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# The importin-β binding domain of snurportin1 is responsible for the Ran- and energy-independent nuclear import of spliceosomal U snRNPs in vitro

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The nuclear localization signal (NLS) of spliceosomal U snRNPs is composed of the U snRNA's 2,2,7-trimethylguanosine ($m_3G$)-cap and the Sm core domain. The $m_3G$-cap is specifically bound by snurportin1, which contains an $NH_2$-terminal importin-β binding (IBB) domain and a COOH-terminal $m_3G$-cap–binding region that bears no structural similarity to known import adaptors like importin-α (impα). Here, we show that recombinant snurportin1 and importin-β (impβ) are not only necessary, but also sufficient for U1 snRNP transport to the nuclei of digitonin-permeabilized HeLa cells. In contrast to impα–dependent import, single rounds of U1 snRNP import, mediated by the nuclear import receptor complex snurportin1–impβ, did not require Ran and energy. The same Ran- and energy-independent import was even observed for U5 snRNP, which has a molecular weight of more than one million. Interestingly, in the presence of impβ and a snurportin1 mutant containing an impα IBB domain ($IBB_{impα}$), nuclear U1 snRNP import was Ran dependent. Furthermore, β-galactosidase (βGal) containing a snurportin1 IBB domain, but not $IBB_{impα}$-βGal, was imported into the nucleus in a Ran-independent manner. Our results suggest that the nature of the IBB domain modulates the strength and/or site of interaction of impβ with nucleoporins of the nuclear pore complex, and thus whether or not Ran is required to dissociate these interactions.

## Introduction

The trafficking of macromolecules between cytoplasm and nucleus is mediated by nuclear pore complexes (NPCs),* large supramolecular structures spanning the nuclear envelope. NPCs, with an estimated molecular mass of 125 MDa in vertebrates, are comprised of ~50 unique proteins, termed nucleoporins (for reviews see Stoffler et al., 1999; Ryan and Wente, 2000; Rout and Aitchison, 2001). Whereas molecules smaller than 40 kD can passively diffuse through the NPC, most macromolecules traverse the NPC by temperature- and signal-dependent mechanisms. The translocation of macromolecules is generally mediated by saturable transport receptors that recognize specific nuclear localization signals (NLSs) (for review see Mattaj and Englmeier, 1998; Görlich and Kutay, 1999).

Transport receptors involved in nuclear import and export identified thus far form a family of proteins termed the importin-β (impβ)superfamily (Fornerod et al., 1997; Görlich et al., 1997). Although they exhibit a low sequence similarity, members of this family share common properties like binding to the small GTPase Ran, NPC proteins termed nucleoporins, and cargo, which most of them bind directly. In contrast, impβ/Karyopherin-β, the receptor required for proteins carrying a so-called classical NLS, requires an adaptor termed importin-α(impα)/Karyopherin-α (Görlich et al., 1994, Moroianu et al., 1995; Radu et al., 1995; Weis et al., 1995). Impα consists of an $NH_2$-terminal impβ binding (IBB) domain that mediates complex formation between impβ and impα (Görlich et al., 1996a; Moroianu et al., 1996; Weis et al., 1996; for review see Mattaj and Englmeier, 1998; Görlich and Kutay, 1999). The COOH-terminal domain of impα provides the NLS binding activity and consists of ten so-called arm motif repeats (Weis et al., 1995; Görlich et al., 1996a; Moroianu et al., 1996).

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*Abbreviations used in this paper: aa, amino acid(s); βGal, β-galactosidase; IBB, importin-β binding; impα, importin-α; impβ, importin-β; NPC, nuclear pore complex; NLS, nuclear localization signal; SPN1, snurportin1; TPN1, transportin1; wt, wild-type.

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A nucleocytoplasmic shuttling protein, the small GTPase Ran, plays a key role in determining the directionality of nuclear transport (Izaurralde et al., 1997). The GTPase activating enzyme for Ran, RanGAP, is sequestered in the cytoplasm (Matunis et al., 1996; Mahajan et al., 1997), and the Ran guanine nucleotide exchange factor (RCC1) is restricted to the nucleus (Ohtsubo et al., 1989). As a consequence, the distribution of Ran is unequal, leaving most nuclear Ran in the GTP-bound form, whereas most cytoplasmic Ran is presumed to be in the GDP-bound form. Import complexes formed between cargoes and their receptors are assembled in the cytoplasm, transferred into the nucleus, and then dissociated in the presence of Ran-GTP. Conversely, complexes formed between export receptors and their cargoes form in the nucleus only in the presence of RanGTP (for review see Mattaj and Englmeier, 1998; Görlich and Kutay, 1999). The adaptor impα, for example, is bound and exported by CAS1 (also a member of the impβ family) and Ran-GTP.

The Ran requirements during single rounds of import have been investigated under experimental conditions where the recycling of import factors is not required. These studies revealed that import pathways differ in their Ran requirements. In particular, nuclear import of the receptors impβ and transportin alone does not depend on Ran and GTP hydrolysis (Kose et al., 1997; Nakielny and Dreyfuss, 1998). The import of cargo bound directly to transportin (Englmeier et al., 1999) or the single round import of the adaptor snurportin1 (see below) bound to impβ does not require RanGTP hydrolysis in vitro (Ribbeck et al., 1999), although the latter study did not address whether loading of the snurportin1/impβ receptor with cargo would require Ran and energy. In contrast, the import of classical NLS cargoes by impα/β strictly depends on the presence of RanGDP and free GTP or a nonhydrolysable equivalent (Schwoebel et al., 1998). Nuclear RanGTP triggers disassembly of the NLS cargo–importinα/β complex and presumably its release from the NPC, which terminates the import process by releasing the NLS cargo into the nucleoplasm (Rexach and Blobel, 1995; Görlich et al., 1996b). Thus, Ran does not appear to play a role in the actual translocation itself, but rather in the proper termination of the transport process (Englmeier et al., 1999; Ribbeck et al., 1999) and the recycling of the import factors.

In contrast to protein import, the mechanism of spliceosomal U snRNP import is less well understood. Each snRNP particle consists of one (U1, U2, U5) or two (U4/U6) snRNA molecules, a common set of seven core proteins (B/B′, D1, D2, D3, E, F, G, also denoted Sm proteins) and a number of particle-specific proteins. With the exception of U6 snRNP, which is thought not to leave the nucleus (Vankan et al., 1990), the biogenesis of these U snRNPs requires the bidirectional transport of the snRNAs across the nuclear envelope. The U1, U2, U4, and U5 snRNAs are synthesized in the nucleus with a 5′-terminal monomethyl-guanosine ($m^7G$) cap structure and exported into the cytoplasm. There, the Sm proteins bind to the U snRNAs Sm site to form a ribonucleoprotein complex referred to as the Sm core (Mattaj et al., 1985). Stable association of all Sm proteins is essential for hypermethylation of the m7G-cap to the 2,2,7-trimethyl-guanosine ($m_3G$)-cap structure (Mattaj, 1986; Plessel et al., 1994). After this event and 3′ end processing of the snRNAs (Neuman de Vegvar and Dahlberg, 1990), the mature snRNP particles are transported back into the nucleus in a receptor-dependent manner.

The NLS of U1 snRNPs is complex. The $m_3G$-cap structure is one essential signaling component (Fischer and Lührmann, 1990; Hamm and Mattaj, 1990), and a second component is located at the Sm core (denoted Sm core NLS), but has not yet been precisely defined (Fischer et al., 1993). Not all spliceosomal snRNAs have the same $m_3G$-cap requirement for nuclear transport in *Xenopus* oocytes. Whereas nuclear import of U1 and U2 snRNPs absolutely requires an intact and accessible $m_3G$-cap, U4 and U5 snRNPs can enter the nucleus as ApppG-capped derivatives, although with significantly reduced transport kinetics (Fischer et al., 1991; Michaud and Goldfarb, 1992). Even though the $m_3G$-cap is not essential for the nuclear import of any U snRNAs in somatic cells, it accelerates their transport, indicating that it still plays a signaling role for nuclear targeting of U snRNPs (Fischer et al., 1994; Marshallsay and Lührmann, 1994).

Investigations using somatic cells in vitro and in vivo (Marshallsay et al., 1996) have indicated that, in contrast to cargoes containing a classical NLS, efficient import of U1 snRNPs can occur in the presence of nonhydrolysable GTP analogues or a mutant form of Ran deficient in GTP hydrolysis (RanQ69L). This observation supports competition analyses which showed that the import of U snRNPs is independent of peptide NLS–dependent pathways (Michaud and Goldfarb, 1992). Interestingly, GTP hydrolysis was needed for U1 snRNP import when using *Xenopus* egg extract for in vitro nuclear import (Palacios et al., 1997). This indicated differential requirements of U snRNP nuclear import in different cell systems.

Recently we characterised a factor termed snurportin1 (SPN1), a nuclear import factor like impα, required as a bridging molecule between the receptor impβ and the $m_3G$-cap of U snRNPs (Huber et al., 1998). SPN1 is composed of two domains, an $NH_2$-terminal domain required for binding to the import receptor, and a COOH-terminal $m_3G$-cap–binding region. Whereas the COOH-terminal $m_3G$-cap–binding region of SPN1 bears no obvious structural similarity to the arm repeat domain found in impα the $NH_2$-terminal domain exhibits a high degree of homology to the IBB domain of impα. The IBB domain of SPN1 was shown to have a stimulatory effect on nuclear import of U1 snRNPs, arguing for a direct involvement of impβ in nuclear import of U snRNPs. This idea is further supported by the observations that impβ depletion from *Xenopus* egg extract significantly inhibits snRNP import (Palacios et al., 1997) and our finding that SPN1 translate binds impβ in vitro (Huber et al., 1998). The addition of recombinant SPN1 to an in vitro import system using HeLa cell nuclei and cytosol significantly stimulates the import of U1 snRNPs, indicating a direct function of SPN1 in import. However, the relative contributions of SPN1 and the putative Sm core NLS receptor to snRNP import (i.e., whether they act autonomously or synergistically) remains to be elucidated.

Here, we investigated U snRNP import in vitro using recombinant transport factors. These studies revealed that

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SPN1–impβ is essential and sufficient for transport of mature snRNPs into the nuclei of digitonin-permeabilized HeLa cells. The nuclear uptake of U1 snRNPs by SPN1–impβ was strictly dependent on the $m_3$G-cap, and thus independent of the presence of an Sm core NLS receptor. This finding enabled us to address the energy requirements of nuclear U snRNP import more closely. Interestingly, single nuclear U1 and U5 snRNP import events were neither dependent on hydrolysable NTPs nor the presence of (non) hydrolysable NTPs and Ran. In contrast, under the same conditions but using impα/β as the adaptor/receptor, a cargo with a classical NLS was not imported and accumulated at the nuclear pores. In subsequent experiments we pinpointed these differences in Ran and energy requirement to the IBB domain of the two adaptors, SPN1 and impα. Our results suggest that the nature of the IBB domain determines whether or not Ran is required to dissociate impβ/cargo interactions with the NPC.

## Results

### SPN1 and impβ mediate the nuclear import of U1 snRNPs in an autonomous manner

Using an in vitro nuclear import assay, we previously showed that intact U1 snRNPs, as well as U1 snRNPs lacking the $m_3$G-cap structure, accumulate in the nucleus in the presence of cytosol. This result indicated that the receptor recognizing the Sm core NLS in HeLa cytosol can act in the absence of the SPN1–$m_3$G-cap interaction (Huber et al., 1998). However, whether SPN1 can also act independently of the Sm core NLS was not known. To investigate the mechanism of action of SPN1 in more detail, we first established an in vitro import assay completely dependent on the addition of recombinant factors.

When HeLa cells were digitonin permeabilized and used without any further treatment, the addition of solely recombinant Ran and an energy regenerating system was sufficient for the import of significant amounts of U1 snRNPs into the nucleus (Fig. 1 A). This U1 snRNP import was an active and temperature-dependent process (unpublished data), and was presumably mediated by snRNP import factors either remaining within the nucleus or bound to the nuclear membrane after cell permeabilization. Indeed, in situ immunostaining revealed a significant amount of SPN1 and impβ still bound to the nuclear membrane after digitonin permeabilization (Fig. 1 C; unpublished data). It was previously reported that the presence of an ATP-regenerating system and a shift to 30°C during permeabilisation, followed by an incubation in transport buffer at room temperature for 15 min, strongly reduces the amount of residual endogenous transport factors (Schwoebel et al., 1998; Englmeier et al., 1999; Nachury and Weis, 1999). As shown in Fig. 1 (compare panels C and D), when HeLa cells were treated accordingly, a strong reduction in residual SPN1 bound to the cy-

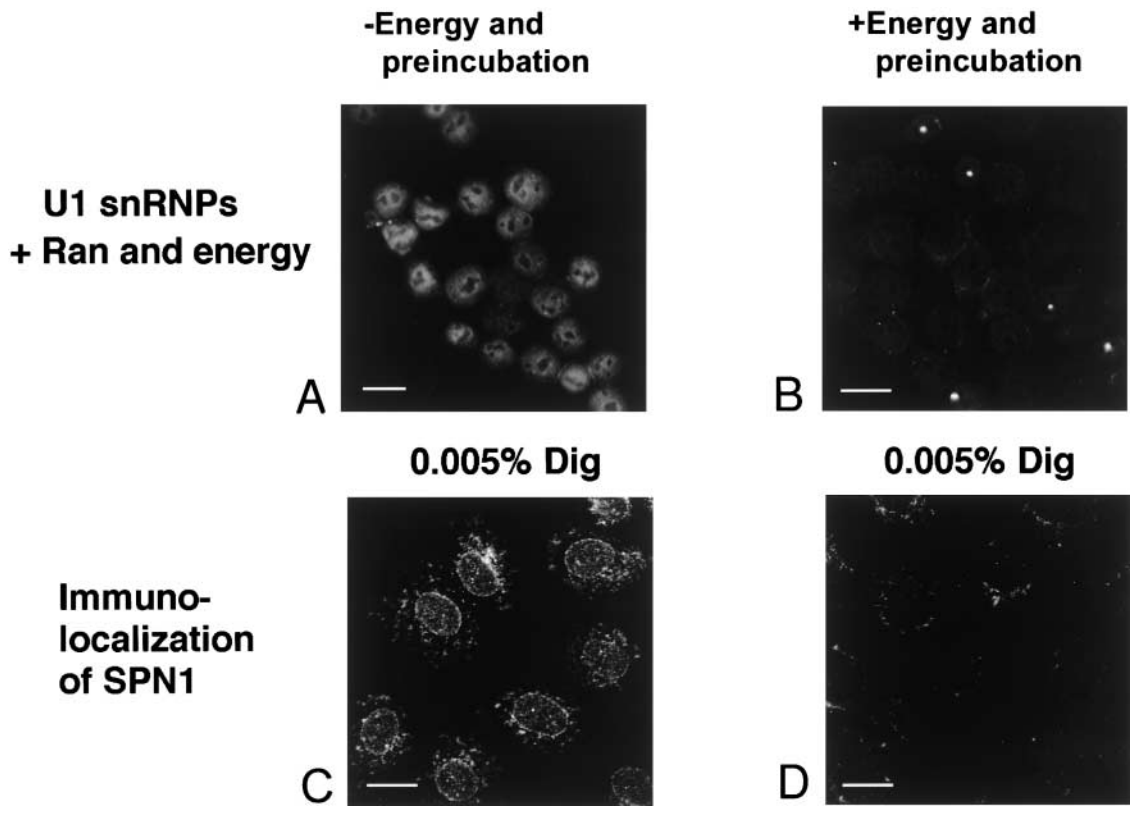


Figure 1. **Depletion of endogenous HeLa cell transport factors by modifying the permeabilization and preincubation conditions strongly reduces the import rate of U1 snRNPs.** HeLa cells were prepared for in vitro import assays by either permeabilizing with digitonin alone (A and C) or in the presence of an energy-regenerating system followed by a 15-min incubation at room temperature (B and D). Transport reactions were performed for 15 min with fluorescently labeled U1 snRNPs and import determined by fluorescence microscopy (A and B). The amount of SPN1 still bound to the cells was determined by in situ immunostaining (C and D). Bars, 20 μm.

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toplasmic side of NPCs was observed. Consistent with this result, the basal import of U1 snRNPs was dramatically reduced (Fig. 1 B).

These changes in the permeabilization and preincubation conditions allowed us to investigate factor requirements for U1 snRNP import using exogenously added recombinant transport factors. To test for the requirement of both SPN1 and impβ in the nuclear transport of U1 snRNPs in vitro, import experiments were performed in the presence of Ran and an energy-regenerating system. Neither SPN1 nor impβ alone led to an increase in nuclear accumulation of U1 snRNPs (Fig. 2, A and B) as compared with the control (unpublished data). Significant U1 snRNP import was only observed in the presence of both SPN1 and impβ (Fig. 2 C). U1 snRNP import mediated by SPN1 and impβ strictly requires the 5′ terminal $m_3$G-cap, because Δ5′U1 snRNPs (in which the 5′$m_3$G-cap had been removed) themselves were not imported (Fig. 2 E). Consistent with this observation, a 100-fold excess of Δ5′U1 snRNPs, could not inhibit import of intact U1 snRNPs (Fig. 2 D). These data clearly illustrate that SPN1 cooperates with impβ in U1 snRNP nuclear import, and that both are sufficient to import $m_3$G-cap–bearing U snRNPs. Thus, they act independently of the Sm core recognizing factor in vitro.

### Ran is not required for the SPN1-mediated translocation of U1 snRNPs into the nucleus

Next, the role of Ran and the influence of the state of Ran loading with various guanosine nucleotides was investigated. Ribbeck et al. (1999) recently showed that free SPN1–impβ import receptors were translocated to the nucleus in a Ran-independent manner; however, they did not address whether this also applied to the SPN1-mediated snRNP import. To allow the analysis of "single round" transport events, nuclear import factors and Ran were added in excess over the import cargos (see Materials and methods). In the presence of Ran preloaded with GDP, GTP, or the nonhydrolysable analogue GMP-PNP, a BSA-NLS conjugate (BSA-NLS) was efficiently imported by impα/β (Fig. 3, F and G). As a control we determined the effect of addition of Ran and the nonhydrolysable GDP analogue GDPβS, or hexokinase/glucose to deplete remaining endogenous NTPs. This resulted in an accumulation of BSA-NLS at the nuclear membrane of digitonin-permeabilized cells (Fig. 3, H and I). A similar effect was observed when only impα/β was added (Fig. 3 K). Surprisingly, SPN1 and impβ alone were sufficient to mediate import of U1 snRNPs. Neither the presence or absence of Ran, nor GTP by itself (or an energy-regenerating system) had any stimulatory effect on U1 snRNP import (Fig. 3, A–E). These data are consistent with previous studies showing that U1 snRNP import into the nucleus of somatic cells is independent of Ran and Ran-dependent GTP hydrolysis (Marshallsay et al., 1996).

### The SPN1-mediated import pathway of U1 snRNPs occurs independent of energy

Although the addition of RanGTP and exogenous NTPs is not required for U1 snRNP import, it is still possible that NTPs that remain bound to the permeabilized cells are used as a source of energy. Therefore, we investigated whether U1 sn-

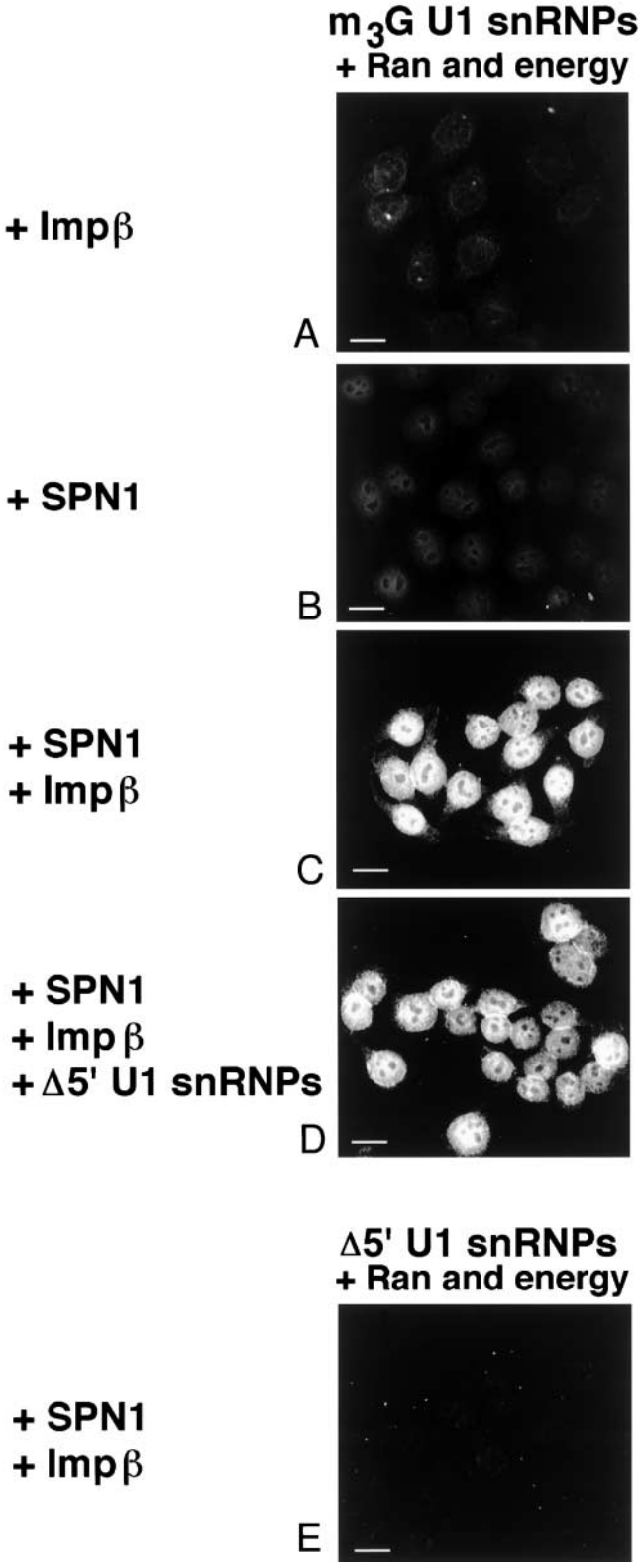


Figure 2. **Import of U1 snRNPs is dependent on exogenously added SPN1 and importin-β, but not the Sm core NLS receptor.** The import of fluorescently labeled U1 snRNPs in the presence of Ran and energy was monitored in the presence of either importin-β (A), SPN1 (B), or both (C). To investigate the contribution of the Sm core receptor to the import of U1 snRNPs, unlabeled Δ5′U1 snRNPs lacking the SPN1 binding site, but still containing the Sm core NLS were added in 100-fold molar excess (D). Comparative analysis of the import of fluorescently labeled Δ5′U1 snRNPs (E). Bars, 20 μm.

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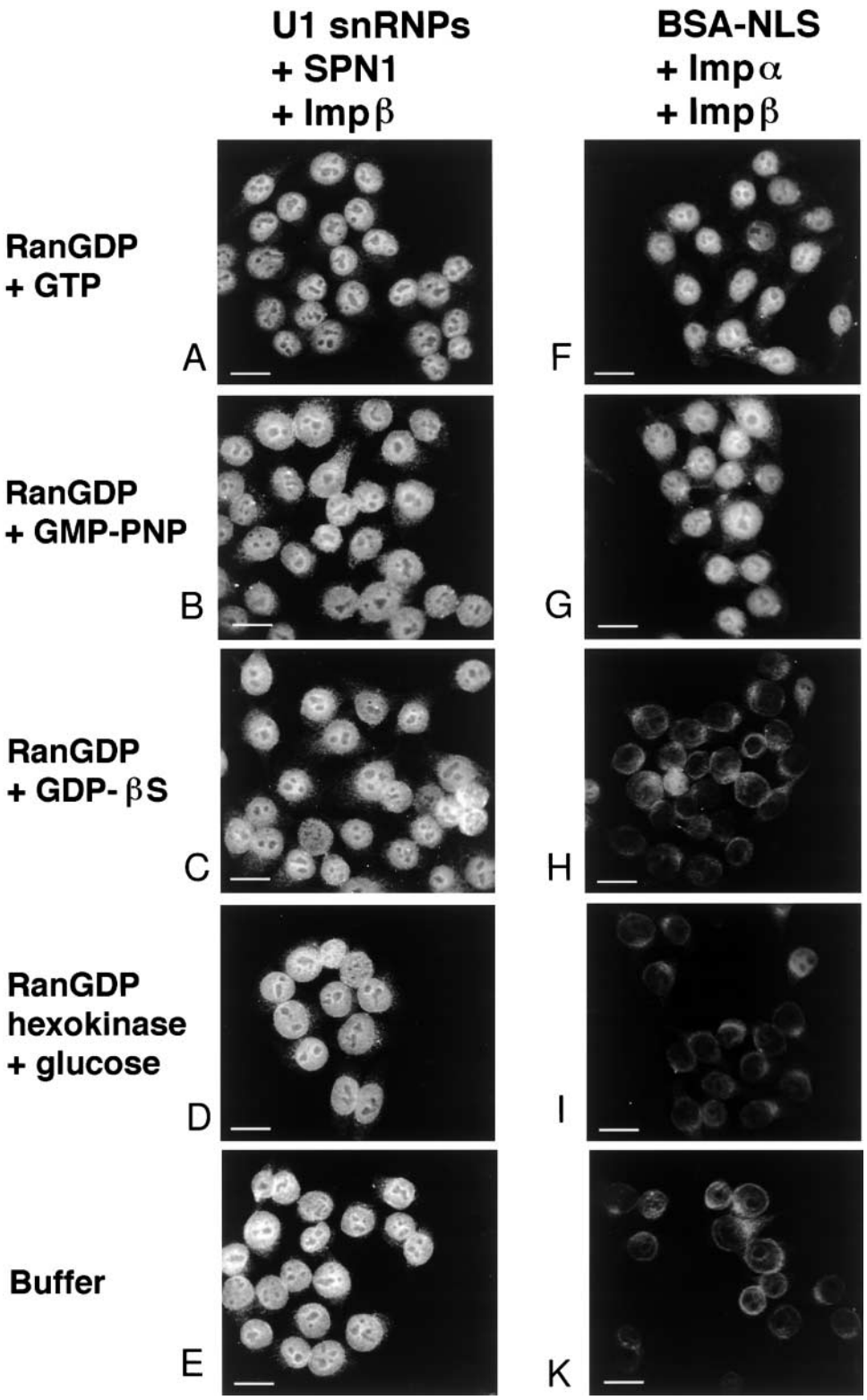


Figure 3. **Ran and Ran-dependent hydrolysis of NTPs are not required for SPN1-mediated translocation of U1 snRNPs through the NPC.** The nuclear import of Cy3-labeled U1 snRNPs (0,04 μM) (A–E) or FLUOS-labeled BSA-NLS (0,1 μM) (F–K) in the presence of preformed adaptor–importin-β complex (0.5 μM SPN1 or 0.6 μM importin-α, respectively and 0.2 μM importin-β) was performed for 15 min at 20°C. The permeabilized cells were preincubated in T buffer (E and H) or in the presence of 2 μM Ran GDP (A–D and F–I) and 1 mM nucleotide as indicated on the left (A–C and F–H) or 20 u/ml hexokinase/glucose (D and I). Bars, 20 μm.

RNPs are still imported after depletion of NTPs by hexokinase (Fig. 4 C), and also whether import could be blocked by non-hydrolysable NTP-analogues like GMP-PNP and AMP-PNP (Fig. 4 B) which would also prevent other NTP/GTPases from acting at the translocation step. Neither experimental condition had any effect on the SPN1–impβ–mediated pathway,

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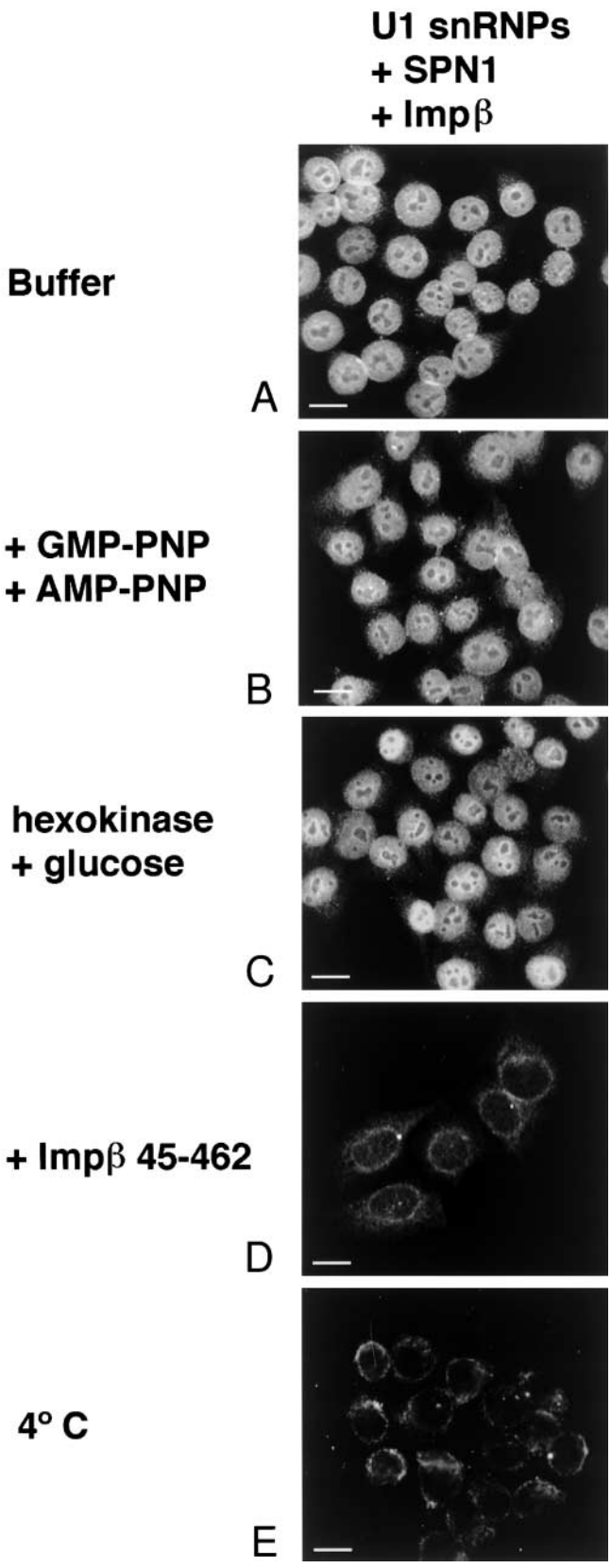


Figure 4. **Hydrolysis of NTPs is not required for the transport of U1 snRNPs into the nucleus.** HeLa cells were preincubated for 15 min in the presence of buffer (A, D, and E), 1 mM each AMP-PNP and GMP-PNP (B), or 20 U/ml hexokinase/glucose (C) before the addition of Cy3-labeled U1 snRNPs (0,04 μM) in combination with a preformed complex of 0.5 μM SPN1 and 0.2 μM importin-β (A–E) or additionally, 0.5 μM importin-β 45–462 was added (D). The cells were incubated for 15 min at room temperature (A–D) or at 0°C (E) before they were fixed. Bars, 20 μm.

compared with the control where buffer alone was added (Fig. 4 A). Thus, ATP or GTP hydrolysis is not required for the SPN1–impβ–mediated translocation of U1 snRNPs into the nucleus. These results also exclude the possibility that a second NTP/GTPase might take part in U1 snRNP import. An impβ deletion mutant lacking the $NH_2$-terminal Ran binding domain (amino acids [aa] 1–44) and the COOH terminus (aa 462–876) was previously shown to accumulate at the nucleoplasmic side of the NPC (Görlich et al., 1996a), thereby blocking multiple import and export pathways (Kutay et al., 1997). As shown in Fig. 4 D, U1 snRNP import was also blocked by the same mutant, demonstrating that the SPN1–impβ import pathway shares at least one intermediate binding site at the nuclear pore with other members of the impβ superfamily. This, and the fact that no U1 snRNP import is observed at 4°C (Fig. 4 E), strongly argue against passive diffusion of U1 snRNPs into the nucleus (Kutay et al., 1997).

### Ran- and energy-independent nuclear import of U5 snRNPs by SPN1 and impβ

Next, we investigated whether Ran and GTP independence are specific for U1 snRNPs, or are a more general feature of the SPN1–impβ–dependent import pathway. Therefore, we investigated the nuclear import of U5 snRNPs which contain, in addition to the U5 snRNA, 15 proteins (Fig. 5 A). The molecular mass of U5 snRNPs exceeds one million daltons, which is about four times the size of a native U1 snRNP. Fluorescence labeling with the dye Cy3 was performed and the integrity of the U5 snRNPs tested by glycerol gradient centrifugation. U5 snRNPs were efficiently labeled (Fig. 5 C) and clearly remain intact, as evidenced by cosedimentation of the RNA and the proteins (Fig. 5, compare A and B). The in vitro nuclear import of those flourescently labeled U5 snRNPs was subsequently tested. As an internal control, the Cy3-labeled U5 snRNPs were mixed with an equal molar amount of FLUOS-labeled U1 snRNPs, and their import behavior was analyzed. In the absence of recombinant SPN1 and impβ, neither U5 snRNPs nor U1 snRNPs accumulate in the nucleus (Fig. 6, A and B). Efficient import was only observed upon addition of both SPN1 and impβ (Fig. 6, C and D), and occurred in the absence of exogenously added Ran and nucleotides. The import rate could not be increased by addition of RanGDP and GTP (unpublished data). Thus, U5 snRNPs and U1 snRNPs are imported efficiently into the nucleus in the same Ran-independent manner. To determine the energy requirements of SPN1–impβ–mediated U5 snRNP nuclear import, assays were performed in the presence of apyrase in order to deplete any remaining endogenous ATP and GTP. As observed with U1 snRNPs, NTP depletion did not influence nuclear uptake U5 snRNPs, demonstrating that SPN1–impβ–mediated translocation of U5 snRNPs also does not depend on ATP or GTP hydrolysis (Fig. 6, compare C and D with E and F). Taken together, these results demonstrate that the mechanism of SPN1–impβ–mediated U snRNP import does not vary with snRNP composition or size.

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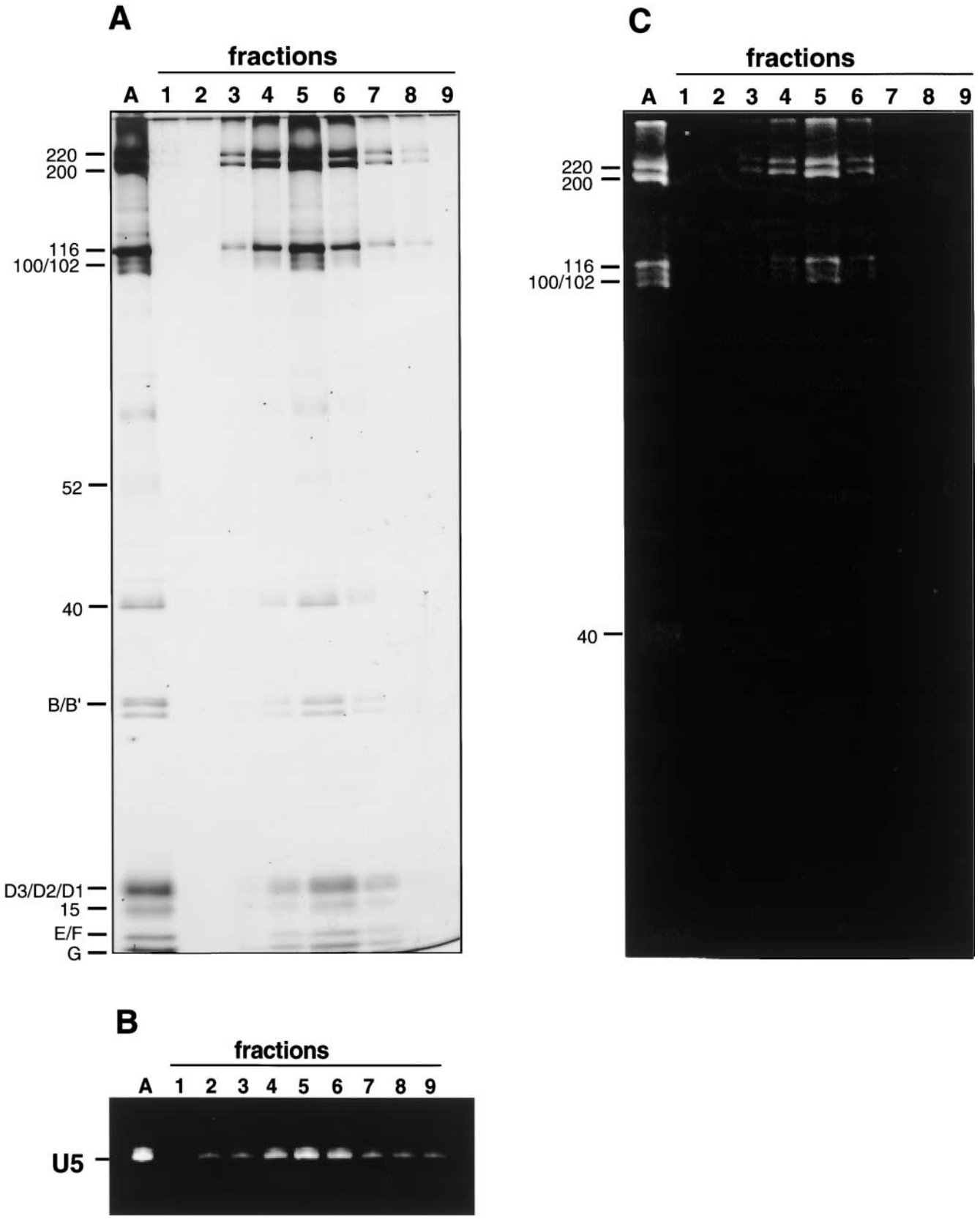


Figure 5. **U5 snRNPs are also imported in a Ran- and energy-independent fashion by SPN1 and importin-β.** (A, B, and C) RNA and protein analysis, and integrity test of U5 snRNPs. 10 μg Cy3-labeled U5 snRNPs were centrifuged on a 1.5-ml 10–30% glycerol gradient (260,000 *g*, 4°C, 3 h). 150-μl fractions were taken and the RNA and protein content analyzed. (A) Proteins were fractionated on a 10/13% step, high-TEMED polyacrylamide gel, and visualized by silver staining. (B) RNA was separated on a 10% denaturing polyacrylamide gel containing 7 M urea and visualized by ethidium bromide staining. (C) The protein gel from panel A illuminated with UV light to visualize the Cy3 fluorescence label bound to the U5 snRNP proteins. Lane A, 40% of the input applied to the gradient. Lanes 1 (top) to 9 (bottom) are the fractions taken from the gradient. The molecular mass (in kD) of the U5 proteins is indicated on the left.

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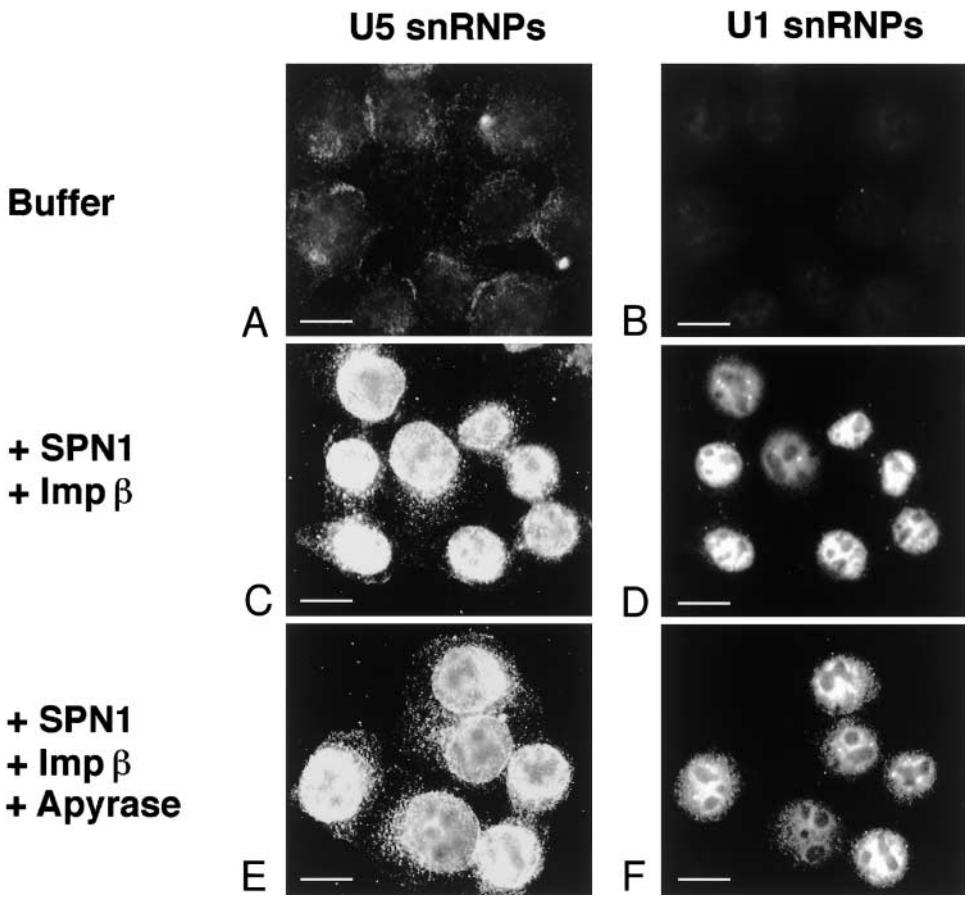


Figure 6. **In vitro import of Cy3-labeled U5 snRNPs (0,012 μM) (A, C, and E) and FLUOS- labeled U1 snRNPs (0,04 μM) (B, D, and F).** Permeabilized HeLa cells were preincubated in buffer (A, B, C, and D) or buffer containing 20 u/ml apyrase (E and F) before the simultaneous addition of Cy3-labeled U5 snRNPs and FLUOS-labeled U1 snRNPs. U snRNPs alone (A and B); U snRNPs in the presence of 0.5 μM SPN1 and 0.2 μM importin-β (preincubated for 10 min at 0°C) (C–F). Bars, 20 μm.

### The IBB domain is responsible for the Ran- and energy-independent translocation of U1 snRNPs

As shown above, BSA-NLS and snRNP nuclear import differ in their Ran and energy requirements, although both depend on the same receptor, impβ. This difference might be due to the nature of the cargo or the adaptor itself. In the former case, U snRNPs could have an indirect effect on impβ, thereby changing the mechanism of translocation of the impβ–adaptor–substrate complexes through the NPC. Alternatively, the difference in import behavior could be due to a qualitative difference in the interaction of the respective IBB domains of the adaptor (i.e., SPN1 or impα) with impβ. The latter possibility is consistent with the observations of Ribbeck et al. (1999) that SPN1 was imported into the nucleus by impβ, also in the absence of snRNP cargo. To distinguish between these possibilities, domain swap experiments were performed by fusing the IBB domain of impα to the transport inactive form of snurportin1, Δ1–65 SPN1. As a control, we first verified that recombinant $IBB_{impα}$-SPN1 protein recognizes efficiently the $m_3G$-cap (unpublished data). U1 snRNP import was tested in the presence of impβ and either wild-type (wt) SPN1 or the $IBB_{Impα}$-SPN1 domain swap mutant in a cytosol free in vitro assay. Strikingly, in contrast to wt SPN1 (Fig. 7, A and B), in the presence of $IBB_{Impα}$-SPN1, U1 snRNPs accumulated at the NPC but not within the nucleus, if Ran and energy were absent (Fig. 7 E), or only RanGDP and GDP (Fig. 7 F) were present. Accumulation of U1 snRNPs in the nucleus was only observed with $IBB_{Impα}$-SPN1 when RanGDP and GTP (Fig. 7 G) or RanGDP and an energy-regenerating system (unpublished data) were added. The loading of Ran with the nonhydrolysable GTP analogue GMP-PNP before the transport reaction led to import inhibition in the presence of wt SPN1 or the $IBB_{impα}$-SPN1 mutant (Fig. 7, D and H). Most likely, this is due to a RanGTP-dependent disassembly of the impβ–adaptor complexes on the cytoplasmic side of the NPC, as has been described for impα/β (Rexach and Blobel, 1995; Görlich et al., 1996a; unpublished data). The U snRNP import in the presence of the $IBB_{Impα}$-SPN1 mutant thus exhibits the same behavior as impα-mediated import of BSA-NLS conjugate (Fig. 3 F). This result demonstrates that the IBB domain of SPN1, either alone or in conjunction with the cargo, but not the U snRNP cargo alone, is responsible for the Ran and energy independence of the SPN1–impβ–mediated pathway.

### Translocation of an $IBB_{SPN1}$–βGal fusion protein requires impβ, but not Ran and energy

To determine whether the SPN1 IBB domain by itself is responsible for the observed Ran and energy independence, and thus exclude a possible role of the cargo, we fused the

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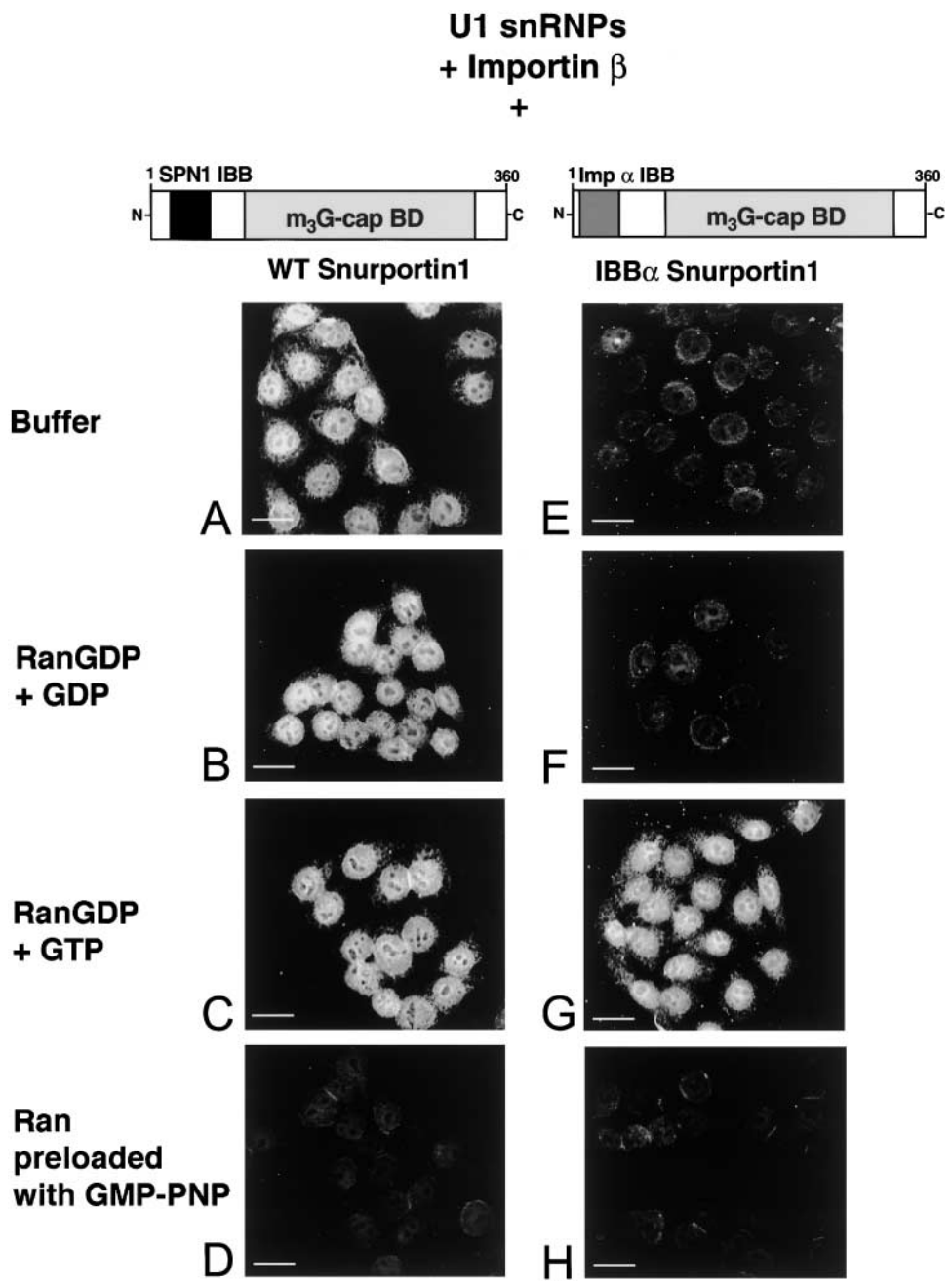


Figure 7. **The SPN1 IBB domain is responsible for the Ran and energy independence of SPN1-mediated nuclear U snRNP import.** Nuclear import of U1 snRNPs was performed with SPN1–importin-β (A–D) or $IBB_{Impα}$-SPN1–importin-β (E–H) for 15 min at 20°C. During the preincubation, either buffer (A and E) or Ran-GDP + GDP (B and F), Ran-GDP + GTP (C and G), or Ran preloaded with GMP-PNP (D and H) was present. The final concentration of transport factors and nucleotides was as follows: 0,04 μM U snRNPs, 0.5 μM SPN1 or $IBB_{Impα}$-SPN1, 0.2 μM importin-β, 2 μM Ran-GDP or Ran-GMPPNP, and 1 mM GDP or GTP. Bars, 20 μm.

SPN1 IBB domain to the reporter protein β-galactosidase ($IBB_{SPN1}$–βGal) and performed in vitro import assays. The IBB domain of impα (aa 1–65) fused to βGal was used as a control. The purified recombinant fusion proteins were fluorescently labeled and tested in the cytosol-free in vitro assay. As shown in Fig. 8 A and consistent with earlier reports (Weis et al., 1996), the $IBB_{Impα}$–βGal fusion protein was effectively imported into the nucleus only in the presence of impβ, Ran, and energy, whereas $IBB_{α}$–βGal by itself did not accumulate in the nucleus (Weis et al., 1996; unpublished data). In the absence of Ran and/or energy, nuclear import was not observed and $IBB_{Impα}$–βGal accumulated at the nuclear pore (Fig. 8, B–D). In contrast, nuclear import of the $IBB_{SPN1}$–βGal fusion in the presence of impβ was as efficient with or without Ran and/or energy (Fig. 8, F–I). Again, $IBB_{SPN1}$–βGal by itself did not accumulate in the nucleus (unpublished data). This result indicates that the IBB-domain of SPN1 is both necessary and sufficient to confer the ability to translocate into the nucleus in the absence of Ran and energy. Thus, the different transport behavior of U

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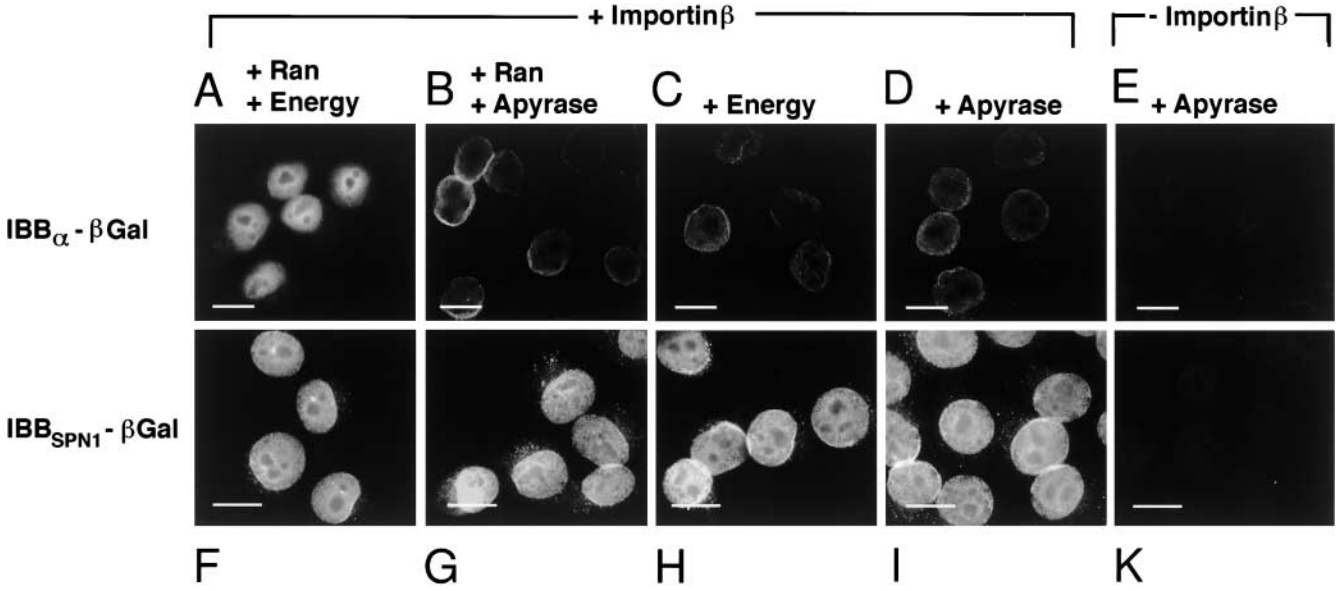


Figure 8. **The IBB domain of SPN1 alone determines the Ran- and energy-independent translocation of IBB–importin-β complexes.** Import of $IBB_{Impα}$–βGal (A–E) and $IBB_{SPN1}$–βGal (F–K) (both at a final concentration or 0.4 μM) were analyzed after a 5-min incubation at 20°C. As indicated, 3 μM RanGDP, 20 u/ml apyrase or energy were added to the import reaction or importin-β (0,2 μM) was omitted (E and K). Bars, 20 μm.

snRNPs and a classical NLS cargo can be attributed to the different IBB domains present in SPN1 and impα.

## Discussion

The investigation of SPN1-dependent import in vitro has provided new insight into the mechanism of nuclear uptake of U snRNPs. The $m_3$G-cap–dependent nuclear import pathway using SPN1–impβ acts independently of the Sm core receptor and is sufficient for the import of U snRNPs in vitro. Although impα and SPN1-mediated nuclear import use the same receptor, namely impβ, significant differences are observed in the nuclear import behavior of their cargoes. Whereas impα–mediated transport requires the presence of Ran and energy, single nuclear import events of U1 snRNPs, and even larger particles like the U5 snRNPs, are not dependent on Ran, energy hydrolysis, or the presence of energy itself. We demonstrate that the major determinant of this difference is the IBB domain of the two adaptors, SPN1 and impα.

### The SPN1-mediated pathway acts independently of the Sm core NLS pathway

By altering the permeabilization and preincubation conditions (Fig. 1), the import of native U1 snRNPs into HeLa cell nuclei was rendered strictly dependent on exogenously added SPN1 and impβ (Fig. 2 C). impβ and SPN1 alone are sufficient for nuclear import and act independently of the Sm core NLS, as indicated by the following observations: (a) the import of U1 snRNPs is not competed by a U1 snRNP that lacks the $m_3$G-cap region (Δ5′U1snRNPs), but containing the Sm-core NLS (Fig. 2); (b) Δ5′U1 snRNPs were not imported above background levels in the presence of exogenously added SPN1, impβ, Ran, and energy (Fig. 2. E).

The fact that the SPN1-dependent import pathway acts autonomously also has implications regarding the second snRNP import pathway. Previous results, namely that Δ5′U1 snRNPs are imported in vitro using unfractionated S100 extract (Huber et al., 1998), did not exclude the possibility that SPN1 recognizes both the $m_3$G-cap NLS and the Sm core NLS. Our finding that Δ5′U1 snRNPs are not imported by SPN1 and impβ (Fig. 2 E) clearly demonstrates that SPN1 recognizes only the $m_3$G-cap structure. This indicates that the Sm coreNLS pathway requires a second import receptor/adaptor distinct fromSPN1, and further suggests that both pathways can act independently of each other.

The precise role and the importance of the two import pathways, and also whether they are tissue-specific or developmentally regulated, is unclear. Whether the two pathways act synergistically or independently of each other in vivo also remains to be solved. In oocytes, both NLSs are required for maximum U1 snRNP import efficiency (Fischer et al., 1991). A likely explanation for this is that SPN1 and possibly the Sm core NLS receptor are present at a low concentration in oocytes, therefore, both are required for efficient import.

### The SPN1-mediated import of large snRNPs is Ran and energy independent

Interestingly, our results show that neither GTP nor any other source of energy are required for SPN1-dependent nuclear import of U1 snRNPs (Fig. 3). Furthermore, the presence of Ran itself is not necessary (Fig. 4). This was somewhat unexpected, as SPN1 interacts with impβ. The import of cargo dependent on impα/β also does not require triphosphate hydrolysis, and thus is independent of energy, but strictly requires the presence of Ran and GTP (Schwoebel et al., 1998). Therefore, the adaptors (i.e., the IBB domains) act as modulators of impβ (see below). The SPN1–impβ pathway resembles the transportin-dependent pathway, which also requires neither Ran nor energy for the import of cargo (Nakielny and Dreyfuss, 1998; Englmeier et al., 1999; Ribbeck et al., 1999).

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In the current import model, Ran-GTP is solely required for the dissociation of the import complexes on the nucleoplasmic side of the NPC. The nucleoporin Nup153, the terminal binding site at the nuclear basket, was found to bind receptors differentially. Interestingly, the receptors bind to different regions of Nup153 depending on the receptor's Ran requirement. Transportin, which imports cargo in a Ran-independent manner, binds to an $NH_2$-terminal region of Nup153. When cargo is bound to transportin, this interaction is not observed indicating a lower affinity for NUP153 (Shah and Forbes, 1998; Nakielny et al., 1999). In contrast, impα/β and cargo, which are dependent on the presence of Ran and GTP for release from the NPC, interact with a COOH-terminal region of Nup153 (Shah and Forbes, 1998; Shah et al., 1998). The results presented in Figs. 3 and 4 clearly suggest a different strategy for the import of complexes containing SPN1 as opposed to impα. The U snRNP containing import complex is released from the NPC independent of Ran and diffuses to its site of function. In sum, the different Ran and energy requirements are consistent with the idea that different complexes have altered affinities and/or binding sites at the nuclear basket.

It has been shown recently (Ribbeck et al., 1999) that free SPN1 was translocated to the nucleus in an Ran-independent manner. The data presented here further demonstrate that import in the absence of NTP hydrolysis and Ran is not restricted to isolated transport receptors. It also occurs with large RNA/protein complexes like the U5 snRNP, which has a molecular mass of ca. 1,000 kD (Fig. 6). To our knowledge, U5 snRNPs are presently the largest RNA–protein complexes so far for which in vitro nuclear import in a Ran- and energy-independent manner has been demonstrated. This is particularly interesting when it is considered that the U5 snRNP–snurportin1–impβ complex has a diameter exceeding significantly the inner nuclear pore diameter in the resting state, which may either be completely closed or a channel of ca. 9 nm (Feldherr et al., 1984). This raises the question of how this expansion is achieved without the expenditure of energy.

### The IBB domain is responsible for different energy and Ran requirements

That the SPN1 IBB domain alone was responsible for the differences in the energy and Ran requirements of impβ–mediated snRNP import was an unexpected observation. As we have shown, an impβ–$IBB_{Impα}$–SPN1 complex is unable to import U1 snRNPs into the nucleus in the absence of Ran and energy, whereas the wt SPN1 is capable of doing so. The results in Fig. 7 indicate that the nature of the adaptor itself modulates the Ran and energy requirements of the impβ–mediated nuclear import pathway. Additionally, our results clearly demonstrate that the differences in the import behavior are solely due to the IBB domain (Figs. 7 and 8), as different COOH termini (β-Gal or $m_3G$-cap binding domain of SPN1) do not interfere with the Ran and energy requirements of IBB domains tested (Figs. 7 and 8).

Recently, an additional member of the family of import adaptors containing an IBB domain has been described (Jullien et al., 1999). The COOH-terminal region of Ripα binds RPA (replication protein A), a single-stranded DNA binding protein complex, and shows no homology to either SPN1 or impα. The $NH_2$-terminal IBB domain binds to impβ, which in turn mediates the import of the RPA–RIPα complex into the nucleus. Sequence alignments of the IBB domains of SPN1, RIPα, and impα reveal a higher degree of identity between Ripα and SPN1 than impα (unpublished data), and suggest that Ripα might interact with impβ like SPN1. In view of the similarities of the IBB domains, it would be interesting to determine whether the Ripα-IBB domain, like that of SPN1, is responsible for Ran-independent nuclear import of RPA.

The interaction of the import complexes with the NPC is exclusively performed by impβ, but the mode or strength of interaction resulting in a Ran-independent release from the nuclear basket is modulated by the IBB domain, which presumably induces conformational changes in impβ. The crystal structure of the impα IBB domain complexed with impβ has been solved (Cingolani et al., 1999). The $IBB_{Impα}$ domain can be divided into two parts, an $NH_2$-terminal extended moiety and a COOH-terminal helix. Interestingly, two different structures were obtained. Wheras the overall shape of impβ is similar in crystal form I and II, there are substantial differences in conformation resulting in a more compact protein in crystal from II (Cingolani et al., 1999). In crystal form I, both domains of $IBB_{Impα}$ are interacing with impβ in an orderly fashion. In crystal form II, the $NH_2$-terminal moiety was poorly ordered and the COOH-terminal helix of the $IBB_{Impα}$-domain showed an unusually high B factor, suggesting that $IBB_{Impα}$ is not the right substrate. The COOH-terminal region of $IBB_{SPN1}$ exhibits a high degree of homology to $IBB_{Impα}$ within the carboxy terminal region. Within the $NH_2$-terminal region, the homology is lower which, in contrast to $IBB_{Impα}$, might allow for a proper interaction of $IBB_{SPN1}$ with impβ of crystal form II. These two crystal structures, as well as the other two crysal structures of impβ obtained thus far, indicate that impβ is highly flexible and can exist in a large variety of conformations (Cingolani et al., 1999; Vetter et al., 1999; Bayliss et al., 2000).

Taken together, our results show that SPN1 interacts with impβ in a manner distinct from impα, which renders U snRNP import independent of Ran and energy. The determinant for this different interaction is localized solely in its IBB domain. Future information obtained from crystal structure of the SPN1–impβ complex should help to explain these mechanistic differences more accurately.

## Materials and methods

All enzymes used for DNA manipulations were purchased from New England Biolabs. Pfu Polymerase was obtained from Stratagene and RNase H from Roche.

#### Nuclear transport factors

**Cloning.** The GST-PreScission-SPN1 constructs pGex-6P1-SPN1 and pGex-6P1-$IBBα_{(1–53)}$SPN1 (Δ1–65) were cloned as follows: pGex-6P1 (Amersham Pharmacia Biotech) was digested by EcoRI and NotI and ligated with the SPN1 insert obtained by PCR amplification using SPN1-for (5′-CCG GAA TTC CCC ATG GAA GAG TTG GAG TCA AGG CC-3′) and SPN1-rev (5′-TTT GCG GCC GCC CCT TAA TTC TCC ATG AGG CAT CC-3′) which introduces an EcoRI and NotI site, respectively. The pGex-6P1-$IBBα_{(1–53)}$SPN1 (Δ1–65) was cloned using the same strategy by first constructing pGex-6P1-SPN1(Δ1–65) lacking the $NH_2$-terminal–most 65 aa with the primers SPN1-rev and Δ1–65SPN1-for (5′-GCG GAA TTC CCC GCT GAA GAT GAC TGG ACA GGG-3′) and subsequent introduction of the $IBB_{impα}$ domain (aa 1–53) amplified from pKW228 (hSRP1 clone kindly

provided by K. Weis, UCSF, CA, USA) using the primers IBBα-for (5′-TTG GGA TCC GGG ATG TCC ACC AAC GAG AAT GCT AAT ACA CC-3′) and IBBα-rev (5′-GGG GAA TTC CCC TAC ATT TCT CCT CTT CAG CAT CTG-3′).

The DNA sequences of all constructed clones were verified with an automated sequencer (Applied Biosystems) using Taq polymerase and double-stranded templates (PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit; Amersham Pharmacia Biotech).

### Expression and purification

Wild-type RanGDP was prepared as described by Melchior et al. (1995). impα and impβ were prepared as described by Hu et al. (1996).

**Expression of the His and GST fusion proteins.** Cells were grown at 20°C until the $OD_{600}$ reached 0.4–0.6. Expression was then induced with IPTG at a final concentration of 1 mM. After incubating for an additional 6–14 h, cells were harvested, washed once with ice-cold PBS, and the pellets were frozen in liquid nitrogen and stored at −80°C until further use.

impβ–His fusion proteins and deletion mutants were purified using a Talon affinity resin (Clontech). Cell pellets were resuspended in ZSP (20 mM Tris/HCl, pH 8, 100 mM NaCl, 2 mM β-mercaptoethanol, and aprotinin, pepstatin, and leupeptin, 1 μg/ml each), sonicated, and the supernatant obtained after centrifuging at 10,000 *g* was added to the resin. After mixing for 1 h at 4°C, the beads were washed three times with WP buffer (ZSP + 15 mM imidazole). Protein was then eluted in two steps with buffers E1 (ZSP + 100 mM imidazole) and E2 (ZSP + 500 mM imidazole), and then dialyzed against T-buffer (see below). Cell pellets of GST fusion proteins were resuspended in GST-ZSP buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml of aprotinin, pepstatin, and leupeptin), and cell disruption and binding to the resin performed as described for His-tagged proteins. After washing, an equal volume of GST-ZSP was added, and precision protease was added (according to the manufacturer's recommendations) and incubated overnight at 4°C. The supernatant, which contained recombinant protein, was pooled with the two subsequent washes, and dialyzed and concentrated against T-buffer.

**Cargoes.** Preparation of U snRNPs was performed as previously described (Huber et al., 1998). To remove the 5′ end of U1 snRNA in order to create Δ5′U1 snRNPs, U1 snRNPs (250 μg) were incubated with 20 U RNase H and a DNA oligonucleotide (5′-CAGGTAAGTAT-3′, final concentration 1.4 μg/μl) in a total volume of 100 μl as described by Lamond and Sproat (1994). Residual amounts of $m_3$G-capped U1 snRNPs were removed from the reaction mixture by immunoprecipitation with 25 μl mAb H20-Sepharose beads in a final volume of 100 μl PBS, pH 8. After a 2-h head-over-tail incubation at 4°C, the sample was briefly centrifuged, and the 5′ U1 snRNPs in the supernatant were concentrated to 2 mg/ml using a Microcon-100 concentrator (Amicon). U1 and U5 snRNPs or Δ5′ U1 snRNPs were fluorescently labeled with Cy3 monofunctional reactive dye (Amersham) or Fluos (Roche) according to the manufacturer's protocol. Due to the instability of U5 snRNPs under low salt conditions (below 250 mM), cross-linking with Cy3 reactive dye was performed in the presence of 500 mM NaCl and 250 mM sucrose. BSA was coupled with the SV40 T-antgen NLS and linked with dye according to Fischer et al. (1995). The pRSET A $IBB_{SPN1(19–85)}$-βGal construct was constructed using the plasmid pKW319, provided by K. Weis (University of California San Francisco, San Francisco, CA). $IBB_{impα}$ was excised from this plasmid using XhoI and KpnI, and was replaced by the sequence coding for aa 19–85 of SPN1. This SPN1 fragment was PCR amplified using SPN1–19-for (5′-GAG CTC GAG ATC AAC AGC ACA GCT GCC CCA CAC CCC CGC-3′) and SPN1–85-rev (5′-GGG CGG TAC CAG TTC TTC ATC ATC ATC TTT CTT ATT TTC TTC-3′), digested with the appropriate restriction enzymes, and ligated into the linearised vector $pKW319_{lin}$. The βGal fusion constructs $IBB_{impα(1–65)}$–βGal and $IBB_{SPN1(19–85)}$–βGal (in pRSET A) were then expressed and purified as described for the His-tagged nuclear transport factors and labeled with FLUOS (Roche) as described for U snRNPs.

**Nuclear import assay.** Nuclear import reactions were performed as described by Huber et al. (1998) except for the following changes. During permeabilization and preincubation, the cells were kept in P-buffer (50 mM Hepes/KOH, pH 7.5, 50 mM KOAc, 8 mM $Mg[OAc]_2$, 2 mM EGTA, 1 mM DTT, and 1 μg/ml each aprotinin, leupeptin, and pepstatin). Permeabilization was performed for 5 min at room temperature in the presence of an energy-regenerating system, followed by a 15-min incubation at room temperature in P buffer. Cells were then transferred to T buffer (20 mM Hepes/KOH, pH 7.5, 80 mM KOAc, 4 mM $Mg[OAc]_2$, 1 mM DTT, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin) before performing the import reaction. A standard 25-μl import reaction generally contained 0.2 mg/ml tRNA, 0.2 mg/ml BSA, 1 mM ATP, 10 mM creatine phosphate, 50 μg/ml creatine phosphokinase [Roche], 40 nM fluorescently labeled U1 snRNPs, and 100 nM BSA-NLS or 12 nM U5 snRNPs. Additional reagents were added as indicated in the figure legends. In general, import factors were in at least fivefold molar excess over U snRNPs (see figure legends for details). Thus, we have chosen experimental conditions which allow the analysis of "single round" transport events. The import mix was depleted of ATP by preincubating for 30 min at 25°C in the presence of 20 U/ml apyrase (Sigma-Aldrich) or 20 U/ml hexokinase/1 mM Glucose. Import reactions were incubated at 25°C for 15 min, terminated as described by Marshallsay and Lührmann (1994), and further processed as described by Huber et al. (1998). Immunofluorescence microscopy for the in situ localisation of SPN1 was performed as previously described (Dickmanns et al., 1996) and mounted and analyzed as in Huber et al. (1998).

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