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Importin Provides a Link between Nuclear Protein Import and U snRNA Export

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Summary

Importin-α mediates nuclear protein import by binding nuclear localization signals and importin-β. We find approximately 30% of SRP1p, the yeast importin-α, in a nuclear complex with the Saccharomyces cerevisiae nuclear cap-binding protein complex (CBC). Similarly, a large fraction of Xenopus CBC is associated with importin-α in the nucleus. CBC promotes nuclear export of capped U snRNAs and shuttles between nucleus and cytoplasm. The CBC–importin-α complex binds specifically to capped RNA, suggesting that CBC might shuttle while bound to importin-α. Strikingly, importin-β binding displaces the RNA from the CBC–importin-α complex. Thus, the commitment of CBC for nuclear reentry triggers the release of the export substrate into the cytoplasm. We provide evidence for a mechanism that ensures that importin-mediated RNA release is a specifically cytoplasmic event.

Introduction

Nuclear protein import proceeds through the nuclear pore complex (NPC), requires energy and soluble factors, and is triggered by nuclear localization signals (NLS; reviewed by Fabre and Hurt, 1994; Powers and Forbes, 1994; Davis, 1995; Görlich and Mattaj, 1996). In the first step of import, protein substrates bind to a heterodimeric complex in the cytoplasm. In this study, we refer to the two components of the heterodimer as importin-α and -β. These proteins have also been referred to as NLS receptor and p97, the nuclear pore targeting complex, or karyopherin α and β (for references, see Görlich and Mattaj, 1996). The yeast homolog of importin-α will be referred to as SRP1p (Yano et al., 1992). The α subunit of the importin complex provides the NLS binding site. The trimeric NLS–recognition complex docks to the nuclear pore via importin-β and is subsequently transferred as a single entity through the nuclear pore complex by a mechanism that requires the small GTPase Ran, a protein of unknown function called pp15/p10/NTF2, and energy (reviewed by Moore and Blobel, 1994; Powers and Forbes, 1994; Melchior and Gerace, 1995; Görlich and Mattaj, 1996).

To promote import, cytoplasmic Ran has to be in the GDP–bound form (Görlich et al., 1996b). The direct binding of nuclear RanGTP to importin-β dissociates the importin–NLS complex on the nucleoplasmic side of the nuclear pore, releasing import substrate and importin-α into the nucleoplasm (Görlich et al., 1996b). In contrast to importin-α, importin-β does not normally accumulate inside the nucleus, perhaps because its recycling to the cytoplasm is too rapid (Görlich et al., 1995b; Moroianu et al., 1995). The different rates of reexport of the α and β subunits indicate that they return separately to the cytoplasm.

Recent data suggest that importin-α binds to β via a basic 41 amino acid motif, the importin-β binding (IBB) domain. The IBB domain not only mediates binding but, when fused to a heterologous protein, is sufficient to confer importin-β–dependent entry into the nucleus. However, whereas importin-α shuttles rapidly between nucleus and cytoplasm, the IBB fusion appears to be unable to leave the nucleus (Görlich et al., 1996a; Weis et al., 1996). This indicates that reexport of importin-α is not the reversal of its entry and suggests that recycling of the α subunit is not dependent on importin-β. In fact, nothing is known about the reexport of importin-α out of the nucleus, but some mechanism must ensure that it normally leaves the nucleus without the import substrate.

SRP1p (suppressor of RNA polymerase I) is the importin-α homolog in Saccharomyces cerevisiae. It was identified first in a screen for suppressors of ts alleles of RNA polymerase I (Yano et al., 1992) and subsequently by its genetic interaction with the nuclear pore proteins Nup1p and Nup2p (Belanger et al., 1994). SRP1p is encoded by an essential gene. Its depletion or inactivation has pleiotropic effects on various nuclear functions. It is not yet clear whether all of these effects are consequences of a defect in nuclear protein import or if SRP1p might have more than one function.

The yeast homolog of importin-β was identified in various ways: as a sequence tag similar to the human importin-β sequence in the GenBank/EMBL DNA sequence database (Görlich et al., 1995a), on the basis of amino acid sequence information from the SRP1p-bound purified protein (Enenkel et al., 1995; this study), via its physical interaction with a yeast nuclear pore protein (Iovine et al., 1995), and as the product of the RSL-1 gene found in a synthetic lethal screen with *rna1–1* (Koepp et al., 1996). Yeast importin-β, like SRP1p, is encoded by an essential gene (Iovine et al., 1995).

The small GTPase Ran/TC4 appears to play a central

role in nuclear transport. Mutations in Ran or mutations in its effectors RanGAP1 (RNA1p in S. cerevisiae), the GTP exchange factor RCC1 (PRP20p in S. cerevisiae), or the Ran binding protein-1 affect both protein import and RNA export (Atkinson et al., 1985; Forrester et al., 1992; Kadowaki et al., 1992; Amberg et al., 1993; Tachibana et al., 1994; Cheng et al., 1995; Corbett et al., 1995; Schlenstedt et al., 1995a, 1995b). An interesting feature of the Ran system is the asymmetric distribution of its constituents between nucleoplasm and cytoplasm. The principal GTP exchange factor for Ran, RCC1, is a nuclear chromatin-bound protein (Ohtsubo et al., 1989; Bischoff and Ponstingl, 1991), whereas the major GTPase-activating protein RanGAP1 is cytoplasmic (Hopper et al., 1990; Melchior et al., 1993; Bischoff et al., 1995). This implies that nuclear Ran is mainly in its GTP–bound form, but cytoplasmic Ran is in the GDP form. From this and from the fact that Ran is highly concentrated in the nucleus, one would predict a steep gradient of RanGTP concentration across the nuclear envelope. As mentioned above, nuclear protein import does indeed require cytoplasmic RanGDP and nuclear RanGTP (Görlich et al., 1996b).

Little is known about the mechanism of RNA export out of the nucleus (reviewed by Gerace, 1995; Izaurralde and Mattaj, 1995; Görlich and Mattaj, 1996). A likely model, however, is that this process may be achieved by shuttling transporter molecules. Protein mediators of RNA export would bind to an RNA and contain a signal to direct nuclear export. The only examples of proteins that have been directly shown to mediate RNA export are the HIV-1 Rev protein (Fischer et al., 1994) and the nuclear cap-binding complex (CBC), which consists of two subunits, CBP80 and CBP20 (Izaurralde et al., 1995). Rev has a specific RNA-binding domain and also a nuclear export signal (Fischer et al., 1995; Wen et al., 1995; references therein). The Rev nuclear export signal allows interaction with nucleoporins and proteins of related structure (Bogerd et al., 1995; Fritz et al., 1995; Stutz et al., 1995). It is likely, but not yet proven, that cellular mediators of RNA export will contain similar functional domains. An implication of this model is that continuous protein import is required to replenish the nucleus with export factors. Export factors appear to fall into at least two categories: those, like Rev and CBC, that have a classical basic NLS and therefore enter the nucleus via the importin-dependent pathway (Malim et al., 1989; Perkins et al., 1989; Izaurralde et al., 1996) and those whose import is directed by other sequences, such as the M9 domain that directs hnRNPA1 to the nucleus via a transcription-dependent pathway (Piñol-Roma and Dreyfuss, 1991; Siomi and Dreyfuss, 1995; Weighardt et al., 1995). A fundamental question arising from this model is why these factors bind RNA only during export and not while being imported into the nucleus.

In this study, we analyze complexes formed by SRP1p (importin-α) in yeast. Surprisingly, a large fraction of SRP1p is bound to yCBC, the yeast counterpart of the human cap-binding protein complex. We find both SRP1p and yCBC at about 10-fold higher concentration in the nucleus than in the cytoplasm, indicating that the SRP1p–yCBC complex is mainly or entirely nuclear. We also detect a homologous CBC–importin-α complex in the nuclei of Xenopus oocytes. Both CBC alone and the trimeric complex it forms with importin-α bind RNA in a cap-dependent fashion, consistent with CBC being exported from the nucleus as a complex with importin-α. Importin-β, however, is shown to release the RNA from the importin-α–CBC complex. This suggests that after CBC has delivered an RNA to the cytoplasm, the export complex is dissociated by binding importin-β. This simultaneously commits CBC to nuclear reentry and releases the exported RNA into the cytoplasm. The ability of importin-β to dissociate the export complex is blocked by RanGTP, whose concentration is predicted to be high in the nucleus but low in the cytoplasm. This would explain why RNA release is a cytoplasmic event. These results implicate importin in RNA export and illustrate how single transporter molecules can cycle through the pore in both directions but bind the transport substrate only during movement in one direction.

Results

Identification of SRP1p-Associated Proteins

Antibodies raised against Xenopus importin-α (Görlich et al., 1994) precipitate importin-β as a second subunit migrating at 90 kDa. Xenopus importin-β can be dissociated from the α subunit by 0.4 M magnesium chloride (Görlich et al., 1995a). Performing the analogous immunoprecipitation experiment in yeast with antibodies against the yeast importin-α, SRP1p, results in the coprecipitation of several major bands. Figure 1A shows an anti-SRP1p immunoaffinity column loaded with yeast extract, then washed, and subsequently eluted with a 0–2 M magnesium chloride gradient, followed by a pH 2.2 step. Fractions were collected and analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie staining. This procedure clearly separates five SRP1p-associated polypeptides.

Quantitative amino acid analysis and microsequencing revealed that all but one correspond to known proteins. SRP1p itself (Figure 1A, band 6) was not released by magnesium chloride but was eluted during the pH 2.2 step. The identification of bound Nup1p (band 2) and Nup2p (band 5) confirms previous reports that these two proteins interact genetically and physically with SRP1p (Belanger et al., 1994). The binding of the yeast importin-β homolog (band 1) to SRP1p was expected by analogy to the Xenopus and mammalian systems and was previously reported (Enenkel et al., 1995). The observation that these three known interactions were detected by this approach emphasizes the significance of the novel SRP1p interaction partners described in the next section.

The Yeast Homologs of Cap-Binding Proteins CBP80 and CBP20 Form an Abundant Complex with SRP1p

Figure 1A, band 3, corresponds to a 100 kDa protein that is 16.7% identical to human CBP80 (cap-binding protein). hCBP80 is known to be involved in splicing and U snRNA export (Izaurralde et al., 1994, 1995). We will refer to the yeast protein as yeast (y)CBP80. The corresponding gene has previously been isolated in various

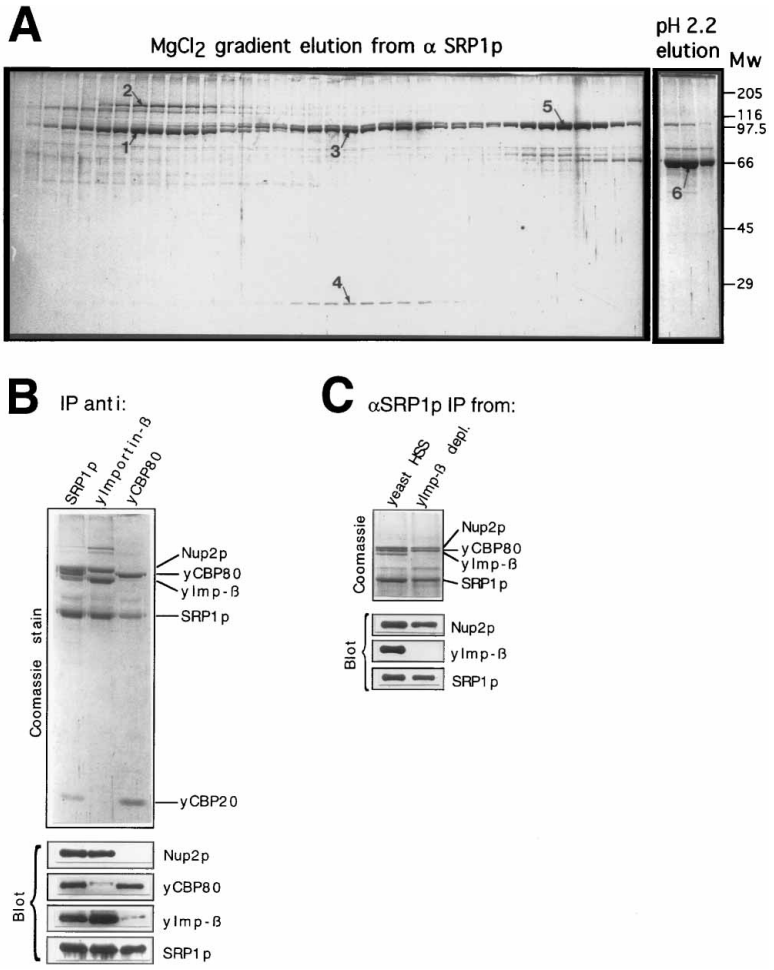

Figure 1. SRP1p-Containing Complexes

(A) Identification of SRP1p-associated proteins. Protein complexes were bound to an anti-SRP1p immuno-affinity column. The column was washed and then eluted with a linear 0–2 M $MgCl_2$ gradient followed by a pH 2.2 step. Eluted proteins were as follows: 1, yeast homolog of importin-β; 2, Nup1p; 3, yeast CBP80 homolog; 4, the yeast CBP20 homolog; 5, Nup2p; 6, SRP1p.

(B) SRP1p is part of several different complexes. Indicated antibodies were used for immunoprecipitations from an S. cerevisiae postribosomal supernatant. Bound proteins were analyzed by SDS–PAGE and Coomassie staining or Western blotting with the indicated antibodies. Nup2p was detected by MAb 414 (Davis and Blobel, 1986).

(C) SRP1p and associated proteins immunoprecipitated from yeast high speed supernatant or high speed supernatant depleted of y–importin-β.

suppressor screens and called GCR3, STO1, or SUT1 (Uemura and Jigami, 1992; GenBank/EMBL accession numbers L07650 and L27744).

The peptide sequence of the 25 kDa protein (Figure 1A, band 4) indicated that its gene had not been identified before. It was therefore cloned and found to encode a 23.8 kDa protein. The most striking homology found was to human CBP20, with which it shares approximately 63% identity in the overlap of 113 amino acids (Figure 2). CBP20 is a member of a family of proteins with a shared domain called the RNP motif or RNA recognition motif (Izaurralde et al., 1995; Kataoka et al., 1995), and this segment of the yeast and human proteins, together with the carboxy-terminal flanking region, is particularly highly conserved (Figure 2). It should be noted that the yeast homologs of CBP80 and CBP20 coeluted together from the antibody column shown in Figure 1A, and they also precisely coeluted from a Mono Q column (data not shown), suggesting that yeast CBP80 and CBP20 form a complex, like their human counterparts.

Three Distinct SRP1p Complexes

Having identified the polypeptides that associate with SRP1p, we wanted to ask if they were all members of a single complex or if multiple complexes existed. We also wanted to obtain information about which proteins might interact directly with SRP1p.

A first indication came from the order of elution in Figure 1A. Yeast importin-β eluted before the yCBC. yCBP20 and yCBP80 in turn eluted earlier than Nup2p. Thus, one can conclude that the importin-β homolog is

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yCBP20 MSLEEFDEVKYDHSTKRLDTPSRYLLRKARRNPNGLQELRESMKSSTIYVGNLSFYTSEEQIYELFSKCGT
                .    .: :. .  .    R..: . :. :: :  .KS.T:YVGNLSFYT:EEQIYELFSK.G.
hCBP20        MSGGLLKALRSDSYVELSQYRDQHFRGDNEEQEKLLKKSCTLYVGNLSFYTTEEQIYELFSKSGD

yCBP20 IKRIIMGLDRFKFTPCGFCFIIYSCPDEALNALKYLSDTKLDEKTITIDLDPGFEDGRQFGRGKSGGQVSD
       IK:IIMGLD:.K T:CGFCF: Y ...:A NA::Y:::T:LD::.I .D D:GF.:GRQ:GRG:SGGQV.D
hCBP20 IKKIIMGLDKMKKTACGFCFVEYYSRADAENAMRYINGTRLDDRIIRTDWDAGFKEGRQYGRGRSGGQVRD

yCBP20 ELRFDFDASRGGFAIPFAERVGVPHSRFDNSSSQSNTNNYIPPPDAMGTFRPGFDEEREDDNYVPQ
       E R D:DA:RGG::    .::
hCBP20 EYRQDYDAGRGGYGKLAQNQ
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Figure 2. The 25 kDa SRP1p-Associated Protein Is Similar to CBP20

The figure shows the aligned protein sequences of CBP20 from S. cerevisiae (yCBP20) and human (hCBP20) in single-letter code.

not required to maintain either the CBC–SRP1p interaction or the Nup2p–SRP1p interaction. The bulk of Nup2p appears to bind directly to SRP1p.

To investigate these complexes further, we compared the precipitates obtained with antibodies raised against SRP1p, yeast importin-β, and yCBP80 (Figure 1B). Next, complexes were precipitated with SRP1p antibodies before and after depleting yeast importin-β from the starting material (Figure 1C).

Nup2p is coprecipitated with either SRP1p or y–importin-β, but not when yCBP80 antibodies are used (Figure 1B). Nup2p efficiently coprecipitates with SRP1p even after depletion of y–importin-β (Figure 1C). Taken together, these results establish that there is a heterotrimeric complex containing SRP1p, y–importin-β, and Nup2p and a heterodimeric complex containing Nup2p and SRP1p.

Yeast importin-β and SRP1p coprecipitated either with anti-SRP1p or anti-yeast importin-β antibodies (Figure 1B, lanes 1 and 2; Figure 1C, lane 1). Quantitation of the experiment in Figure 1C showed that less than half the SRP1p was bound to y–importin-β. In the reverse experiment, however, most soluble importin-β could be codepleted with anti-SRP1p (data not shown). In this context, it should also be noted that when SRP1p is enriched by binding to a bovine serum albumin–NLS conjugate, only yeast importin-β is copurified; neither Nup2p nor the yCBC proteins are recovered in this fraction (data not shown).

The Complex between SRP1p and yCBC

Antibodies raised against recombinant yCBP80 efficiently precipitated yCBP20 (Figure 1B, lane 3), again suggesting that they form a stable complex. The coprecipitation of SRP1p indicated that about one-third of the SRP1p was bound to yCBC at a given timepoint (Figure 1B, lanes 1 and 3). Conversely, about one-third of CBC is bound to SRP1p (Figure 1B, lanes 1 and 3).

When yCBC was precipitated with a different antibody, raised against the amino-terminal 10 amino acids of yCBP80, yCBP20 copurified stoichiometrically but SRP1p hardly at all (data not shown). Peptide elution from this antibody provided a convenient way to obtain pure yCBC. This antibody and SRP1p appeared unable to bind to yCBP80 at the same time, and their binding sites therefore probably overlap at the amino-terminus of yeast CBP80. In support of this view, recombinant yCBP80 bound SRP1p as a full-length protein but not if it lacked the amino-terminal 22 amino acids (data not shown).

The very amino-terminus of yCBP80 was characterized by arginine clusters that resembled a bipartite NLS. In addition, the yCBC–SRP1p complex could bind y–importin-β (see below). Thus, the yCBC–SRP1p interaction is probably related to an NLS–receptor interaction. Similarly, human CBP80 has a functional bipartite NLS close to its amino-terminus (Izaurralde et al., 1996) that is required for its binding to Rch1p, one of the human importin-α homologs (Cuomo et al., 1994; Weis et al., 1995). However, the fact that 1 M magnesium chloride is needed for dissociation of SRP1p from yCBC indicates that their interaction is far stronger than a standard receptor–NLS interaction. Even 20 SV40-NLS peptides conjugated to bovine serum albumin are not a strong enough ligand to remain complexed with SRP1p under these conditions.

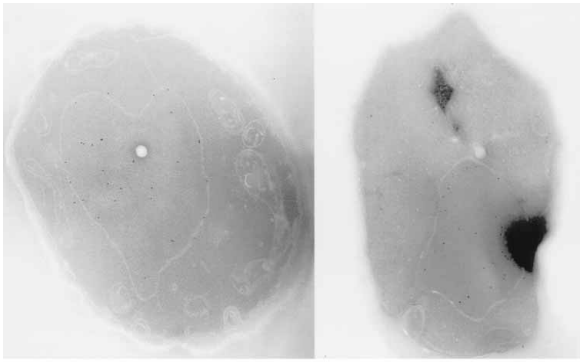

Figure 3. SRP1p and CBP80 Are Predominantly Nuclear in Yeast

Frozen sections of S. cerevisiae wild-type cells (Kaergel et al., 1996) were stained with affinity-purified anti-SRP1p or -yCBP80 antibodies followed by Protein A–10 nm gold. For quantitation, see Table 1.

The SRP1p/yCBC Complex Is Mainly Nuclear

Does the SRP1p–yCBC complex represent just an import intermediate formed between the NLS receptor and a karyophilic protein, or does this complex have a specific function? An import intermediate would be associated with importin-β and would localize in the cytoplasm and at nuclear pores. The distribution of SRP1p and yCBP80 in yeast cells was determined by immunoelectron microscopy (Figure 3). Besides labeling close to the nuclear envelope, probably corresponding to nuclear pores, the anti-SRP1p antibody gave a prominent staining inside the nucleus. yCBP80 is, like human CBC, predominantly nuclear (Figure 3). The extent of background yCBP80 staining was determined by using the same antibody with a yCBP80 deletion strain, which is viable, though with a severe growth defect (Uemura and Jigami, 1992; our unpublished data). Quantitation of the immuno-gold staining (Table 1) shows that both SRP1p and yCBP80 are about 10-fold concentrated in the nucleus. Taking the volumes into account, about two-thirds of total SRP1p and yCBC are nuclear. This strongly suggests that the complex of the two also has a nuclear localization. It is particularly remarkable that yCBC is the only nucleoplasmic protein detectably bound to SRP1p under stringent binding conditions (Figure 1A), even though we can estimate by immunoprecipitation that it accounts for less than 0.1% of the total nuclear protein.

The yCBP80 antibodies also precipitated a small, but significant, amount of y–importin-β. In turn, y–importin-β antibodies precipitated a small fraction of the yCBC complex (see Figure 1B). This small amount of yCBC–y–importin-β–SRP1p complex probably represents the fraction of yCBC–SRP1p complex that is on its way into the nucleus (see also below).

Yeast CBC Binds Capped RNA

Although the sequence conservation of the yeast and human CBP20 homologs is striking, the CBP80 proteins

Table 1. Quantitation of Antibody Labeling

Strain	Antibody against	Cytoplasmic gold particles/μm^2	Nuclear gold/μm^2	Nuclear: cytoplasmic ratio
Wild-type	SRP1p	1.4	14.4	10.3
Wild-type	yCBP80	4.5	33	
ΔyCBP80	yCBP80	1.4	2.2	
(Background) Minus background	yCBP80	3.1	30.8	9.9

are much less well conserved. To determine whether the two complexes, yCBC and hCBC, were functionally related, we compared their binding to RNA in vitro. Recombinant hCBC bound specifically to capped RNA in vitro in an electrophoretic mobility shift assay (Figure 4, lanes 1 and 2). This complex was sensitive to competition by cap dinucleotide analogs carrying either a 7-methyl or 7-ethyl group, while it was insensitive to competition by 2,2,7-trimethyl guanosine, adenosine, or unmethylated guanosine cap analogs. 2,7-dimethyl guanosine cap showed an intermediate behavior (Figure 4, lanes 3–8).

Purified yeast CBC (see Experimental Procedures) gave rise to a retarded complex of similar mobility (Figure 4, lanes 9 and 10). When the same cap analogs were used in competition experiments (lanes 11–16), one important difference in specificity was observed. Whereas hCBC was not affected by unmethylated diguanosine triphosphate, yCBC responded similarly to either 7-methylated or unmethylated cap analog (lanes 11 and 12) over a range of concentrations (data not shown). Recombinant yCBC behaved similarly to the native yCBC, both in terms of cap-dependent binding and in its response to the cap analog competitors (data not shown). These results demonstrate that yCBC does indeed bind RNA in a cap-dependent way but that it does not require the 7-methyl group for recognition.

The CBC/Importin-α Complex Binds Capped RNA, But the Importin-α/-β Heterodimer Dissociates CBC from RNA

Given the roles of CBC and the importin heterodimer in nucleocytoplasmic transport and the nuclear association of CBC with importin-α/SRP1p (see also Figure 6), it was of interest to determine the consequence of interaction of CBC with importin-α, -β, or both. The effect of interaction on the binding of hCBC or yCBC to RNA was therefore analyzed. Complexes between yCBC and RNA were formed as before (Figure 5A, lanes 1 and 2). Addition of y–importin-β alone to these complexes was without effect (Figure 5A, lanes 9–12). In contrast, addition of SRP1p alone resulted in the formation of a new complex of lower mobility, as expected if SRP1p could bind to the yCBC–RNA complex (lanes 3–4). SRP1p did not detectably bind to RNA on its own (data not shown). Strikingly, the addition of a mixture of y–importin-β and SRP1p to the CBC–RNA complex caused a concentration-dependent dissociation of yCBC from the RNA (lanes 5–8). The same result was obtained independent of the order of mixing; i.e., y–importin-β addition to a preformed SRP1p–CBC–RNA complex also resulted in RNA release, whereas the simultaneous addition of all four proteins did not lead to RNA complex formation (data not shown).

Since this result has implications for the mechanism of action of CBC in RNA export, we wished to determine whether this effect was specific to the yeast system. The experiment was therefore repeated using hCBC, h–importin-β, and Rch1p, a human homolog of importin-α. Just as in yeast, Rch1p caused the production of a retarded band in a concentration-dependent manner when added to hCBC–RNA (Figure 5B, lanes 1–4), while h–importin-β on its own had no effect on the hCBC–RNA complex (lanes 9–12). Addition of h–importin-β caused the complete dissociation of RNA from complexes containing hCBC and Rch1 (lanes 5–8). Again, the order of addition of the proteins did not affect the result (see Figure 5D). Thus, interaction of either human or yeast

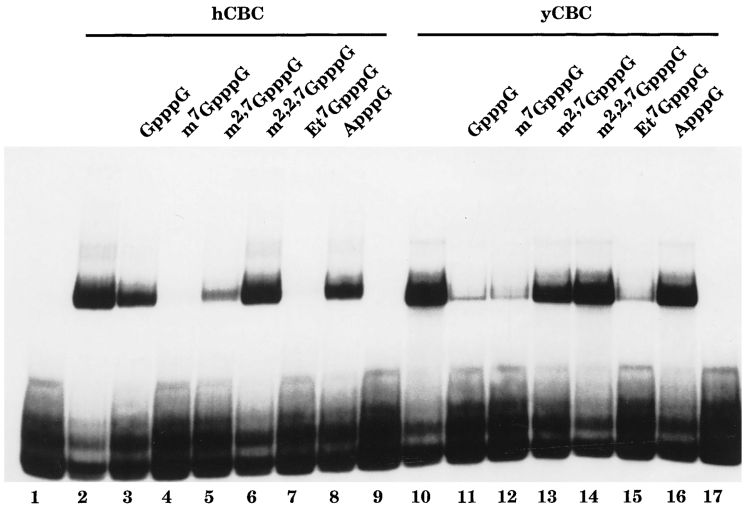

Figure 4. Yeast CBC Binds to Capped RNA

An m^7GpppG-capped RNA probe was subjected to native gel electrophoresis either alone (lanes 1, 9, and 17), together with recombinant human CBC (lanes 2–8), or purified yeast CBC (lanes 10–16). Cap dinucleotide analogs were added to the mixtures as indicated above the lanes.

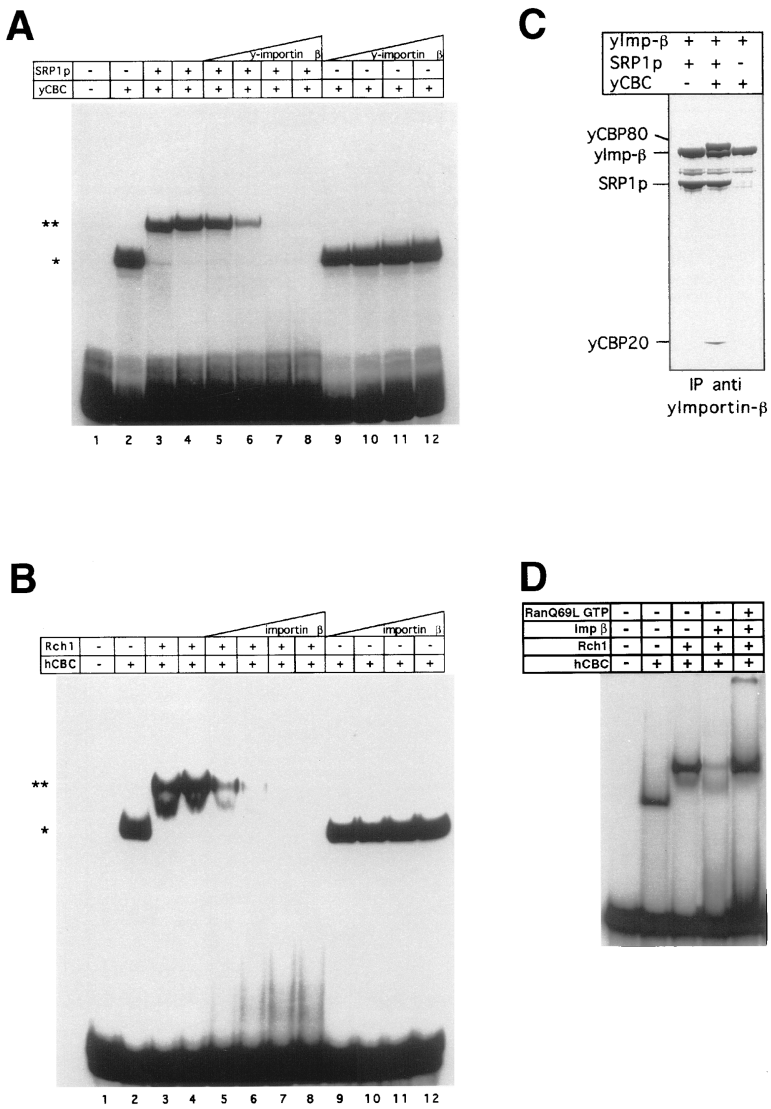

Figure 5. The CBC–Importin-α (SRP1p) Complex Binds to Capped RNA, But Importin-β Dissociates the Complex from the RNA

(A) A yCBC-capped RNA complex was formed and analyzed as in Figure 4 (lanes 1 and 2). Purified recombinant SRP1p was added to the CBC–RNA mixture either alone (lanes 3 and 4, 1 or 2 μg) or together with increasing amounts of y–importin-β (lanes 5–8, 1–4 μg). The same quantities of y–importin-β were also added in the absence of SRP1p (lanes 9–12).

(B) As in (A), except that purified recombinant hCBC, Rch1p, and h–importin-β were used. Lane 1, probe alone; lane 2, hCBC (400 ng); lanes 3–4, hCBC plus recombinant Rch1p (1, 1.5μg); lanes 5–8, hCBC plus 1.5 μg Rch1p and increasing amounts of h–importin-β (1.4, 2, 2.8, and 3.5 μg, respectively). The same quantities of h–importin-β were also added in the absence of Rch1p (lanes 9–12).

(C) Yeast CBP20, CBP80, SRP1p, and importin-β form a tetrameric complex. Yeast importin-β was prebound to immobilized anti-yeast importin-β antibodies and incubated with SRP1p, yCBC, or both. Bound fractions were washed, eluted with SDS, and analyzed by SDS–PAGE and Coomassie staining.

(D) RanGTP protects the CBC–importin-α–RNA complex against dissociation by importin-β. A mixture of hCBC, Rch1p, and RNA was incubated to allow complex formation. Subsequently, either importin-β or a mixture of importin-β and RanQ69L was added. The reaction was then fractionated as in Figure 4. Protein mixtures contained 400 ng of hCBC, 1.25 μg of Rch1, 6 μg of importin-β, and 6 μg of RanQ69L (GTP form) as indicated.

importin heterodimers with CBC–RNA complexes causes their dissociation.

To determine the fate of the proteins in this situation, the coimmunoprecipitation of yCBC with y–importin-β was analyzed in the presence and absence of SRP1p. While no CBC associated with y–importin-β in the absence of SRP1p, the tetrameric complex was coprecipitated in the presence of SRP1p (Figure 5C).

RanGTP Prevents Importin from Dissociating the CBC–RNA Complex

Importin should release the RNA from CBC only in the cytoplasm but not inside the nucleus. This predicts that this release mechanism must be regulated in some way. Importin-β is a Ran-binding protein, and binding of the GTP form of Ran has been reported to dissociate the yeast or Xenopus importin-α/β heterodimer (Rexach and Blobel, 1995, Görlich et al., 1996b). This predicts that RanGTP might protect the importin-α–CBC–RNA complex against dissociation by importin-β. Capped RNA was therefore first incubated with CBC and importin-α to allow complex formation. Then either importin-β or a mixture of importin-β and the GTP–loaded RanQ69L mutant, which is stabilized in its GTP–bound form, were added. As seen from Figure 5D, importin-β could not release the RNA from the importin-α–CBC–RNA complex if RanGTP was present. As expected, RanGDP, whose affinity for importin-β is roughly 30,000-fold lower than that of RanGTP (Görlich et al., 1996b), did not prevent the importin-β–mediated dissociation of RNA from the complex (data not shown).

Xenopus CBC Remains Bound to Importin-α after Nuclear Entry

To find out whether the nuclear complex of CBC with importin-α was specific for yeast, we isolated nuclei from Xenopus oocytes and precipitated importin-α with a specific polyclonal antiserum (Görlich et al., 1995a). As seen from Figure 6A, lanes 1–5, roughly 18% of the nuclear CBP20 was coprecipitated by these antibodies. To determine whether a conventional nonshuttling nuclear protein would be associated with importin-α in the

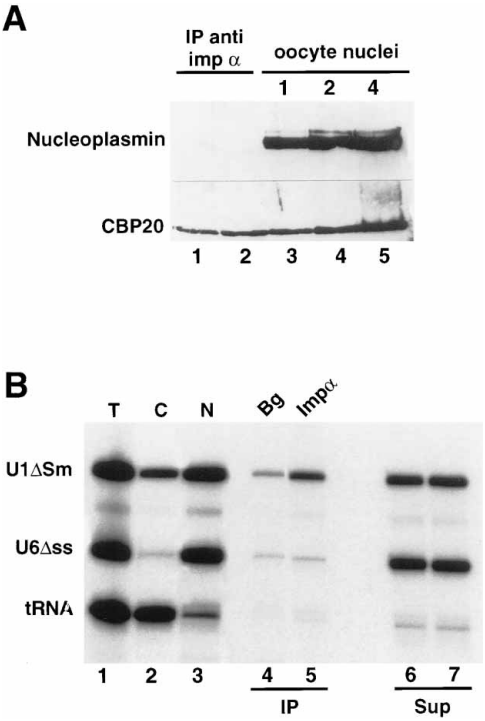

Figure 6. Importin-α Is Bound to CBC and Capped RNAs in the Nuclei of Xenopus Oocytes

(A) Soluble nuclear fractions from oocytes were immunoprecipitated with anti–importin-α antiserum. Proteins were eluted, fractionated by SDS–PAGE, and analyzed by Western blotting. Lanes 1 and 2, bound fractions from four and eight nuclei, respectively. Lanes 3–5, total protein extracted from one, two, and four nuclei, respectively. (B) A mixture of U1ΔSm, U6Δss, and tRNA was microinjected into Xenopus oocyte nuclei. After 1 hr, RNA was extracted from either total or fractionated oocytes (lanes 1–3). Extract from 20 isolated nuclei was prepared and selected on beads either with or without anti–importin-α antibodies. Bound RNA was extracted from control (lane 4) or anti–importin-α beads (lane 5) corresponding to eight oocyte nuclei. RNA corresponding to two nuclei from the supernatants (lanes 6 and 7) was also loaded.

nucleus, we also probed the immunoprecipitate with anti-nucleoplasmin antiserum. No nucleoplasmin was detected in the precipitate (Figure 6A, lanes 1 and 2). Thus, CBC escapes the mechanism that normally causes dissociation of nuclear proteins from the import receptor.

Importin-α Is Associated with U snRNAs Destined for Nuclear Export

If the CBC–importin-α complex binds to capped RNAs in vivo, it should be possible to precipitate capped RNAs microinjected into oocyte nuclei with anti–importin-α antibodies. To test this, a mixture of three labeled RNAs, U1ΔSm RNA, U6Δss RNA, and a tRNA, was injected into nuclei, and after 1 hr immunoprecipitations were performed (Figure 6B). Roughly 12% of the injected U1 snRNA was precipitated with the anti–importin-α antibody (average of two experiments). The amount of RNA precipitated is likely to be an underestimate, because the association with export factors is probably a rate-limiting step for export (Jarmolowski et al., 1994), and the half-life of completely assembled RNPs in the nucleus may well be short. Additionally, export RNPs contain many components, and steric interference with antibody binding is likely to occur. Importantly, U6 snRNA, which is neither capped nor exported, and tRNA, which is exported but not capped, were not precipitated with the antibody at levels above background. While this shows that importin-α associates with export U1 snRNPs in the nucleus, it should be noted that in addition to CBC, other factors might constitute importin-α binding sites on the particles.

U snRNA Export Requires Continuous Importin-Dependent Protein Import

CBC is mainly nuclear at steady state (Izaurralde et al., 1994; Kataoka et al., 1994; Visa et al., 1996; see Figure 3). However, it traverses the NPC in association with RNPs and can be visualized on the cytoplasmic face of the NPC (Visa et al., 1996). This suggests that CBC actually shuttles between the nucleus and cytoplasm while promoting export of the RNAs to which it is bound. The same probably applies to other export factors and predicts that RNA export should depend on the continuous reimport of these specific factors.

To test whether U snRNAs rely on importin for recycling of their export factors, we used an isolated domain of importin-α that binds importin-β (IBB domain; Görlich et al., 1996a). The IBB domain competes with importin-α for binding to importin-β and in this way blocks not only import of nuclear proteins but also the nuclear entry of importin-α itself. The wild-type or a nonfunctional truncated IBB domain were expressed as fusions to one copy of the IgG-binding domain of protein A and injected into the cytoplasm of Xenopus oocytes. After 1 hr, a second injection of a mixture of U1ΔSm and U5ΔSm RNAs, both of which depend on CBC for export, U6Δss RNA, which remains in the nucleus after injection, and tRNA, whose export is CBC–independent (Izaurralde et al., 1995), was performed. While the truncated IBB domain had no effect on RNA export (Figure 7A, lanes 4–6 and 7–9), the wild-type IBB inhibited U snRNA export (Figure 7A, lanes 10–12). Importantly, when the IBB domain was injected immediately after the RNA, U snRNA export was not affected (data not shown), demonstrating that the IBB domain is not unspecifically toxic for U snRNA export but inhibits by time-dependent depletion of at least one essential export factor from the nucleus. CBC and importin-α are good candidates to have become limiting factors in the nucleus; however, it is quite possible that they are not the only cycling factors involved in U snRNA export. It is important to note that the IBB injection had no effect on the export of tRNA, which has previously been shown to leave the nucleus by a pathway that relies on factors distinct from those required for U snRNA export (Jarmolowski et al., 1994; Cheng et al., 1995).

Discussion

We have analyzed complexes formed by the yeast nuclear protein import receptor SRP1p, the homolog of

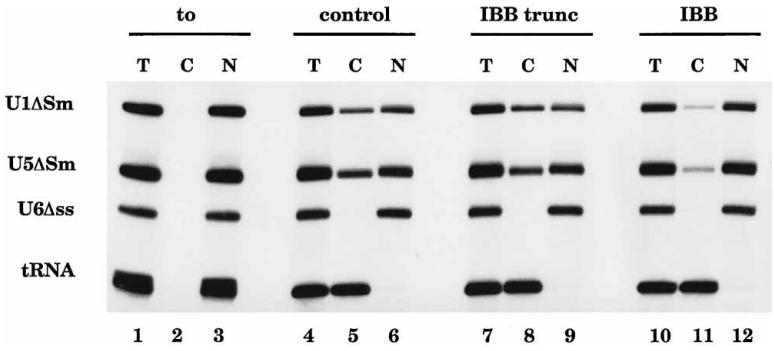

Figure 7. U snRNA Export Requires Importin-Dependent Protein Import

Xenopus laevis oocytes were injected into the cytoplasm with truncated (lanes 7–9) or functional (lanes 10–12) IBB domains or with phosphate-buffered saline alone (lanes 1–6). After 1 hr, U1ΔSm, U5ΔSm, U6Δss, and tRNA were injected into their nuclei. In lanes 4–12, RNA was extracted 180 min after injection; in lanes 1–3, RNA was extracted immediately after injection. T, C, and N indicate RNA extracted from total oocytes or from cytoplasmic or nuclear fractions, respectively.

importin-α. The observed interaction with yeast importin-β corroborates previous work (Enenkel et al., 1995) and provides evidence that nuclear protein import in lower and higher eukaryotes is a mechanistically conserved process. We could also confirm previous reports that SRP1p interacts with the nuclear pore proteins Nup1p and Nup2p (Belanger et al., 1994). Nup2p binds SRP1p directly in an importin-β–independent manner. Until now, the only established role for importin-α homologs was to bind nuclear proteins via their NLS and thereby mediate their interaction with importin-β. In mammalian cells, binding to importin-β is not only essential, but also sufficient, to import a protein substrate through the pore into the mammalian nucleus (Görlich et al., 1996a; Weis et al., 1996; see Introduction). If the same holds true in yeast, the direct SRP1p–Nup2p interaction is not likely to be important during translocation of the NLS–recognition complex from the cytoplasm through the NPC into the nucleus, but it may play an alternative role, e.g., in the disassembly of the NLS–SRP1p–importin-β complex.

A Yeast Nuclear Cap-Binding Complex

A striking novel finding is the existence in yeast extract of an abundant complex between SRP1p and yeast CBC, which we show by sequence homology and by binding to capped RNA to be the yeast counterpart of human CBC. One obvious difference between the yeast and the vertebrate system is that S. cerevisiae contains approximately equal concentrations of SRP1p and CBC, whereas in HeLa cells, for example, CBC is far less abundant than the protein import receptor.

CBC is involved in at least two aspects of nuclear RNA metabolism, pre-mRNA splicing and RNA export. CBC is not absolutely essential for splicing, but its depletion from HeLa nuclear extracts inhibits pre-mRNA splicing at an early step of splicing complex assembly (Izaurralde et al., 1994; Lewis et al., 1996a). The defect can be restored by CBC readdition. Yeast CBP20 was independently discovered as the product of MUD 13, a gene whose mutation caused synthetic lethality in combination with a mutant form of U1 snRNA. Genetic depletion of yCBP20 from yeast cells or extracts was shown to cause a reduction in the efficiency of splicing and early splicing complex (commitment complex) assembly (Colot et al., 1996), and immunodepletion of yCBC has a similar effect (Lewis et al., 1996b).

A variety of studies have led to the conclusion that the m^7G cap structure of RNA polymerase II transcripts plays a role in their export from the nucleus in vertebrates (reviewed by Izaurralde and Mattaj, 1995). The cap-dependence of export is strong for U snRNAs but much weaker for mRNAs (Jarmolowski et al., 1994) and is mediated by CBC (Izaurralde et al., 1995). The similar behavior of yeast and human CBC in the presence of the importin subunits is a strong indication that the yeast complex will also be involved in nucleocytoplasmic transport, but direct evidence is lacking. As expected from the vertebrate data, deletion of the yCBP80 gene does not result in the accumulation of polyadenylated RNA in the nucleus (our unpublished data). Owing to the low abundance of yeast U snRNAs and the difficulty in obtaining clean nuclear and cytoplasmic fractions, U snRNA transport has not yet been studied in yeast. It is therefore not known if U snRNP assembly occurs in a similar way in yeast and in vertebrates.

Neither CBP20 nor CBP80 is essential in yeast, although their deletion causes a severe growth defect (Uemura and Jigami, 1992; Colot et al., 1996; our unpublished data). This suggests either that CBC function in splicing and (presumably) RNA export is stimulatory, rather than absolutely required, or that these functions can be compensated in some way in the absence of yCBC. In the case of transport, this might be explained by the complex composition of nuclear RNPs. Especially in the case of mRNPs, multiple hnRNP proteins (Dreyfuss et al., 1993), at least some of which contain independent nuclear export signals (Piñol-Roma and Dreyfuss, 1992; Michael et al., 1995), are part of each transport substrate. This might explain why a single contributor to RNP export can be dispensable. U snRNAs, the other major class of capped RNAs that are exported from the nucleus in multicellular eukaryotes, also form sizable RNPs prior to export (Terns et al., 1992).

The CBC Transport Cycle

At steady state, CBC is found mainly in the nucleus (Izaurralde et al., 1994b; Kataoka et al., 1994; Visa et al., 1996), although it can also be seen, for example, attached to the giant Balbiani ring mRNPs on the cytoplasmic face of the NPC, but not on free cytoplasmic Balbiani ring mRNPs or polysomes (Visa et al., 1996).

There are several reasons why CBC must dissociate from capped RNA in the cytoplasm: first, the cap of mRNAs needs to be unmasked to allow eIF4E or eIF4F to bind and mediate translation initiation (Shatkin, 1985; Sonenberg, 1988). In the case of U snRNAs, the cap must undergo cytoplasmic hypermethylation as part of

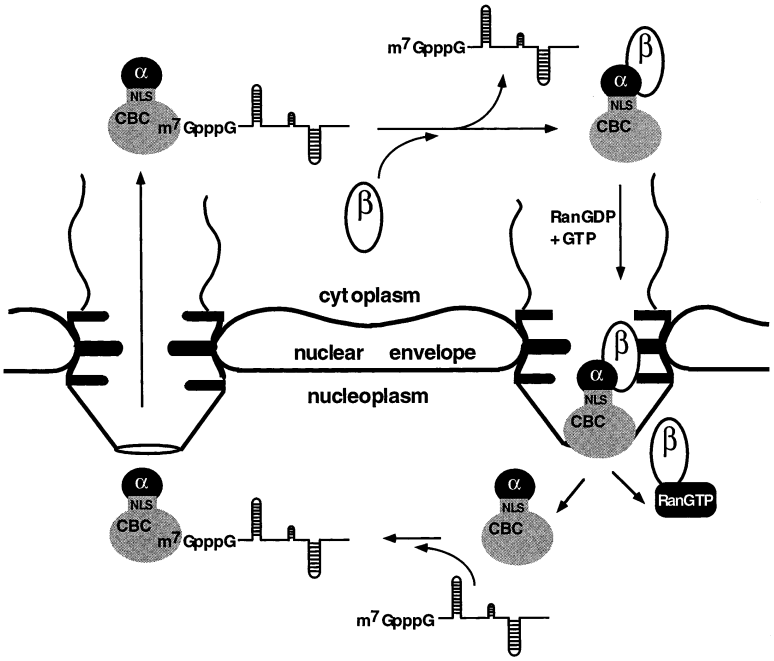

Figure 8. A Model of the CBC Nuclear Transport Cycle

the process of formation of a mature U snRNP (reviewed by Lührmann et al., 1990; Izaurralde and Mattaj, 1992). Finally, CBC has to be recycled back to the nucleus, leaving its export substrate behind, if it is to function again in splicing or transport. The need for CBC reimport is one possible explanation why U snRNA export requires continuous importin-dependent protein import. The finding that a high proportion of yeast or Xenopus CBC remains stably associated with importin-α inside the nucleus directly implies that the importin-α–CBC interaction is not equivalent to a normal NLS binding interaction and suggests that CBC might remain importin-α–bound during its entire transport cycle. This view is further supported by the observation that the importin-α–CBC complex can bind to capped RNA. The attachment of importin-α to the export complex would ensure the fastest possible recycling of CBC to the nucleus, possibly even by importin-β that is prebound to the cytoplasmic face of the NPC. Further study is required to examine what is the export signal on exported U snRNA–protein complexes. However, an attractive possibility is that CBC might utilize the return of importin-α to the cytoplasm to promote RNA export. The CBC transport cycle is summarized in Figure 8.

CBC binds to mRNAs in the nucleus and is translocated with them through the NPC (Visa et al., 1996). Importin-mediated dissociation of CBC should occur independently of whether it is bound to a U snRNA or an mRNA, although CBC is not essential for mRNA export (Jarmolowski et al., 1994; Izaurralde et al., 1995). The possibility of a role for importin in the release and recycling of other RNA export factors remains to be investigated.

Asymmetry of Transport across the Nuclear Pore Complex

One of the major questions in nucleo-cytoplasmic transport is how shuttling transport receptors achieve transport in one direction. For example, why do RNA-export mediators export RNA out of the nucleus rather than import RNA from the cytoplasm? One explanation is certainly the asymmetry of the NPC itself. However, transport receptors like CBC or importin bind their transport substrates before contacting the NPC, and these receptors must therefore "sense" a cytoplasmic or nucleoplasmic environment.

We show here that binding of importin-β triggers the dissociation of the importin-α–CBC complex from a capped RNA. Probably, two conditions synergize to make the importin-mediated RNA release a specifically cytoplasmic event. First, the importin-β concentration is higher in the cytoplasm than in the nucleus. Second, the high nucleoplasmic RanGTP concentration prevents importin-α/-β interaction and thereby protects the nuclear CBC–importin-α–RNA complex against dissociation by importin-β (Figure 8).

Importin-β can bind either RanGTP or importin-α but not both at the same time (Rexach and Blobel, 1995; Görlich et al., 1996b). The high nucleoplasmic RanGTP concentration dissociates importin-β from importin-α inside the nucleus, and the low levels of RanGTP in the cytoplasm allow the importin heterodimer to form. CBC enters the nucleus as a complex with importin-α and -β and can therefore not carry RNA into the nucleus. When the nucleoplasmic side of the NPC is reached, the importin heterodimer is dissociated by binding of nuclear RanGTP to importin-β (Görlich et al., 1996b), releasing the importin-α–CBC complex into the nucleoplasm and simultaneously restoring the capacity of CBC to bind RNA. CBC can then participate in splicing and, for example, promote export of U snRNA out of the nucleus, perhaps in a complex with importin-α. The cycle ends when importin-β releases the RNA from CBC in the cytoplasm. Dissociation from the capped RNA generates a complex between importin-α, importin-β, and CBC and thus simultaneously commits CBC to reimport into the nucleus.

The RanGTP binding site in importin-β can, in this model, be seen as a built-in sensor for nucleoplasmic or cytoplasmic identity. This would explain why it is not the binding of importin-α alone, but the binding of importin-β that determines whether CBC remains bound to the RNA. The detailed mechanism by which the importin heterodimer causes CBC dissociation from RNA is currently unknown, but conformational changes need to be transmitted through importin-α from its IBB domain to its NLS binding site and then via the NLS on CBC to the site of cap binding. The result is an economical and elegant method for accomplishing the two goals of RNA release and recycling of the RNA-export mediator to the nucleus. It seems likely that other RNA-binding proteins that are removed from RNA during or subsequent to export and returned to the nucleus may be subject to the same, or a similar, process.

Experimental Procedures

Antibodies

Antibodies were raised in rabbits against recombinant yeast SRP1p, yeast importin-β, yCBP80, nucleoplasmin-core, and a peptide corresponding to the 10 amino-terminal residues of yCBP80. Antibodies were affinity-purified on appropriate antigen columns and are monospecific on blots of total cell lysate. Covalent cross-linking to protein A Sepharose FF was by dimethyl pimelimidate.

Purification of SRP1p-Bound Proteins

S. cerevisiae was grown and homogenized as described (Panzner et al., 1995). The medium speed supernatant was adjusted to 50 mM Hepes/KOH (pH 7.5), 50 mM Tris–HCl (pH 7.5), 50 mM potassium acetate, 200 mM NaCl, 5 mM magnesium acetate, 5 mM mercaptoethanol, protease inhibitors, 10% glycerol and spun for 3 hr at 370,000 g. Lysate (250 ml, from 50 g cells) was loaded over 5 hr to a 5 ml anti-SRP1p column. After an overnight wash with 500 ml equilibration buffer (50 mM Tris, 200 mM NaCl, 2 mM mercaptoethanol), a 30 ml linear gradient ending at 2 M magnesium chloride was applied, and 1 ml fractions were collected, aliquots of which were precipitated with 90% isopropanol. Each fraction (1%) was loaded onto the 11% SDS–PAGE gel shown in Figure 1. Fractions containing the yCBC were dialyzed and purified further on Mono Q.

Analytical immunoprecipitations in batch mode were with 20 μl of immobilized antibody and 1 ml of yeast lysate. Binding and washing times were as for the preparative scale. Equilibration buffer contained 0.005% digitonin to prevent sticking of the beads to the plastic tubes. Elution was with SDS sample buffer minus dithiothreitol.

Identification of the SRP1p Bound Proteins

The identity of Nup2p was confirmed by its reactivity with MAb 414 (Davis and Blobel, 1986) and by its amino-terminal sequence. Internal sequence information on the other peptides was obtained after Lys-C digest. A total of 77, 158, 89, and 58 residues of sequence were obtained from yeast importin-β, yCBP80p, yCBP20p, and Nup1p, respectively, and amino acid compositions matched closely the calculated values (data not shown, available on request).

Molecular Cloning of yCBP20

A search of the GenBank database identified yeast expressed sequence tag (ACC T36583) coding for some yCBP20p, allowing isolation of several full-length clones from a yeast genomic library.

Protein Purification

Immobilized yCBP80p peptide antibody (1 ml) was loaded with S. cerevisiae lysate as above. After extensive washing, yCBC was competitively eluted by 1 mg/ml of antigenic peptide in 50 mM Tris–1M NaCl at room temperature. yCBC was further purified on Superdex 200 equilibrated in 50 mM Tris–HCl, 100 mM NaCl, 3 mM mercaptoethanol.

Recombinant protein purification was as described (Görlich et al., 1995a; 1995b, 1996; Izaurralde et al., 1995), except for yeast importin-β, which was expressed from a pQE30 vector (Qiagen) and purified by its his-tag and recombinant yCBC, which was as for the human complex (Izaurralde et al., 1995).

Gel Shift Analysis

The binding buffer contained 5 mM HEPES (pH 7.9), 125 mM KCl, 1 mM EDTA, 0.6 mM dithiothreitol, 0.1% v/v digitonin, 0.1 μg/μl of bovine serum albumin, 0.8 μg/μl of tRNA, 0.5 μg/μl of uncapped pBluescript T3 transcript, 2.5% v/v glycerol, and 1 U/μl of RNAsin. Approximately 100 ng of the native proteins were used per reaction. Other conditions were as described (Izaurralde et al., 1995).

Oocyte Injections

Oocyte injection experiments were performed as described (Jarmolowski et al., 1994). The concentration of the recombinant IBB fusion proteins injected was 20 μg/μl.

Direct immunoprecipitation of CBC or of U1ΔSm RNA 60 min after its injection was from nuclear fractions from 20 oocytes in 500 μl of TNE buffer (10 mM Tris–HCl [pH 8], 50 mM NaCl, 1 mM EDTA, 1 U/μl RNAsin, 0.1 mM phenylmethylsulfonyl fluoride, and 1% v/v Trasylol). Precipitation was with affinity-purified antibodies against recombinant Xenopus importin-α (Görlich et al., 1995a) on Protein A beads.

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Note Added in Proof

The Genbank/EMBL accession number for the gene encoding yCBP20 is U39665.