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Export of Importin α from the Nucleus Is Mediated by a Specific Nuclear Transport Factor

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Summary

NLS proteins are transported into the nucleus by the importin α/β heterodimer. Importin α binds the NLS, while importin β mediates translocation through the nuclear pore complex. After translocation, RanGTP, whose predicted concentration is high in the nucleus and low in the cytoplasm, binds importin β and displaces importin α. Importin α must then be returned to the cytoplasm, leaving the NLS protein behind. Here, we report that the previously identified CAS protein mediates importin α re-export. CAS binds strongly to importin α only in the presence of RanGTP, forming an importin α/CAS/RanGTP complex. Importin α is released from this complex in the cytoplasm by the combined action of RanBP1 and RanGAP1. CAS binds preferentially to NLS-free importin α, explaining why import substrates stay in the nucleus.

Introduction

Bidirectional transport of macromolecules between nucleus and cytoplasm occurs through the nuclear pore complexes (NPCs). It is generally energy-dependent and triggered by specific signals. These signals are recognized by saturable transport receptors that are thought to shuttle between nucleus and cytoplasm (for recent reviews, see Corbett and Silver, 1997; Nigg, 1997). The first identified signal is the "classical" nuclear localization signal (NLS), which is characterized by clusters of basic amino acids and found in many nuclear proteins (reviewed in Dingwall and Laskey, 1991). Importin is the nuclear import receptor for NLS-containing proteins (for recent reviews, see Koepp and Silver, 1996; Schlenstedt, 1996; Görlich, 1997). The M9 domain is the import signal for hnRNP proteins such as A1 (Siomi and Dreyfuss, 1995; Weighardt et al., 1995) and confers import along the transportin-dependent pathway (Aitchison et al., 1996; Nakielny et al., 1996; Pollard et al., 1996; Fridell et al., 1997). Further transport signals are the leucine-rich nuclear export signal (NES) (Fischer et al., 1995; Wen et al., 1995; Fridell et al., 1996; Richards et al., 1996) and the KNS shuttling domain of the hnRNP K protein, which allows both import and export (Michael et al., 1997).

A fundamental question is how it is possible for the shuttling transport receptors to carry their cargo in only one direction. An import receptor, for example, has to bind its cargo initially in the cytoplasm, carry the import substrate into the nucleus, and release the cargo there. To accomplish multiple rounds of transport, the import receptor has to return to the cytoplasm, leaving the cargo behind. Conversely, a nuclear export receptor has to bind its substrate in the nucleus, carry it out, and release it in the cytoplasm. This model predicts asymmetric import/re-export cycles and implies that the binding of cargoes to their transport receptors is regulated by the different environments of nucleus and cytoplasm.

The small GTP binding protein Ran (Drivas et al., 1990; Bischoff and Ponstingl, 1991a) is a key component in nucleocytoplasmic transport (Melchior et al., 1993a; Moore and Blobel, 1993; Schlenstedt et al., 1995; Görlich et al., 1996b; Palacios et al., 1996; Richards et al., 1997) and probably the major source of asymmetry across the nuclear envelope (discussed in Görlich et al., 1996c). Like all G proteins, Ran switches between a GDP- and a GTP-bound state by nucleotide exchange and GTP hydrolysis. The intrinsic rates for these reactions are very low and are stimulated by specific factors. RCC1 is Ran's major nucleotide exchange factor (Bischoff and Ponstingl, 1991b). It is chromatin-bound (Ohtsubo et al., 1989) and thus generates RanGTP inside the nucleus. So far, RanGAP1 (Rna1p) is the only known RanGTPase-activating protein and causes conversion of RanGTP into RanGDP (Bischoff et al., 1994, 1995a; Becker et al., 1995; Corbett et al., 1995). This GTPase activation is facilitated by the RanGTP binding protein RanBP1 (Coutavas et al., 1993; Bischoff et al., 1995b; Richards et al., 1995). RanGAP1 and RanBP1 are excluded from the interior of the nucleus (Hopper et al., 1990; Melchior et al., 1993b; Matunis et al., 1996; Richards et al., 1996; Mahajan et al., 1997) and thus deplete RanGTP only from the cytoplasm. Because Ran is concentrated in the nucleus (Bischoff and Ponstingl, 1991a) and because of the asymmetric distribution of its GAP and nucleotide exchange factor, one would predict a steep RanGTP gradient across the nuclear envelope with a low cytoplasmic RanGTP concentration and a high concentration inside the nucleus. As detailed below, the RanGTP concentration gradient is used to make interactions of shuttling import receptors, such as importin β, specific for the cytoplasmic or the nuclear compartment.

The initial, cytoplasmic event of NLS-dependent nuclear protein import is the binding of the import substrate to the importin α/β heterodimer. Importin α provides the NLS-binding site (Adam and Gerace, 1991; Weis et al., 1995), whereas importin β accounts for the subsequent interactions with the NPC that drive translocation into the nucleus (Görlich et al., 1995b, 1996a; Moroianu et

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al., 1995; Weis et al., 1996a). The transfer of the trimeric NLS/importin α/β complex to the nucleoplasmic side of the NPC is energy-dependent and appears to require GTP hydrolysis by Ran (Melchior et al., 1993a; Moore and Blobel, 1993, 1994; Palacios et al., 1996; Weis et al., 1996b), but the detailed mechanism is not understood. The direct binding of RanGTP to importin β finally terminates the translocation (Görlich et al., 1996b) and disassembles the importin heterodimer (Rexach and Blobel, 1995; Chi et al., 1996; Görlich et al., 1996b), thereby releasing importin α with the import substrate into the nucleoplasm. As detailed above, free RanGTP should be available only inside the nucleus, and this would make the RanGTP-mediated dissociation of the importin heterodimer a specific nuclear event that follows cargo translocation into the nucleus.

After delivering the NLS substrate into the nucleus, importin α must be returned to the cytoplasm. For three reasons, we can rule out the possibility that importin α is exported by importin β. First, the importin β binding (IBB) domain of importin α confers very efficient import into the nucleus, but not nuclear export (Görlich et al., 1996a; Weis et al., 1996a). Second, importin β is exported much faster than the α subunit (Görlich et al., 1995b; Moroianu et al., 1995). Third, the high nuclear RanGTP concentration should prevent any stable association between importin α and β inside the nucleus. In fact, the normal high levels of nuclear RanGTP are essential for export of importin α. Importin α accumulated inside the nucleus when nuclear RanGTP production was impaired in S. cerevisiae strains defective in Prp20p, the yeast nucleotide exchange factor for Ran (Koepp et al., 1996). Likewise, depleting nuclear RanGTP by microinjecting RanGAP1 into nuclei of Xenopus oocytes blocked nuclear export of importin α (Görlich et al., 1997), suggesting this process to be dependent on nuclear RanGTP.

Here, we identify the mediator of importin α export from the nucleus as CAS (Cellular Apoptosis Susceptibility gene), a protein originally implicated in apoptosis and cell proliferation (Brinkmann et al., 1995, 1996). During NLS-dependent import, importin α strongly accumulates in the nuclei of permeabilized cells in the absence of CAS but is efficiently returned to the cytoplasm when CAS is added. CAS interacts with NPCs, crosses the nuclear envelope rapidly, and binds RanGTP and importin α simultaneously in a highly cooperative manner. As CAS requires RanGTP for high affinity binding to importin α, CAS should bind importin α inside the nucleus and the trimeric importin α/CAS/RanGTP complex probably represents the species that is transferred through the NPC to the cytoplasm. The disassembly of the complex requires RanGAP1 and RanBP1, which are normally excluded from the nucleoplasm. This disassembly therefore provides a mechanism by which importin α is specifically released from its export factor into the cytoplasm. We propose that CAS is the prototype of a RanGTP-regulated shuttling transport receptor that mediates export from the cell nucleus. We suggest that export receptors of this type be collectively called "exportins."

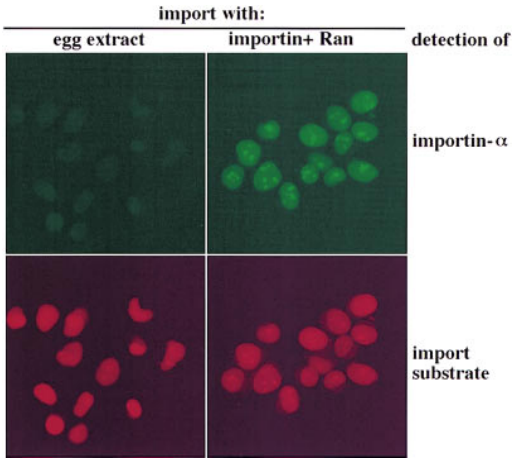

Figure 1. Re-export of Importin α from the Nucleus Requires a Soluble Factor

Nuclear import into nuclei of permeabilized cells was carried out with 1 μM fluorescein-labeled importin α and 1 μM Texas Red–labeled nucleoplasmin, in the presence of either a Xenopus egg extract (left-hand panels) or with recombinant factors, namely 1 μM importin β, 2 μM Ran, 0.2 μM RanBP1, 0.2 μM Rna1p, and 0.2 μM NTF2 (right-hand panels). An energy-regenerating system was present in both reactions. After 15 minutes of incubation, importin α and nucleoplasmin were simultaneously detected by laser scanning microscopy in the fluorescein and Texas Red channels, respectively. Note that nucleoplasmin had accumulated in the nuclei of both incubations. In contrast, importin α showed nuclear accumulation only in the import reaction with recombinant factors, but not in the egg extract, suggesting that the extract contains an additional factor required for re-export of importin α.

Results

Export of Importin α from the Nucleus Requires a Soluble Transport Factor

Importin α is the shuttling nuclear import receptor for NLS-containing proteins. It binds the import substrate initially in the cytoplasm and carries its cargo through the NPC into the nucleoplasm where the import substrate is finally released. To accomplish another round of import, importin α has to be returned to the cytoplasm. To study this re-export of importin α from the cell nucleus, we incubated permeabilized HeLa cells with a Texas Red–labeled NLS-import substrate, fluorescein-labeled importin α, an energy regenerating system, and cytosol or various transport factors. We then measured the cytoplasmic and nuclear concentrations of importin α and import substrate by scanning with a confocal fluorescence microscope directly through the unfixed samples. When using a Xenopus egg extract as the source of soluble transport factors, the import substrate strongly accumulated in the nuclei, whereas importin α was so efficiently returned to the cytoplasm that it did not accumulate in the nuclei (Figure 1, left panels). The formal possibility that importin α had vanished from the nuclei by proteolysis can be excluded because importin α is entirely stable even during prolonged import reactions (not shown). When the egg extract was replaced by a mixture of the import factors importin β, Ran, RanBP1, NTF2, and RanGAP1, nuclear import of nucleoplasmin

was fully reconstituted, but the re-export of importin α was not. Instead, importin α strongly accumulated inside the nuclei (Figure 1, right panels). This could be explained if importin α was exported by a soluble export factor that is present in the complete cytosol but is distinct from the added recombinant factors and limiting in the permeabilized cells.

Identification of CAS as an Importin α–Binding Factor

Previous studies have shown that the presence of nuclear RanGTP is essential for nuclear export of importin α (Koepp et al., 1996; Görlich et al., 1997). We therefore reasoned that the putative export factor might have to interact with RanGTP to bind and export importin α. To identify a candidate, we looked in egg extracts for a protein that would simultaneously bind RanGTP and importin α and by these properties mediate importin α binding to a RanGTP column. Because wild-type Ran would not remain in the GTP-bound form in the presence of cellular extracts that contain RanGAP, we used the GTPase-deficient RanQ69L mutant (Klebe et al., 1995). The extract was applied to either immobilized wild-type Ran (GDP-bound form) or RanQ69L (in the GTP-bound form). The columns were extensively washed, and bound proteins were eluted with 2 M magnesium chloride and analyzed by SDS–PAGE followed by Western blotting or Coomassie staining and sequence analysis of the stained bands. The immunoblot revealed that importin α did not bind to the GDP-bound form of wild-type Ran. However, importin α bound to RanQ69L GTP specifically and as efficiently as to the anti–importin α antibody. Peptide sequencing identified the "standard" importin α and a related protein ("importin α*") as major bands among the RanGTP-bound proteins (Figure 2). The importin α binding to the RanQ69L column had to be indirect via another factor because purified importin α alone could not bind under these conditions (not shown). The crucial hint to the identity of this Ran-binding and importin α–binding factor came from the observation that 1 M NaCl disassembled the bound complex and eluted importin α from the column. Protein sequencing identified the predominant protein in this fraction and thus the putative binding factor as a previously described protein, CAS (Brinkmann et al., 1995). We have recently shown CAS to contain an importin β–like RanGTP-binding motif and to bind RanGTP (Görlich et al., 1997).

We then cloned CAS from HeLa mRNA into an in vitro transcription vector, translated it in vitro, and compared its importin α binding to that of importin β (Figure 3). Without further addition or with addition of wild-type RanGDP, importin β bound efficiently to importin α, but CAS only weakly. The opposite pattern was observed when the permanently GTP-bound Ran mutants RanQ69L or RanQ69LΔC were added: importin β binding was completely abolished, whereas the CAS binding to importin α was greatly enhanced. This suggested that the CAS/RanGTP complex is the high affinity form for importin α binding. However, the experiment did not rule out that an additional factor from the reticulocyte lysate was also involved.

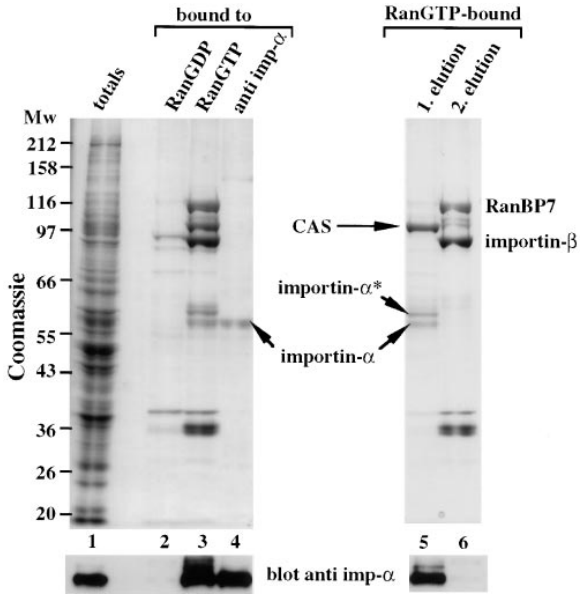

Figure 2. Identification of Importin α and CAS among the Proteins That Bind RanGTP Specifically

A postribosomal supernatant prepared from a Xenopus egg extract (lane 1) was applied to either immobilized wild-type Ran GDP (lane 2), to the GTPase-deficient RanQ69L mutant in its GTP form (lanes 3, 5, and 6), or to an antibody raised against the N terminus of importin α (lane 4). After extensive washing, bound proteins were eluted with either 2 M $MgCl_2$ (lanes 2 and 3), 0.2 M glycine (pH 2.2, lane 4), or 1 M NaCl ("1st elution," lane 5), followed by 2 M $MgCl_2$ ("2nd elution," lane 6). The eluates were precipitated with 90% isopropanol (final), analyzed by SDS–PAGE followed by Coomassie staining and by immunoblotting with antibodies raised against importin α. RanBP7, importin α, and importin β were identified by immunoblotting. CAS was identified as such by sequencing an internal peptide (AAEEAFEDNSEEYI, in single-letter code) that was identical to residues 356–370 of human CAS. "Importin α*" represents a protein that is roughly 70% identical to the "standard" importin α as judged by sequencing several internal peptides (LLHHDDREVLADT, VGNIVTGTFQTQV, NVTXTL NLC, LVAFLAHSDCSPIQFEAAAA). Note that importin α and CAS were specifically recovered with RanQ69L, and that importin α, the importin α–related factor, and CAS together represent the major bands in the NaCl eluate from RanQ69L.

To show a direct interaction between importin α, RanGTP, and CAS, we performed the gel filtration experiment shown in Figure 4, using recombinant proteins. Ran and importin α applied alone eluted in two distinct peaks and showed no interaction with each other. However, when CAS was also added, importin α and Ran were incorporated into a larger, 200–250 kDa complex that also contained CAS. Thus, importin α, CAS, and RanGTP efficiently form a heterotrimeric complex.

The Binding of Importin α and RanGTP to CAS Is Highly Cooperative

Binding of importin β to RanGTP has striking effects on the RanGTPase in that GTPase activation by RanGAP1 and nucleotide exchange by RCC1 become blocked (Floer and Blobel, 1996; Görlich et al., 1996b; Lounsbury and Macara, 1997). As CAS has an importin β–like Ran binding motif (Görlich et al., 1997), it was of interest to see if CAS would have similar effects. In addition,

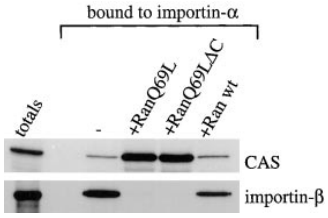

Figure 3. CAS Binding to Importin α Depends on RanGTP

CAS and importin β were translated in vitro (totals), and 20 μl aliquots were incubated for 10 minutes with 5 μM human importin α (Rch1p) and 15 μM of the indicated forms of Ran. For binding, the samples were diluted 50-fold in binding buffer. The added importin α had been tagged with the IgG-binding z domain from Protein A, allowing importin α–containing complexes to be retrieved with IgG-Sepharose ("bound to importin α"). Analysis was by SDS–PAGE followed by fluorography. Load in the bound fractions corresponds to twice the amount of the totals. Note that the permanently GTP-bound RanQ69L and RanQ69LΔC mutants abolish importin β binding to α, whereas they greatly stimulate the CAS–importin α interaction. The regulation of importin α binding by RanGTP is thus opposite for CAS and importin β.

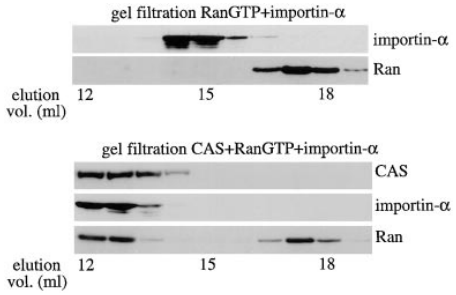

Figure 4. Importin α, CAS, and RanGTP Form a Stable Heterotrimeric Complex

Xenopus importin α (1 nmol) plus RanQ69L GTP (2 nmol) either alone or together with CAS (1.5 nmol) was incubated in a total volume of 100 μl for 30 minutes on ice. These samples were applied at 0.4 ml/min to a 25 ml Superdex 200 column (Pharmacia) equilibrated in 50 mM HEPES/KOH (pH 7.5), 150 mM NaCl, 5 mM magnesium acetate, 2% w/v glycerol, 0.005% digitonin. Fractions (0.75 ml) were collected, of which 10 μl was analyzed by Western blotting with antibodies (Qiagen) that recognize the N-terminal His tags of CAS and Ran, and with an anti–importin α antibody. The void volume is at approximately 8 ml. Note that importin α and Ran alone show no interaction with each other and elute in distinct peaks after 15 ml and 18 ml, respectively. With CAS, a larger CAS/importin α/RanGTP complex is formed that elutes between 12 and 13 ml, corresponding to an approximate size of 200–250 kDa.

nucleotide exchange and GTP hydrolysis can easily be quantitated and used as a measure for the proportion of Ran that is associated with the respective factor. From the dose dependence of the effects, one can estimate dissociation constants for the complex formation. For comparison, the dissociation constants for the importin β/RanGTP and transportin/RanGTP complexes are around 1 nM (Görlich et al., 1996b; F. R. B., S. Nakielny, and G. Dreyfuss, unpublished data).

We first tested the effects of CAS on the RCC1-mediated nucleotide exchange on RanGTP (Figure 5A). Surprisingly, micromolar concentrations of CAS were required to see any stabilization of Ran's GTP-bound state, suggesting that CAS alone has a very low affinity for RanGTP and/or that the RanGTP/CAS complex is kinetically unstable. The situation, however, changed dramatically when 1 μM importin α was added. Now 1000 times less CAS was required for half maximum binding of RanGTP, suggesting a constant of approximately 1 nM for dissociation of RanGTP from the RanGTP/CAS/importin α complex. Figure 5A also demonstrates that the interaction is specific for the GTP-bound form of Ran, as CAS has no effect on RanGDP.

Figure 5B shows that the RanGTP/CAS/importin α complex is resistant toward GTPase activation by RanGAP. It confirms that the affinity for RanGTP is very low in the case of CAS alone but is high when CAS and importin α are present simultaneously. Taken together, the affinity of CAS for importin α is greatly enhanced by simultaneous binding of RanGTP (Figure 3). Conversely, the affinity of CAS for RanGTP becomes 2–3 orders of magnitude higher when importin α is simultaneously bound (Figure 5). The formation of the trimeric complex is thus highly cooperative.

CAS Preferentially Binds to NLS-Free Importin α

The function of the NLS as a strictly unidirectional import signal (Michael et al., 1995; Shulga et al., 1996) implies that importin α is normally returned to the cytoplasm without the import substrate. If CAS is responsible for importin α re-export it should discriminate between free importin α and an NLS–importin α complex. To test this directly, we measured kinetically the association between importin α and CAS/RanGTP in the absence or presence of NLS-import substrates. As a control, we first confirmed that nucleoplasmin with a deleted NLS or BSA did not influence the importin α/CAS interaction and behaved exactly as the buffer control (not shown). Figure 5C shows that the presence of either 0.5 μM nucleoplasmin pentamers or 2.4 μM BSA-NLS conjugate made importin α a 10 times weaker ligand for CAS binding compared to the control. This probably reflects the decrease in the concentration of free importin α by complexing it with the NLS. Taken together, the experiment confirms that CAS has a preference for NLS-free importin α.

RanBP1 and RanGAP1 Together Release Importin α from Its Complex with CAS and RanGTP

The RanGTP/CAS/importin α complex is kinetically very stable, indicating that its disassembly has to be facilitated by some factor. In the case of the RanGTP complexes of importin β, RanBP5, RanBP7, or transportin, this is accomplished by the concerted action of RanBP1 and RanGAP1 (Deane et al., 1997; Görlich et al., 1997; Lounsbury and Macara, 1997; F. R. B. and D. G., unpublished data). Figure 5D confirms that the RanGTP/CAS/importin α complex alone entirely resisted GTPase activation by RanGAP1. However, the addition of subnanomolar concentrations of RanBP1 allowed GTP hydrolysis to take place. Because CAS has no detectable affinity for RanGDP (Figures 2, 3, and 5A), the GTP hydrolysis would be predicted to result in the disassembly of the trimeric complex. To show this directly, the experiment shown in Figures 5E and 5F was performed. CAS was

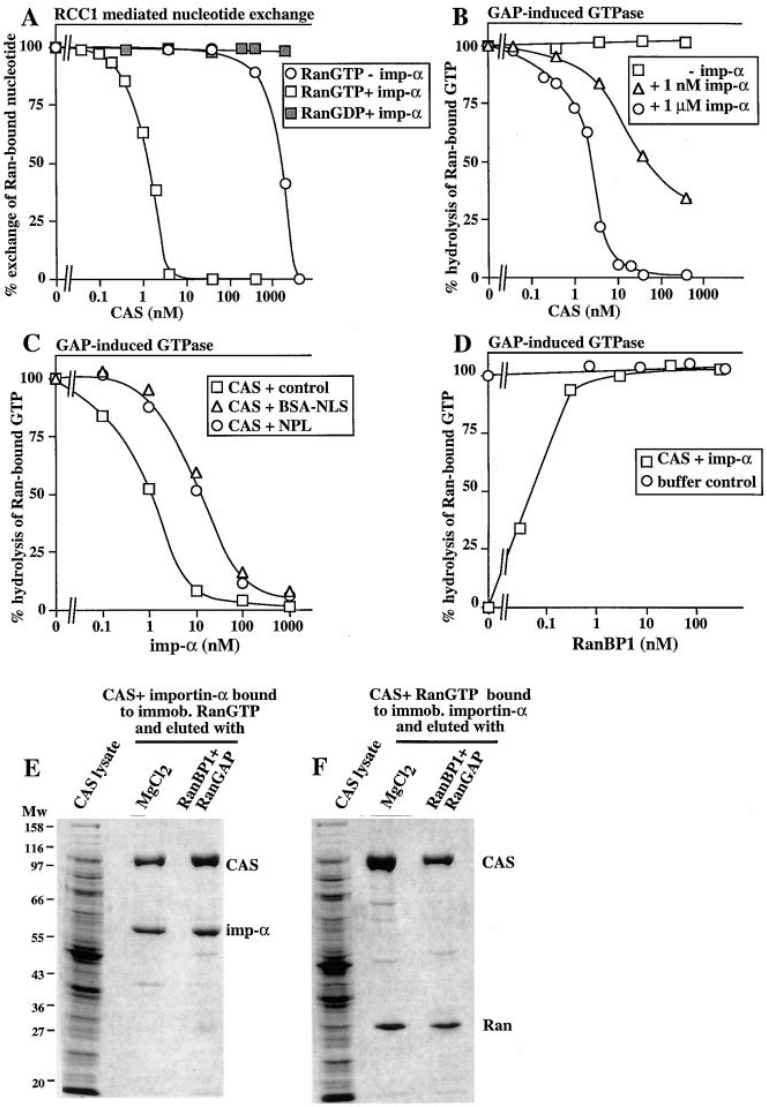

Figure 5. Kinetic Characterization of the CAS/Importin α/RanGTP Interaction

(A) RCC1-induced nucleotide exchange on Ran is prevented in the trimeric CAS/importin α/RanGTP complex. Ran-[γ-^{32}P]GTP (50 pM) or Ran-[α-^{32}P]GDP (50 pM) were incubated for 15 min either with indicated final concentrations of CAS or a mixture of CAS and 1 μM importin α (human Rch1p). Nonradioactive GDP was added to 200 μM, and a 2 min exchange reaction was started by the addition of 10 nM RCC1. Nucleotide exchange was determined as the decrease in protein-bound radioactivity. The dose dependence of nucleotide exchange inhibition can be used to estimate the dissociation constant of RanGTP from the complexes, which is roughly 1 nM for the trimeric CAS/importin α/RanGTP complex. Note that Ran and importin α binding to CAS is highly cooperative, with importin α increasing CAS' affinity for RanGTP apparently 1000-fold.

(B) CAS and importin α cooperatively bind RanGTP and thereby prevent GTPase activation on RanGTP. Ran-[γ-^{32}P]GTP (50 pM) was preincubated for 15 min with buffer, 1 nM or 1 μM importin α, and indicated final concentrations of CAS. Rna1p (20 nM; RanGAP1 from S. pombe) was added and the reaction was allowed to proceed for 2 min. Hydrolysis of Ran-bound GTP was determined as released [^{32}P]phosphate.

(C) CAS binds preferentially to NLS-free importin α. The formation of the CAS/importin α/RanGTP complex was measured as the decrease in GAP sensitivity of RanGTP as described in (B). The final concentration was 200 nM for CAS, 50 pM for Ran-[γ-^{32}P]GTP. Importin α was titrated either alone (control), or in the presence of 0.5 μM nucleoplasmin pentamers or 2.4 μM BSA-NLS conjugate. Note that the presence of the import substrates decreases the apparent affinity of importin α for CAS more than 10-fold.

(D) The CAS/importin α/RanGTP complex becomes disassembled by RanBP1 plus RanGAP1. Ran-[γ-^{32}P]GTP (50 pM) was preincubated for 15 min with 400 nM CAS plus 200 nM importin α or with buffer as control. Then 20 nM Rna1p was added followed by RanBP1 at the indicated final concentrations. After 2 min, hydrolysis of Ran-bound GTP was determined as released [^{32}P]phosphate. Note that the CAS/importin α/RanGTP complex is entirely GAP-resistant in the absence of RanBP1 but GTP hydrolysis occurs in its presence.

(E) CAS was expressed in E. coli, a lysate was prepared in 50 mM HEPES/KOH (pH 7.5), 200 mM NaCl, 10 mM magnesium acetate, and importin α was added to 1 μM. 1 ml of this mixture was then rotated for 1 hour with z-tagged Ran wild-type (GTP form) pre-bound to 20 μl of IgG Sepharose. After extensive washing, elution was performed with either 100 μl of 1 M $MgCl_2$ or 100 μl of binding buffer containing 80 ng of each Rna1p (RanGAP) and RanBP1. Analysis was by SDS–PAGE followed by Coomassie staining. 0.2% of the starting material and 10% of the eluates were loaded. Note that CAS was bound specifically out of the complex lysate and that $MgCl_2$ and Rna1p plus RanBP1 were equally effective in releasing CAS and importin α from the Ran column.

(F) The experiment was identical to (E), except that importin α and not Ran was the factor immobilized to the IgG Sepharose. RanGTP was added to the lysate at 2 μM.

expressed in E. coli and a lysate was prepared. Trimeric RanGTP (wild-type)/CAS/importin α complexes were formed by binding CAS from this lysate either together with importin α to immobilized Ran wild-type GTP (Figure 5E), or together with RanGTP to immobilized importin α. The resins were extensively washed to remove loosely bound material. The disassembly of the complexes was then detected as the release of CAS and importin α from the RanGTP column (Figure 5E) or by the release of CAS and Ran from the importin α column. As seen from the figure, 1 M magnesium chloride or trigger of GTP hydrolysis by catalytic amounts of Rna1p (RanGAP) plus RanBP1 was equally effective in the disassembly of the complexes into their constituents.

CAS Binds to NPCs and Can Rapidly Cross the Nuclear Membrane

A crucial criterion for being a shuttling transport receptor is the ability to cross the nuclear envelope and to interact with nuclear pore complexes. To test this, we labeled

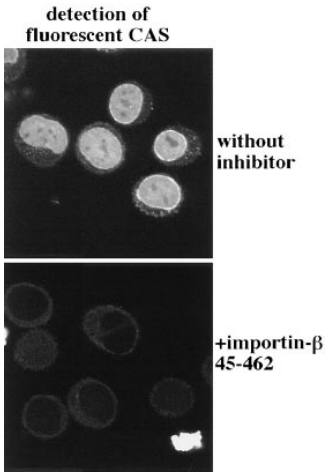

Figure 6. CAS Binds to Nuclear Pore Complexes and Efficiently Enters the Nucleus

CAS was expressed in E. coli, purified, and modified at a 1:1 molar ratio with fluorescein 5′ maleimide. Labeled CAS (1 μM) was incubated for 8 minutes with permeabilized cells in the presence of an energy-regenerating system. In the lower panel, the nuclei were preincubated with 1 μM of the dominant-negative 45–462 importin β mutant. Nuclei were fixed, spun onto coverslips, and examined by confocal fluorescence microscopy. Note that without the inhibitor, CAS accumulated at the nuclear envelope and inside the nucleus, whereas the importin β fragment prevented NPC binding and nuclear entry of CAS.

CAS with fluorescein and incubated it with permeabilized cells in the presence of an energy regenerating system, but without addition of any other soluble transport factor. After 8 minutes, the nuclei were fixed, spun onto coverslips, and examined by confocal microscopy. As seen from the upper panel of Figure 6, CAS gave a prominent staining of the nuclear envelope and had accumulated inside the nuclei while sparing nucleoli. If the permeabilized cells were pretreated with the dominant-negative importin β mutant 45–462, which binds almost irreversibly to NPCs (Kutay et al., 1997), the accumulation of CAS at the NPC and inside the nuclei was entirely prevented. Thus, CAS can rapidly cross the nuclear envelope on its own, and it uses similar sites at the NPC as importin β for this passage.

CAS Is a Functional Nuclear Export Factor for Importin α

To test directly if CAS is the nuclear export factor for importin α, we compared the nuclear accumulation of an NLS substrate and importin α in three different extracts. The first extract was an untreated, unfractionated Xenopus egg extract. The second one was obtained by passing the extract through a RanQ69L GTP column that depleted essentially all RanGTP binding proteins, including importin β, RanBP7, and CAS (not shown, but see Figure 2). To restore import activity of this extract, importin β had to be replenished, and 0.2 μM RanBP1 was also re-added. The third extract was the same as the second, but 2 μM CAS was re-added. The extracts were then supplemented with permeabilized cells and an energy-regenerating system, and import was started by the addition of a preformed complex of Texas Red BSA-NLS, fluorescein-labeled importin α, and unlabeled importin β (each 1 μM final). After 15 minutes of incubation, the unfixed samples were scanned with a confocal fluorescence microscope to measure directly the cytoplasmic and nuclear concentrations of the labeled proteins. Figure 7A shows that the import substrate had accumulated in the nuclei of all three samples and that the untreated extract showed no nuclear accumulation of importin α. In contrast, when the extract was depleted of Ran binding proteins (including CAS), importin α strongly accumulated in the nuclei and was particularly bright in the nucleoli, indicating that re-export of importin α was very inefficient. Strikingly, when 2 μM CAS was re-added, importin α no longer accumulated in the nuclei and gave a pattern very similar to that in the complete extract. The fact that the BSA-NLS-conjugate, whose import is strictly importin α–dependent, had accumulated in these nuclei implies that importin α must have entered the nuclei in the first place to deliver the import substrate. This strongly suggested that CAS mediated importin α export, although it did not definitively rule out the possibility that CAS had prevented the nuclear entry of importin α. To show the CAS dependence of importin α's nuclear exit directly, we performed the pulse–chase experiment shown in Figure 7B. We first allowed nuclear accumulation of importin α and NLS substrate in the absence of CAS ("15 minutes import plus importin β"). We then split the sample into three and added buffer, or 2 μM CAS, or 2 μM transportin, and scanned the samples 15 minutes later. In all three samples, nuclear accumulation of the import substrate continued. With addition of buffer or transportin, importin α also became brighter in the nuclei. However, when CAS was added, importin α became efficiently exported from the nuclei and, in particular, the bright staining of the nucleoli disappeared completely.

Finally, we tested if the export of importin α from the nuclei can be reconstituted with recombinant transport factors. We first allowed import of Texas Red–labeled nucleoplasmin and fluorescein-importin α in the presence of importin β, Ran, RanBP1, NTF2, and Rna1p ("15 minutes import"). The sample was split and either buffer or CAS was added. After 15 minutes, the samples were fixed and nuclei were spun onto coverslips and scanned at high magnification. As seen from Figure 7C, CAS again greatly stimulated importin α export. In this sample, importin α is also visible at the nuclear envelope. This probably represents both the population entering the nucleus via importin β and the one leaving the nucleus while bound to CAS/RanGTP.

Discussion

Importin α and β together accomplish nuclear import of proteins along the classical NLS pathway. After the translocation into the nucleus is terminated by dissociation of the importin heterodimer, the two subunits are returned to the cytoplasm to participate in the next round of import.

Here, we show that export of importin α from the cell nucleus requires a specific export factor, namely CAS. The initial observation was that in the presence of a

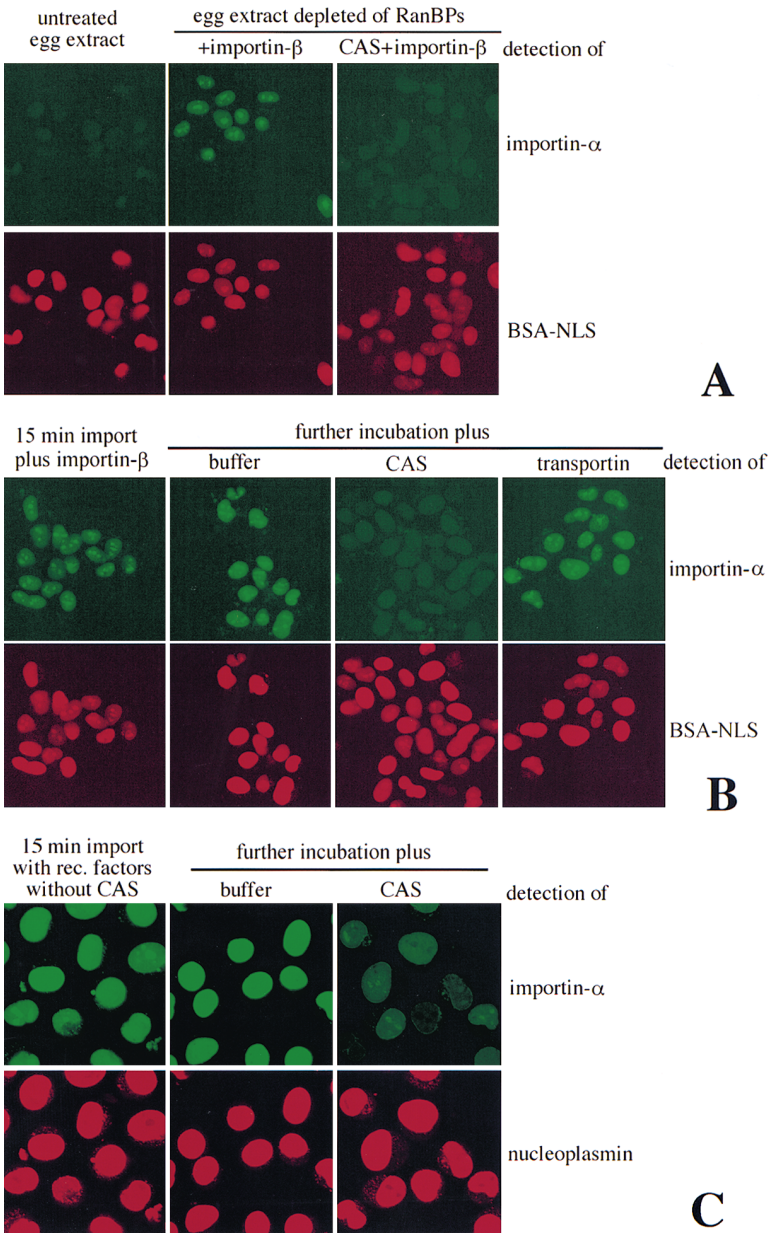

Figure 7. CAS Is a Functional Export Factor for Importin α

(A) Nuclear accumulation of fluorescein importin α and a Texas Red–labeled BSA-NLS conjugate was studied using three different extracts. The first was an untreated egg extract. The second was depleted of RanGTP-binding proteins (RanBPs) including importin β and CAS. The third extract was also depleted of RanBPs, but CAS was re-added. To restore import activity of the depleted extracts, they had to be replenished with importin β. Import was therefore started by addition of a preformed BSA-NLS/importin α/importin β complex (each 1 μM final). Analysis was as in Figure 1. Note that the import substrate accumulated in the nuclei of all three samples. Re-export of importin α was efficient in the untreated extract and when CAS was re-added to the depleted extract, whereas importin α showed strong nuclear accumulation in the depleted extract without CAS addition.

(B) Nuclear import of BSA-NLS and importin α was allowed in the depleted egg extract supplemented with importin β as described in (A). After 15 minutes, nuclear accumulation of importin α and import substrate was monitored by laser scanning microscopy. The remaining sample was divided into three, and either buffer, 2 μM CAS, or 2 μM transportin were added. After 15 minutes, the samples were analyzed exactly as the earlier time point. Note that in all three samples, the import substrate continued to accumulate and so did importin α after addition of buffer or transportin. In contrast, addition of CAS resulted in efficient export of importin α from the nuclei.

(C) Nuclear import of 2 μM Texas Red nucleoplasmin and 2 μM fluorescent importin α was allowed for 15 minutes in the presence of recombinant import factors, namely importin β, Ran, RanBP1, Rna1p, and NTF2. After 15 minutes, one aliquot sample was fixed. The remaining sample was split into two, either buffer or 2 μM CAS was added, and the incubation continued for 15 minutes before fixation. Nuclei were spun onto coverslips and scanned at high magnification (63× oil objective) with a confocal microscope. Note that CAS promoted nuclear export of importin α and that importin α in this sample is concentrated at the nuclear envelope.

complete cytosol, only the import substrate accumulated in the nuclei of permeabilized cells, but importin α was efficiently re-exported. In contrast, when import was performed in a reconstituted assay in the presence of importin, Ran, RanGAP1, RanBP1, RanGAP1, and NTF2, then both the NLS substrate and importin α accumulated. Efficient importin α recycling requires the presence of CAS. We would estimate that CAS' concentration in egg extracts is approximately 3 μM—roughly the same as importin β. Considering that for every imported NLS protein one molecule of importin α needs to be returned to the cytoplasm by CAS, this re-export has to be a tremendous activity that requires the high abundance of CAS.

CAS associates at high affinity with importin α (K_D ~1 nM), provided that RanGTP is simultaneously bound. As detailed in the Introduction, RanGTP should be available only inside the nucleus, and hence, CAS should bind importin α only in the nuclear compartment. We have shown before that the depletion of nuclear RanGTP by injecting RanGAP1 into oocyte nuclei blocks importin α export (Görlich et al., 1997; Izaurralde et al., 1997), probably by preventing the interaction between CAS and importin α.

CAS can enter the nucleus in the absence of any other soluble factor and binds similar sites at the NPCs as does importin β. This suggests that CAS can make a direct contact to the NPC and probably accounts for the interactions with the NPC that drive translocation of the importin α/CAS/RanGTP complex out of the nucleus.

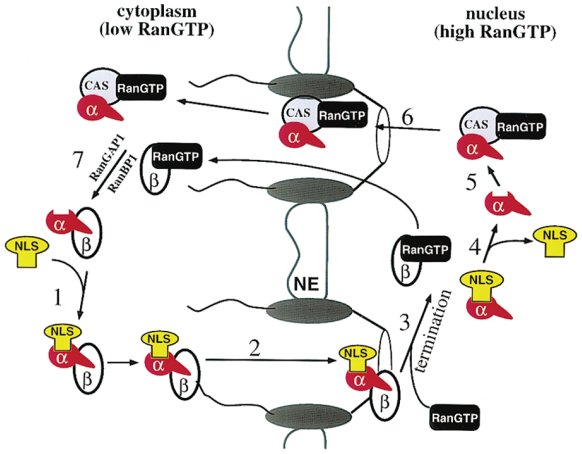

Figure 8. Scheme of the Importin α Transport Cycle

(1) The initial, cytoplasmic event in NLS-dependent import is the binding of the import substrate to the importin α/β heterodimer, where the importin α subunit provides the NLS binding site. (2) The translocation of the resulting trimeric complex is mediated by importin β and is (3) finally terminated at the nuclear side of the NPC by direct binding of nuclear RanGTP to importin β, which disassembles the importin heterodimer. (4) The NLS protein is released, and (5) importin α binds cooperatively with RanGTP to CAS. RanGTP should be stable only inside the nucleus; therefore the dissociation of importin α from β and the assembly of importin α into the importin α/CAS/RanGTP complex should be specifically nuclear events. (6) This trimeric complex is exported to the cytoplasm. CAS probably accounts for the interactions that drive translocation out of the nucleus. (7) In the cytoplasm, RanGAP1 and RanBP1 trigger GTP hydrolysis and thereby release importin α from its export receptor.

It is unclear what the energy source for this export is. However, the observation that it is resistant toward inhibition by the GTPase-deficient RanQ69L mutant (Izaurralde et al., 1997) could suggest that it is not GTP hydrolysis by Ran.

On the cytoplasmic side, importin α needs to be released from the export complex. We show here that RanBP1 and RanGAP1 together can trigger GTP hydrolysis in the importin α/CAS/RanGTP complex and thereby disassemble the complex into its constituents. RanBP1 is actively excluded from nuclei (Richards et al., 1996). RanGAP1 has two localizations in higher eukaryotes (Matunis et al., 1996; Mahajan et al., 1997), soluble in the cytoplasm and bound to RanBP2 (Wu et al., 1995; Yokoyama et al., 1995), a protein of the cytoplasmic periphery of the NPC with four domains that are functional equivalents of RanBP1. Thus, the release of the exported importin α could take place at RanBP2 and/or in the cytoplasm.

The importin α transport cycle provides an explanation for why an NLS is an import signal only and how importin α can achieve nuclear accumulation of a molar excess of import substrate. On the way into the nucleus, importin α's affinity for the NLS is enhanced by importin β binding (Rexach and Blobel, 1995; Görlich et al., 1996b). On the way out, CAS preferentially binds and thus exports NLS-free importin α. A schematic representation of the "updated" transport cycle of importin α is depicted in Figure 8.

Human CAS is 40% identical to S. cerevisiae Cse1p (Xiao et al., 1993). We have not directly addressed if Cse1p functions in importin α export in yeast. However, the available genetic data is consistent with this assumption. *Cse1* is an essential gene (Xiao et al., 1993). Cse1p has been reported to give an NPC-like staining pattern (cited in Irniger et al., 1995), and we have shown that Cse1p binds yeast Ran (Gsp1p) (Görlich et al., 1997). Furthermore, the cold-sensitive *cse1-1* allele could be suppressed (Xiao et al., 1993) by overexpression of SRP1p (Yano et al., 1992), the S. cerevisiae importin α. If this mutation in Cse1p causes defective SRP1p export, then SRP1p should accumulate inside the nucleus and become limiting in the cytoplasm. Overexpression of SRP1p would then be expected to compensate the defect. The *cse1-22* allele was identified as causing a defect in B-type cyclin degradation (Irniger et al., 1995) which, in yeast, takes place in the nucleus. Interestingly, the *srp1-31* allele has a similar phenotype (Loeb et al., 1995). This would now suggest that the interruption of the importin cycle at different points is the primary cause of both cell cycle defects and that protein import is instrumental for progression through mitosis.

CAS was originally identified as a human gene whose moderate suppression by antisense mRNA would protect cultured human cells against TNF-triggered apoptosis (Brinkmann et al., 1995, 1996). A possible explanation for this observation might be that components of the suicide machinery need to be delivered by the importin-dependent pathway into the nucleus. High level expression of the CAS antisense RNA was lethal, indicating that CAS is essential also in higher eukaryotes.

An obvious question is whether nuclear export of molecules other than importin α is accomplished by factors that are related to and function similarly as CAS. Support for this assumption comes from experiments in which RanGAP1 was mislocalized to the nucleus by microinjection of Xenopus oocytes or cultured cells. The treatment should deplete RanGTP from the nucleus and was found to block export of NES-containing proteins (Richards et al., 1997), importin α, tRNA, and that of several mRNAs (Izaurralde et al., 1997). Export of all of these substrates could be restored by coinjecting the GTPase-deficient RanG19V or RanQ69L mutants. This suggests that the respective export factors function similarly to CAS in that they require nuclear RanGTP to bind and export these substrates.

Recently, a superfamily of RanGTP binding factors was identified that share a sequence motif related to the Ran-binding site of importin β and that also show some overall similarity to importin β and to CAS (Fornerod et al., 1997a; Görlich et al., 1997). Some of them have been experimentally confirmed to interact with the NPC and/or to bind RanGTP. One of them, Crm1p, has recently been suggested to account for nuclear export of NES-containing proteins (see Fornerod et al., 1997b and Stade et al., 1997, this issue of *Cell*). The other members of this importin β/Crm1p/CAS superfamily might well represent transport factors that carry unknown cargoes. Their interaction with RanGTP is most likely used to regulate cargo binding. Import factors such as importin β and transportin bind their cargo in the cytoplasm and release it upon RanGTP binding, i.e., in the nucleus (Rexach and Blobel, 1995; Chi et al.,

1996; Görlich et al., 1996b; Izaurralde et al., 1997). Export factors would be regulated the other way around and would stably bind their cargo only if RanGTP is simultaneously bound, i.e., in a nuclear environment, and release the export substrate into the cytoplasm upon GTP hydrolysis triggered by RanGAP1 and RanBP1. CAS could serve as the paradigm for the function of these "exportins."

Experimental Procedures

Recombinant Protein Expression

CAS was cloned from HeLa mRNA as follows: mRNA was prepared using the Oligotex mRNA isolation kit (Qiagen), and first-strand cDNA synthesis was performed with AMV reverse transcriptase according to the manufacturer's (Boehringer Mannheim) instructions. The *CAS* gene was then amplified by PCR using a proofreading enzyme and primers that introduced a BamHI site at the 5′ end and a XmaI site after the stop codon. The fragment was cloned into the BamHI/XmaI sites of pQE30 (Qiagen). The construct was used to express CAS with an N-terminal His tag in E. coli. CAS was purified on nickel-NTA-agarose, followed by chromatography on MonoQ.

Constructs for the expression of zz-tagged Ran wild type and RanQ69L were obtained by cloning the Ran-coding fragments into the SphI/HindIII sites of the zz70 vector. Expression was in E. coli. Affinity columns for the binding experiments described below were prepared by saturating IgG Sepharose with the zz-tagged proteins from the E. coli lysates. This resulted in approximately 4 mg of z-tagged protein per ml of matrix. The columns were prewashed in 50 mM HEPES, 1 M NaCl, 10 mM magnesium acetate to remove loosely bound proteins and equilibrated in binding buffer (50 mM HEPES, 200 NaCl, 50 mM potassium acetate, 10 mM magnesium acetate).

RanQ69LΔC is a RanQ69L derivative lacking the six C-terminal residues. Purification was performed via the N-terminal His tag provided by the pQE32 vector.

zz-tagged human importin α (Rch1p) was prepared by cloning the coding sequence into the zz-60 vector. Expression was in E. coli and purification via the C-terminal His tag.

The expression of the following proteins has been described: transportin, Xenopus importin α, human importin α (Rch1p), importin β, the 45–462 fragment of importin β, NTF2, S. pombe Rna1p, untagged and N-terminally His-tagged Ran and RanQ69L, and RanBP1 (Bischoff et al., 1995a, 1995b; Görlich et al., 1996a, 1996b, 1997; Kutay et al., 1997).

In Vitro Translation

CAS and importin β were cloned into the NcoI/BamHI sites of the T7 60 vector (Görlich et al., 1997), which contains a T7 promoter and the 5′ UTR from Xenopus β-globin to enhance translation efficiency. ^{35}S-labeled proteins were synthesised in the TNT coupled transcription/translation system (Promega).

Fluorescence Labeling

Fluorescent importin α has been described before (Görlich et al., 1996a). Nucleoplasmin (dissolved in 50 mM Tris [pH 7.5], 200 mM NaCl) was modified with Texas Red C_2 maleimide (Molecular Probes, dissolved in DMSO) at a 1:1 molar ratio (referring to nucleoplasmin monomers) for 30 minutes at 37°C. Free fluorophore was removed by gel filtration on a PD10 column (Pharmacia). CAS (MonoQ fraction in ~50 mM HEPES [pH 7.5], 300 mM NaCl) was modified with fluorescein 5′ maleimide (dissolved in DMF) at a 1:1 molar ratio for 2 hours on ice. The BSA-NLS was labeled with Texas Red NHS ester (Molecular Probes).

Permeabilized Cell Assays

The basic method, such as preparation of permeabilized cells, has been described before. Xenopus egg extract (low speed supernatant) was prepared as described (Leno and Laskey, 1991). Import reactions with recombinant factors contained: 2 mg/ml nucleoplasmin core to block nonspecific binding, 20 mM HEPES/KOH (pH 7.5), 140 mM potassium acetate, 4 mM magnesium acetate, 250 mM sucrose, and unless noted otherwise, 2 μM RanGDP, 0.2 μM RanBP1, 0.2 μM Rna1p from S. pombe, 0.2 μM NTF2. All import reactions were supplemented with an energy-regenerating system (0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 50 μg/ml creatine kinase) and performed at 20°C. For scanning unfixed samples, 2 μl of each sample was placed onto a 10-well multitest slide (ICN) and covered with a 10 mm circular-shaped coverslip. Scanning was with a 40× dry objective of a Leika TCS NT confocal microscope. Fixation with paraformaldehyde was as described. Fixed samples were examined at higher magnification using a 63× oil objective.

Binding Assays and Depletions

To prepare a high speed supernatant (HSS) from Xenopus eggs, the LSS was diluted four times in binding buffer (see above) and supplemented with an energy-regenerating system (see above) and 3 μM Rna1p. It was spun at 4°C in TLA 100.3 rotor (Beckmann) for 45 minutes at 75,000 rpm. Each 1 ml of HSS was bound o/n to either 25 μl of immobilized zz Ran wild type or zz RanQ69L (GTP form), or to immobilized antibodies raised against the N terminus of Xenopus importin α (forms 1 and 2, Görlich et al., 1995a). The beads were washed three times with 1.5 ml of binding buffer, and elution was performed either with 50 mM Tris/HCl (pH 7.5), 1 M NaCl, or with 50 mM Tris/HCl (pH 7.5), 2 M $MgCl_2$, or with 0.2 M glycine (pH 2.2), as indicated in the legends.

Binding of in vitro translated CAS and importin β was essentially the same. Further details are given in the legend to Figure 3 and in the main text. Elution was with SDS sample buffer (minus reducing agent), and 50 mM DTT was added before loading the gels.

To deplete an egg LSS of RanBP, 0.5 ml of LSS (plus energy-regenerating system) was rotated for 1 hour with 100 μl (spin-dried) of immobilized RanQ69L that had been equilibrated in 20 mM HEPES/KOH (pH 7.5), 80 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose. The extract was recovered by spinning it through a column, and the procedure was repeated. Western blotting confirmed that at least 95% of importin β and RanBP7 had been removed, whereas the concentration of other proteins such as nucleoplasmin remained unaffected.

Kinetic measurement of the RanGTPase was as described in previous publications (Bischoff et al., 1995a, 1995b; Görlich et al., 1997).

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