An ATP-dependent, Ran-independent Mechanism for Nuclear Import of the U1A and U2B′′ Spliceosome Proteins

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Abstract. Nuclear import of the two uracil-rich small nuclear ribonucleoprotein (U snRNP) components U1A and U2B′′ is mediated by unusually long and complex nuclear localization signals (NLSs). Here we investigate nuclear import of U1A and U2B′′ in vitro and demonstrate that it occurs by an active, saturable process. Several lines of evidence suggest that import of the two proteins occurs by an import mechanism different to those characterized previously. No cross competition is seen with a variety of previously studied NLSs. In contrast to import mediated by members of the importin-β family of nucleocytoplasmic transport receptors, U1A/U2B′′ import is not inhibited by either non-hydrolyzable guanosine triphosphate (GTP) analogues or by a mutant of the GTPase Ran that is incapable of GTP hydrolysis. Adenosine triphosphate is capable of supporting U1A and U2B′′ import, whereas neither nonhydrolyzable adenosine triphosphate analogues nor GTP can do so. U1A and U2B′′ import in vitro does not require the addition of soluble cytosolic proteins, but a factor or factors required for U1A and U2B′′ import remains tightly associated with the nuclear fraction of conventionally permeabilized cells. This activity can be solubilized in the presence of elevated MgCl2. These data suggest that U1A and U2B′′ import into the nucleus occurs by a hitherto uncharacterized mechanism.

Key words: uracil-rich small nuclear ribonucleoproteins (U snRNP) • nucleocytoplasmic transport • nuclear import • Ran GTPase • U1A protein

Introduction

Nucleocytoplasmic transport of proteins and RNPs is a major cellular function. Transport occurs through large proteinaceous structures called nuclear pore complexes (NPCs)1 (Corbett and Silver, 1996; Nigg, 1997; Ohno et al., 1998). In vertebrates, NPCs have a size of ∼125 million D and are composed of 30–100 distinct subunits (Rout and Blobel, 1993; Doye and Hurt, 1997). These proteins, the nucleoporins, form an aqueous channel through which small ions, metabolites, and small polypeptides can freely diffuse (Bonner, 1978). However, the transport of most cellular proteins and RNPs, even that of small molecules such as histones (Breeuwer and Goldfarb, 1990), ribosomal proteins (Rout et al., 1997; Schlenstedt et al., 1997; Jäkel and Görlich, 1998), and transfer RNAs (Achts et al., 1998) is an active, signal-mediated process. A ctively transported proteins are targeted to the nucleus by specific nuclear localization signals (NLSs). In the first discovered or classical nuclear import pathway, the NLSs were characterized as short, positively charged sequences exemplified by that of the simian virus (SV) 40 T antigen or as the longer bipartite basic signals such as that of nucleoplasmin (Dingwall, 1991). Later, a second apparently unrelated type of signal was identified in uracil-rich small nuclear (U sn) RNPs. These RNPs carry a complex import signal partly composed of a trimethyl guanosine cap structure, and partly of an as-yet poorly characterized element formed on binding of the U snRNA to the seven U snRNP core or Sm proteins (Mattaj and De Robertis, 1985; Mattaj, 1988). A third class of NLS, the M9 domain of heterogeneous-nuclear (hn) RNP A1, is rich in glycine and aromatic residues, and unlike the two forms of classical NLS, can also function as a nuclear export signal (Soni and Dreyfuss, 1995; Michael et al., 1995). Recently, two additional distinct classes of import signal that, like the classical NLS, are composed mainly of basic amino acids (aa), were identified in ribosomal proteins and in histone H1 (Jäkel and Görlich, 1998; Jäkel et al., 1999).

As predicted from the diversity of import signals, import

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1Abbreviations used in this paper: aa, amino acid(s); GDP, guanosine diphosphate; GST, glutathione S-transferase; hn, heterogeneous-nuclear; KNS, hnRNP K nuclear entry signal; NLS, nuclear localization signal; NPC, nuclear pore complex; Nplc, nucleoplasmin core; RBD, RNA binding domain; U sn, uracil-rich small nuclear.
into the cell nucleus is mediated by several distinct import receptors. The currently characterized import receptors form a family of related proteins named for the founding member the importin-β family (for review see M attaj and E nglmeyer, 1998). Nuclear import of the two forms of classical NLS is mediated by the importin-α/β heterodimer, where importin-α acts as an adaptor that binds the NLS and mediates interaction with importin-β. Importin-β targets the complex to and through the NPC (A dam and G erace, 1991; A dam et al., 1991; G örl ich et al., 1994, 1995a; W eis et al., 1995; I mamoto et al., 1995; R adu et al., 1995). The M 9 signal can bind directly to its receptor, transportin, without need for an adaptor (P ollard et al., 1996). Virtually all of the characterized forms of protein nuclear import studied to date involves the importin-β receptor family. These receptors bind to and are regulated by the small GTPase R an. R an in the GTP-bound state interacts with import receptors of the importin-β family, and in all reported cases but one (see below), causes them to release their cargo, i.e., either bound substrate or bound adaptor plus substrate. GTP-bound R an is the form favored in the nucleus, whereas guanosine diphosphate (GDP)-bound R an is the major cytoplasmic form of R an. This leads to import receptors interacting with their cargo in the cytoplasm and dissociating from their cargo in the nucleus. Thus, the asymmetric distribution of R anGTP lends directionality to nuclear import (G örl ich, 1998; M attaj and E nglmeyer, 1998). The requirement for GTP hydrolysis by R an in the cytoplasm to maintain the asymmetric distribution of R anGTP and R anGDP across the nuclear envelope is what makes at least some forms of transport mediated by receptors of the importin-β family an active process (S chwoeb el et al., 1998; E nglmeyer et al., 1999; R ibebeck et al., 1999).

Occasional exceptions to this paradigm of active protein transport to the nucleus have been reported. Some proteins do not themselves contain an NLS but piggyback to the nucleus by binding to another protein which does (M attaj and E nglmeyer, 1998). Calmodulin import into the nucleus was initially proposed to occur by facilitated diffusion (P rusch y et al., 1994) but recent persuasive data suggest that in fact calmodulin is imported by simple diffusion (L iao et al., 1999). Receptors of the importin-β family have also been shown to enter and/or leave the nucleus by a process that is most likely facilitated diffusion (K ose et al., 1997; N aki elny et al., 1997). In addition, a number of nuclear proteins have been characterized whose NLS sequences are not identifiable recognition signals for any member of the importin-β family (for review see M attaj and E nglmeyer, 1998). Among those are U 1A and U 2B′′, two closely related R NA -binding proteins that are components of the U 1 and U 2 sn R N P, respectively (S cheryl y et al., 1991; K ambach and M attaj, 1992, 1994).

Both U 1A and U 2B′′ enter the nucleus independently of interactions with their cognate sn R N A s or with other proteins (F een ey and Z lieve, 1990; J antsch and G all, 1992; K ambach and M attaj, 1992, 1994). Both proteins consist of two R NA binding domains (R NP motifs, R NA binding domains [R BD s] or R NA recognition motifs) situated at the NH₂- and COOH-terminal ends of the proteins that are highly conserved between U 1A and U 2B′′ (S illekens et al., 1987). The central regions of the proteins that lie between the R BD s were identified as unusually long and complex NLSs, capable of targeting heterologous proteins to the nucleus of X enopus oocytes by an active transport mechanism (K ambach and M attaj, 1992, 1994). The similarity between the U 1A and U 2B′′ proteins is least in the regions encoding their NLSs.

Here, the import of U 1A and U 2B′′ is analyzed in vitro in permeabilized H eLa cells (A dam et al., 1990). A s in vivo, the central region of U 1A and U 2B′′ is shown to be both essential and sufficient for nuclear import. The import mediated by these signals is not competitively inhibited by saturation of several import pathways characterized previously. Furthermore, dominant inhibitors of the R an cycle are shown to be without effect on import of U 1A/U 2B′′, suggesting that their nuclear entry is R an-independent. In contrast to the nuclear import of previously studied proteins, U 1A/U 2B′′ transport exhibits a requirement for hydrolyzable ATP, suggesting that import of these proteins does not involve a member of the importin-β receptor family, but instead requires a novel alternative mediator of nuclear protein import.

Materials and Methods

Plasmid Construction, Expression of Recombinant Proteins

The NLSs of U 1A and U 2B′′ were ligated as B amH I fragments (K ambach and M attaj, 1992, 1994) into the expression vector pGE 60N plc. To obtain glutathione S-transferase (GST) fusions, the same fragments were inserted into pGE H islus (G underson et al., 1994). Proteins were expressed in E. coli BL 21 (L ysS) and T G 1, respectively. The cultures were induced with 1 mM isopropyl-β-D-thiogalactopyranosid at OD 0.4–0.6 and grown for 4–5 h at 37°C. Nucleoplasmin core (N p lc) fusions were purified by nickel nitrilo-triacetic acid (Ni-NT A) chromatography (In vitro and eluted with 400 mM imidazole. GST fusions contain a COOH-terminal His-tag and were isolated by N i-NT A followed by glutathione-agarose chromatography (A mersham Pharmacia Biotech). BSA-NLS was prepared as described in P alacios et al. (1996). A ll proteins were labeled with fluorescein isothiocyanate (F L U O S; B oehringer M annheim) following the manufacturer’s instructions. N p l c and N p l c–hn R N P K nuclear entry signal (K NS) expression constructs were gifts from D irk G örl ich (H eidelberg University, H eidelberg, G ermany). N p l c–M 9 is described in E nglmeyer et al. (1999).

In Vitro Transport Assay

H eLa cells were permeabilized and X enopus egg extracts prepared as described in P alacios et al. (1996). To obtain the high-salt nuclei, the protocol was slightly modified (see below). The in vitro transport reactions (15 μl) contained 0.5 mM A TP, 0.5 mM G TP, 10 mM creatine phosphate (Sigma Chemical Co.), 50 μg/ml creatine phosphokinase (Sigma Chemical Co.), 1 × 10⁴ H eLa nuclei (A dam et al., 1990), and 0.8 mg/ml N p lc (G örl ich et al., 1994). For the initial import experiments with cytosol, FIT C-labeled import substrates were incubated with nuclei and 4 μl X enopus egg extract. Incubations were carried out at 25°C and fixation, mounting, and monitoring were as described by P alacios et al. (1996). To monitor import in unfixed cells, import reactions were incubated on ICN slides in the dark and directly monitored under the laser scanning microscope. To predeplete endogenous NTPs, nuclei were incubated for 5–10 min with apyrase (1 mg/ml; Sigma Chemical Co.) at room temperature. The settled nuclei were recovered and incubated with transport buffer.

Preparation of High-Salt Nuclear Extract and Extract Depletion

H eLa cells (10⁶/ml) were permeabilized with digitonin (60 μg/ml) for 5 min on ice. To extract U 1A import activity, the nuclei were washed for 2 min with an ice-cold buffer (50 mM H epesK O H , pH 7.3, 50 mM K AC, 2 mM E GTA) containing 80 mM M gCl₂. Subsequently, it was essential to wash...
the nuclei three times in low salt buffer (8 mM MgCl\textsubscript{2}) before freezing and storing at −80°C. To obtain high-salt nuclear extract 10\textsuperscript{10}-10\textsuperscript{12} HeLa nuclei were permeabilized and incubated with high-salt buffer for 5 min under agitation on ice. The nuclei were spun at 2,000 rpm and the supernatant was recovered. The extract was concentrated to 10 mg/ml using spin columns (10 K; Pallfiltron) and dialyzed against transport buffer.

For depletion studies the concentrated extracts were passed through glutathione-Sepharose beads to which GSTU1ANLS or GSTU1-ANLStrunc had been prebound. Columns were equilibrated with transport buffer before use. The flow-through fraction was directly assayed in import reactions.

**Results**

**Nuclear Import of U1A and U2B'’ In Vitro**

The overall primary structure of U1A and U2B’’ with the central NLS-containing region located between two conserved RBDs is shown in Fig. 1 (Sillekens et al., 1987). Functional versions of the human U1A (aa 94–204) and U2B’’ (aa 91–146) NLSs (Kambach and Mattaj, 1992, 1994) were fused to Nplc to generate U1ANLS and U2B’’NLS, respectively (Fig. 1). As a negative import control, a truncated form of the U1ANLS (aa 94–119) was also fused to Nplc (U1ANLStrunc). These fusion proteins formed pentameric structures large enough (>250 kD) to exceed the passive diffusion limit of NPCs (≈60 kD). To analyze the requirements for nuclear import of the two fusion proteins, digitonin-permeabilized HeLa cells were used (Adam et al., 1990). The import substrates were labeled with fluorescein and incubated with permeabilized cells and an energy-regenerating system.

A positive control import substrate, consisting of the simian virus (SV) 40 T antigen NLS cross-linked to BSA, whose transport is mediated by importin-α/β (see Introduction), was efficiently imported (Fig. 2 A). Both U1A and U2B’’ fusion substrates also accumulated in the nuclei at similar rates to BSA-NLS. The truncated form of the U1A NLS did not detectably accumulate in the nucleus (data not shown; see below). Nuclear import of the substrates was greatly reduced when the endogenous NTP pool was predepleted with apyrase (Fig. 2 A) or when the import reaction was carried out on ice (Fig. 2 B). Whereas BSA-NLS accumulated at the nuclear periphery under these conditions, little rim staining was observed in the case of U1A or U2B’’ (Fig. 2), suggesting that interaction between these proteins and the NPC is less stable than importin-β-mediated NPC binding of BSA-NLS.

Next, we tested the effect of a truncated version of importin-β from which parts of both the Ran and importin-α binding domains have been deleted. This mutant, ΔN44 (aa 45–461), appears to irreversibly bind to sites on the NPC and has been shown to block multiple import and export pathways (Kutay et al., 1997). Nuclear import of BSA-NLS, U1A, and U2B’’ was inhibited in the presence of a 2 μM ΔN44 (Fig. 2 A).

Another inhibitor of diverse nuclear transport events is WGA. This lectin binds specifically to glycosylated residues on several nucleoporins, thereby blocking essential binding sites for many import receptors without restricting passive diffusion (Finlay et al., 1987; Palacios et al., 1996). In the presence of WGA, nuclear import not only of BSA-NLS but also of U1A (Fig. 2 B) and U2B’’ (data not shown).

![Figure 1. Schematic representation of the U1A and U2B’’ fusion proteins. The overall structure of U1A and U2B’’ is schematized at the top of the figure. To generate the two import substrates, U1A NLS and U2B’’NLS, the central region of U1A (aa 94–204) and U2B’’ (aa 91–146) were fused to Nplc. A s a negative control (U1A_NLStrunc), a truncated form of the U1A NLS (aa 94–119) was fused to Nplc. Nplc serves as a transport-deficient pentamerization module. For affinity chromatography the same fragments were fused to GST.](image-url)
shown) was efficiently blocked. This inhibition was relieved by addition of an excess of N-acetylglucosamine to titrate the WGA (Fig. 2 B).

Taken together, these results indicate that the nuclear import of U1A and U2B' is a temperature- and energy-dependent process that requires functional NPCs, and is in these respects indistinguishable from importin-α/β-mediated BSA-NLS import.

**U1A and U2B’ Require the Same Saturable Import Mediator**

The RBDs of U1A and U2B’ are very closely related, whereas similarity between the NLS-containing central segment of the two proteins is less (Sillekens et al., 1987; Fig. 1). Nevertheless, both U1A and U2B’ import was inhibited when import was carried out in the presence of either a 40-fold molar excess of unlabeled U1A NLS or of unlabeled U2B’ NLS (Fig. 3 A). BSA-NLS import was not affected by the fusion proteins (Fig. 3 A). The import-deficient truncated form of U1A NLS did not inhibit U1A or U2B’ import (data not shown; Fig. 5 C). Thus, import of U1A and U2B’ appears to require interaction with the same saturable mediator.

In an initial step towards characterizing this saturable factor, attempts were made to inhibit U1A import with saturating amounts of other well-defined nuclear import signals. Saturating concentrations of BSA-NLS did not block U1A import in vitro or in vivo (Fig. 3 B; data not shown), nor did saturating amounts of IBB, the importin-β binding domain of importin-α (Görlich et al., 1995b; Weis et al., 1996; data not shown). Similarly, the M9 domain of hnRNP A1, which is recognized by transportin (Siomi and Dreyfuss, 1995; Pollard et al., 1996), had no effect on U1A import at a concentration that blocked transportin-mediated import (Fig. 3 B).

Recently, a novel import signal was identified in the hnRNP K protein called KNS (Michael et al., 1997). The receptor for KNS has not been identified, but is different from either importin-α/β or transportin. As shown in Fig. 3 B, saturating concentrations of KNS had no effect on U1A import. These results demonstrate that U1A and
U2B'' import, although saturable, does not appear to be mediated by importin-α/β, transportin, or the factor responsible for hnRNP K import. The ability of two other members of the importin-β family of import receptors, importin-7 and importin-8 (Jäkel and Görlich, 1998), to mediate U1A import was also tested in vitro, but neither stimulated U1A import (data not shown). We conclude that U1A and U2B'' import is not mediated by any of the so-far characterized vertebrate import receptors.

U1A Import Does Not Require Cytosol

As a first step in identifying the U1A and U2B'' import receptor, we decided to assay the import activity in fractionated cytosol. Surprisingly, we found that U1A and U2B'' import was cytosol-independent and that the proteins accumulated in nuclei in the presence of transport buffer alone (Fig. 4A; data not shown). Consistent with previous studies (Adam et al., 1990), import of BSA-NLS was strictly dependent on addition of the cytosolic fraction. To rule out the possibility that the U1A fusion protein was able to enter and leave the nucleus by diffusion in the absence of cytosol, we monitored nuclear accumulation of fluorescein-labeled U1A substrate without washing or fixing the cells. Under these conditions U1A was able to accumulate in the nucleus against a concentration gradient at a rate even greater than that seen in the presence of cytosol (Fig. 4B; data not shown). Efficient accumulation in the nuclei...
A Nuclear Fraction that Mediates U1A Import

The lack of requirement for cytosol indicated that any factors required for U1A/U2B′′ import remained associated with the nuclear fraction of permeabilized cells. To investigate this possibility, several more stringent procedures were used in an attempt to obtain functional, intact nuclei with reduced U1A import activity. Among the conditions tested, only the addition of high MgCl2 to the nuclear extraction buffer resulted in significant reduction in U1A import (Fig. 5 A). Nuclear integrity under these conditions was dependent on the presence of an energy-regenerating system (Fig. 4 C). In the absence of energy, U1A clearly entered the nuclei, but did not accumulate there, and this entry was reduced by WGA (Fig. 4 C). These results indicated that U1A accumulation in the nucleus in the absence of cytosol was not simply the result of diffusion, but rather an active, vectorial process.

Figure 4. U1A import does not require soluble cytosolic factors in vitro. (A) Import of U1ANLS and BSA-NLS under standard conditions (+ cytosol) or with an energy-regenerating system and buffer alone (− cytosol). (B) U1A import against a concentration gradient. U1ANLS import reaction was carried out without fixing the nuclei and images were taken at various time points as indicated. (C) Import was carried out as in B, except that either no energy was added (− ATP) or import was blocked with 2 μg/ml WGA, respectively (+ WGA).

Figure 5. An alternative permeabilization protocol. (A) BSA-NLS and GSTU1ANLS were incubated with nuclei that had been washed with buffers containing different MgCl2 concentrations as indicated on the left. GSTU1ANLS import into nuclei prepared by the standard permeabilization protocol (control) or after extraction in 80 mM MgCl2 buffer. The latter nuclei were incubated in the absence or presence of extract prepared by washing nuclei in 80 mM MgCl2 buffer (−/+/M80). (C) GST-U1ANLS import into nuclei extracted in high MgCl2 in the absence of added extract (control) or with 80 mM MgCl2 nuclear extract after passage over either a control column (GSTU1ANLS/trunc) or a U1ANLS column (GSTU1ANLS/trunc).
(80 and 100 mM MgCl₂ extraction) was tested in several ways. First, neither BSA-NLS nor BSA attached to the reverse NLS peptide was detected in the nuclei prepared in 80 mM MgCl₂ in the absence of added cytosol, indicating that the nuclei were still intact (Fig. 5 A; data not shown). In contrast, after washing in 100 mM MgCl₂ both proteins were detected in the nuclei in the absence of cytosol, indicative of damage to the nuclear envelope (Fig. 5 A; data not shown). Second, adding either Xenopus egg extract or recombinant importin-α/β and Ran to the nuclei prepared in 80 mM MgCl₂ efficiently promoted BSA-NLS import, but not BSA-reverse NLS import, indicating that the nuclei were still functional (data not shown). Transportin-mediated M9 import could also be efficiently restored in these nuclei using recombinant proteins (data not shown).

Having established conditions in which U1A import activity could be depleted without affecting other import pathways, we next assayed the nuclear extract prepared by washing with 80 mM MgCl₂ (M80 wash) for its ability to reconstitute U1A import. As shown in Fig. 5 B, dialyzed and concentrated M80 wash fraction was able to partially restore U1A import activity to nuclei washed in 80 mM MgCl₂. This M80 fraction did not stimulate BSA-NLS import (data not shown). These data suggest that a nuclear-associated U1A import factor could be isolated from permeabilized cells in an active form. The fact that this fraction was unable to fully restore U1A import activity indicated that the extraction procedure resulted in loss of some activity. We attempted to further purify the import activity, first by using affinity chromatography. Dialyzed M80 wash fraction was incubated with GSTU1ANLS (Fig. 1) immobilized on glutathione-Sepharose beads. This resulted in specific depletion of U1A import activity (Fig. 5 C). However, in spite of multiple attempts using various elution conditions, import activity could not be recovered even when the flow-through and bound fractions of the affinity column were combined. Similarly, U1A import activity could be efficiently depleted from the M80 wash fraction by passing it over immobilized WGA (data not shown). Again, no stable import activity could be recovered from the bound fraction after elution with N-acetylglucosamine in the conditions tested.

**U1A Import Is Not Inhibited by RanQ69L or Nonhydrolyzable GTP Analogues**

The characteristics of the U1A import fraction, nuclear association in permeabilized cells, and affinity for both the U1ANLS and WGA, distinguish it from the nuclear import receptors of the importin-β family characterized to date. The activity of the importin-β receptor family is regulated by the small GTPase Ran. Dominant-negative Ran mutants like RanQ69L, which are unable to hydrolyze GTP (Kliebe et al., 1995), as well as wild-type Ran loaded with nonhydrolyzable GTP analogues, inhibit import mediated by these receptors. For example, BSA-NLS import was efficiently blocked by addition of GTPγS, in the presence of wild-type Ran or by 2 μM RanQ69L (Fig. 6, A and B). In clear contrast, the import of U1A and U2B′′ was not affected by either treatment (Fig. 6, A and B). No significant reduction of U1A or U2B′′ import was seen even in the presence of 10 μM RanQ69L or after extensive pre-incubation of nuclei with RanQ69L before assaying U1A or U2B′′ import (data not shown). These data strongly suggest that Ran does not play a role in U1A or U2B′′ import.

**U1A Import Requires Hydrolyzable ATP**

Previous studies with permeabilized HeLa cells have shown that repeated cycles of import mediated by the importin-β family of receptors requires GTP hydrolysis. In contrast, NPC translocation of the receptors themselves, and at least in some cases, of receptors together with bound cargo, can occur without energy consumption (see Introduction). The block of U1A import after apyrase treatment (Fig. 2 A) suggested an essential role for a nucleotide triphosphate at some step in U1A import. Since Ran did not seem to be involved in U1A import, and since a nonhydrolyzable GTP analogue had no inhibitory effect on the process, the energy requirement for U1A import...
U1A and U2B’’ import and mechanisms of nuclear import characterized previously.

Discussion

We have used an in vitro system to study the nuclear import mechanism of two U snRNP proteins, U1A and U2B’’. A s previously shown to be the case in vivo (Kambach and Mattaj, 1992, 1994), the relatively poorly conserved central regions of the two otherwise very closely related proteins are active NLSs. The NLS spans 110 aa in U1A and 56 aa in U2B’’ (Kambach and Mattaj, 1992, 1994). These sequences were shown to target heterologous proteins into the nucleus of semipermeabilized HeLa cells. Import was temperature- and energy-dependent and saturable. Both WGA and a dominant-negative importin-β mutant, both of which block sites on the NPC (Finlay et al., 1987; Kutay et al., 1997), inhibited nuclear entry of U1A and U2B’’, indicating that like all previously described actively transported nuclear proteins, U1A and U2B’’ are translocated through the NPC.

U1A and U2B’’ import is an active process that can cause efficient accumulation of these proteins in the nucleus against a concentration gradient. For the following reasons, we can rule out that U1A and U2B’’ import is due to diffusion. First, the Nplc U1A and U2B’’ fusions form very stable pentamers of $>250$ kD, even at very low concentration. This size is significantly above the NPC diffusion limit of 60 kD. Second, WGA, which has no effect on passive diffusion through the NPC (Palacios et al., 1996), strongly inhibited U1A and U2B’’ import. Third, experiments that monitored nuclear accumulation of U1A in unfixed cells showed that import occurred against a concentration gradient. Finally, as discussed later, U1A and U2B’’ import required the presence of hydrolyzable ATP. Nuclear import of U1A and U2B’’ is saturable, and therefore, by definition, requires a mediator or receptor that recognizes the NLSs of the proteins. The NLSs of the two snRNP proteins showed efficient cross competition, and behaved similarly in all other assays, suggesting that they require the same import receptor. No cross competition was seen between U1A and U2B’’ and NLSs that access other characterized import pathways, including importin-α/β, importin-β, and transportin-mediated import (Fig. 3; data not shown).

Further strong evidence that members of the importin-β family of nuclear import receptors are not required for U1A and U2B’’ import came from investigation of the effects of nonhydrolyzable GTP analogues and of hydrolysedeficient Ran mutants on U1A and U2B’’ import. Both of these reagents, when present on the cytoplasmic side of the NPC, inhibit import mediated by importin-β family members (Palacios et al., 1996). This is because Ran in the GTP-bound state binds to these import receptors and causes them to dissociate from their cargo (Rexach and Blobel, 1995; Görlich et al., 1996; Izaurralde et al., 1997; Görlich, 1998; Mattaj and Englemier, 1998). There is one reported exception to this general rule that will be discussed in more detail below. Finally, U1A and U2B’’ import does not require cytosolic fractions from permeabilized cells for import, but instead needs nuclear-associated factors. This is in contrast to all previously characterized

![Figure 7](image_url)
forms of nuclear transport that depend on the importin-β family of receptors.

**A Nucleus-associated Import Mediator for U1A and U2B**

Efficient U1A and U2B’’ import into conventionally prepared permeabilized cell nuclei (Adam et al., 1990) did not require the addition of soluble cytosolic fractions. This observation suggests that the U1A and U2B’’ import receptor may be either stably nucleoplasmic, tightly associated with the NPC, or perhaps might even be a nucleoporin. Rather stringent extraction of nuclear fractions was necessary to render U1A and U2B’’ import dependent upon solubilized factor readdition. Nuclear extract prepared in 80 mM MgCl2 was able to restore import activity to stringently washed semipermeabilized cells, showing that at least one import mediator could be solubilized. No restoration activity was seen when conventionally prepared cytosolic (S100) extracts from HeLa cells were assayed (data not shown). Even with the fraction extracted from nuclei, import restoration was never complete. Furthermore, import activity rapidly decayed on storage of the fraction and was lost on freezing and thawing, suggesting that the factor(s) involved in U1A and U2B’’ import is easily inactivated. Import activity could be specifically depleted from active fractions by depletion with either the U1A NLS or WGA. The former result indicates that the active extracted fraction contains the factor(s) that specifically recognizes the U1A and U2B’’ NLS, whereas the latter provides weak evidence in support of the notion that U1A and U2B’’ might interact directly with a nucleoporin, rather than with a soluble nuclear factor. Further characterization of the U1A and U2B’’ import mediator(s) was frustrated by the fact that no activity could be recovered from either affinity column. Furthermore, multiple attempts to fractionate the active import fraction by conventional column chromatography also resulted in the loss of activity. At present, further biochemical characterization of this novel activity is therefore beyond our technical capabilities, and we are focusing on alternative approaches.

**Comparison with Other Unconventional Examples of Nuclear Protein Import**

A number of other cases have been reported in which nuclear proteins are imported by mechanisms that either do not require soluble cytosolic factors or that are not inhibited by cytoplasmic RanGTP. However, it appears likely that these examples are distinguishable from U1A and U2B’’ nuclear import, as will now be discussed.

Protein kinase Ca (PK Ca) is cytoplasmic in resting cells but can be activated to enter the nucleus by a number of stimuli. In cultured cells, PK Ca import is an energy-dependent process that is not inhibited by nonhydrolyzable GTP analogues under conditions in which import of a protein carrying a classical basic NLS is severely reduced (Schmalz et al., 1998). Since this form of import is not inhibited by WGA, or by antinucleoporin antibodies, it is distinct from U1A and U2B’’ import. Indeed, a plausible model for PK Ca import is that activation of the protein for import requires energy, whereas nuclear import occurs by simple diffusion, since diffusion through the NPC is not sensitive to WGA (Palacios et al., 1996). The import of human cyclin B1 has also been reported to be Ran-independent in vitro and is not inhibited by a hydrolysis-deficient Ran mutant or by nonhydrolyzable GTP analogues (Takizawa et al., 1999). In spite of these characteristics, it appears that cyclin B1 import is mediated by importin-β, in distinction to U1A and U2B’’, whose import is reduced in the presence of importin-β (see below). A further difference between cyclin B1 and U1A and U2B’’ import, and indeed a feature so far unique to cyclin B1 import, is the fact that dominant-negative forms of importin-β do not inhibit cyclin B1 import. Unlike many cargos that are transported by importin-β, cyclin B1 binds near the NH2 terminus of the receptor. This raises the possibility that the lack of inhibition of cyclin B1 import by cytoplasmic RanGTP is due to overlap between the binding sites of the two on importin-β, that would lead to cyclin B1 sterically blocking RanGTP interaction with importin-β. Takizawa et al. (1999) did not determine whether a trimeric complex of cyclin B1, importin-β, and RanGTP can form. After the cyclin B1-importin-β complex enters the nucleus, cyclin B1 must somehow dissociate from the receptor. Once this happens, importin-β will be able to bind nuclear RanGTP and will presumably leave the nucleus in this heterodimeric form (Izaurralde et al., 1997). Regenerating free importin-β will therefore require hydrolysis of GTP by Ran, making it possible that multiple rounds of cyclin B1 import in vivo or in vitro will actually not be independent of Ran GTPase activity.

Like U1A and U2B’’ import, β-catenin import into the nucleus in vitro is also independent of the presence of Ran (Y okoya et al., 1999) and also does not require soluble cytosolic factors. β-Catenin is distantly related to both importin-α and the importin-β receptor family, and it has been suggested that it may be imported by a mechanism related to that used by these import receptors. A though it was reported that a dominant-negative form of Ran did not inhibit β-catenin import in vivo (Y okoya et al., 1999), both a hydrolysis-deficient Ran mutant and nonhydrolyzable GTP analogues strongly inhibited β-catenin import in vitro (Fagotto et al., 1998), in contrast to their lack of effect on U1A or U2B’’.

The nuclear import of the H1V-1 Vpr protein is mediated by two redundant signals (Jenkins et al., 1998). Vpr import in vitro does not require soluble cytosolic factors and is not inhibited by a dominant-negative form of Ran (Jenkins et al., 1998). Nevertheless, Vpr import is mechanistically distinguishable from U1A and U2B’’ import by several criteria. First, Vpr import is partially resistant to inhibition by both WGA and a dominant-negative form of importin-β. Second, both Vpr signals are competitive inhibitors of both M9- and classical NLS-mediated import. Finally, although cold-sensitive, Vpr import is not inhibited by apyrase treatment, suggesting that it does not require nucleotide triphosphate hydrolysis (Jenkins et al., 1998).

One form of reported nuclear import with which U1A and U2B’’ import may be mechanistically related, at least on the basis of current information, is that mediated by the KNS signal. Human hNRNP K has two signals that can mediate nuclear import: a standard bipartite basic NLS that interacts with importin-α/β; and the KNS, which can mediate both nuclear import and nuclear export (Michael et al.,
References

A dam, S.A., and L. Gerace. 1991. Cytosolic proteins that specifically bind nu-
clear location signals are receptors for nuclear import. Cell. 66:837–847.

A dam, S.A., R.S. Marr, and L. Gerace. 1990. Nuclear protein import in perme-
abilized mammalian cells requires soluble cytoplasmic factors. J. Cell Biol.
111:807–816.

A dam, S.A., R. Sterne-Marr, and L. Gerace. 1991. In vitro nuclear protein im-
port using permeabilized mammalian cells. Methods Cell Biol. 35:469–482.

A rts, G. J. M., Fornerod, and I.W. M. Matte. 1998. Identification of a nuclear ex-
port receptor for tRNA. Curr. Biol. 8:305–314.

B onner, W. M. 1978. Protein migration and accumulation in nuclei. In The Cell
Nucleus, Vol. 6. H. Busch, editor. A cademic Press, New York. 97–148.

B reuwer, M., and D.S. Goldfarb. 1990. Facilitated nuclear transport of histone
H1 and other small nuclearophilic proteins. Cell. 60:1099–1008.

C orbett, A. H., and P.A. Silver. 1996. The NTF2 gene encodes an essential,
highly conserved protein that functions in nuclear transport in vivo. J. Cell
Chem. 271:18477–18484.

D ingwall, C. 1991. Transport across the nuclear envelope: enigmas and expla-
nations. Bioessays. 13:213–218.

D oye, V., and E. Hurt. 1997. From nucleoporins to nuclear pore complexes.
Curr. Opin. Cell Biol. 9:401–411.

E ngmeier, L., J.C. O’livo, and I.W. Matte. 1999. Receptor-mediated substrate
translocation through the nuclear pore complex without nucleotide triphos-
phate hydrolysis. Curr. Biol. 9:30–41.

F aggotto, F., U. Gluck, and M. B. Guminer. 1988. Nuclear localization signal-
dependent and importin/karyophilin-independent nuclear import of beta-
catenin. Curr. Biol. 8:181–190.

F eeney, R. J., and G. W. Zieve. 1990. Nuclear exchange of the U1 and U2 sn-
RNP-specific proteins. J. Cell Biol. 110:971–981.

F inlay, D. R., D.D. Newmeyer, T. M. Price, and D. J. Forbes. 1987. Inhibition of
in vitro nuclear transport by a lectin that binds to nuclear pores. J. Cell Biol.
104:189–200.

G örlch, D. 1988. Transport into and out of the cell nucleus. EMBO (Eur. Mol.
Biol. Organ.) J. 17:2721–2727.

G örlch, D., S. Prehn, R.A. Laskey, and E. Hartmann. 1994. Isolation of a pro-
tein that is essential for the first step of nuclear protein import. Cell. 79:767–778.

G örlch, D., F. Vogel, A.D. Mills, E. Hartmann, and R.A. Laskey. 1995a. Distin-
tinct functions for the two importin subunits in nuclear protein import. Na-
ture. 377:246–248.

G örlch, D., S. Kostka, R. Kraft, C. Dingwall, R.A. Laskey, E. Hartmann, and
S. Prehn. 1995b. Two different subunits of importin cooperate to recognize
nuclear localization signals and bind to the nuclear envelope. Curr. Biol.
5:383–392.

G örlch, D., N. Pante, U. Kutay, U. Aebi, and F.R. Bischoff. 1996. Identifica-
tion of different roles for RanGTP and RanBP7 in nuclear protein import.
EMBO (Eur. Mol. Biol. Organ.) J. 15:5594–5594.

G underson, S.I., K., Beyer, G. Martin, W. Kellar, W.C. Boelens, and I.W. Mat-
taj. 1994. The human U1A snRNP protein regulates polyadenylation via a
direct interaction with poly(A) polymerase. Cell. 76:531–541.

I纳米oto, N., T. Shimamoto, S. Kose, T. Takao, T. Tachibana, M. Matsubae, T.
Sekimoto, Y. Shimoshini, and Y. Oneda. 1995. The nuclear pore-targeting
complex binds to nuclear pores after association with a karyophile. FEBS
Lett. 368:415–419.

I saurralde, E., U. Kutay, C. von Kobbe, I.W. Matte, and D. Görlch. 1997. The
asymmetric distribution of the constituents of the Ran system is essential for
transport into and out of the nucleus. EMBO (Eur. Mol. Biol. Organ.) J.
16:6533–6547.

J äkel, S., and D. Görlch. 1998. Importin beta, transportin, RanBP5 and
RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells.
EMBO (Eur. Mol. Biol. Organ.) J. 17:4493–4502.

J äkel, S., W. A. Bigg, U. Kutay, F.R. Bischoff, K. Schwamborn, D. Donecke,
and D. Görlch. 1999. The importin beta/importin 7 heterodimer is a func-
tional nuclear import receptor for histone H1. EMBO (Eur. Mol. Biol.
Organ.) J. 18:2411–2423.

J antsch, M.F., and J.G. Gell. 1992. A semibol and localization of the U1-specific
snRNP C protein in the amphibian oocyte. J. Cell Biol. 119:1037–1046.

J enkins, Y., M. McEntee, K. Weis, and W.C. Greene. 1998. Characterization of
HI-V1 vpr nuclear import: analysis of signals and pathways. J. Cell Biol.
143:875–885.

K ambach, C., and I.W. M. Matte. 1992. Intracellular distribution of the U1 protein
depends on active transport and nuclear binding to U1 snRNA. J. Cell
Biol. 118:11–21.

K ambach, C., and I.W. M. Matte. 1994. Nuclear transport of the U2 snRNP-
specific U2 B’ protein is mediated by both direct and indirect signaling mecha-
nisms. J. Cell Sci. 107:1807–1816.

K liebe, C., H. Prinz, A. Wittinghofer, and R.S. Goody. 1995. The kinetic mecha-
nism of Ran–nucleotide exchange catalyzed by RCC1. Biochemistry. 34:
12543–12552.

K ose, S., N. Imamoto, T. Tachibana, T. Shimamoto, and Y. Oneda. 1997. Ran-
unassisted nuclear migration of a 97-kD component of nuclear pore-target-
ing complex. J. Cell Biol. 139:841–849.

K utay, U., E. Izaurralde, F.R. Bischoff, I.W. Matte, and D. Görlch. 1997. Dim-
nitant-negative mutants of importin-beta block multiple pathways of
import and export through the nuclear pore complex. EMBO (Eur. Mol.
Biol. Organ.) J. 16:1153–1163.

L iao, B., B. M. Paschal, and K. Luby-Phelps. 1999. Mechanism of Ca2+–depend-
ent nuclear accumulation of calmodulin. Proc. Natl. Acad. Sci. USA. 96:
6217–6222.

M atte, I.W. 1998. Rбинucleoprotein assembly: clues from spinal muscular at-
rophy. Curr. Biol. 8:93–95.

M atte, I.W., and E.M. De Robertis. 1985. Nuclear segregation of U2 snRNA
requires binding sites. Cell. 40:111–118.

M atte, I.W., and L. Engmeier. 1998. Nucleocytoplasmic transport: the soluble
phase. Annu. Rev. Biochemistry. 67:265–306.

M atte, I. W. 1988. U snRNA assembly and transport. In Structure and Function
of hnRNP A1 and hnRNP A2. M. Brimell, editor. Springer-Verlag, Heidelberg,
Germany. 100–114.

M ichael, W. M., M. Choi, and G. Dreyfuss. 1995. A nuclear export signal in
hnRNP A1: a signal-mediated, temperature-dependent nuclear protein ex-
port pathway. J. Cell Biol. 132:413–422.

M ichael, W. M., P.S. Eder, and G. Dreyfuss. 1997. The K nuclear shuttling do-
main: a novel signal for nuclear import and nuclear export in the hnRNP K
protein. EMBO (Eur. Mol. Biol. Organ.) J. 16:3587–3598.

N akielny, S., U. Fischer, W.M. Michael, and G. Dreyfuss. 1993. DNA transport:
Annu. Rev. Neurosci. 16:269–301.

Nigg, E. A. 1997. Nucleocytoplasmic transport: signals, mechanisms and regu-
lation. Nature. 386:779–787.

O hno, M., M. Fornerod, and I.W. Matte. 1998. Nucleocytoplasmic transport:

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the last 200 nanometers. Cell. 92:327–336.
Palacios, I., K. Weis, C. Klebe, I.W. Mattaj, and C. Dingwall. 1996. R A N / T C 4
mutants identify a common requirement for snR N P and protein import into
the nucleus. J. Cell Biol. 133:485–494.
Pollard, V.W., W.M. Michael, S. Nakielny, M.C. Siomi, F. Wang, and G. Drey-
fuss. 1996. A novel receptor-mediated nuclear protein import pathway. Cell.
86:985–994.
Pruschy, M., Y. Ju, L. Spitz, E. Carafoli, and D.S. Goldfarb. 1994. Facilitated
nuclear transport of calmodulin in tissue culture cells. J. Cell Biol. 127:1527–
1536.
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 G. Blobel. 1993. Isolation of the yeast nuclear pore complex. J.
Cell Biol. 123:771–783.
Rout, M.P., G. Blobel, and J. D. Aitchison. 1997. A distinct nuclear import path-
way used by ribosomal proteins. Cell. 89:715–725.
Scherly, D., C. Kambach, W. Boelens, W.J. van Venrooij, and I.W. Mattaj.
1991. Conserved amino acid residues within and outside of the N-terminal
ribonucleoprotein motif of U1A small nuclear ribonucleoprotein involved in
U1 R N A binding. J. M ol. Biol. 219:577–584.
Schlenstedt, G., E. Smirnova, R. Deneke, J. Solsbacher, U. Kutay, D. Görlich, H.
Ponstingl, and F.R. Bischoff. 1997. Y rb4p, a yeast ran-GTP-binding protein
involved in import of ribosomal protein L25 into the nucleus. E M B O (E u r. M ol.
Biol. Organ.) J. 16:6237–6249.
Schmalz, D., F. Hucho, and K. Buchner. 1998. Nuclear import of protein kinase
C occurs by a mechanism distinct from the mechanism used by proteins with
a classical nuclear localization signal. J. Cell Sci. 111:1823–1830.
Schwoebel, E.D., B. Talcott, I. Cushman, and M.S. Moore. 1998. Ran-depen-
dent signal-mediated nuclear import does not require GTP hydrolysis by
Ran. J. Biol Chem. 273:35170–35175.
Siomi, H., and G. Dreyfuss. 1995. A nuclear localization domain in the hnR N P
A1 protein. J. Cell Biol. 129:551–560.
Takizawa, C.G., K. Weis, and D.O. Morgan. 1999. Ran-independent nuclear
import of cyclin B1-Cdc2 by importin beta. Proc. Natl. Acad. Sci. USA. 96:
7938–7943.
Weis, K., I.W. Mattaj, and A.J. Lamond. 1995. Identification of hSR P 1a as a
functional receptor for nuclear localization sequences. Science. 268:1049–1053.
Weis, K., U. Ryder, and A.J. Lamond. 1996. The conserved amino-terminal do-
main of hSR P 1a is essential for nuclear protein import. E M B O (E u r. M ol.
Biol. Organ.) J. 15:1818–1825.
Yokoya, F., N. Imanoto, T. Tachibana, and Y. Y oneda. 1999. Beta-catenin can
be transported into the nucleus in a Ran-unassisted manner. M ol. Biol. Cell.
10:1119–1131.