* Oho 31/pendulin (Küssel and Frasch, 1995; Török *et al.*, 1995) and yeast SRP1 (Yano *et al.*, 1992) were aligned. A consensus was generated when at least three out of five residues at the respective amino acid position were indentical. Bold letters were used when the respective amino acid residue was identical in all five homologues.

the nucleus in an *in vitro* transport system. For this assay, the recombinant human transport factors p97, Ran and p10 were used. The fusion protein alone does not enter the nucleus (Figure 7A). Addition of recombinant p97 led to an accumulation of the fusion protein at the nuclear envelope (Figure 7B). Addition of all three transport factors, p97, Ran and p10, resulted in efficient nuclear import of the fusion protein (Figure 7C). Import of the fusion protein is energy dependent (data not shown). Incubation of β-galactosidase without the amino-terminal domain of hSRP1α did not result in nuclear import even in the presence of all three transport factors (Figure 7D). We note that p97, Ran and p10 alone were not sufficient to promote nuclear import of an NLS-containing substrate (Figure 7E). Consistent with earlier studies (Görlich *et al.*, 1994; Moroianu *et al.*, 1995a; Weis *et al.*, 1995), import of NLS-containing substrates is strictly dependent on the presence of an NLS-binding protein, such as hSRP1α (Figure 7F).

The above data indicate that the amino-terminal domain of hSRP1α is able to promote NLS-independent nuclear transport when fused to a cytoplasmic reporter protein. This predicts that a deletion of the amino-terminal domain of hSRP1α should abolish its ability to promote nuclear transport of NLS-containing substrates by preventing interaction with p97. This was confirmed in the *in vitro* transport assay where an NLS-containing substrate was not imported in the presence of p97, Ran, p10 and the deletion mutant of hSRP1α lacking amino acids 1–65 (Figure 7G). Further confirmation of the essential role of the amino-terminal domain in nuclear import was provided by a domain-swap experiment where fusion of the amino-terminus of a second human SRP1-homologue, NPI, to the inactive deletion mutant of hSRP1α restored nuclear import of the NLS-containing substrate (Figure 7H).

### *hSRP1α can both enter and exit the nucleus*

Immunofluorescence analysis using antisera specific for hSRP1α (Weis *et al.*, 1995) shows that the protein is localized both in the nucleus and cytoplasm (Figure 8A). This is supported both by biochemical fractionation studies (K.Weiss and A.I.Lamond, unpublished data) and by previous immunofluorescence analysis (Görlich *et al.*, 1995b; Imamoto *et al.*, 1995a; Moroianu *et al.*, 1995b). To demonstrate directly that hSRP1α can exit as well as enter the nucleus, we microinjected His-tagged hSRP1α together with fluorescently labelled dextran sulfate (mol. wt 70 000) as a control into HeLa cell nuclei. The distribution of hSRP1α was analysed with an anti-$His_6$ monoclonal antibody (Figure 8B). This shows that nuclear hSRP1α is able to translocate into the cytoplasm. As expected from earlier studies (Görlich *et al.*, 1995b; Imamoto *et al.*, 1995a; Moroianu *et al.*, 1995b), hSRP1α can also enter the nucleus when microinjected into the cytoplasm (data not shown). We have shown above that the amino-terminal domain of hSRP1α is sufficient to promote nuclear import when fused to a cytoplasmic reporter protein. To see whether this amino-terminal domain is also sufficient to promote exit from the nucleus, we microinjected the amino-terminal β-galactosidase fusion protein into either nuclei or cytoplasm of HeLa cells. Again, the injection was monitored by the co-injection of fluorescent dextran sulfate. Immunofluorescence analysis revealed that the fusion protein exclusively localizes to the nucleus, irrespective of its site of injection (Figure 8C and D). We conclude from this that the amino-terminal domain of hSRP1α can promote β-galactosidase entry but not exit from the nucleus. To further confirm this result, we examined the behaviour of these proteins using permeabilized cells. For these experiments, the

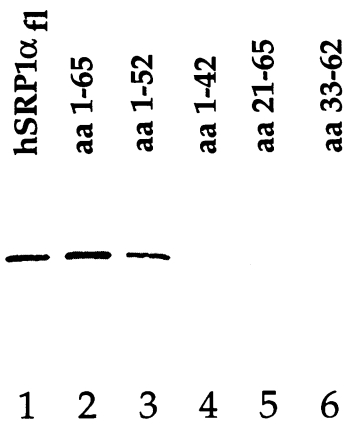


**Fig. 5.** Definition of the interaction domain between hSRP1α and p97. Various deletion fragments of hSRP1α were tested for their ability to bind to *in vitro* translated $^{35}S$-labelled p97 protein. Around 10 pmol of either full-length hSRP1α (lane 1), residues 1–65 of hSRP1α (lane 2), residues 1–52 of hSRP1α (lane 3), residues 1–42 of hSRP1α (lane 4), residues 21–65 of hSRP1α (lane 5) or residues 33–62 of hSRP1α (lane 6) were incubated with $^{35}S$-labelled p97 protein in 100 μl IPP buffer. Co-selection with Ni-NTA agarose and analysis of the bound proteins were as described in Figure 3.

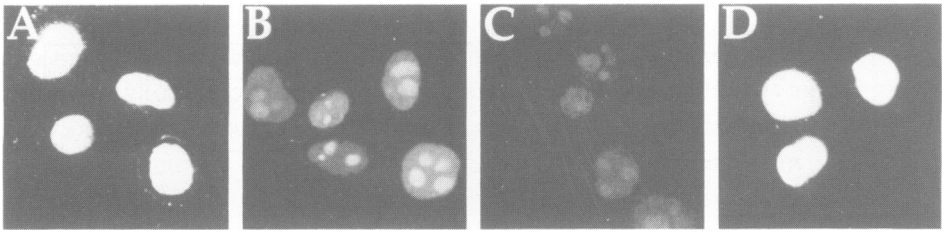


**Fig. 6.** Inhibition of *in vitro* nuclear import by the amino-terminal domain of hSRP1α. *In vitro* transport assays were performed with digitonin-permeabilized HeLa cells and *Xenopus* egg extracts either in the absence (**A**) or in the presence of a 2 μM (**B**) or 20 μM (**C**) concentration of the amino-terminal domain of hSRP1α. In (**D**), a 20 μM concentration of the fragment containing amino acids 1–42 of hSRP1α was added. Import reactions shown in the panels were incubated for 30 min at RT using FITC-labelled BSA–NLS conjugate as an import substrate. Images were recorded with a confocal fluorescence microscope.

proteins were introduced into the nucleus in an *in vitro* import reaction, then external factors removed by washing, followed by incubation of the nuclei with fresh *Xenopus* egg extract (Figure 8E–H). This showed that >50% of the imported hSRP1α protein was able to leave the nucleus (Figure 8E and F), while the fusion protein remained concentrated in the nucleus (Figure 8G and H). We conclude that the amino-terminal domain of hSRP1α, when fused to a reporter protein, can direct entry into, but not exit out of, the nucleus.

**Table I.** Inhibition of nuclear uptake of BSA–NLS conjugates in the presence of the amino-terminal domain of hSRP1α

| Concentration of inhibitor (μM) | Transport (%) |
|---|---|
| 0 | 100 ± 8.4 |
| 2 | 58 ± 3.2 |
| 5 | 27 ± 2.2 |
| 10 | 21 ± 0.5 |
| 20 | 5 ± 2.6 |

The uptake of fluorescent BSA–NLS was quantified after *in vitro* transport reactions. The value obtained in the absence of inhibitor was taken as 100% transport. The table shows the average nuclear fluorescence of >50 cells in each experiment. The errors indicate the SD values.

## Discussion

In this study, we have shown that the human NLS receptor subunit hSRP1α (Weis *et al.*, 1995) has a modular structure, with separable functional domains that are involved in binding to the NLS motif and to the import factor p97, respectively. The interaction domain required for binding to p97 was mapped to a conserved amino-terminal region of hSRP1α. The domain is located between amino acids 1 and 51, with residues between amino acids 21 and 51 being particularly important for binding. The presence of this amino-terminal domain is essential for the function of hSRP1α in promoting nuclear transport of NLS-containing substrates. Furthermore, fusion of this amino-terminal domain to a cytoplasmic reporter protein was sufficient to promote its accumulation in the nucleus in a reaction that circumvented the usual requirement for an NLS.

These data lead us to conclude that hSRP1α functions as an adaptor which tethers NLS-containing substrates to p97. It is the interaction with p97 and other downstream transport factors which then conducts the transport of the substrate through the NPC. Neither the NLS substrate nor other parts of hSRP1α seem to be directly required for the translocation step. Considering this role as an adaptor, it is interesting that there is a large family of SRP1-like proteins with multiple homologues in different species. At least two human SRP1 homologues, hSRP1α/Rch1 and hSRP1/NPI, have been shown to bind NLS motifs (Moroianu *et al.*, 1995a,b; Weis *et al.*, 1995) and, as shown here, both of these human proteins interact through their amino termini with p97. The use of a family of adaptor proteins which interact with a common downstream transport machinery could allow the recognition of diverse NLS motifs in different nuclear proteins and facilitate their nuclear transport by a common mechanism. It will be interesting now to investigate whether different SRP1 homologues preferentially recognize distinct subsets of NLS-containing substrates.

The conserved amino-terminal region of hSRP1α that interacts with p97 is highly basic and includes a core region which resembles a typical NLS motif. Although we did not detect any binding of this region to hSRP1α, the fact that its fusion to a cytoplasmic protein causes nuclear transport shows that it represents a type of nuclear localization signal itself. Since in this case the substrate can interact with p97 directly, without the requirement of an additional NLS adaptor, we speculate that this basic amino-terminal domain may resemble an archetypal NLS. The use of the family of NLS adaptor proteins may have evolved because it confers greater flexibility in the recognition of diverse NLS motifs in different nuclear proteins.

The essential role of the amino-terminal domain has been demonstrated here in several ways. First, a deletion mutant of hSRP1α lacking the amino-terminal domain is no longer able to support the import of NLS-containing substrates, despite the fact that it can still bind the NLS motif. The function can be rescued in a domain-swap experiment between the amino terminus of NPI and the hSRP1α deletion mutant. Additionally, the amino terminus itself acts as a dominant inhibitor of protein import in the *in vitro* transport assay. In a parallel study, Görlich *et al.* (1996) report that amino acids 10–50 in *Xenopus* importin α, which is homologous to hSRP1α, are essential for interaction with importin β (p97) and thus required for mediating nuclear import of NLS-containing substrates. The fact that the data of Görlich *et al.* are in close agreement with our present findings underlines the functional conservation of the amino-terminal domain. How-

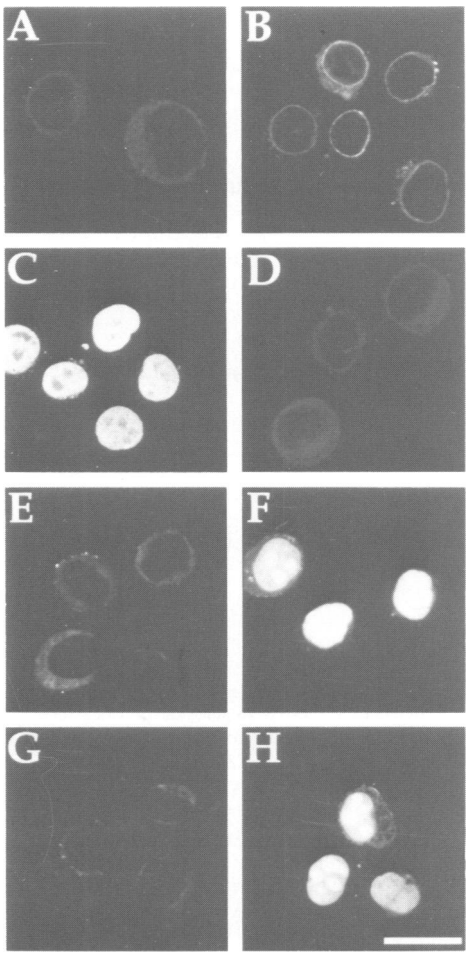


**Fig. 7.** A fusion of the amino terminus of hSRP1α to β-galactosidase enters the nucleus. To test its ability to mediate nuclear protein import, a fusion protein between the first 65 amino acids of hSRP1α and β-galactosidase was generated. The fusion protein alone is not able to enter the nucleus (**A**). Addition of recombinant human p97 causes docking of the fusion protein at the nuclear envelope (**B**). When recombinant Ran, p10 and p97 are present, the fusion protein is able to enter the nucleus (**C**). β-galactosidase without the amino-terminal fragment of hSRP1α is not able to enter the nucleus even in the presence of Ran, p10 and p97 (**D**). Ran, p10 and p97 alone were also not able to promote import of a BSA–NLS conjugate (**E**). Import of an NLS-containing substrate is dependent on the presence of an NLS-binding protein, such as hSRP1α, together with these three transport factors (**F**). However, a truncated variant of hSRP1α lacking the first 65 amino acids is no longer able to support import of the BSA–NLS conjugate even in the presence of Ran, p10 and p97 (**G**). The ability to promote import together with Ran, p10 and p97 is completely restored by fusing the first 71 amino acids of NPI to the carboxy-terminal fragment of hSRP1α (**H**). All import reactions were performed for 30 min at RT in the presence of energy. The following concentrations of recombinant proteins were used: 50 μg/ml BSA–NLS conjugate (E–H), 30 μg/ml hSRP1α (F), 30 μg/ml amino-terminal β-galactosidase fusion (A–C), 30 μg/ml β-galactosidase (D), 30 μg/ml carboxy-terminal fragment of hSRP1α (G), 30 μg/ml of the NPI–hSRP1α fusion protein (H), 50 μg/ml p97 (B–H), 5 μg/ml p10 (C–H), 100 μg/ml Ran (C–H).

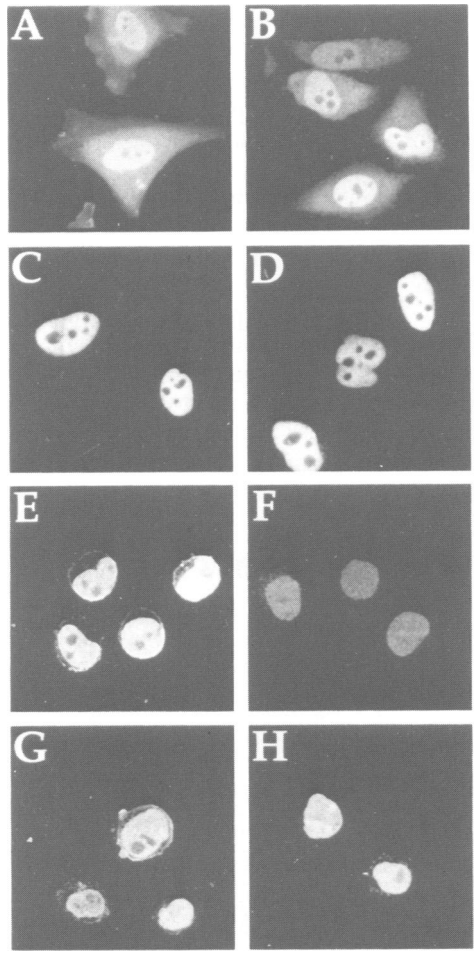


**Fig. 8.** hSRP1α is able to enter and leave the nucleus. (**A**) A confocal section through HeLa cells fixed with paraformaldehyde and immunostained with an antibody specific for hSRP1α. (**B**) To test whether hSRP1α is able to leave the nucleus, recombinant hSRP1α was microinjected into the nuclei of HeLa cells. Cells were incubated for 2 h at 37°C and then fixed with paraformaldehyde. Injected hSRP1α was detected with a monoclonal antibody directed against the amino-terminal His tag with indirect immunofluorescence using a fluorescently labelled secondary antibody. (**C** and **D**) The fusion of the first 65 amino acids of hSRP1α to β-galactosidase was microinjected either into nuclei (C) or into the cytoplasm (D) of HeLa cells. Microinjected proteins were detected with a monoclonal antibody against β-galactosidase by indirect immunofluorescence. (**E–H**) *In vitro* transport assays were performed in the presence of recombinant human p97, Ran, p10 and either BSA–NLS and hSRP1α (E and F) or the amino-terminal β gal fusion protein (G and H) for 30 min at RT. Cells were washed to remove external factors and either fixed immediately (E and G) or incubated for another hour after addition of fresh *Xenopus* egg extract and then fixed (F and H). hSRP1α was detected with a monoclonal antibody directed against the His tag, the β-galactosidase fusion with a monoclonal antibody directed against β-galactosidase.

ever, in separate studies, Moroianu *et al.* (1995a,b) present a conflicting result. In these studies, the authors report efficient import of NLS-containing substrates using truncated NLS-binding proteins (karyopherin $\alpha_1$/hSRP1/NPI and karyopherin $\alpha_2$/hSRP1α/Rch1) lacking either part or all of the amino-terminal interaction domain, which in our assay system is essential. At present, the reason for this discrepancy is unclear.

It was suggested that a cytoplasmic NLS receptor should shuttle between the nucleus and the cytoplasm (Adam and Gerace, 1991). Consistent with this prediction, we show here in a combination of immunofluorescence and microinjection experiments that a human NLS receptor subunit, hSRP1α, is able to exit as well as enter the nucleus. However, the amino-terminal domain, which is sufficient to facilitate entry of a reporter protein into the nucleus, is

not sufficient to promote its exit from the nucleus. This points to a difference in the mechanism whereby hSRP1α enters and leaves the nucleus. More generally, the data suggest that movement in opposite directions through the NPC may involve distinct, although possibly overlapping, factors or mechanisms.

We have shown here that a fragment of hSRP1α including the amino-terminal interaction domain inhibits nuclear transport of NLS-containing substrates in the *in vitro* transport assay. The mechanism of this dominant inhibition is most likely competition between this fragment and NLS-binding proteins in the extract for binding to p97. The use of this fragment as an inhibitor of transport will therefore provide a useful experimental tool for future studies on the transport machinery. For example, it will help to define which nuclear transport events depend on a common pathway involving p97.

## Materials and methods

### *cDNA cloning and recombinant protein expression*

The complex between hSRP1α and the 97 kDa transport factor was purified from cytosolic HeLa cell extracts as described previously (Weis *et al.*, 1995). After the purification by FPLC on a MonoQ column, the band with an apparent mol. wt of ~90 kDa was excised from the SDS gel and subjected to peptide analysis with a novel electrospray mass spectrometry approach (Mann and Wilm, 1995; Wilm *et al.*, 1996). Six peptides were identified by database analysis on peptide mass and sequence information [(1) TVSPDRLELEAAQK; (2) WLAIDANAR; (3) LAATNALLNASLEFTK; (4) VAALQNLVK; (5) LETTDRPDGHQNNLR; (6) SDYDMVDYLNELR] which matched to four database entries (GenBank accession numbers L38951, L39793, D45836 and L38644) and to three overlapping expressed sequence tags deposited in the ATCC (GenBank accession numbers T05916, T07554 and T09278, respectively). The internal *Hpa*I site in clone T07554 and the *Not*I in the polylinker of pBluescript were used to generate a full-length clone (pKW275) by subcloning the *Hpa*I–*Not*I fragment of clone T09278 into clone T07554. For expression of the full-length untagged 97 kDa protein in *E.coli*, the *Nco*I–*Pst*I fragment of pKW275 was subcloned together with the *Pst*I–*Hin*dIII fragment of T09278 into pQE60 (Qiagen) cut with *Nco*I and *Hin*dIII. The generated plasmid pKW291 was transformed into *E.coli* strain M15[pRep4], which was grown to an $OD_{600}$ of 0.4 and then induced for 4 h at 23°C with 1 mM IPTG. The expressed protein was purified by FPLC on a MonoQ column and when indicated with an additional hSRP1α affinity column.

Full-length hSRP1α with an amino-terminal His tag was expressed as described previously (Weis *et al.*, 1995). The amino-terminal deletion mutant lacking amino acids 1–65 was constructed by subcloning the *Pst*I–*Kpn*I fragment of pBShSRP1α (Weis *et al.*, 1995) into pRSET C (Invitrogen) carboxy-terminal to the $His_6$ portion of the vector to generate clone pKW313. The construct pKW312 containing the first 65 amino acids of hSRP1α was made by subcloning a *Pst*I–*Pst*I fragment of pBShSRP1α into pRSET A, and the correct orientation was confirmed by restriction digestion. The construct containing amino acids 21–65 of hSRP1α was derived by subcloning a *Pst*I–*Pst*I fragment of p21 (Weis *et al.*, 1995) into pRSET C. All the other deletion mutants of hSRP1α and NPI were generated with PCR using Pfu polymerase (Stratagene) and standard conditions. All PCR-derived constructs were verified by sequencing and subcloned into pRSET with the appropriate restriction enzymes. The following primers were used for the PCR amplification: (i) construct containing amino acids 1–51 of hSRP1α: primers 879 [CTCGAATTCCATGTCCACCAACGAGAAT] and 876 [TTCTCCTCTTCAGCATCTGG]; (ii) construct containing amino acids 1–42 of hSRP1α: primers 879 and 1050 [GGTTAAGCTTAGCTTTCCTCAGCTCCAC]; (iii) construct containing amino acids 33–63 of hSRP1α: primers 1049 [GACGAATTCAGAGGTCAATGTGGAGCTG] and 1035 [GGTTAAGCTTGGAGAAGTAGCATCATCAGG]; (iv) construct containing amino acids 1–72 of NPI: primers 943 [CCCCCAACGCGATGGATCCGAAATATGACCACCCCAG] and 1036 [CCCCTGCAGCTGAGCCTCATGAAAGCC].

The fusion between the amino terminus of hSRP1α and β-galctosidase was generated by subcloning a *Kpn*I–*Hin*dIII fragment of pKW312 in front of a pRSET-bGal cassette. The fusion between NPI and hSRP1α was constructed by cloning the *Bam*HI–*Pst*I PCR fragment containing the first 72 amino acids of NPI (see above) into the same sites of pKW313.

All pRSET derived constructs were transformed into BL21[LysS]. Cells were grown to an $OD_{600}$ of 0.4 and then induced for 4 h at 23°C. After lysis in a French press the overexpressed, $His_6$-tagged proteins were bound to Ni-NTA agarose, washed and eluted with 500 mM imidazole in 20 mM HEPES (pH 7.6), 100 mM NaCl, 2 mM β-mercaptoethanol, 8.7% glycerol.

Ran was expressed and purified as described previously (Weis *et al.*, 1995). Untagged p10 was expressed from a pET construct (a gift from R.Bischoff) in BL21[LysS] using the same induction and lysis protocol as described above. The active p10-dimer was purified with FPLC Sephadex S100 gel filtration.

### *Co-selection experiments with p97*

Recombinant or *in vitro* translated p97 protein was incubated with full-length hSRP1α or the various truncated variants of hSRP1α, respectively, in 100 µl IPP buffer [10 mM Tris (pH 7.2), 150 mM NaCl, 5 mM $MgCl_2$, 10 mM imidazole] for 1 h at 4°C in the presence of 20 µl Ni-NTA agarose beads. After three washes with 1 ml IPP buffer, the bound proteins were eluted with IPP buffer containing 500 mM imidazole and the eluate was analysed by SDS–PAGE.

### *In vitro transport assays*

Transport assays were basically performed as described previously (Weis *et al.*, 1995) and the uptake of fluorescent BSA–NLS conjugate was analysed with confocal microscopy. The uptake of the β-galactosidase reporter proteins was monitored by indirect immunofluorescence with a monoclonal antibody against β-galactosidase (Boehringer Mannheim). Fixed cells were permeabilized for 10 min with phosphate-buffered saline (PBS) + 0.5% Triton X-100. After three washes with PBS + 0.05% Tween 20, the cells were incubated with the anti-β-galactosidase antibody followed by an incubation with an anti-mouse fluorescein isothiocyanate (FITC)-conjugated antiserum.

Unless otherwise stated, the import reactions contained the following amount of recombinant proteins: 50 µg/ml BSA–NLS conjugate, 30 µg/ml hSRP1α, 50 µg/ml p97, 5 µg/ml p10, 100 µg/ml Ran. The import reactions were performed in import buffer containing 100 µM ATP, 100 µM GTP, 10 mM creatine phosphate, 5 µg/ml creatine kinase, 110 mM potassium acetate, 5 mM magnesium acetate and 2 mM dithiothreitol (DTT) for 30 min at room temperature (RT).

Nuclear fluorescence was quantitated on a Zeiss Axiovert 10 equipped with a cooled CCD camera. Image acquisition was controlled with a SUN workstation with the software package KHOROS. The area of the nuclei was localized by DAPI staining and an automatic labelling and counting procedure was applied (Palacios *et al.*, 1996).

### *Microinjection*

Microinjection into the nucleus or the cytoplasma of HeLa cells was performed using the AIS microinjection system (Zeiss). Correct injection was monitored by co-injection of Texas Red-labelled dextran sulfate (mol. wt 70 K). After the injection, the cells were incubated for 2 h at 37°C and analysed by immunofluorescence as described above.

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