RAN/TC4 Mutants Identify a Common Requirement for snRNP and Protein Import into the Nucleus

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Abstract. Kinetic competition experiments have demonstrated that at least some factors required for the nuclear import of proteins and U snRNPs are distinct. Both import processes require energy, and in the case of protein import, the energy requirement is known to be at least partly met by GTP hydrolysis by the Ran GTPase. We have compared the effects of nonhydrolyzable GTP analogues and two mutant Ran proteins on the nuclear import of proteins and U snRNPs in vitro. The mutant Ran proteins have different defects; Q69L (glutamine 69 changed to leucine) is defective in GTP hydrolysis while T24N (threonine 24 changed to asparagine) is defective in binding GTP. Both protein and snRNP import are sensitive either to the presence of the two mutant Ran proteins, which act as dominant negative inhibitors of nuclear import, or to incubation with nonhydrolyzable GTP analogues. This demonstrates that there is a requirement for a GTPase activity for the import of U snRNPs, as well as proteins, into the nucleus. The dominant negative effects of the two mutant Ran proteins indicate that the pathways of protein and snRNP import share at least one common component.

The study of macromolecular import into the nucleus has concentrated on the transport of karyophilic proteins, whose uptake is generally mediated by a nuclear localization signal (NLS) in the primary sequence. This uptake is a two step process, the first step is energy independent docking of the karyophile at the outer face of the nuclear envelope in the vicinity of nuclear pore complexes (NPCs). The second step is energy dependent translocation of the karyophile through the NPC (Newmeyer and Forbes, 1988; Richardson et al., 1988; for reviews see Powers and Forbes, 1994; Sweet and Gerace, 1995; Melchior and Gerace, 1995).

The development of a permeabilized-cell in vitro system that accurately recapitulates nuclear protein import (Adam et al., 1990) has permitted the fractionation of activities essential for either docking or the complete import reaction (Moore and Blobel, 1992). Further fractionation led to the subsequent purification and characterization of four soluble components essential for nuclear import. Two of these are part of a heterodimeric complex that binds, in the cytoplasm, to NLS-containing proteins and mediates their docking to NPCs (see below). This complex thus corresponds to the original definition of an NLS receptor. Although there currently is a number of alternative nomenclatures for the two subunits (see below), we will use the term NLS receptor to mean any of the functional heterodimeric complexes.

The smaller NLS receptor subunit has been characterized in various organisms. While in yeast it appears that there is a single protein called Srplp (Yano et al., 1992) in each of the vertebrate species, a family of genes encode related, but quite diverse, proteins. These have been characterized from bovine, Xenopus, mouse, human, and Drosophila sources and called NLS receptor, (Adam and Adam, 1994), importin α (or 60) (Görliech et al., 1994), hSRP1 (Cortes et al., 1994), Rch1/hSRP1α (Cuomo et al., 1994; Weis et al., 1995), Karyopherin α (Moroihau et al., 1995a), m-importin (Imamoto et al., 1995a), and Pendulin/OHO31 (Kussel and Frasch, 1995; Török et al., 1995). There is general agreement that it is this subunit that makes the critical binding interaction with the NLS. Whether the individual members of the vertebrate families are functionally different remains to be tested. In contrast, the larger of the two receptor subunits appears to be a single protein in each species. Again, homologues from various species including human, Xenopus, mouse, and yeast have been characterized (Chi et al., 1995; Enenkel et al., 1995; Görliech et al., 1995; Imamoto et al., 1995b; Radu et al., 1995) and named p97, importin β, karyopherin β, PTAC97, etc. This subunit functions to target the complex

1. Abbreviations used in this paper: 3mG, trimethyl guanosine cap structure; NLS, nuclear localization signal; NPC, nuclear pore complex.
formed in the cytosol between the NLS-receptor and the NLS-containing protein to the NPC (Chi et al., 1995; Görlich et al., 1995a,b; Imamoto et al., 1995b; Moroianu et al., 1995b; Radu et al., 1995); and direct interaction between this subunit and nucleoporins has been reported (Moroianu et al., 1995b; Radu et al., 1995). Interestingly, in complete import assays, the small subunit of the receptor has been shown to enter the nucleoplasm with the NLS-containing protein while the large subunit apparently translocates through the pore, and is found in association with the inner face of the NPC but not in the nucleoplasm (Moroianu et al., 1995b; Görlich et al., 1995b). This indicates that the two subunits dissociate at this point. When, or how, the small subunit dissociates from the NLS is not yet known.

A third soluble factor, required only for the translocation step in vitro, is the GTP-binding protein Ran/TC4 (Moore and Blobel, 1993; Melchior et al., 1993). Ran is a mainly nuclear protein in which the domains important for guanine nucleotide binding and GTP hydrolysis are conserved with respect to Ras and other small GTPases (Driivas et al., 1990). Experiments using nonhydrolyzable GTP analogues in the in vitro import system, or overexpression of a mutant version of yeast Ran that is stabilized in the GTP-bound form, indicate that hydrolysis of GTP by Ran is a prerequisite for nuclear import (Moore and Blobel, 1993; Melchior et al., 1993; Schlenstedt et al., 1995). The guanine nucleotide exchange factor for Ran is RCC1 (Bischoff et al., 1991a,b; for review see Dasso, 1993) while the Ran specific GTPase-activating protein is homologous to a previously known yeast protein, Rna1p (Bischoff et al., 1994, 1995). Both RCC1 and Rna1p are required for RNA export from the nucleus although, interestingly, RCC1 is mainly found in the nucleus and Rna1p in the cytoplasm, indicating that Ran would have to cross the nuclear envelope to interact with both proteins (for review see Izaurralde and Mattaj, 1995). There is disagreement as to whether RCC1 is required for protein import in to the nucleus (Kadowaki et al., 1993; Tachibana et al., 1994), but recently it has been shown that Rna1p is required for nuclear import in yeast (Corbett et al., 1995).

A fourth soluble factor has been shown to be required in vitro to give rates of nuclear import equal to those seen with unfractionated cytosol. It is a small, previously identified protein initially called pp15, but also known as p10 or NTF 2 (Moore and Blobel, 1994; Paschal and Gerace, 1995). This protein was identified in two ways, first as an activity that stimulated import when present in a partially purified import system (Moore and Blobel, 1994), and as a factor required for import that interacted with the mammalian nucleoporin protein p62 (Paschal and Gerace, 1995). pp15, like Ran, is only required for the translocation step.

In contrast to protein import, the mechanism of nuclear import of the spliceosomal snRNPs, U1, U2, U4, and U5 has been much less well characterized. Kinetic competition experiments in vivo have shown that at least some of the factors required for their nuclear import are distinct from those needed for protein import (Fischer et al., 1991, 1993; Michaud and Goldfarb, 1991, 1992). This is explicable in terms of the distinct nature of the NLSs in these particles. An essential, and apparently ubiquitously required signal, is formed when the snRNA is bound in the cytoplasm by a group of common or core snRNP proteins (Mattaj and DeRobertis, 1985; Fischer et al., 1993). The nature of the signal formed by snRNP core assembly is not defined, but it is distinct from karyophilic protein NLSs (Fischer et al., 1991, 1993; Michaud and Goldfarb, 1991). In addition, the trimethyl guanosine cap structure (3mG) of these RNAs, formed in the cytoplasm by hypermethylation of the monomethyl guanosine cap, is also part of their bipartite NLS (Fischer and Luhrmann, 1990; Hamm et al., 1990). In some cell types and for some snRNPs the 3mG cap is also essential for import, while in others it provides a less important accessory function to stimulate the rate of import (Fischer et al., 1991, 1993). An in vitro system similar to that used in the study of nuclear protein import has recently been applied to the study of U snRNP import (Marshallsay et al., 1994). Many of the in vivo characteristics of the snRNP import process were reproduced in this in vitro system, including the cell type specificity of 3mG cap dependence for import, which was shown to be conferred by the source of the cytosolic fraction used in the import experiments (Marshallsay et al., 1994).

All these data are consistent with the hypothesis that snRNP import relies on a qualitatively distinct NLS receptor from that used in protein import. To determine whether these differences extend to a difference in a requirement for a functional Ran GTPase cycle, we made use of dominant negative mutants modeled on equivalent mutations made in Ras (Der et al., 1986; Feig and Cooper, 1988; Klebe et al., 1995). The results of these experiments, and others using nonhydrolyzable GTP analogues, indicate that Ran is required for nuclear snRNP import. There are, however, quantitative differences in the inhibition pattern of protein vs snRNP import in the presence of nonhydrolyzable GTP analogues which may indicate that the role of GTPases in the two processes is not identical.

**Materials and Methods**

**Preparation of Karyophiles**

The use of bacteriophage T7 RNA polymerase and T7 polymerase with the SV40 NLS in nuclear targeting has been described previously (Dunn et al., 1988). The proteins were expressed in E. coli and purified as described (Davanloo et al., 1984). Rabbit antisera specific for T7 RNA polymerase was a generous gift from P. Fisher (SUNY Stony Brook). Fluorescently labeled histidine tagged Xenopus nucleoplasmin was prepared as described (Görlich et al., 1994a).

BSA (Sigma) was labeled with fluorescein isothiocyanate (FLUOS, Boehringer Mannheim, Indianapolis, IN) and cross-linked to 50-fold molar excess of a synthetic peptide (CGGGPKKKRKVED) containing the SV40 large T antigen NLS (Melchior et al., 1993). 15-20 NLS peptides were cross-linked to each BSA molecule. A control karyophile was made in the same way using the reverse NLS peptide sequence.

**U snRNPs**

U snRNPs were isolated from HeLa nuclear extracts in a one step purification process as described by Bach et al. (1990). The anti-3mG antibody was commercial (Oncogene Science, Uniondale, NY). The isolated U snRNPs were modified by 100-fold excess of FLUOS (Boehringer Mannheim) using the manufacturer's instructions. The labeled U snRNPs were repurified by 3mG affinity chromatography, but eluted in transport buffer (20 mM Hepes/KOH, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EDTA, 2 mM DTT, 1 mM PMSE, and aprotinin, leupeptin, and pepstatin A, 1 μg/ml each). snRNP
components were analyzed as follows. The RNA was extracted from nuclear extracts or purified fractions by phenol-chloroform extraction and ethanol precipitated, separated on an 8% denaturing gel and visualized by staining with ethidium bromide. The corresponding proteins were precipitated by acetone treatment, separated on a 20% SDS-polyacrylamide gel, and silver stained.

Nuclear Import Assay
The in vitro transport reactions (50 μl) all contain 2 mM ATP, 2 mM creatine phosphate (Sigma Chem. Co., St. Louis, MO) and 1 U per ml creatine phosphokinase (Sigma Chem. Co.). 14 μl Xenopus egg extract (Newmeyer et al., 1986), and 104 digitonin-permeabilized HeLa nuclei (Adam et al., 1990). T7-NLS, BSA-NLS, nucleoplasmin, or U snRNPs were used as karyophiles. Final concentrations ~20 μg/ml, 60 μg/ml, 10 μg/ml, and 20 μg/ml, respectively.) Nonhydrolyzable GTP analogues and mutant Ran protein additions were made to the basic import reaction plus karyophile. Fluorescent dextrins were obtained from Sigma Chem. Co.

Incubations were carried out at 25°C and samples were taken at various times, diluted rapidly with transport buffer (Adam et al., 1990) and fixed with paraformaldehyde on ice. The fixed reaction was layered over 30% sucrose in transport buffer in a tube containing a polylysine-coated coverslip. After centrifugation (1,000 g for 10 min) the coverslip was recovered. When fluorescently labeled substrates were used, the coverslip was washed in PBS containing DAPI dye (330 ng/ml) and mounted on top of a drop of transport buffer and then placed on a coverslip sealed with nail varnish (Brown et al., 1990) and viewed directly. Unlabeled substrates were detected by indirect immunofluorescence using specific rabbit antisera and fluorescent second antibodies. ATP-depleted conditions were obtained omitting ATP and the ATP-regenerating system and by preincubation with hexokinase and glucose (Newmeyer et al., 1986).

Quantitative Fluorescence Microscopy
Quantitation of nuclear fluorescence was performed on a ZEISS inverted fluorescence light microscope (Axiosvert 10) equipped with a cooled CCD camera (Photometrics CH250, 1317 × 1035 pixels). Image acquisition was completely controlled from a Sun Microsystems workstation (SPARC Station 10/41) with the software package KHOROS. Two separate images were taken for each field of cells on a coverslip, one image of the DAPI staining of cell nuclei and one image of the fluorescein of the stained cells. The area of the cell nuclei was then localized and determined by adjusting an appropriate threshold. Image pixels with intensities lower than the threshold were considered as background and those above the threshold as cell nuclei. On this image an automatic labeling procedure was applied that counted the found objects, numbered them, calculated the area, and discarded those below a certain size limit to exclude small fluorescent spots in the image. The setting of the threshold was controlled by superimposing the labeling image with the fluorescein image and comparison of cell nuclei areas in both images. Mean intensities of cell nuclei was then calculated by integrating the pixel intensities of the fluorescein image in the regions of the labeled objects divided by the object area. For fluorescence microscopy, samples were visualized using 63× or 100× objectives on a Zeiss Axiovert microscope equipped with epifluorescence. Photographs were taken on T max 400 film (Kodak). For confocal fluorescence microscopy, the modular confocal microscope, developed and constructed at EMBL, was used. Excitation wavelengths of 488 nm were selected from an Argon-ion laser (Carmo-Fonseca et al., 1991). Images were analyzed by Adobe Photoshop version 3.0.

GTase Activity Assay
Wild-type Ran/TC4 and the Q69L mutant proteins were incubated for 60 min at 25°C with a 10-fold excess of [γ-32P]GTP (specific activity 480 cpm/pmol) in 30 mM potassium phosphate, pH 7.4, 2 mM EDTA, 5 mM DTE, and 50 mM KCl. After the removal of the noncomplexed [γ-32P]GTP, the concentration of the complexed protein was determined by filtering duplicate aliquots through nitrocellulose membranes and measuring the membrane bound radioactivity by liquid scintillation counting. 320 μl of 1 μM Ran- or RanQ69L-[γ-32P]GTP complex were then mixed with 30 μl of Xenopus egg extract (30 mg/ml protein). 32P release was determined by diluting 30 μl aliquots of the reaction mixture in 1 ml charcoal suspension (Leupold et al., 1983). The mixture was vortexed for 15 s, centrifuged, and 700 μl of the supernatant was counted by liquid scintillation counting.

Results
snRNP Import is Inhibited by Cap Analogues In Vitro
U snRNPs were purified using immuno-affinity chromatography with an antibody specific for the trimethyl guanosine cap of the U snRNA and then fluorescently labeled (Materials and Methods). To circumvent possible problems arising from dissociation of proteins during fluorescent labeling, the labeled snRNPs were repurified by cap affinity chromatography before use. The RNA and protein composition of the U snRNP fractions at each stage in their preparation is shown in Fig. 1, a and b. The in vitro system used throughout this paper combines the use of digitonin-permeabilized mammalian cells (Adam et al., 1990) and cytosol fractions derived from Xenopus eggs (Newmeyer et al., 1986).

In this in vitro system fluorescently labeled U snRNPs accumulate in the nuclei of permeabilized cells (Fig. 1 c). Consistent with previous reports (Marshallsay et al., 1994), we find that the rate of protein import is more rapid than snRNP import, maximum protein accumulation is observed within an hour, while maximal snRNP accumulation takes between 1 and 2 h (data not shown). snRNP import is efficiently blocked by the tri-methyl guanosine cap analogue, m7,2,2,7GTP (Fig. 1 d), but is not significantly affected by the mono-methyl guanosine cap analogue m7GTP (Fig. 1 e). Quantitation of the levels of snRNP and Xenopus nucleoplasmin accumulation in the presence of 2 mM m7,2,2,7GTP show that snRNP import is reduced to approximately one third of control levels in a 2-h incubation while nucleoplasmin accumulation is not significantly affected (Table I). Lower concentrations of m7,2,2,7GTP had proportionately lower inhibitory effects (data not shown) but higher concentrations (up to 10 mM) did not lead to greater inhibition of snRNP or protein accumulation.

The specific inhibition of snRNP import by cap analogues argues against the observed accumulation being due to protein that has dissociated from the labeled U snRNPs during the import reaction. In addition the common or core U snRNPs proteins, which represent the bulk of the labeled proteins in our preparations (Fig. 1 b) do not enter the nucleus unless bound to RNA (Mattaj and De Robertis, 1985), and have been shown not to exchange at a detectable rate between different snRNPs in vivo (Fischer et al., 1993). The other class of U snRNP proteins, those specific for a particular snRNP, are a not major fraction of the labeled protein in most of our preparations and cannot contribute significantly to the observed accumulation; those U snRNP-specific proteins that were studied have a conventional protein NLS and their uptake would not be in-
Table I. Quantitation of the In Vitro Nuclear Uptake of Nucleoplasmin and U snRNPs in the Absence or Presence of Cap Analogues

|                | Control | m2,2'7GTP | 7mGTP |
|----------------|---------|-----------|-------|
| Nucleoplasmin  | 100 ± 9.9 | 86 ± 5.50 | 100 ± 9.0 |
| snRNPs         | 100 ± 7.24 | 29 ± 4.90 | 86 ± 9.80 |

The nuclear uptake of nucleoplasmin and U snRNPs after 120 min incubation in the absence (control) or presence of m2,2'7GTP and 7mGTP cap analogues (both 2 mM) was quantified. The value obtained in the transport reaction for both karyophiles in the absence of any cap analogue (control) was taken as 100% import. The values obtained in the import reactions in the presence of m2,2'7GTP and 7mGTP cap analogues (both 2 mM) are relative to the control. The table shows the average figures for more than 50 cells per experiment in two independent experiments. The errors are standard deviations. The transport reactions were as described in Materials and Methods. Fluorescently labeled nucleoplasmin and affinity-purified fluorescently labeled snRNPs were used as karyophiles.

Figure 1. In vitro import of U snRNPs is specifically inhibited by the 3mG cap analogue m2,2'7GTP. (a) Analysis of RNA from labeled snRNPs. RNAs were analyzed by denaturing electrophoresis. Total RNA from HeLa nuclear extract (left lane), U snRNPs purified on a 3mGcap antibody column (center lane), and fluorescently labeled U snRNPs repurified on the same column (right lane). (b) Analysis of proteins from labeled snRNPs. Proteins were separated on high percentage polyacrylamide gels to resolve the small E, F, and G proteins. Total protein from HeLa nuclear extract (lane 1). U snRNP proteins extracted after a single round of purification on a 3mG cap antibody column (lane 2). The same proteins on a lower percentage gel (lane 3). High percentage gel analysis of proteins from fluorescently labeled U snRNPs repurified on the cap affinity column visualized by silver stain (lane 4) or by fluorescence (lane 5). The most intensely labeled bands correspond to the A and D proteins. (c-d) In vitro import of U snRNPs. Digitonin-permeabilized HeLa cells were incubated with affinity-purified fluorescently labeled U snRNPs for 120 min in the presence of Xenopus egg extract, ATP and an ATP regeneration system (see Materials and Methods). The transport reaction was performed in the absence (c) or presence of m2,2'7GTP (d) or 7mGTP (e) cap analogues both at 2 mM final concentration.

Nonhydrolyzable GTP Analogues Inhibit Both Protein and snRNP Import

Studies of the role of GTPases in cellular processes has relied extensively on the use of nonhydrolyzable GTP analogues and their use in studies of protein import led to the identification and characterization of Ran as the essential factor for the translocation step of nuclear protein import (Melchior et al., 1993; Moore and Blobel, 1993).

The analogues GTPγS, GDPβS, GMPPNP (guanylyl imidodiphosphate), and GMPPPCP (guanylyl methylene-diphosphonate) all inhibited nuclear protein accumulation in vitro (Table II, Fig. 2, upper panels). The maximal inhibitory effect of all of these analogues was observed at final concentrations between 1 and 5 mM. However, quantitatively, GMPPNP is the most potent inhibitor, reducing the level of accumulation by a factor of fifty (Table II). GMPPPCP was the least effective inhibitor, a concentration of 1 mM causing an approximately fourfold reduction in accumulation (Table II). The same analogues also inhibited snRNP nuclear accumulation in vitro (Table II, Fig. 2, lower panels). A comparison of the quantitative effects of the analogues on snRNP and protein import (Table II) indicates that snRNP is less sensitive than protein import, an
approximately threefold reduction occurring with all three analogues.

An additional, possibly significant difference between snRNP import and protein import is the apparent absence of docking of snRNPs at the nuclear envelope under conditions where nuclear proteins can be detected bound to the nuclear envelope. Thus, when snRNP import was assayed in the absence of ATP no accumulation at the envelope was observed (data not shown). We have observed some nuclear envelope binding of nuclear proteins in the presence of GTPγS and GMPPNP with a small amount of intranuclear fluorescence (Fig. 2, upper panels a–c), however, the effect of GTPγS was variable, causing only very weak perinuclear accumulation in some experiments. Consistent with previously published data weak perinuclear accumulation could be detected in the presence of GDPβS but was always less than that seen under conditions of ATP depletion (data not shown). In contrast, when U snRNP import was assayed, these three analogues (GTPγS, GMPPNP, and GDPβS) were inhibitory but none caused a detectable accumulation of snRNPs at the nuclear envelope (Fig. 2, lower panels a–c).

**Mutant Ran Proteins Inhibit Both Protein and U snRNP Import**

The results of the experiments with nonhydrolyzable GTP analogues suggested that at least one GTPase is involved in snRNP nuclear import. Ran is the only nuclear GTPase known to be essential for nuclear protein import and a mutant Ran protein expressed in yeast acts as a dominant negative inhibitor simultaneously inhibiting protein import and RNA export (Schlenstedt et al., 1995). We have used two mutant Ran proteins to investigate the role of Ran in protein and snRNP import. In one glutamine 69 is changed to leucine (Q69L). This protein is insensitive to Ran GTPase-activating protein (Ran GAP) and persists in the GTP bound state and is functionally equivalent to the mutant used by Schlenstedt et al. (1995); (Bischoff et al., 1995). To ensure that the Q69L mutant protein exhibited the same properties in the conditions of the nuclear transport assay, its GTPase activity was compared with that of wild-type Ran in the presence of Xenopus egg extract. The GTPase activity of the two proteins in a phosphate release assay (Leupold et al., 1983) in the presence of Xenopus egg extract is shown in Fig. 3. The wild-type protein hydrolyzed GTP rapidly at rates comparable to those measured in other systems (Klebe et al., 1995) while the Q69L mutant exhibited only weak activity.

Direct addition of Q69L mutant protein to the in vitro transport reaction completely inhibited the import of nucleoplasmin and a T7 RNA polymerase fusion protein having a single copy of the SV40 T antigen NLS (Fig. 4, a–d). A titration to determine the concentration of mutant protein required to inhibit nuclear protein import indicated that the import of both the T7 RNA polymerase-NLS fusion and of nucleoplasmin were progressively inhibited up to a concentration of 2 μM. Additional increases in concentration caused no significant increase in inhibition (data not shown). The concentration of Ran in the egg extracts used in these experiments was determined by quantitative immunoblotting and found to be 2–3 μM, consistent with previously reported estimates for oocyte extracts (Moore and Blobel, 1993). This gives a final concentration of Ran in the assay of ~0.5–1 μM indicating that the inhibitory effect of Q69L occurs in the presence of approximately stoichiometric amounts of endogenous wild-type protein.

The import of fluorescently labeled purified U snRNPs in the presence of 2, 5, and 10 μM Q69L was then investigated and compared to the uptake of the BSA-SV40 NLS conjugate under identical conditions. For both karyophiles the addition of 2 μM Q69L caused a drastic inhibition of import which was unchanged by further addition of mutant protein (Figs. 5 and 6, a–d, Table III). As a control the addition of the wild-type Ran protein had only a small effect on either protein or snRNP import even at a maximum concentration of 10 μM (Figs. 5 and 6, panel e, Table III). As further controls for the specificity of the inhibitory

![Figure 3. Stimulation of the GTPase activity of purified recombinant Ran and RanQ69L proteins by Xenopus egg extracts. The GTPase activities of recombinant Ran and RanQ69L are measured by a phosphate release assay in which [γ-32P]GTP bound forms of the proteins were incubated with Xenopus egg extract and the release of 32Pi was plotted versus time. The phosphate release by wild-type Ran/TC4 protein (circles) and RanQ69L protein (triangles) is shown. (RanQ69L is a mutant of Ran/TC4 in which glutamine 69 has been changed to leucine.)](image-url)
Inhibition of nuclear uptake of T7-NLS fusion protein and nucleoplasmin by the addition of RanQ69L protein. The import of T7-NLS fusion protein (a and b) and nucleoplasmin (c and d) was assayed in the absence (a and c) or presence of 10 μM purified recombinant RanQ69L protein (b and d) after 60 min incubation. The transport reactions were as described in Fig. 1. Nucleoplasmin was fluorescently labeled while T7-NLS fusion protein was detected by immunofluorescence.

Effect of the Q69L mutant, equivalent versions of either Ras (Q61L Der et al., 1986) or Rab5 (Q79L Stenmark et al., 1994) were added to the in vitro system and we found that neither protein had any detectable effect on nuclear protein import (data not shown).

In the other mutant protein (T24N) threonine 24 is changed to asparagine. This protein binds GTP poorly but has wild-type GTPase activity in the presence of the Ran GAP and thus accumulates in the GDP-bound form (Klebe et al., 1995). When added to nuclear reconstitution experiments in Xenopus egg extracts this protein suppresses nuclear growth and prevents DNA replication and has a slight effect on nuclear protein import which was not quantitated (Kornbluth et al., 1994). We have found that this protein also inhibits the accumulation of the BSA-SV40 NLS conjugate and snRNPs (Figs. 7 and 8 and Table IV). Quantitatively the effects are significantly less pronounced than those observed with Q69L, the level of inhibition increasing as the concentration of mutant Ran protein is increased to 10 μm (compare Tables III and IV). In the presence of Q69L, no clear nuclear envelope staining is observed (Figs. 4 and 5). In the presence of T24N, the small amount of protein which does enter the nucleus is preferentially associated with the nucleoli (Fig. 7).

The inhibitory effects of Q69L and T24N mutant Ran proteins differ in the extent to which the inhibition can be relieved by the subsequent addition of wild-type protein. The inhibition of both nuclear protein and snRNP import brought about by the presence of 2 μM Q69L Ran could not be reversed by 10 μM wild-type protein, and only weak restoration was observed using 20 μM wild-type protein in the presence of 200 μM GTP (data not shown). By contrast the inhibition of protein and snRNP import observed in the presence of 2 μM T24N Ran could be restored by the addition of 2 μM wild-type protein (data not shown).

The inhibition of U snRNP import by the mutant Ran proteins could be indirect, being due to either blockage of nuclear pores by improperly imported nuclear substrates derived from the added cytosol, or to an effect on the import of a hypothetical protein factor whose uptake would be required for U snRNP import to occur. To show that there is not a generalized blockage of the nuclear pores, we studied the entry of a small (4.4 kD) fluorescently labeled dextran. The nuclear entry of this dextran was unaffected by the addition of either wild-type Ran or the T24N or Q69L mutant proteins (Fig. 9, a-d). The integrity of the

Figure 4. Inhibition of nuclear uptake of T7-NLS fusion protein and nucleoplasmin by the addition of RanQ69L protein. The import of T7-NLS fusion protein (a and b) and nucleoplasmin (c and d) was assayed in the absence (a and c) or presence of 10 μM purified recombinant RanQ69L protein (b and d) after 60 min incubation. The transport reactions were as described in Fig. 1. Nucleoplasmin was fluorescently labeled while T7-NLS fusion protein was detected by immunofluorescence.

Figure 5. The in vitro import of a fluorescently labeled BSA-NLS conjugate is inhibited by the addition of RanQ69L protein. In vitro nuclear uptake of fluorescently labeled BSA-NLS conjugate was performed in the absence (a) or presence of 2 μM, 5 μM, and 10 μM purified recombinant RanQ69L protein (b, c, and d, respectively). e shows the import of BSA-NLS conjugate when purified recombinant wild-type Ran protein was added to the reaction to a final concentration of 10 μM.
nuclei used in this experiment was demonstrated by the exclusion of a larger dextran (Fig. 9 e). To address the possibility that snRNP import could be dependent upon the import of a nuclear protein, we added an excess of the BSA-NLS peptide synthetic karyophile such that the uptake of nucleoplasmin was efficiently competed (Table V). The same concentration of the BSA-reverse NLS conjugate was not inhibitory. Under these conditions, where a greater than tenfold reduction of nucleoplasmin uptake occurred, U snRNPs were still efficiently imported (Table V).

Discussion

Docking of snRNPs at the Nuclear Envelope Cannot be Detected

Previous studies of the nuclear import of proteins and snRNPs have emphasized the differences between these two classes of karyophile. Competition experiments have shown that these karyophiles are accumulated in the nucleus by biochemically distinct pathways (Michaud and Goldfarb, 1991; Fischer et al., 1991, 1993). A further indication of the distinct nature of the early steps of the import of proteins and snRNPs is the apparent lack of docking at the nuclear envelope when U snRNPs were used as a substrate for import. Docking of nuclear proteins occurs most prominently when high energy phosphate (ATP) is lacking (Newmeyer et al., 1986; Richardson et al., 1988), or in the absence of Ran (Moore and Blobel, 1993). Docking can also be observed at a low level in the presence of the GDP analogue GDPβS (Moore and Blobel, 1993; this paper). We find no detectable accumulation of U snRNPs at the nuclear envelope under conditions where nuclear protein docking is observed. It is possible that docking of snRNPs at the NPC does occur but it is either too transient or occurs at too low a level to be detected.

Nonhydrolyzable GTP Analogues Inhibit Both Protein and snRNP Import

The addition of nonhydrolyzable GTP analogues to the in vitro import assay inhibited both nuclear protein and snRNP import. Quantitatively, however, the analogues all had a detectably greater effect on protein import than snRNP import. One possible explanation is that given the slower rate of snRNP import, the contribution of GTPase activities is less than the case of nuclear proteins, i.e., fewer cycles of GTP hydrolysis and nucleotide exchange are required per mole of substrate. Alternatively, additional GTPases specific for each karyophile and having different sensitivities to the analogues may be involved.

Our results with nuclear proteins differ from published results because we observe docking in the presence of GTPyS and GMPPNP. In this respect the effects of GTPyS were variable and may be explained by the observation that the ratio of the number of permeabilized cells to the concentration of extract determines whether nuclear protein import can be inhibited by GTPyS (Melchior et al., 1993). For comparative purposes we used a fixed ratio of nuclei

Table III. Quantitation of the Nuclear Uptake of BSA-NLS Conjugate and U snRNPs In Vitro in the Absence or Presence of Different RanQ69L Mutant Proteins

|          | Control | Q69L 2 μM | Q69L 10 μM | wt 10 μM |
|----------|---------|-----------|------------|---------|
| BSA-NLS  | 100 ± 0.97 | 0.72 ± 0.04 | 0.64 ± 0.03 | 61 ± 1.18 |
| snRNPs   | 100 ± 10.9 | 12 ± 1.34  | 13 ± 1.84  | 65 ± 3.16 |

Quantitation of the import of BSA-NLS conjugate and U snRNPs after 120 min in the absence (control) or presence of RanQ69L (2 and 10 mM) and wild-type Ran (10 mM) is shown. Both the BSA-NLS conjugate and the purified U snRNPs were fluorescently labeled. The value obtained in the transport reaction for both karyophiles in the absence of any added Ran protein (control) was taken as 100% import. The values obtained in the import reactions in the presence of RanQ69L and wild-type Ran proteins are relative to the control. The table shows the average figures for more than 50 cells per experiment. The errors are standard deviations.
and extract as we found that dilution of the egg extract significantly reduced snRNP import (data not shown).

**Mutant Ran Proteins Inhibit Both Protein and snRNP Import**

The dominant negative inhibition of both protein and snRNP import by two different mutant Ran proteins implicates Ran/TC4 as a common factor in the import of both karyophiles, contrasting with the clear differences in the cytosolic and possibly nuclear pore complex components responsible for their import. Interestingly, there are detectable differences in the affects of the two mutant Ran proteins. Q69L has a quantitatively larger inhibitory affect on protein import while T24N affects protein and snRNP import almost equally. The Q69L Ran mutant protein that is insensitive to GAP activity is quantitatively and qualitatively the most potent and least reversible inhibitor of both protein and snRNP import.

**Table IV. Quantitation of the Nuclear Uptake of BSA-NLS Conjugate and U snRNPs In Vitro in the Absence or Presence of RanT24N Mutant Protein**

|            | Control | 2 μM | 5 μM | 10 μM |
|------------|---------|------|------|-------|
| BSA-NLS    | 100 ± 3.8| 55 ± 6.0| 29 ± 7.5| 19 ± 3.8|
| snRNPs     | 100 ± 11.8| 38 ± 3.7| 25 ± 3.1| 24 ± 9.4|

Quantitation of the import of BSA-NLS conjugate and U snRNPs after 120 min in the absence (control) or presence of RanT24N (2, 5, and 10 μM). Both the BSA-NLS conjugate and the purified U snRNPs were fluorescently labeled. The value obtained in the transport reaction for both karyophiles in the absence of any added Ran protein (control) was taken as 100% import. The values obtained in the import reactions in the presence of RanT24N proteins are relative to the control. The table shows the average figures for more than 50 cells per experiment. The errors are standard deviations.

While the direct role of Ran/TC4 in nuclear protein import has been unequivocally demonstrated, our results using mutant Ran proteins to implicate Ran in snRNP import could be explained as a secondary consequence of the inhibition of protein import or blocking of the pore. We have eliminated these possible explanations by showing that pores are not blocked; for example, by the presence of improperly imported substrates from the cytosol because they remain permeable to dextran. U snRNP import appears not to be dependent upon nuclear protein import as, inhibition of nuclear protein uptake with an excess of synthetic karyophile does not significantly inhibit U snRNP import.
import. There is probably a single pathway for nuclear protein import (Michaud and Goldfarb, 1993), and the results described here demonstrate that U snRNPs import is not dependent upon the operation of this pathway and is consistent with in vivo competition experiments (Fischer et al., 1991; Michaud and Goldfarb, 1991) and experiments in which a cytoplasmically anchored nuclear protein specifically interferes with nuclear protein import but not U1 snRNA import (van Zee et al., 1993). All of these data are consistent with the existence of different classes of receptor that recognize protein or U snRNPs, but do not rule out that steps of import subsequent to NLS receptor binding (i.e., docking and/or translocation through the NPC) might occur by a common mechanism. It is possible that the requirement for Ran for the import step of both karyophiles is indicative of such a common step.

While our results establish Ran/T4C as the first known factor required for the import of both nuclear proteins and U snRNPs, the differences we observe in the sensitivity of import of the two classes of karyophiles to nonhydrolyzable GTP analogues and mutant Ran proteins suggest that in addition to Ran, other GTPases may be involved. A more direct biochemical approach to the identification of factors involved in snRNPs import should clarify the basis of these differences.

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Table V. Competitive Inhibition of Nuclear Protein Import Does Not Inhibit U1 snRNP Import In Vitro

| Control | BSA-NLS | BSA-NLSRev |
|---------|---------|------------|
|         |         | 0.35 mg/ml |
| Nucleoaplasmin | 100 ± 2.80 | 8 ± 1.60 | 112 ± 11.40 |
| snRNPs   | 100 ± 2.30 | 66 ± 3.40 | 93 ± 3.00 |

Quantitation of the import of fluorescently labeled nucleoaplasmin and fluorescently labeled U snRNPs in the presence of reverse BSA-NLS conjugate or the BSA-NLS conjugate in vitro after 120 min. The table shows the average figures for more than 50 cells in an experiment. The errors are standard deviations.

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