

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

Jochen Huber,¹ Achim Dickmanns,^{1,2} and Reinhard Lührmann¹

¹Department of Cellular Biochemistry, Max Planck Institute of Biophysical Chemistry, D-37077 Göttingen, Germany

²RNA Metabolism and Neuronal Diseases, Max Planck Institute of Biochemistry, D-82152 Martinsried, Germany

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 NH₂-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 ($imp\alpha$). Here, we show that recombinant snurportin1 and importin- β ($imp\beta$) are not only necessary, but also sufficient for U1 snRNP transport to the nuclei of digitonin-permeabilized HeLa cells. In contrast to $imp\alpha$ -dependent import, single rounds of U1 snRNP import, mediated by the nuclear import receptor complex snurportin1- $imp\beta$,

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 $imp\beta$ and a snurportin1 mutant containing an $imp\alpha$ IBB domain ($IBB_{imp\alpha}$), nuclear U1 snRNP import was Ran dependent. Furthermore, β -galactosidase (β Gal) containing a snurportin1 IBB domain, but not $IBB_{imp\alpha}$ - β Gal, 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 $imp\beta$ 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 \sim 50 unique proteins, termed nucleoporins (for reviews see Stoffler et al., 1999; Ryan and Wenthe, 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 ($imp\beta$)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, $imp\beta$ /Karyopherin- β , the receptor required for proteins carrying a so-called classical NLS, requires an adaptor termed importin- α ($imp\alpha$)/Karyopherin- α (Görlich et al., 1994; Moroianu et al., 1995; Radu et al., 1995; Weis et al., 1995). $Imp\alpha$ consists of an NH₂-terminal $imp\beta$ binding (IBB) domain that mediates complex formation between $imp\beta$ and $imp\alpha$ (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 $imp\alpha$ 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).

Address correspondence to Reinhard Lührmann, Max Planck Institute of Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen. Tel.: 49-551-201-1405. Fax: 49-551-201-1197.

E-mail: reinhard.luehrmann@mpi-bpc.mpg.de

J. Huber's present address is Aventis Pharma Deutschland GmbH, DG Cardiovascular, H827, D-65926 Frankfurt/Main, Germany.

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

Key words: spliceosomal U snRNPs; IBB domain; snurportin 1; nucleocytoplasmic transport; energy and Ran requirements

A nucleocytoplasmic shuttling protein, the small GTPase Ran, plays a key role in determining the directionality of nuclear transport (Izaurre 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 imp α , for example, is bound and exported by CAS1 (also a member of the imp β 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 imp β 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 imp β does not require RanGTP hydrolysis *in vitro* (Ribbeck et al., 1999), although the latter study did not address whether loading of the snurportin1/imp β receptor with cargo would require Ran and energy. In contrast, the import of classical NLS cargoes by imp α / 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–importin α / 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 (m⁷G) 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 (m₃G)-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 m₃G-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 m₃G-cap requirement for nuclear transport in *Xenopus* oocytes. Whereas nuclear import of U1 and U2 snRNPs absolutely requires an intact and accessible m₃G-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 m₃G-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 imp α , required as a bridging molecule between the receptor imp β and the m₃G-cap of U snRNPs (Huber et al., 1998). SPN1 is composed of two domains, an NH₂-terminal domain required for binding to the import receptor, and a COOH-terminal m₃G-cap-binding region. Whereas the COOH-terminal m₃G-cap-binding region of SPN1 bears no obvious structural similarity to the arm repeat domain found in imp α the NH₂-terminal domain exhibits a high degree of homology to the IBB domain of imp α . The IBB domain of SPN1 was shown to have a stimulatory effect on nuclear import of U1 snRNPs, arguing for a direct involvement of imp β in nuclear import of U snRNPs. This idea is further supported by the observations that imp β depletion from *Xenopus* egg extract significantly inhibits snRNP import (Palacios et al., 1997) and our finding that SPN1 translate binds imp β *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

SPN1-imp β 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-imp β was strictly dependent on the m₃G-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 imp α / 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 imp α . Our results suggest that the nature of the IBB domain determines whether or not Ran is required to dissociate imp β /cargo interactions with the NPC.

Results

SPN1 and imp β 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 m₃G-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-m₃G-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 imp β 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-

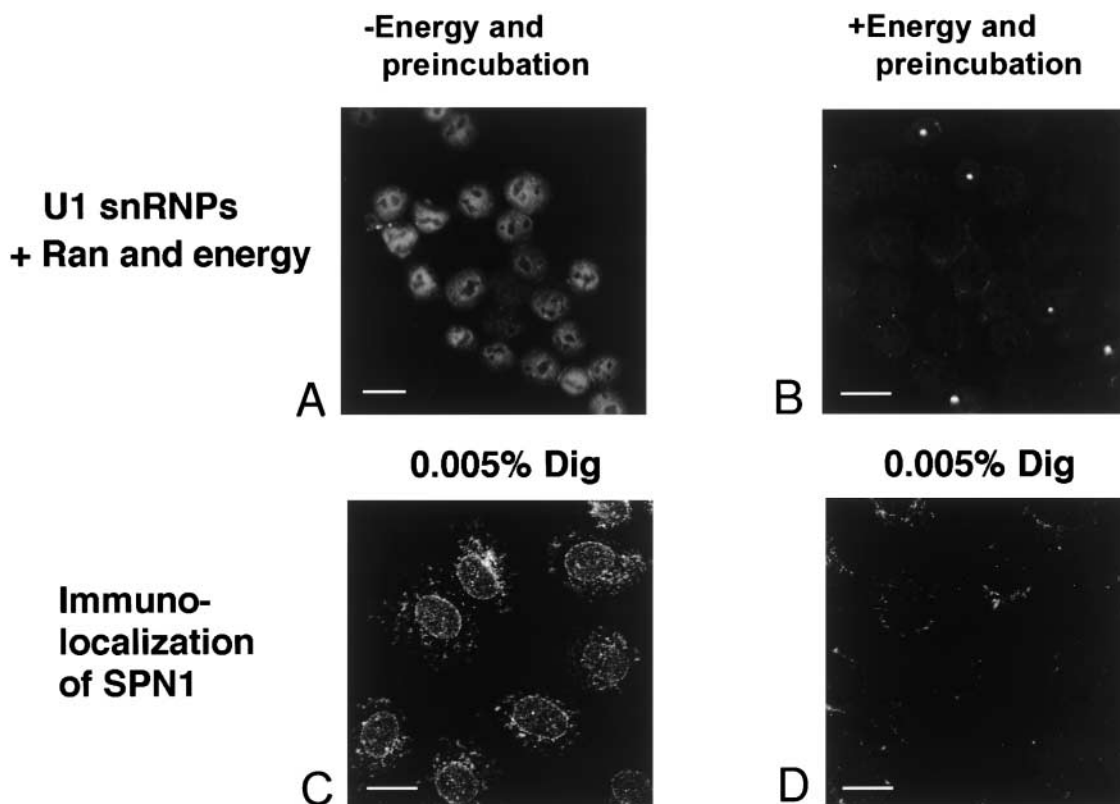


Figure 1. Depletion of endogenous HeLa cell transport factors by modifying the permeabilization and preincubation conditions strongly reduces the import rate of U1 snRNPs. HeLa cells were prepared for *in vitro* import assays by either permeabilizing with digitonin alone (A and C) or in the presence 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 μ m.

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 $\text{imp}\beta$ 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 $\text{imp}\beta$ 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 $\text{imp}\beta$ (Fig. 2 C). U1 snRNP import mediated by SPN1 and $\text{imp}\beta$ strictly requires the 5' terminal m_3G -cap, because $\Delta 5'$ U1 snRNPs (in which the 5' m_3G -cap had been removed) themselves were not imported (Fig. 2 E). Consistent with this observation, a 100-fold excess of $\Delta 5'$ U1 snRNPs, could not inhibit import of intact U1 snRNPs (Fig. 2 D). These data clearly illustrate that SPN1 cooperates with $\text{imp}\beta$ in U1 snRNP nuclear import, and that both are sufficient to import m_3G -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- $\text{imp}\beta$ 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 $\text{imp}\alpha/\beta$ (Fig. 3, F and G). As a control we determined the effect of addition of Ran and the nonhydrolysable GDP analogue GDP β S, 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 $\text{imp}\alpha/\beta$ was added (Fig. 3 K). Surprisingly, SPN1 and $\text{imp}\beta$ 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-

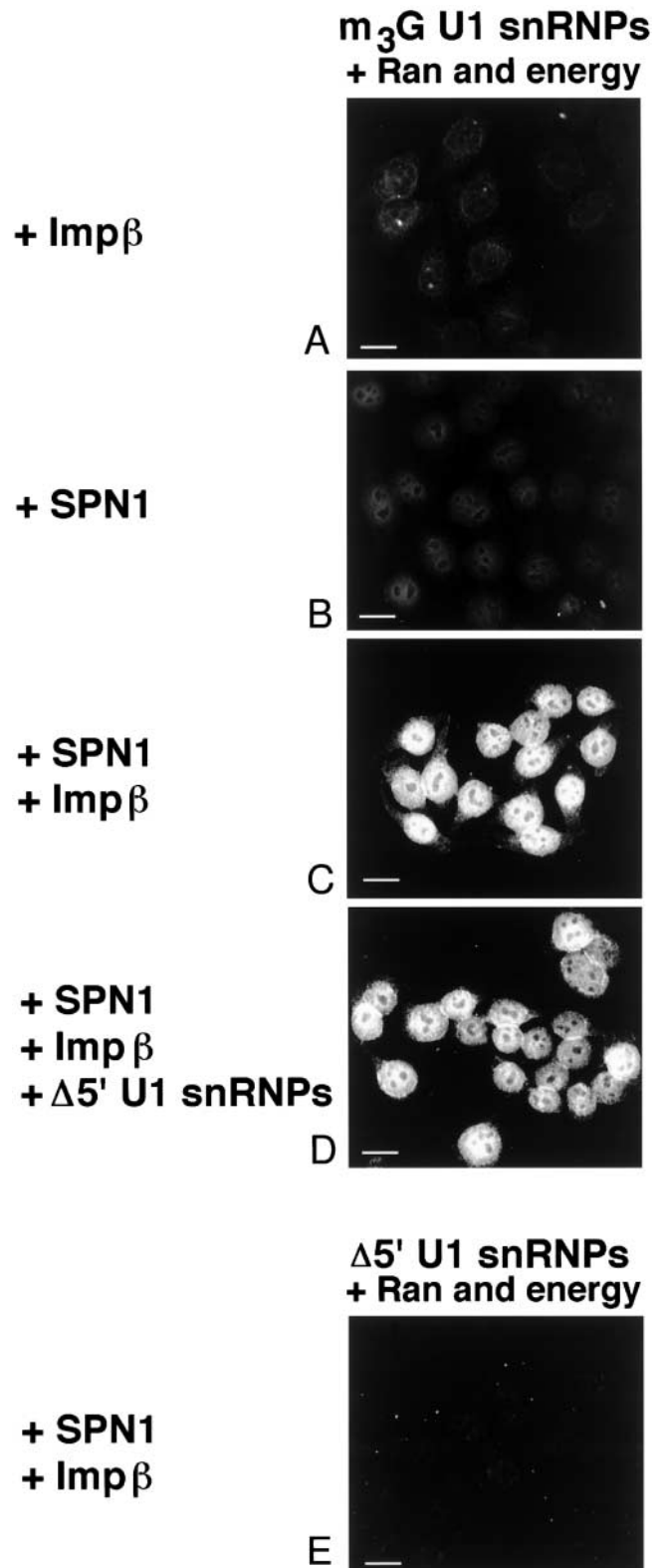


Figure 2. Import of U1 snRNPs is dependent on exogenously added SPN1 and importin- β , but not the Sm core NLS receptor. The import of fluorescently labeled U1 snRNPs in the presence of Ran and energy was monitored in the presence of either importin- β (A), SPN1 (B), or both (C). To investigate the contribution of the Sm core receptor to the import of U1 snRNPs, unlabeled $\Delta 5'$ 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 $\Delta 5'$ U1 snRNPs (E). Bars, 20 μm .

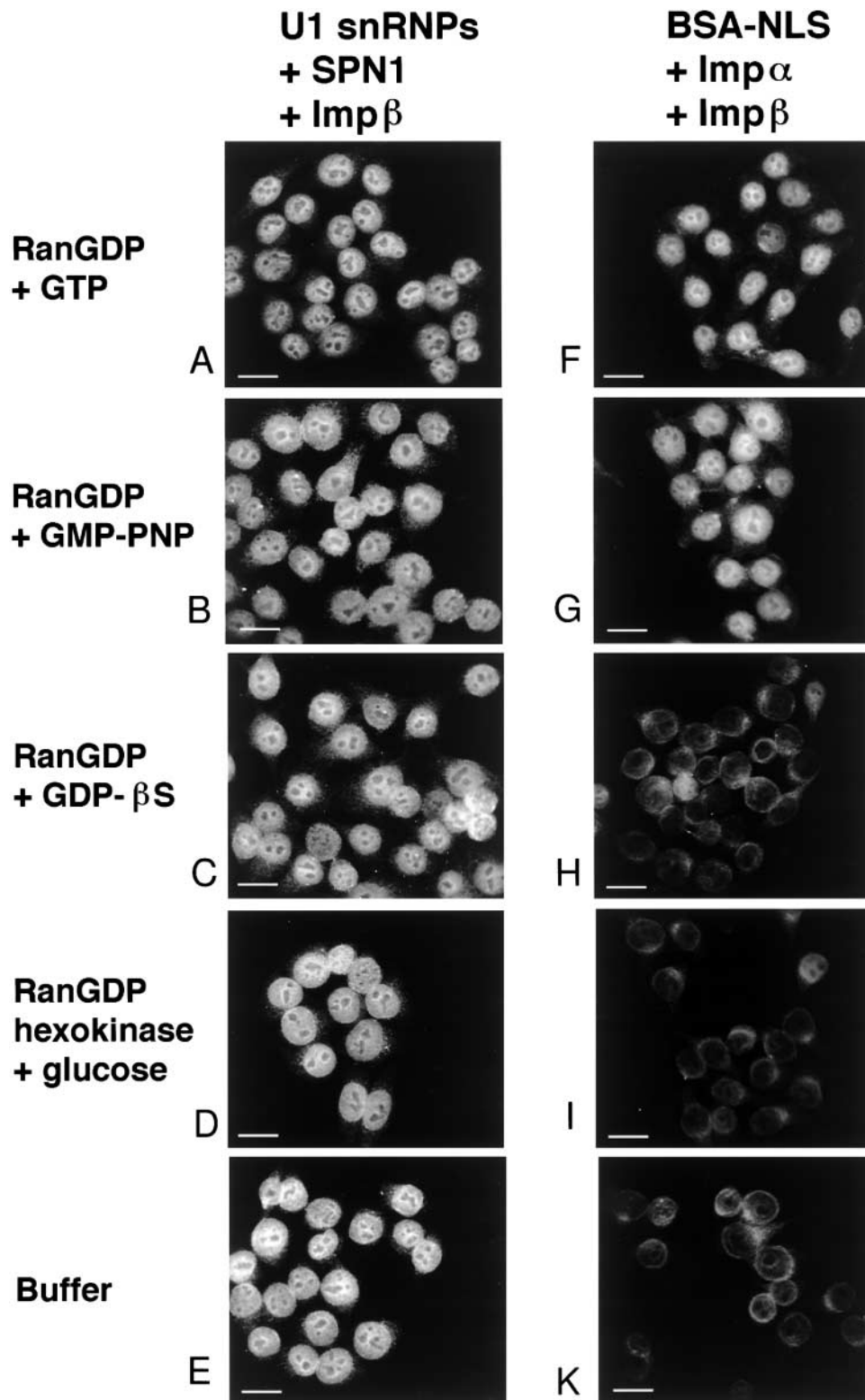


Figure 3. Ran and Ran-dependent hydrolysis of NTPs are not required for SPN1-mediated translocation of U1 snRNPs through the NPC. The nuclear import of Cy3-labeled U1 snRNPs (0,04 μ M) (A–E) or FLUOS-labeled BSA-NLS (0,1 μ M) (F–K) in the presence of preformed adaptor–importin- β complex (0.5 μ M SPN1 or 0.6 μ M importin- α , respectively and 0.2 μ M 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 μ M 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 μ m.

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–imp β –mediated pathway,

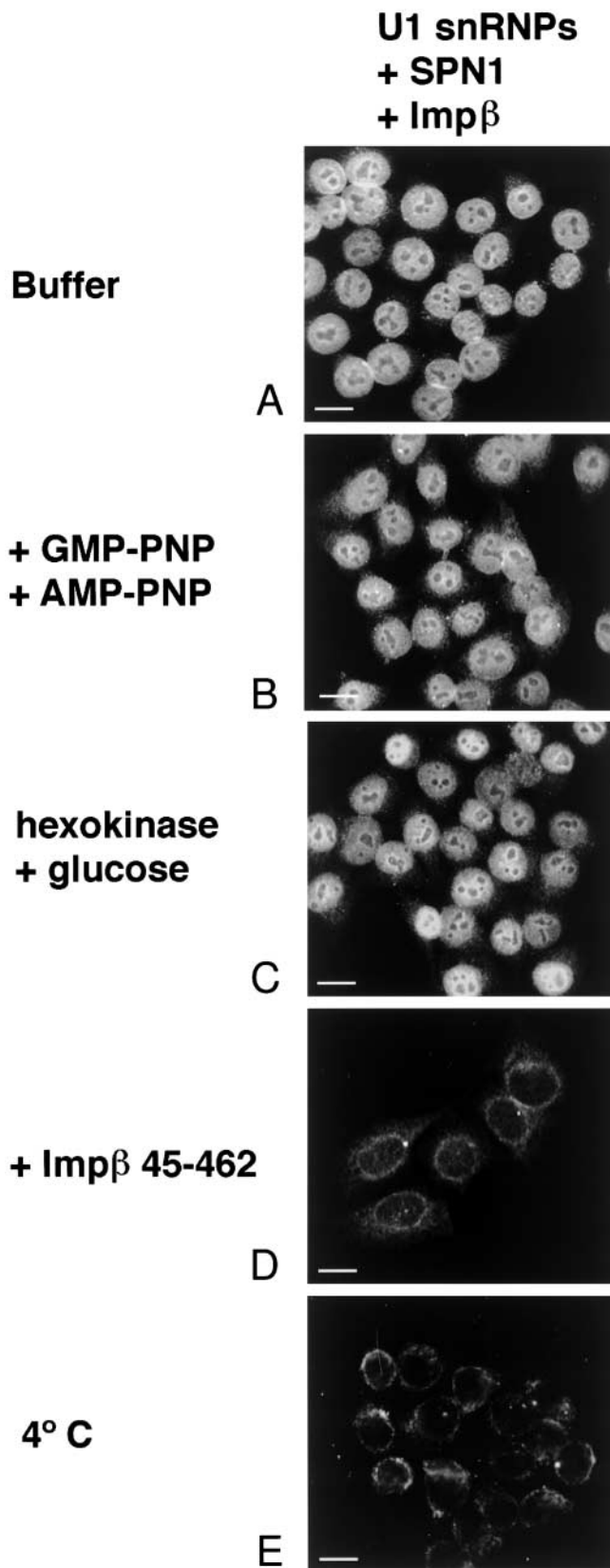


Figure 4. Hydrolysis of NTPs is not required for the transport of U1 snRNPs into the nucleus. HeLa cells were preincubated for 15 min in the presence of buffer (A, D, and E), 1 mM each AMP-PNP and GMP-PNP (B), or 20 U/ml hexokinase/glucose (C) before the addition of Cy3-labeled U1 snRNPs (0,04 μ M) in combination

compared with the control where buffer alone was added (Fig. 4 A). Thus, ATP or GTP hydrolysis is not required for the SPN1–imp β –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 imp β deletion mutant lacking the NH₂-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–imp β import pathway shares at least one intermediate binding site at the nuclear pore with other members of the imp β 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 imp β

Next, we investigated whether Ran and GTP independence are specific for U1 snRNPs, or are a more general feature of the SPN1–imp β –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 fluorescently 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 imp β , 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 imp β (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–imp β –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–imp β –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–imp β –mediated U snRNP import does not vary with snRNP composition or size.

with a preformed complex of 0.5 μ M SPN1 and 0.2 μ M importin- β (A–E) or additionally, 0.5 μ M 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 μ m.

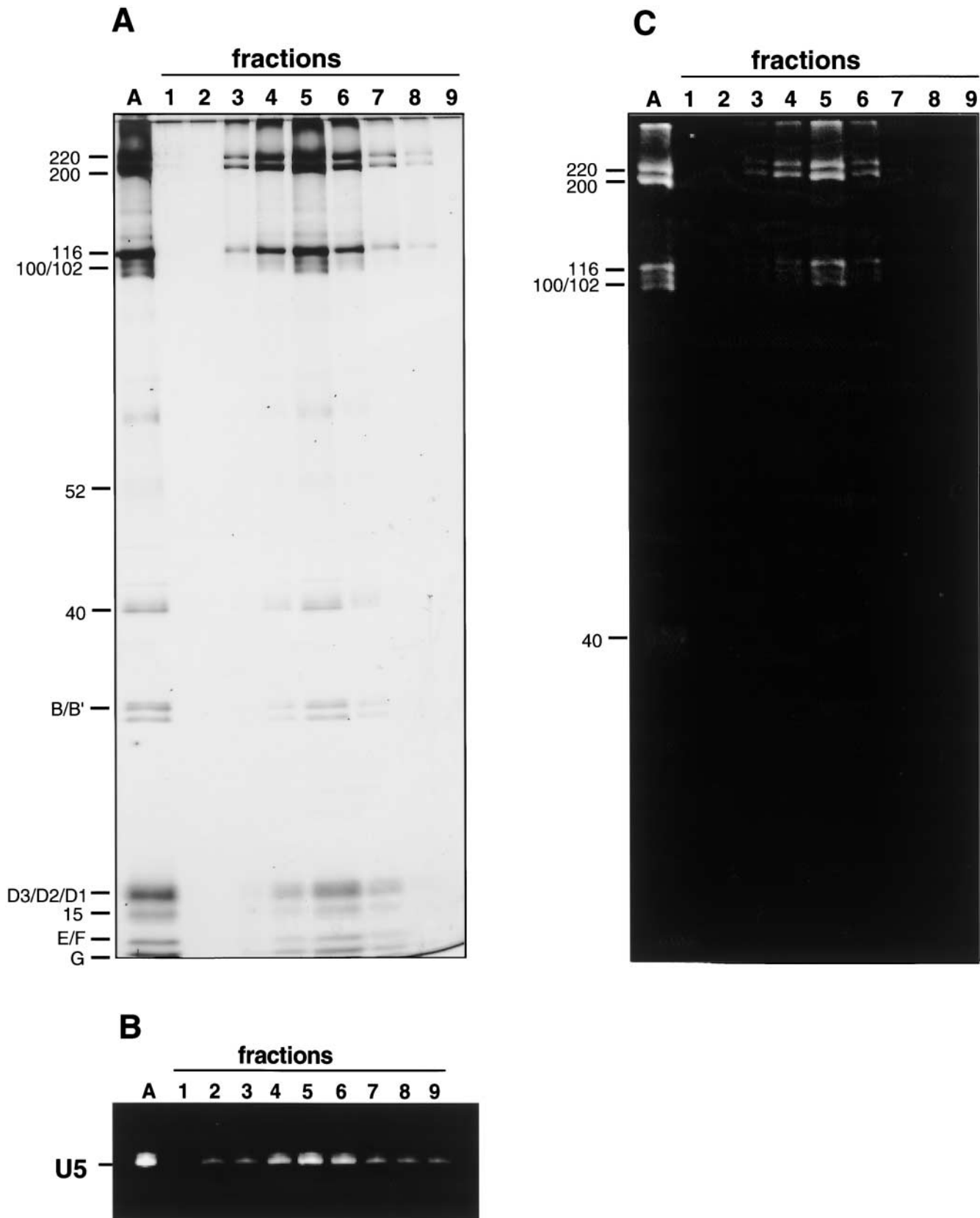


Figure 5. **U5 snRNPs are also imported in a Ran- and energy-independent fashion by SPN1 and importin-β.** (A, B, and C) RNA and protein analysis, and integrity test of U5 snRNPs. 10 μg Cy3-labeled U5 snRNPs were centrifuged on a 1.5-ml 10–30% glycerol gradient (260,000 g, 4°C, 3 h). 150-μl fractions 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.

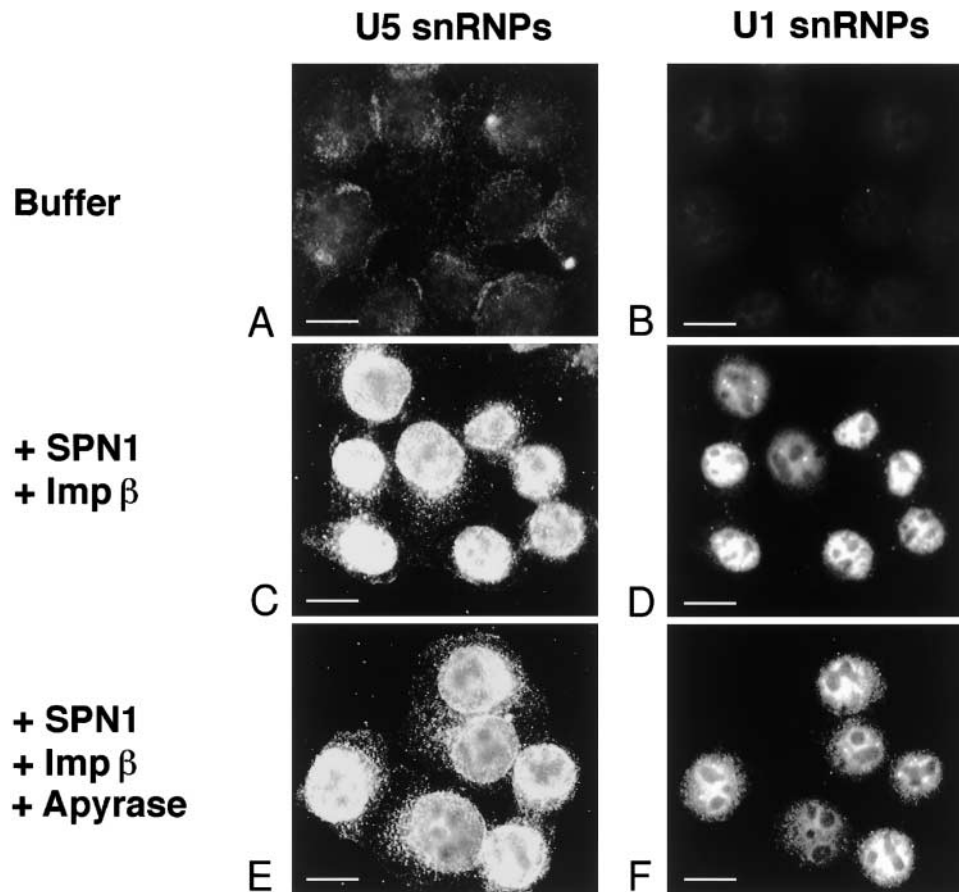


Figure 6. **In vitro import of Cy3-labeled U5 snRNPs (0,012 μ M) (A, C, and E) and FLUOS-labeled U1 snRNPs (0,04 μ M) (B, D, and F).** Permeabilized HeLa cells were preincubated in buffer (A, B, C, and D) or buffer containing 20 u/ml apyrase (E and F) before the simultaneous addition of Cy3-labeled U5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

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, $\text{imp}\beta$. 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 $\text{imp}\beta$, thereby changing the mechanism of translocation of the $\text{imp}\beta$ -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 $\text{imp}\alpha$) with $\text{imp}\beta$. The latter possibility is consistent with the observations of Ribbeck et al. (1999) that SPN1 was imported into the nucleus by $\text{imp}\beta$, also in the absence of snRNP cargo. To distinguish between these possibilities, domain swap experiments were performed by fusing the IBB domain of $\text{imp}\alpha$ to the transport inactive form of snurportin1, $\Delta 1-65$ SPN1. As a control, we first verified that recombinant $\text{IBB}_{\text{imp}\alpha}$ -SPN1 protein recognizes efficiently the m₃G-cap (unpublished data). U1 snRNP import was tested in the presence of $\text{imp}\beta$ and either wild-type (wt) SPN1 or the $\text{IBB}_{\text{imp}\alpha}$ -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 $\text{IBB}_{\text{imp}\alpha}$ -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 $\text{IBB}_{\text{imp}\alpha}$ -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 $\text{IBB}_{\text{imp}\alpha}$ -SPN1 mutant (Fig. 7, D and H). Most likely, this is due to a RanGTP-dependent disassembly of the $\text{imp}\beta$ -adaptor complexes on the cytoplasmic side of the NPC, as has been described for $\text{imp}\alpha/\beta$ (Rexach and Blobel, 1995; Görlich et al., 1996a; unpublished data). The U snRNP import in the presence of the $\text{IBB}_{\text{imp}\alpha}$ -SPN1 mutant thus exhibits the same behavior as $\text{imp}\alpha$ -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- $\text{imp}\beta$ -mediated pathway.

Translocation of an $\text{IBB}_{\text{SPN1}}-\beta\text{Gal}$ fusion protein requires $\text{imp}\beta$, 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

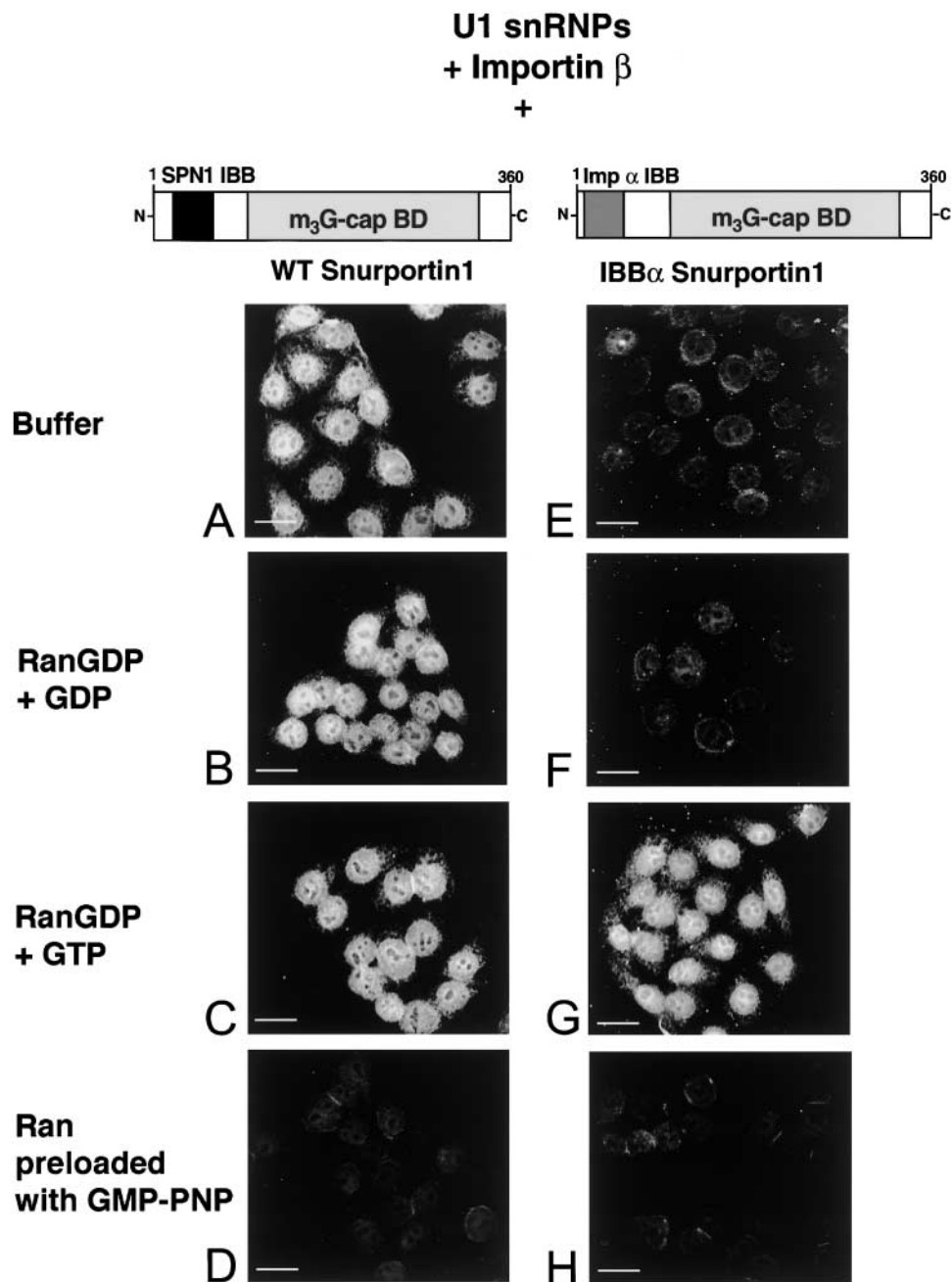


Figure 7. The SPN1 IBB domain is responsible for the Ran and energy independence of SPN1-mediated nuclear U snRNP import. Nuclear import of U1 snRNPs was performed with SPN1–importin- β (A–D) or IBB_{imp α} –SPN1–importin- β (E–H) for 15 min at 20°C. During the preincubation, either buffer (A and E) 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 μ M U snRNPs, 0.5 μ M SPN1 or IBB_{imp α} –SPN1, 0.2 μ M importin- β , 2 μ M Ran-GDP or Ran-GMPNP, and 1 mM GDP or GTP. Bars, 20 μ m.

SPN1 IBB domain to the reporter protein β -galactosidase (IBB_{SPN1}– β Gal) and performed in vitro import assays. The IBB domain of imp α (aa 1–65) fused to β Gal 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_{imp α} – β Gal fusion protein was effectively imported into the nucleus only in the presence of imp β , Ran, and energy, whereas IBB α – β Gal by itself did not accumulate in the nucleus (Weis et al., 1996; unpub-

lished data). In the absence of Ran and/or energy, nuclear import was not observed and IBB_{imp α} – β Gal accumulated at the nuclear pore (Fig. 8, B–D). In contrast, nuclear import of the IBB_{SPN1}– β Gal fusion in the presence of imp β was as efficient with or without Ran and/or energy (Fig. 8, F–I). Again, IBB_{SPN1}– β Gal 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

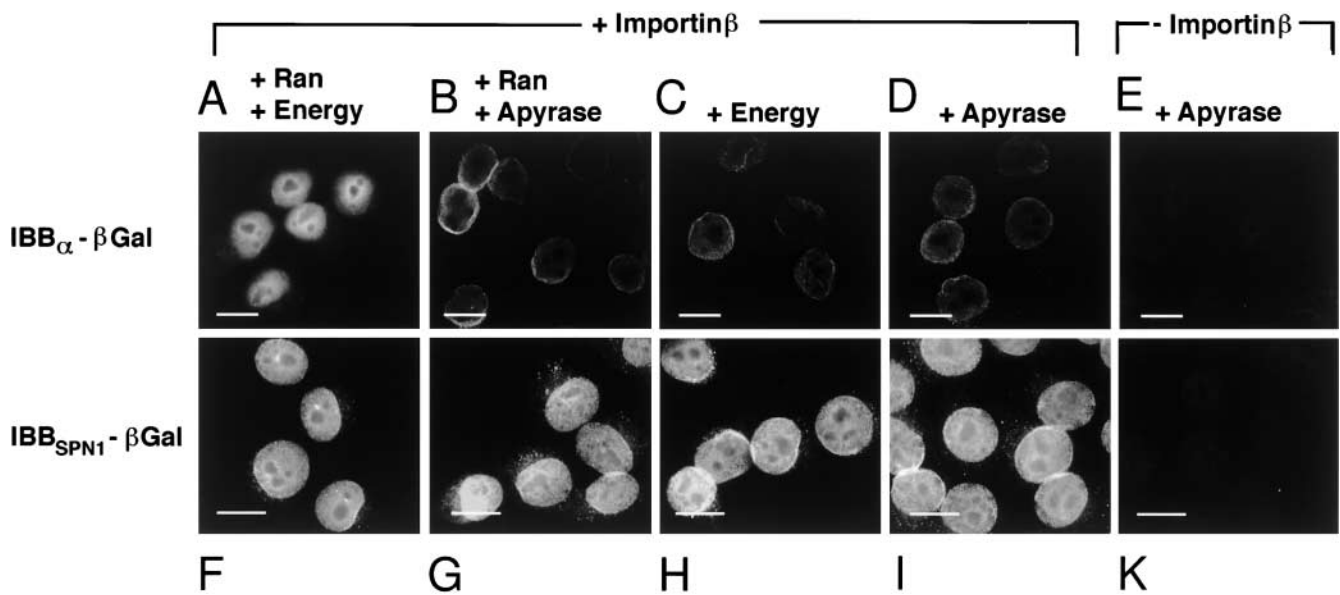


Figure 8. The IBB domain of SPN1 alone determines the Ran- and energy-independent translocation of IBB–importin- β complexes. Import of IBB_{imp α} - β Gal (A–E) and IBB_{SPN1}- β Gal (F–K) (both at a final concentration of 0.4 μ M) were analyzed after a 5-min incubation at 20°C. As indicated, 3 μ M RanGDP, 20 u/ml apyrase or energy were added to the import reaction or importin- β (0.2 μ M) was omitted (E and K). Bars, 20 μ m.

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

Discussion

The investigation of SPN1-dependent import *in vitro* has provided new insight into the mechanism of nuclear uptake of U snRNPs. The m₃G-cap-dependent nuclear import pathway using SPN1–imp β acts independently of the Sm core receptor and is sufficient for the import of U snRNPs *in vitro*. Although imp α and SPN1-mediated nuclear import use the same receptor, namely imp β , significant differences are observed in the nuclear import behavior of their cargoes. Whereas imp α -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 imp α .

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 imp β (Fig. 2 C). imp β 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 m₃G-cap region (Δ 5'U1snRNPs), but containing the Sm-core NLS (Fig. 2); (b) Δ 5'U1 snRNPs were not imported above background levels in the presence of exogenously added SPN1, imp β , Ran, and energy (Fig. 2 E).

The fact that the SPN1-dependent import pathway acts autonomously also has implications regarding the second sn-

RNP import pathway. Previous results, namely that Δ 5'U1 snRNPs are imported *in vitro* using unfractionated S100 extract (Huber et al., 1998), did not exclude the possibility that SPN1 recognizes both the m₃G-cap NLS and the Sm core NLS. Our finding that Δ 5'U1 snRNPs are not imported by SPN1 and imp β (Fig. 2 E) clearly demonstrates that SPN1 recognizes only the m₃G-cap structure. This indicates that the Sm coreNLS pathway requires a second import receptor/adaptor distinct from SPN1, 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 imp β . The import of cargo dependent on imp α / 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 imp β (see below). The SPN1–imp β pathway resembles the transportin-dependent pathway, which also requires neither Ran nor energy for the import of cargo (Nakielnny and Dreyfuss, 1998; Englmeier et al., 1999; Ribbeck et al., 1999).

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 NH₂-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, imp α / 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 imp α . 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 a 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–imp β 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 imp β -mediated snRNP import was an unexpected observation. As we have shown, an imp β –IBB_{imp α} –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 imp β -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 m₃G-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 (Julien et al., 1999). The COOH-terminal region of Rip α binds RPA (replication protein A), a single-stranded DNA binding protein complex, and shows no homology to either

SPN1 or imp α . The NH₂-terminal IBB domain binds to imp β , which in turn mediates the import of the RPA–RIP α complex into the nucleus. Sequence alignments of the IBB domains of SPN1, RIP α , and imp α reveal a higher degree of identity between Rip α and SPN1 than imp α (unpublished data), and suggest that Rip α might interact with imp β like SPN1. In view of the similarities of the IBB domains, it would be interesting to determine whether the Rip α –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 imp β , 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 imp β . The crystal structure of the imp α IBB domain complexed with imp β has been solved (Cingolani et al., 1999). The IBB_{imp α} domain can be divided into two parts, an NH₂-terminal extended moiety and a COOH-terminal helix. Interestingly, two different structures were obtained. Whereas the overall shape of imp β is similar in crystal form I and II, there are substantial differences in conformation resulting in a more compact protein in crystal form II (Cingolani et al., 1999). In crystal form I, both domains of IBB_{imp α} are interacting with imp β in an orderly fashion. In crystal form II, the NH₂-terminal moiety was poorly ordered and the COOH-terminal helix of the IBB_{imp α} -domain showed an unusually high B factor, suggesting that IBB_{imp α} is not the right substrate. The COOH-terminal region of IBB_{SPN1} exhibits a high degree of homology to IBB_{imp α} within the carboxy terminal region. Within the NH₂-terminal region, the homology is lower which, in contrast to IBB_{imp α} , might allow for a proper interaction of IBB_{SPN1} with imp β of crystal form II. These two crystal structures, as well as the other two crystal structures of imp β obtained thus far, indicate that imp β 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 imp β in a manner distinct from imp α , 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–imp β 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-IBB α _(1–53)-SPN1 (Δ 1–65) 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-IBB α _(1–53)-SPN1 (Δ 1–65) was cloned using the same strategy by first constructing pGex-6P1-SPN1(Δ 1–65) lacking the NH₂-terminal-most 65 aa with the primers SPN1-rev and Δ 1–65SPN1-for (5'-GCG GAA TTC CCC GCT GAA GAT GAC TGG ACA GGG-3') and subsequent introduction of the IBB_{imp α} domain (aa 1–53) amplified from pKW228 (hSRP1 clone kindly

provided by K. Weis, UCSF, CA, USA) using the primers IBB α -for (5'-TTG GGA TCC GGG ATG ACC AAC GAG AAT GCT AAT ACA CC-3') and IBB α -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). *imp α* and *imp β* 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 OD₆₀₀ 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.

imp β -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 μ g/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 μ g/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 Δ 5' U1 snRNPs, U1 snRNPs (250 μ g) were incubated with 20 U RNase H and a DNA oligonucleotide (5'-CAGGTAAGTAT-3', final concentration 1.4 μ g/ μ l) in a total volume of 100 μ l as described by Lamond and Sproat (1994). Residual amounts of m₃G-capped U1 snRNPs were removed from the reaction mixture by immunoprecipitation with 25 μ l mAb H20-Sepharose beads in a final volume of 100 μ l 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 Δ 5' 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-antigen NLS and linked with dye according to Fischer et al. (1995). The pRSET A IBB_{SPN1(19–85)}- β Gal construct was constructed using the plasmid pKW319, provided by K. Weis (University of California San Francisco, San Francisco, CA). IBB_{imp α} 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 TTT CTT ATT TTC TTC-3'), digested with the appropriate restriction enzymes, and ligated into the linearised vector pKW319_{in}. The β Gal fusion constructs IBB_{imp α (1–65)}- β Gal and IBB_{SPN1(19–85)}- β Gal (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 μ g/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 μ g/ml each of aprotinin, leupeptin, and pepstatin) before performing the import reaction. A standard 25- μ l import reaction generally contained 0.2 mg/ml tRNA, 0.2 mg/ml BSA, 1 mM ATP, 10 mM creatine phosphate, 50 μ g/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).

We are indebted to Cindy L. Will for critically reading the manuscript, and would like to thank Irene Öchsner, Winfried Loren, and Axel Badouin for excellent technical assistance.

This work was supported by the Gottfried Wilhelm Leibniz Program, grants from the Deutsche Forschungsgemeinschaft (SFB 523/A8) and Fonds der Chemischen Industrie (to R. Lührmann), and a postdoctoral fellowship from the Deutsches Krebsforschungszentrum to A. Dickmanns.

Submitted: 22 August 2001
Revised: 11 December 2001
Accepted: 20 December 2001

References

- Bayliss, R., T. Littlewood, and M. Stewart. 2000. Structural basis for the interaction between. FxFG nucleoporin repeats and importin- β in nuclear trafficking. *Cell*. 102:99–108.
- Cingolani, G., C. Petosa, K. Weis, and C.W. Muller. 1999. Structure of importin- β bound to the IBB domain of importin- α . *Nature*. 399:221–229.
- Dickmanns, A., F.R. Bischoff, C. Marshallsay, R. Lührmann, H. Ponstingl, and E. Fanning. 1996. The thermolability of nuclear protein import in tsBN2 cells is suppressed by microinjected Ran-GTP or Ran-GDP, but not by RanQ69L or RanT24N. *J. Cell Sci.* 109:1449–1457.
- Englmeier, L., J.C. Olivo, and I.W. Mattaj. 1999. Receptor-mediated substrate translocation through the nuclear pore complex without nucleotide triphosphate hydrolysis. *Curr. Biol.* 9:30–41.
- Feldherr, C.M., E. Kallenbach, and N. Schultze. 1984. Movement of a karyophilic protein through the nuclear pores of oocytes. *J. Cell Biol.* 99:2216–2222.
- Fischer, U., E. Darzynkiewicz, S.M. Tahara, N.A. Dathan, R. Lührmann, and I.W. Mattaj. 1991. Diversity in the signals required for nuclear accumulation of U snRNPs and variety in the pathways of nuclear transport. *J. Cell Biol.* 113:705–714.
- Fischer, U., and R. Lührmann. 1990. An essential signaling role for the m3G cap in the transport of U1 snRNP to the nucleus. *Science*. 249:786–790.
- Fischer, U., V. Sumpter, M. Sekine, T. Satoh, and R. Lührmann. 1993. Nucleocytoplasmic transport of U snRNPs: definition of a nuclear location signal in the Sm core domain that binds a transport receptor independently of the m3G cap. *EMBO J.* 12:573–583.
- Fischer, U., J. Heinrich, K. van Zee, E. Fanning, and R. Lührmann. 1994. Nuclear transport of U1 snRNP in somatic cells: differences in signal requirement compared with *Xenopus laevis* oocytes. *J. Cell Biol.* 125:971–980.
- Fischer, U., J. Huber, W.C. Boelens, I.W. Mattaj, and R. Lührmann. 1995. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell*. 82:475–483.
- Fornerod, M., M. Ohno, M. Yoshida, and I.W. Mattaj. 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell*. 90:1051–1060.
- Görlich, D., and U. Kutay. 1999. Transport between the cell nucleus and the cytoplasm. *Annu. Rev. Cell Dev. Biol.* 15:607–660.
- Görlich, D., S. Prehn, R.A. Laskey, and E. Hartmann. 1994. Isolation of a protein that is essential for the first step of nuclear protein import. *Cell*. 79:767–778.
- Görlich, D., P. Henklein, R.A. Laskey, and E. Hartmann. 1996a. A 41 amino acid motif in importin- α confers binding to importin- β and hence transit into the nucleus. *EMBO J.* 15:1810–1817.
- Görlich, D., N. Pante, U. Kutay, U. Aebi, and F.R. Bischoff. 1996b. Identification of different roles for RanGDP and RanGTP in nuclear protein import. *EMBO J.* 15:5584–5594.
- Görlich, D., M. Dabrowski, F.R. Bischoff, U. Kutay, P. Bork, E. Hartmann, S. Prehn, and E. Izaurralde. 1997. A novel class of RanGTP binding proteins. *J. Cell Biol.* 138:65–80.

- Hamm, J., and I.W. Mattaj. 1990. Monomethylated cap structures facilitate RNA export from the nucleus. *Cell*. 63:109–118.
- Hu, T., T. Guan, and L. Gerace. 1996. Molecular and functional characterization of the p62 complex, an assembly of nuclear pore complex glycoproteins. *J. Cell Biol.* 134:589–601.
- Huber, J., U. Cronshagen, M. Kadokura, C. Marshallsay, T. Wada, M. Sekine, and R. Lührmann. 1998. Snurportin1, an m3G-cap-specific nuclear import receptor with a novel domain structure. *EMBO J.* 17:4114–4126.
- Izaurrealde, E., U. Kutay, C. von Kobbe, I.W. Mattaj, and D. Görlich. 1997. The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. *EMBO J.* 16:6535–6547.
- Jullien, D., D. Görlich, U.K. Laemmli, and Y. Adachi. 1999. Nuclear import of RPA in *Xenopus* egg extracts requires a novel protein XRIP α but not importin- α . *EMBO J.* 18:4348–4358.
- Kose, S., N. Imamoto, T. Tachibana, T. Shimamoto, and Y. Yoneda. 1997. Ran-unassisted nuclear migration of a 97-kD component of nuclear pore-targeting complex. *J. Cell Biol.* 139:841–849.
- Kutay, U., E. Izaurrealde, F.R. Bischoff, I.W. Mattaj, and D. Görlich. 1997. Dominant-negative mutants of importin- β block multiple pathways of import and export through the nuclear pore complex. *EMBO J.* 16:1153–1163.
- Lamond, A.I., and B.S. Sproat. (1994). Isolation and characterization of ribonucleoprotein complexes. In *RNA Processing: A Practical Approach*. Higgins, S.J., and B.D. Hames, editors. IRL Press, Oxford. 103–140 pp.
- Mahajan, R., C. Delphin, T. Guan, L. Gerace, and F. Melchior. 1997. A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell*. 88:97–107.
- Marshallsay, C., and R. Lührmann. 1994. In vitro nuclear import of snRNPs: cytosolic factors mediate m3G-cap dependence of U1 and U2 snRNP transport. *EMBO J.* 13:222–231.
- Marshallsay, C., A. Dickmanns, F.R. Bischoff, H. Ponstingl, E. Fanning, and R. Lührmann. 1996. In vitro and in vivo evidence that protein and U1 snRNP nuclear import in somatic cells differ in their requirement for GTP-hydrolysis, Ran/TC4 and RCC1. *Nucleic Acids Res.* 24:1829–1836.
- Mattaj, I.W. 1986. Cap trimethylation of U snRNA is cytoplasmic and dependent on U snRNP protein binding. *Cell*. 46:905–911.
- Mattaj, I.W., and L. Englmeier. 1998. Nucleocytoplasmic transport: the soluble phase. *Annu. Rev. Biochem.* 67:265–306.
- Mattaj, I.W., R. Zeller, A.E. Carrasco, M. Jamrich, S. Lienhard, and E.M. De Robertis. 1985. U snRNA gene families in *Xenopus laevis*. *Oxf. Surv. Eukaryot. Genes.* 2:121–140.
- Matunis, M.J., E. Coutavas, and G. Blobel. 1996. A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J. Cell Biol.* 135:1457–1470.
- Melchior, F., T. Guan, N. Yokoyama, T. Nishimoto, and L. Gerace. 1995. GTP hydrolysis by Ran occurs at the nuclear pore complex in an early step of protein import. *J. Cell Biol.* 131:571–581.
- Michaud, N., and D. Goldfarb. 1992. Microinjected U snRNAs are imported to oocyte nuclei via the nuclear pore complex by three distinguishable targeting pathways. *J. Cell Biol.* 116:851–861.
- Moroianu, J., G. Blobel, and A. Radu. 1995. Previously identified protein of uncertain function is karyopherin α and together with karyopherin β docks import substrate at nuclear pore complexes. *Proc. Natl. Acad. Sci. USA.* 92:2008–2011.
- Moroianu, J., G. Blobel, and A. Radu. 1996. The binding site of karyopherin α for karyopherin β overlaps with a nuclear localization sequence. *Proc. Natl. Acad. Sci. USA.* 93:6572–6576.
- Nachury, M.V., and K. Weis. 1999. The direction of transport through the nuclear pore can be inverted. *Proc. Natl. Acad. Sci. USA.* 96:9622–9627.
- Nakiely, S., and G. Dreyfuss. 1998. Import and export of the nuclear protein import receptor transportin by a mechanism independent of GTP hydrolysis. *Curr. Biol.* 8:89–95.
- Nakiely, S., S. Shaikh, B. Burke, and G. Dreyfuss. 1999. Nup153 is an M9-containing mobile nucleoporin with a novel Ran-binding domain. *EMBO J.* 18:1982–1995.
- Neuman de Vegvar, H.E., and J.E. Dahlberg. 1990. Nucleocytoplasmic transport and processing of small nuclear RNA precursors. *Mol. Cell. Biol.* 10:3365–3375.
- Ohtsubo, M., H. Okazaki, and T. Nishimoto. 1989. The RCC1 protein, a regulator for the onset of chromosome condensation locates in the nucleus and binds to DNA. *J. Cell Biol.* 109:1389–1397.
- Palacios, I., M. Hetzer, S.A. Adam, and I.W. Mattaj. 1997. Nuclear import of U snRNPs requires importin- β . *EMBO J.* 16:6783–6792.
- Plessel, G., U. Fischer, and R. Lührmann. 1994. m3G cap hypermethylation of U1 small nuclear ribonucleoprotein (snRNP) in vitro: evidence that the U1 small nuclear RNA-(guanosine-N2)-methyltransferase is a non-snRNP cytoplasmic protein that requires a binding site on the Sm core domain. *Mol. Cell. Biol.* 14:4160–4172.
- Radu, A., G. Blobel, and M.S. Moore. 1995. Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. *Proc. Natl. Acad. Sci. USA.* 92:1769–1773.
- Rexach, M., and G. Blobel. 1995. Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. *Cell*. 83:683–692.
- Ribbeck, K., U. Kutay, E. Paraskeva, and D. Görlich. 1999. The translocation of transportin-cargo complexes through nuclear pores is independent of both Ran and energy. *Curr. Biol.* 9:47–50.
- Rout, M.P., and J.D. Aitchison. 2001. The nuclear pore complex as a transport machine. *J. Biol. Chem.* 276:16593–16596.
- Ryan, K.J., and S.R. Wentz. 2000. The nuclear pore complex: a protein machine bridging the nucleus and cytoplasm. *Curr. Opin. Cell Biol.* 12:361–371.
- Schwoebel, E.D., B. Talcott, I. Cushman, and M.S. Moore. 1998. Ran-dependent signal-mediated nuclear import does not require GTP hydrolysis by Ran. *J. Biol. Chem.* 273:35170–35175.
- Shah, S., and D.J. Forbes. 1998. Separate nuclear import pathways converge on the nucleoporin Nup153 and can be dissected with dominant-negative inhibitors. *Curr. Biol.* 8:1376–1386.
- Shah, S., S. Tugendreich, and D. Forbes. 1998. Major binding sites for the nuclear import receptor are the internal nucleoporin Nup153 and the adjacent nuclear filament protein Tpr. *J. Cell Biol.* 141:31–49.
- Stoffler, D., B. Fahrenkrog, and U. Aebi. 1999. The nuclear pore complex: from molecular architecture to functional dynamics. *Curr. Opin. Cell Biol.* 11:391–401.
- Vankan, P., C. McGuigan, and I.W. Mattaj. 1990. Domains of U4 and U6 snRNAs required for snRNP assembly and splicing complementation in *Xenopus* oocytes. *EMBO J.* 9:3397–3404.
- Vetter, I.R., A. Arndt, U. Kutay, D. Görlich, and A. Wittinghofer. 1999. Structural view of the Ran-importin- β interaction at 2.3 Å resolution. *Cell*. 97:635–646.
- Weis, K., I.W. Mattaj, and A.I. Lamond. 1995. Identification of hSRP1 α as a functional receptor for nuclear localization sequences. *Science*. 268:1049–1053.
- Weis, K., U. Ryder, and A.I. Lamond. 1996. The conserved amino-terminal domain of hSRP1 α is essential for nuclear protein import. *EMBO J.* 15:1818–1825.