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 nonhydrolyzable 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 $MgCl_2$. 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 snRNPs) • nucleocytoplasmic transport • nuclear import • Ran GTPase • U1A protein

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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 (Arts et al., 1998) is an active, signal-mediated process.

Actively 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 (Siomi 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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[1]*Abbreviations 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.

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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 Mattaj and Englmeier, 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 (Adam and Gerace, 1991; Adam et al., 1991; Görlich et al., 1994, 1995a; Weis et al., 1995; Imamoto et al., 1995; Radu et al., 1995). The M9 signal can bind directly to its receptor, transportin, without need for an adaptor (Pollard 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 Ran. Ran 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 Ran is the form favored in the nucleus, whereas guanosine diphosphate (GDP)-bound Ran is the major cytoplasmic form of Ran. 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 RanGTP lends directionality to nuclear import (Görlich, 1998; Mattaj and Englmeier, 1998). The requirement for GTP hydrolysis by Ran in the cytoplasm to maintain the asymmetric distribution of RanGTP and RanGDP across the nuclear envelope is what makes at least some forms of transport mediated by receptors of the importin-β family an active process (Schwoebel et al., 1998; Englmeier et al., 1999; Ribbeck 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 (Mattaj and Englmeier, 1998). Calmodulin import into the nucleus was initially proposed to occur by facilitated diffusion (Pruschy et al., 1994) but recent persuasive data suggest that in fact calmodulin is imported by simple diffusion (Liao 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 (Kose et al., 1997; Nakielny 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 Mattaj and Englmeier, 1998). Among those are U1A and U2B′′, two closely related RNA-binding proteins that are components of the U1 and U2 snRNP, respectively (Scherly et al., 1991; Kambach and Mattaj, 1992, 1994).

Both U1A and U2B′′ enter the nucleus independently of interactions with their cognate snRNAs or with other proteins (Feeney and Zieve, 1990; Jantsch and Gall, 1992; Kambach and Mattaj, 1992, 1994). Both proteins consist of two RNA binding domains (RNP motifs, RNA binding domains [RBDs] or RNA recognition motifs) situated at the NH_2- and COOH-terminal ends of the proteins that are highly conserved between U1A and U2B′′ (Sillekens et al., 1987). The central regions of the proteins that lie between the RBDs were identified as unusually long and complex NLSs, capable of targeting heterologous proteins to the nucleus of *Xenopus* oocytes by an active transport mechanism (Kambach and Mattaj, 1992, 1994). The similarity between the U1A and U2B′′ proteins is least in the regions encoding their NLSs.

Here, the import of U1A and U2B′′ is analyzed in vitro in permeabilized HeLa cells (Adam et al., 1990). As in vivo, the central region of U1A and U2B′′ 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 Ran cycle are shown to be without effect on import of U1A/U2B′′, suggesting that their nuclear entry is Ran-independent. In contrast to the nuclear import of previously studied proteins, U1A/U2B′′ 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 U1A and U2B′′ were ligated as BamHI fragments (Kambach and Mattaj, 1992, 1994) into the expression vector pQE60Nplc. To obtain glutathione *S*-transferase (GST) fusions, the same fragments were inserted into pGEXHisplus (Gunderson et al., 1994). Proteins were expressed in *E. coli* BL21 (LysS) and TG1, 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 (Nplc) fusions were purified by nickel nitrilotri-acetic acid (Ni-NTA) chromatography (Invitrogen) and eluted with 400 mM imidazole. GST fusions contain a COOH-terminal His-tag and were isolated by Ni-NTA followed by glutathione-agarose chromatography (Amersham Pharmacia Biotech). BSA-NLS was prepared as described in Palacios et al. (1996). All proteins were labeled with fluorescein isothiocyanate (FLUOS; Boehringer Mannheim) following the manufacturer's instructions. Nplc and Nplc–hnRNP K nuclear entry signal (KNS) expression constructs were gifts from Dirk Görlich (Heidelberg University, Heidelberg, Germany). Nplc-M9 is described in Englmeier et al. (1999).

In Vitro Transport Assay

HeLa cells were permeabilized and *Xenopus* egg extracts prepared as described in Palacios 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 ATP, 0.5 mM GTP, 10 mM creatine phosphate (Sigma Chemical Co.), 50 μg/ml creatine phosphokinase (Sigma Chemical Co.), 1×10^4 HeLa nuclei (Adam et al., 1990), and 0.8 mg/ml Nplc (Görlich et al., 1994). For the initial import experiments with cytosol, FITC-labeled import substrates were incubated with nuclei and 4 μl *Xenopus* egg extract. Incubations were carried out at 25°C and fixation, mounting, and monitoring were as described by Palacios 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

HeLa cells (10^6/ml) were permeabilized with digitonin (60 μg/ml) for 5 min on ice. To extract U1A import activity, the nuclei were washed for 2 min with an ice-cold buffer (50 mM Hepes/KOH, pH 7.3, 50 mM KAc, 2 mM EGTA) containing 80 mM $MgCl_2$. Subsequently, it was essential to wash

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the nuclei three times in low salt buffer (8 mM $MgCl_2$) before freezing and storing at −80°C. To obtain high-salt nuclear extract 10^9–10^{10} 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 in the presence of *Xenopus* egg extract 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 U1ANLS 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

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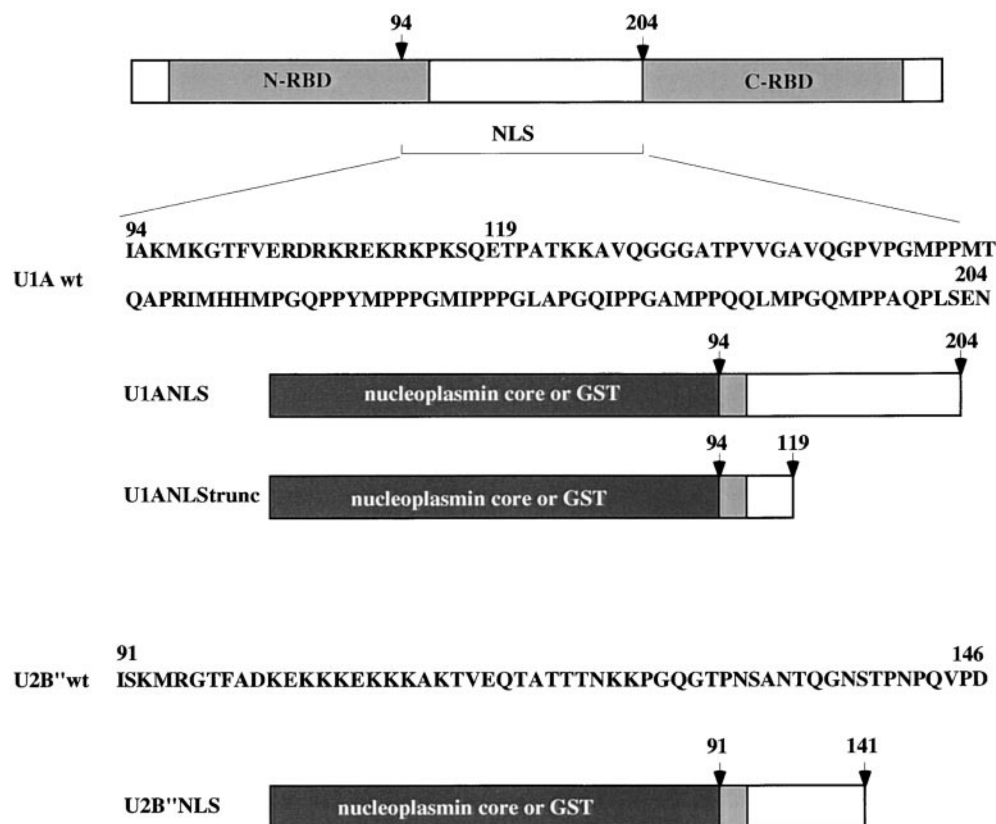

Figure 1. Schematic representation of the U1A and U2B′′ fusion proteins. The overall structure of U1A and U2B′′ is schematized at the top of the figure. To generate the two import substrates, U1ANLS and U2B′′NLS, the central region of U1A (aa 94–204) and U2B′′ (aa 91–146) were fused to Nplc. As a negative control (U1ANLStrunc), a truncated form of the U1ANLS (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.

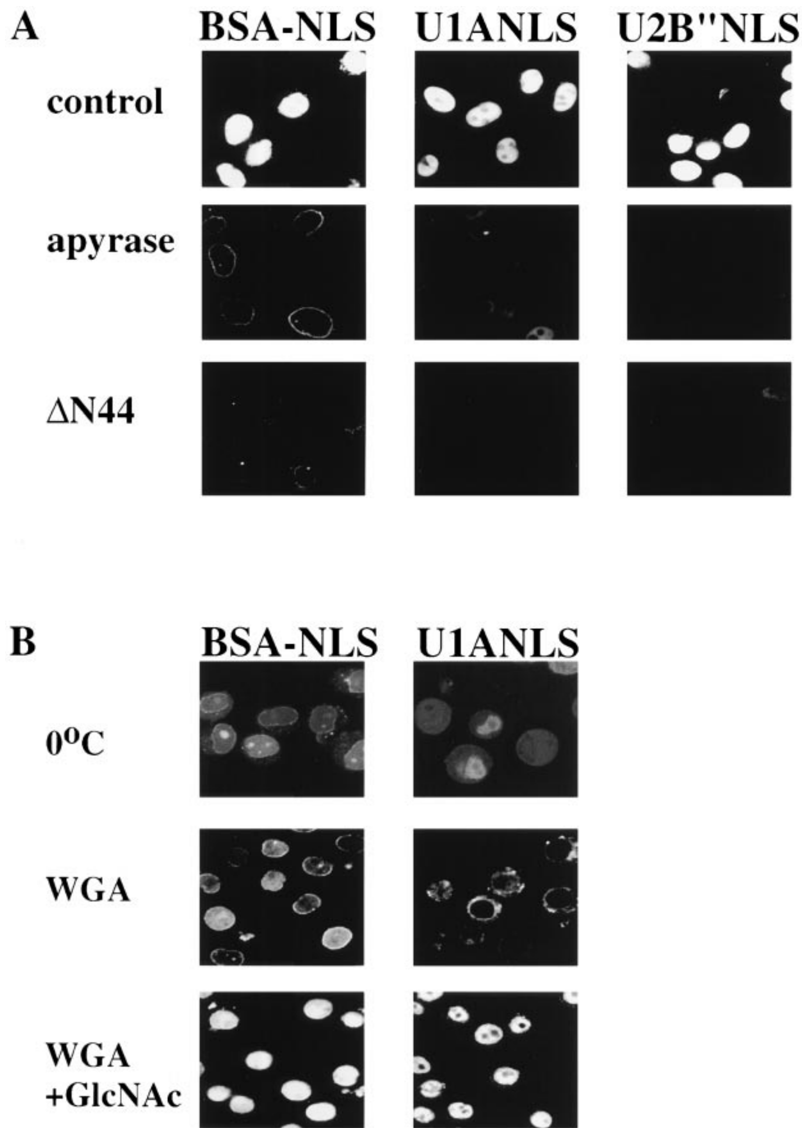

Figure 2. Nuclear import of fluorescein-labeled U1ANLS and U2B′′NLS in permeabilized HeLa cells. (A) Nuclear accumulation of the two proteins was compared with that of BSA-NLS in control conditions or in the presence of apyrase (1 mg/ml) or the dominant-negative importin-β mutant ΔN44 (2 μM) as indicated. (B) Effect of low temperature or of WGA (2 mg/ml) on the import of BSA-NLS (left panels) or U1ANLS (right panels).

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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 U1ANLS 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 U1ANLS 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

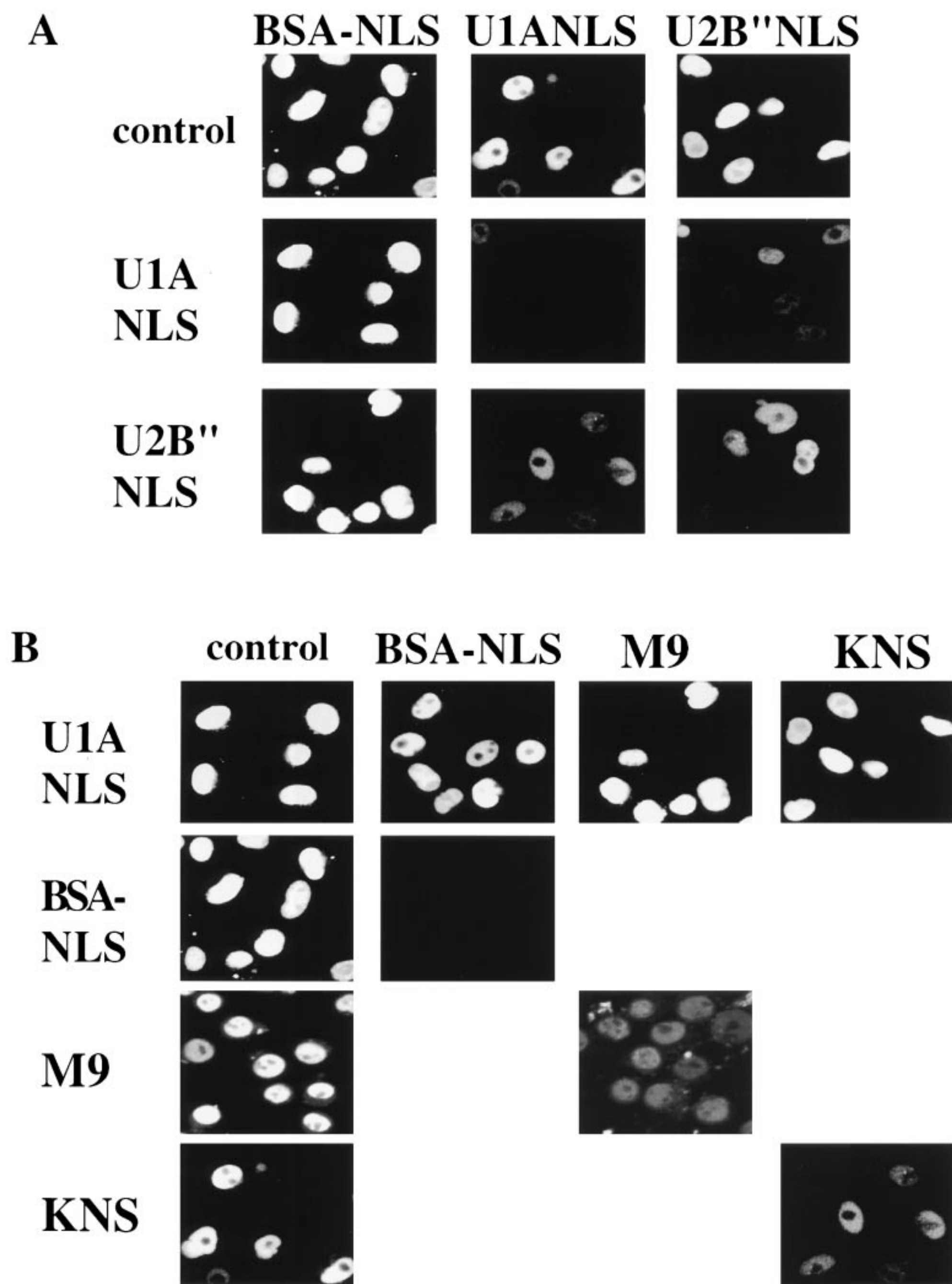

Figure 3. Characterization of cross competition with the U1A and U2B′′ import signals. (A) Import of BSA-NLS, U1ANLS, or U2B′′-NLS under either standard conditions (control) or in the presence of excess unlabeled U1ANLS or U2B′′NLS, respectively. (B) U1A import compared with classical NLS, M9, and KNS import. Fluorescently labeled U1ANLS, BSA-NLS, Nplc-M9, and Nplc-KNS were incubated with saturating amounts of unlabeled import substrates as indicated along the top of the figure.

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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. 4 A; 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. 4 B; data not shown). Efficient accumulation in the nuclei

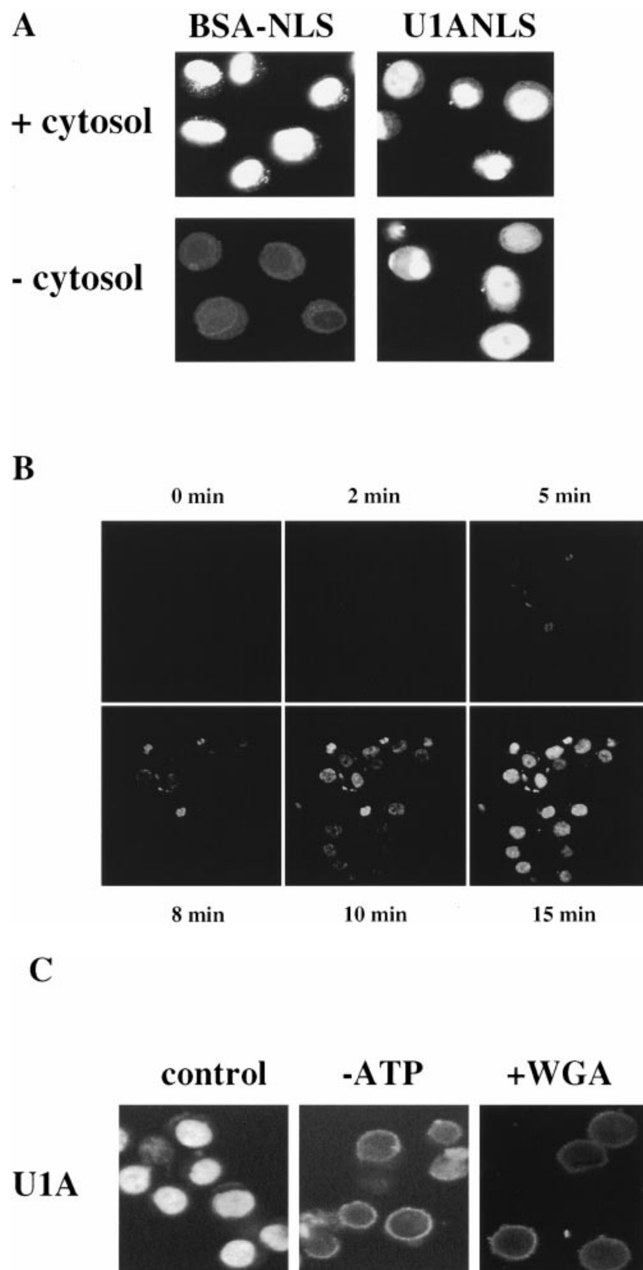

Figure 4. U1A import does not require soluble cytosolic factors in vitro. (A) Import of U1ANLS and BSA-NLS under standard conditions (+ cytosol) or with an energy-regenerating system and buffer alone (− cytosol). (B) U1A import against a concentration gradient. U1ANLS import reaction was carried out 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).

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.

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 $MgCl_2$ to the nuclear extraction buffer resulted in significant reduction in U1A import (Fig. 5 A). Nuclear integrity under these conditions

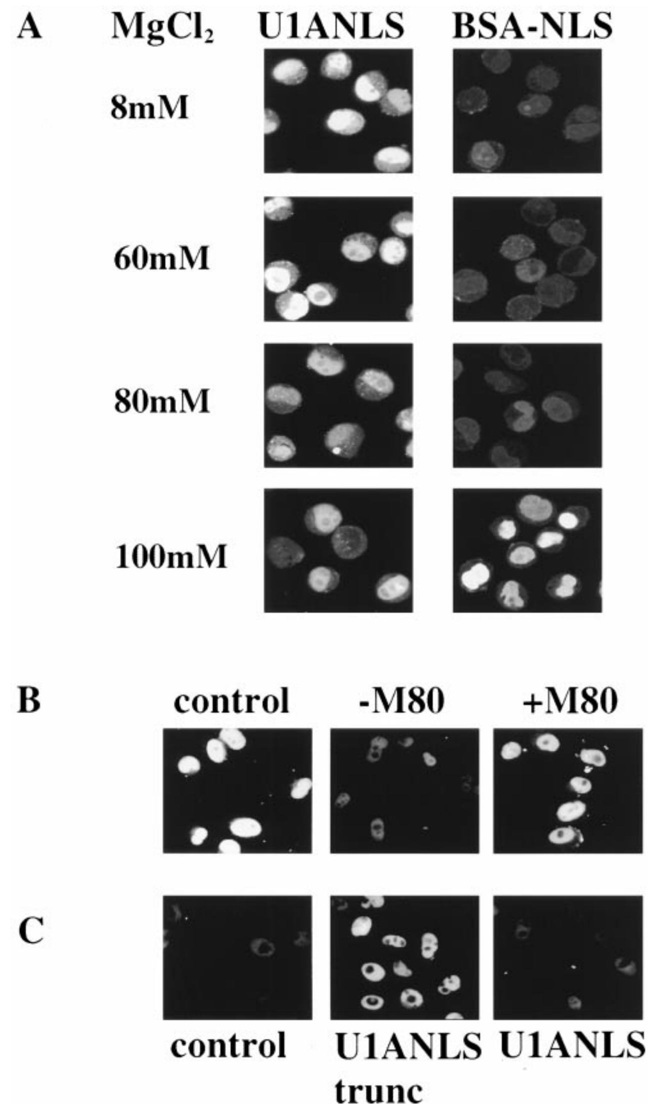

Figure 5. An alternative permeabilization protocol. (A) BSA-NLS and GSTU1ANLS were incubated with nuclei that had been washed with buffers containing different $MgCl_2$ concentrations as indicated on the left. (B) GSTU1ANLS import into nuclei prepared by the standard permeabilization protocol (control) or after extraction in 80 mM $MgCl_2$ buffer. The latter nuclei were incubated in the absence or presence of extract prepared by washing nuclei in 80 mM $MgCl_2$ buffer (−/+ M80). (C) GST-U1ANLS import into nuclei extracted in high $MgCl_2$ in the absence of added extract (control) or with 80 mM $MgCl_2$ nuclear extract after passage over either a control column (GSTU1ANLStrunc) or a U1ANLS column (GSTU1ANLS).

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(80 and 100 mM $MgCl_2$ 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 $MgCl_2$ 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 $MgCl_2$ 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 $MgCl_2$ 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 $MgCl_2$ (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 $MgCl_2$. 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.

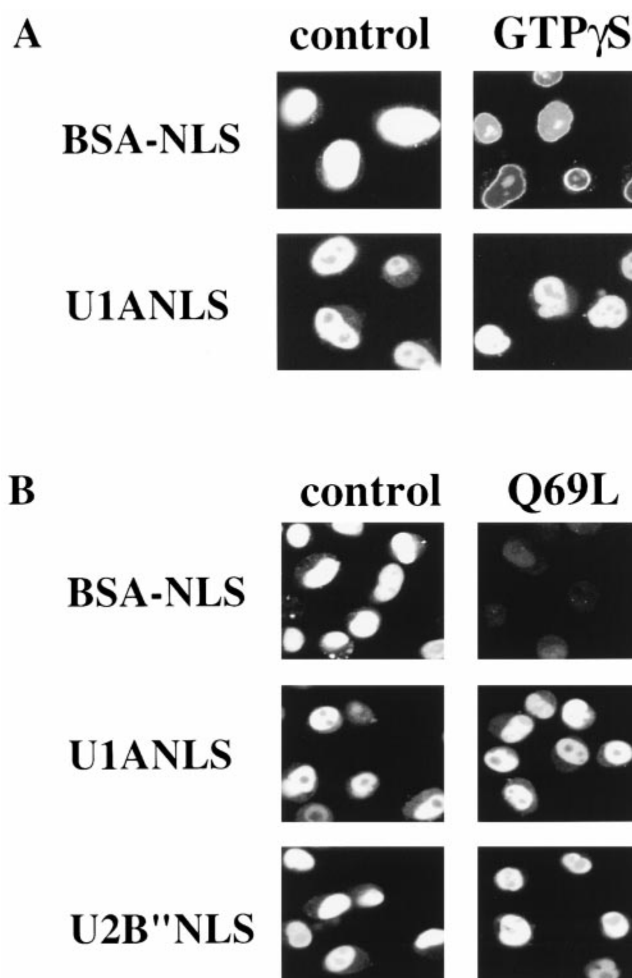

Figure 6. U1A import is not inhibited by nonhydrolyzable GTP analogues or the GTP hydrolysis-deficient Ran mutant Q69L. (A) BSA-NLS and U1ANLS were incubated with permeabilized HeLa cells under standard conditions (control) or in the presence of 2 mM GTPγS. (B) Nuclei were incubated with buffer (control) or 2 μM RanQ69L before addition of labeled import substrates and *Xenopus* extracts.

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 (Klebe 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 preincubation 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

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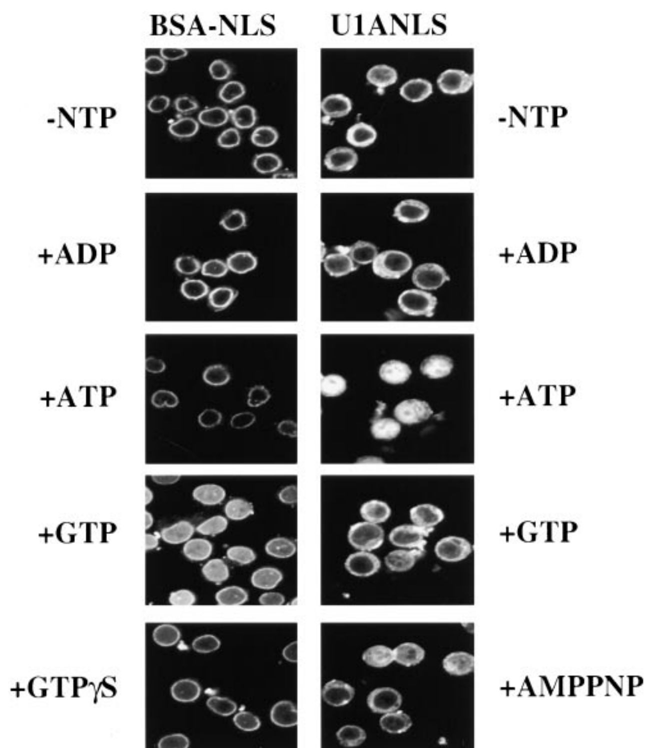

Figure 7. U1A import requires ATP hydrolysis. Endogenous NTP levels were predepleted from permeabilized HeLa cells by incubation with apyrase. The nuclei were subsequently incubated with 2 mM UDP to buffer endogenous phosphotransfer activity. Then import substrates, either BSA-NLS (left panels) or U1ANLS (right panels) were added with either buffer alone (−NTP) or together with 500 μM of either ADP, ATP, GTP, GTPγS, or 5′adenylylimidodiphosphate as indicated.

was examined in more detail. To test the effect of single NTPs in the import assay, the experimental conditions were altered such as to avoid interference by the endogenous phosphotransferase activity (Englmeier et al., 1999). UDP was added in excess over endogenous NTPs to serve as a phosphotransfer sink, so that transfer of the γ-phosphate from ATP or GTP would be directed to the added UDP and not to endogenous ADP or GDP (Englmeier et al., 1999). As expected, the combination of UDP and GTP, but not of UDP and GTPγS, stimulated BSA-NLS import (Fig. 7, left panel). ATP addition did not support BSA-NLS import, demonstrating that the addition of UDP did prevent endogenous phosphotransfer activity from converting GDP to GTP at the expense of ATP (Englmeier et al., 1999). In contrast, U1A import was strongly dependent on the addition of ATP. Neither ADP, the nonhydrolyzable ATP analogue 5′adenylylimidodiphosphate, nor GTP was able to support U1A import activity (Fig. 7). The relatively high cytoplasmic background in this experiment is due to batch-to-batch variation of the permeabilized cells.

These data confirm that GTP hydrolysis cannot support U1A or U2B′′ nuclear import. The finding that ATP hydrolysis is required for efficient import of U1A into the nucleus underlines the qualitative difference between 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′′. As 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 hydrolysis-deficient 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 Englmeier, 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

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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 $MgCl_2$ 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 U1ANLS 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 C α (PKCα) is cytoplasmic in resting cells but can be activated to enter the nucleus by a number of stimuli. In cultured cells, PKCα 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 PKCα 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 NH_2 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 (Yokoya 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. Although it was reported that a dominant-negative form of Ran did not inhibit β-catenin import in vivo (Yokoya 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 HIV-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.,

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1997). Similar to the situation with U1A and U2B″, KNS-mediated import in vitro does not require soluble cytoplasmic factors and does not require addition of exogenous Ran (Michael et al., 1997). As yet, the effect of nonhydrolyzable GTP analogues or of hydrolysis-deficient Ran mutants on KNS-mediated import has not been reported.

A second similarity between KNS- and U1A or U2B″ NLS-mediated import is that both are inhibited by the addition of wild-type importin-β (Michael et al., 1997; our unpublished data). Since this occurs in the absence of soluble cytosolic factors in both cases, it most likely indicates competition for NPC binding sites. KNS and the U1A and U2B″ class of NLS do not cross compete (Fig. 3 B), demonstrating differences between the two forms of import at the step of NLS recognition. Whether there are other mechanistic differences between the two import pathways requires further experimentation.

A feature of U1A and U2B″ import is its requirement for hydrolyzable ATP (Fig. 6), a requirement that has not been demonstrated for any other form of nuclear protein import. Since U1A appears to be able to enter nuclei in the absence of ATP, but not to accumulate there (Fig. 4 C), it seems probable that the energy requirement of U1A import is not for NPC translocation, but rather either for dissociation of U1A from its receptor or for retention of U1A in the nucleus. A better mechanistic understanding of this ATP dependence will have to await a more complete characterization of the mediator(s) of U1A and U2B″ import. Nevertheless, the requirement for ATP distinguishes the nuclear transport of U1A and U2B″ from import mechanisms characterized previously, and therefore warrants further study.

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