Cell, Vol. 90, 1051–1060, September 19, 1997, Copyright ©1997 by Cell Press

CRM1 Is an Export Receptor for Leucine-Rich Nuclear Export Signals

Maarten Fornerod,*‡ Mutsuhito Ohno,*‡ Minoru Yoshida,† and Iain W. Mattaj*§
*European Molecular Biology Laboratory
D-69117 Heidelberg
Germany
†Department of Biotechnology
University of Tokyo
Tokyo 113
Japan

Summary

CRM1 is distantly related to receptors that mediate nuclear protein import and was previously shown to interact with the nuclear pore complex. Overexpression of CRM1 in Xenopus oocytes stimulates Rev and U snRNA export from the nucleus. Conversely, leptomycin B, a cytotoxin that is shown to bind to CRM1 protein, specifically inhibits the nuclear export of Rev and U snRNAs. In vitro, CRM1 forms a leptomycin B–sensitive complex involving cooperative binding of both RanGTP and the nuclear export signal (NES) from either the Rev or PKI proteins. We conclude that CRM1 is an export receptor for leucine-rich nuclear export signals and discuss a model for the role of RanGTP in CRM1 function and in nuclear export in general.

Introduction

The fact that RNA and protein synthesis occur in separate compartments of eukaryotic cells creates a requirement for a very high level of macromolecular trafficking between the nucleus and cytoplasm. For most protein and all known RNA transport substrates, transport into or out of the nucleus is a saturable, energy-dependent, and thus "receptor"-mediated process. The saturability reflects the presence of signals for transport on protein or RNA substrates that are recognized by transport mediators whose concentration can become limiting in the presence of excess substrate (Zasloff, 1983; Goldfarb et al., 1986; Hamm and Mattaj, 1990; Jarmolowski et al., 1994; Fischer et al., 1995).

Transport signals for nuclear protein import have been well characterized. One group of sequences form the basic nuclear localization signal (NLS) family (reviewed by Dingwall and Laskey, 1991). These interact with the α subunit of importin, the "NLS receptor." Importin is a heterodimer with an α and a β subunit that was characterized in several laboratories in parallel (for reviews, see Powers and Forbes, 1994; Görlich and Mattaj, 1996; Nigg, 1997). A second import signal is the M9 domain found in hnRNP A1 and a family of related hnRNP proteins (Michael et al., 1995; Siomi and Dreyfuss, 1995; Weighardt et al., 1995). The receptor that mediates M9 import into the nucleus, transportin, is related to the β subunit of importin (Pollard et al., 1996; Nakielny et al., 1996; Fridell et al., 1997). M9 also functions as a signal for nuclear export in mammalian cells (Michael et al., 1995). Recognition of parts of the hnRNP A1 protein including, but not confined to, the M9 domain, is required for export of some mRNAs (Izaurralde et al., 1997), but it is not yet known whether transportin plays a role in hnRNP A1 or mRNA export. The likely yeast homolog, transportin or Kap104p (Aitchison et al., 1996; Nakielny et al., 1996), has also been implicated in the nuclear import of hnRNP-like proteins (Aitchison et al., 1996). Two additional yeast importin β relatives, Kap123p and Pse1, have recently been proposed to be mediators of ribosomal protein import (Rout et al., 1997).

There are other characterized protein import signals (e.g., Mandell and Feldherr, 1990; Kambach and Mattaj, 1992; Michael et al., 1997) that do not obviously resemble either M9 or an NLS and whose import is likely to involve additional "receptors." In addition, a corresponding variety of nuclear export substrates exists (Jarmolowski et al., 1994; Fischer et al., 1995; Michael et al., 1995; Pokrywka and Goldfarb 1995; Wen et al., 1995). In analogy to import, nuclear export is also thought to be mediated by signals. So far, the best defined nuclear export signals (NESs) are a family of leucine-rich peptide NESs first identified in the HIV-1 Rev protein and in the cellular protein kinase A inhibitor PKI (Fischer et al., 1995; Wen et al., 1995). Saturation of the leucine-rich peptide NES pathway specifically inhibits the export of this class of proteins and of U snRNAs and 5S rRNA (Fischer et al., 1995), a result that suggests that these classes of export substrate may share a common export receptor.

The energy dependence of nucleocytoplasmic transport is explained at least in part by the requirement for the small GTPase Ran for all forms of active import and export thus far studied (Melchior et al., 1993; Moore and Blobel, 1993; Chi et al., 1996; Görlich et al., 1996a; Palacios et al., 1996; Sweet and Gerace, 1996; Weis et al., 1996; E. Izaurralde et al., submitted; Richards et al., 1997). In addition, the components of the Ran GTPase system, that is, the nucleotide exchange factor RCC1/Prp20p, the GTPase activating protein RanGAP1/Rna1p, and the Ran binding protein RanBP1/Yrb1p, are arranged asymmetrically such as to produce a high Ran GTP:Ran GDP ratio in the nucleus and the reverse ratio in the cytoplasm (reviewed by Moore and Blobel, 1994; Görlich and Mattaj, 1996; Koepp and Silver, 1996; Nigg, 1997). This asymmetric distribution of RanGTP has recently been shown to be a requirement not only for NLS protein import (Görlich et al., 1996a) but also for the export of NES proteins (Richards et al., 1997) and for the export and import of a large variety of other transport substrates (E. Izaurralde et al., submitted).

The likely reason for this is illustrated by considering examples. RanGTP binding to importin β dissociates it from importin α (Rexach and Blobel, 1995; Chi et al., 1996; Görlich et al., 1996a). Thus, RanGTP disassembles the NLS import receptor complex, a reaction that terminates NLS protein import (Görlich et al., 1996a; Kutay

‡These authors contributed equally to this work.
§To whom correspondence should be addressed.

et al., 1997). Similarly, RanGTP dissociates transportin from the M9 signal (E. Izaurralde et al., submitted). These interactions are thought to occur in the nucleus and ensure that import substrate:import receptor interaction cannot stably occur there, that is, RanGTP-induced dissociation of the import receptor-substrate complexes prevents reexport by reversal of the import reaction. The same RanGTP–importin β interaction also plays a role in imparting directionality to U snRNA export (Görlich et al., 1996b) although in a more complex way. Possible predictions from these studies are that interactions between export receptors and their substrates might be dissociated by interaction with RanGDP when they reach the cytoplasm or, alternatively, formation of complexes between export receptors and their cargo might be dependent upon RanGTP.

One can predict that export receptors will bind not only their substrate and Ran but also, like importin β or transportin, interact with nucleoporins, proteins of the nuclear pore complex (NPC). Although this would not necessarily involve the same NPC proteins for each transport mediator, reagents that prevent access to nucleoporins block many nuclear import and export pathways (e.g., Finlay et al., 1987; Powers et al., 1997). Also, an importin β mutant that cannot be dislodged from certain NPC sites (Görlich et al., 1996a) is a dominant negative inhibitor of all tested forms of active nucleocytoplasmic transport (Kutay et al., 1997). An extensive family of proteins that resemble importin β has recently been identified (Fornerod et al., 1997; Görlich et al., 1997) and proposed to consist of transport factors based on two arguments. First, the similarity of the family members is greatest near their N termini. This region of importin β is required for Ran binding, and several family members bind Ran in vitro (Görlich et al., 1997). Second, one of the family, human CRM1, interacts directly with the nucleoporin CAN/Nup214 (Fornerod et al., 1996, 1997).

CRM1 was first identified in the fission yeast, Schizosaccharomyces pombe, where its mutation causes abnormal chromosome morphology (Adachi and Yanagida, 1989). S. pombe CRM1 is the target of a cytotoxin, leptomycin B, and mutations in CRM1 can render S. pombe resistant to its effects (Hamamoto et al., 1983; Nishi et al., 1994). In mammalian cells, leptomycin B blocks both HIV-1 Rev-dependent pre-mRNA export from the nucleus and the actinomycin D–dependent relocation of Rev protein from the nucleus to the cytoplasm (Wolff et al., 1997), suggesting that either the viral Rev protein itself or a cellular factor required for Rev export from the nucleus might be a target for leptomycin B.

Here we show that leptomycin B inhibits the nuclear export of both Rev and U snRNAs, suggesting that the cellular target of leptomycin B is involved in their export. An in vitro interaction between leptomycin B and both human and S. pombe CRM1 is demonstrated. Moreover, expression of human CRM1 in Xenopus oocytes is shown to increase the rate of Rev and U snRNA nuclear export and to reverse leptomycin B–induced inhibition of Rev export. In vitro, CRM1 binds in a cooperative way to both RanGTP and either Rev or PKI NES peptides. Thus, CRM1 is an export receptor for leucine-rich NESs.

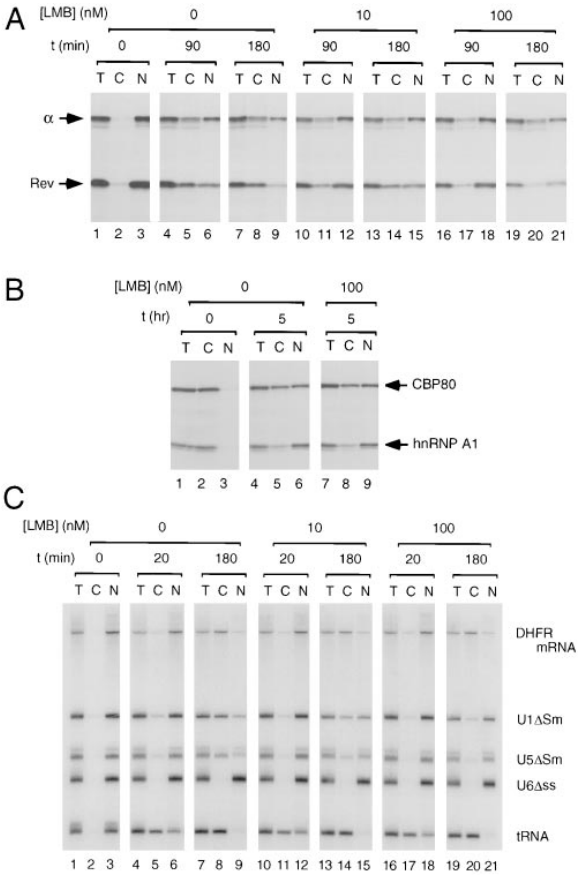

Figure 1. Leptomycin B Specifically Inhibits Rev Protein and U snRNA Export in Xenopus Oocytes

(A) Xenopus laevis oocytes were incubated for 2 hr in the absence (lanes 1–9) or presence (10 nM, lanes 10–15; 100 nM, lanes 16–21) of leptomycin B. Subsequently ^{35}S-labeled HIV-1 Rev and importin α (α) proteins were injected into the nucleus and incubation was continued in the same media. Protein samples from total oocytes (T) or from cytoplasmic (C) or nuclear (N) fractions were collected immediately (lanes 1–3), 90 min (lanes 4–6, 10–12, and 16–18) or 180 min (lanes 7–9, 13–15 and 19–21) after injection and analyzed by SDS-PAGE.

(B) Oocytes were incubated in the absence (lanes 1–6) or presence of leptomycin B (100 nM; lanes 7–9) as in A. ^{35}S-labeled CBP80 and hnRNP A1 proteins were then injected into the cytoplasm. Protein samples were collected immediately (lanes 1–3) or 5 hr (lanes 4–9) after injection.

(C) Xenopus oocytes were incubated with leptomycin B as in (A), then injected into the nucleus with a mixture of ^{32}P-labeled RNAs containing: DHFR mRNA, U1ΔSm, U5ΔSm, U6Δss, and human initiator methionyl tRNA. RNA was extracted immediately (lanes 1–3), 20 min (lanes 4–6, 10–12, and 16–18), or 180 min (lanes 7–9, 13–15, and 19–21) after injection.

Results

Leptomycin B Is a Specific Inhibitor of Rev and U snRNA Export

To determine whether leptomycin B would affect the import or export of cellular factors, the transport of various substrates was analyzed in Xenopus laevis oocytes. The kinetics of export of ^{35}S-labeled Rev protein after its microinjection into the nucleus is shown in Figure 1A (lanes 1–9), where T designates extract of total oocytes

and C and N cytoplasmic and nuclear fractions. Incubation of the oocytes in 10 nM leptomycin B reduced Rev export (lanes 10–15) and 100 nM leptomycin B essentially blocked export (lanes 16–21). Neither the import of an NLS-containing protein (CBP80; Izaurralde et al., 1995a) nor that of the M9-containing hnRNP A1 (Figure 1B, compare lanes 4–6 with lanes 7–9) was detectably affected by leptomycin B at 100 nM. Similarly, leptomycin B had no detectable effect on the export from the nucleus of either importin α or transportin (Figures 1A and 4).

The effect of leptomycin B on the nuclear export of RNA substrates was also tested. A mixture of RNAs including dihydrofolate reductase mRNA, two U snRNAs, U1ΔSm and U5ΔSm, initiator methionyl tRNA, and U6 Δss RNA, was microinjected into oocyte nuclei. U6 RNA remains in the nucleus as a marker of injection accuracy and nuclear integrity. Because the RNAs are exported with very different kinetics (Jarmolowski et al., 1994), their export was examined after 20 and 180 min. Control oocytes (Figure 1C, lanes 1–9) showed rapid export of tRNA and slower export of the mRNA and U snRNAs. Incubation of the oocytes in either 10 or 100 nM leptomycin B did not affect mRNA or tRNA export detectably (Figure 1C, lanes 10–21) but reduced (10 nM) or blocked (100 nM) U1 and U5 snRNA export. These data are reminiscent of the block of export of Rev and U snRNAs by saturating levels of NES peptides (Fischer et al., 1995) and strongly suggest that the cellular target of leptomycin B is involved in both Rev and U snRNA export.

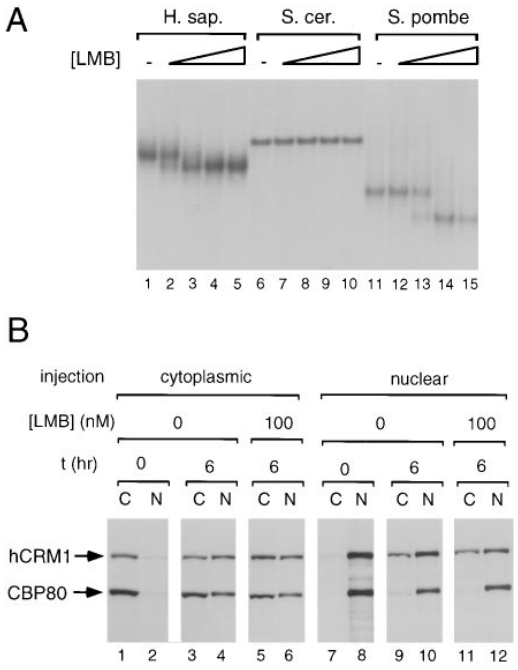

Figure 2. Effect of Leptomycin B on CRM1 Proteins

(A) ^{35}S-labeled human (H. sap.), S. cerevisiae (S. cer.), and S. pombe CRM1 proteins were incubated alone (lanes 1, 6, and 11) or with 50, 100, 200, or 400 nM leptomycin B for 45 min at 30°C, then analyzed by nondenaturing polyacrylamide gel electrophoresis.

(B) Xenopus oocytes were incubated with leptomycin B, then a mixture of ^{35}S-labeled hCRM1 and CBP80 proteins was injected into the cytoplasm (lanes 1–6) or the nucleus (lanes 7–12). Protein was extracted from cytoplasmic (C) and nuclear (N) fractions immediately (lanes 1, 2, 7, and 8) or 6 hr (lanes 3–6 and 9–12) after injection.

Leptomycin B Interacts with CRM1

Since CRM1 is the leptomycin B target in S. pombe (see Introduction), interaction of leptomycin B with ^{35}S-labeled CRM1 protein from human, S. pombe, and Saccharomyces cerevisiae was assayed. The three CRM1 proteins were separated on native polyacrylamide gels either alone (Figure 2A, lanes 1, 6, and 11) or in the presence of leptomycin B. Leptomycin B did not affect the mobility of the S. cerevisiae protein (lanes 7–10), but both the human and S. pombe proteins have a higher mobility in the presence of leptomycin B, suggesting that both proteins change conformation on binding leptomycin B. Direct binding between CRM1 and leptomycin B was confirmed by repeating the experiment using recombinant human CRM1 protein made in an E. coli extract with the identical result (data not shown; see Figure 5C). The apparent lack of binding to the S. cerevisiae CRM1 protein may explain why this yeast species is insensitive to leptomycin B (Hamamoto et al., 1983).

Since leptomycin B affects nuclear export, and since CRM1 distantly resembles importin β, we wished to test whether CRM1 protein, like importin β, would move between the nucleus and cytoplasm and whether leptomycin B would affect its transport. ^{35}S-labeled human CRM1 protein was injected into either the cytoplasm (Figure 2B, lanes 1–6) or the nucleus (lanes 7–12) of Xenopus oocytes, together with CBP80 as an internal control. CRM1 was imported into the nucleus from the cytoplasm (Figure 2B, lanes 1–4). Unlike CBP80, CRM1 was also detected in the cytoplasm after injection into the nucleus (Figure 2B, lanes 7–10). The distribution of CRM1 seen in lanes 3, 4, 9, and 10 therefore represents the steady state of its shuttling between the nucleus and cytoplasm. Leptomycin B had no detectable effect on export of CRM1 (Figure 2B, lanes 11 and 12) and at most a minor effect on its import (lanes 5 and 6).

hCRM1 Expression in Oocytes Increases Rev and U snRNA Export

The effects of leptomycin B on nuclear export and its binding to CRM1 suggest that CRM1 might have a role in Rev and U snRNA export. This was analyzed by (over)-expressing human CRM1 (hCRM1) in Xenopus oocytes and by measuring the rate of export of these substrates. hCRM1 mRNA was microinjected into the cytoplasm of Xenopus oocytes. One day later, expressed hCRM1 protein was readily detected by Western blot using anti-hCRM1 antibodies (Fornerod et al., 1997), although we cannot accurately estimate the level of overexpression since we do not know whether the CRM1 antibody recognizes the endogenous Xenopus protein and human CRM1 identically.

The mixture of RNA export substrates was injected into the nucleus at this time (Figure 3A, lanes 1–3). RNA export was assessed 150 min later in oocytes preinjected into the cytoplasm with hCRM1 mRNA. This had no effect on mRNA or tRNA export, but the export of both

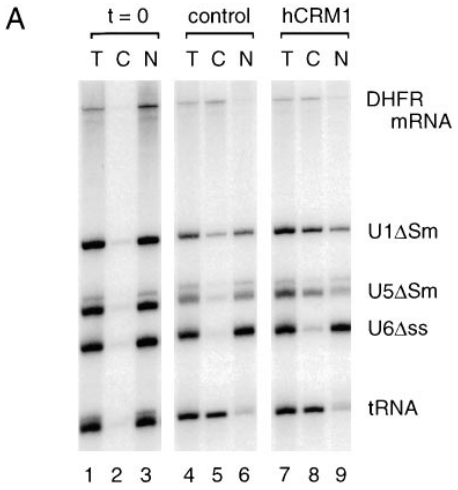

Figure 3. hCRM1 Specifically Accelerates U snRNA Export in Xenopus Oocytes

(A) Unlabeled DHFR mRNA (lanes 1–6) or hCRM1 mRNA (lanes 7–9) was injected into the cytoplasm of Xenopus oocytes. After 24 hr, a mixture of ^{32}P-labeled RNAs was injected into the nucleus. RNA was extracted immediately (lanes 1–3) or 150 min (lanes 4–9) after injection.

(B) The percentage of U1 and U5 snRNA in the cytoplasmic (C) and nuclear (N) fractions of control oocytes or hCRM1 mRNA-injected oocytes was determined by phosphoimager analysis. Control oocytes were preinjected with H_2O. The error bars represent the maximum and minimum values obtained from two separate experiments, both independent of that shown in (A).

U1 and U5 snRNAs was increased in hCRM1-injected oocytes (Figure 3A, lanes 4–9). In control oocytes, roughly two-thirds of the two U snRNAs was still nuclear, while in oocytes injected with hCRM1 mRNA the nuclear fraction was approximately one-third. The result of two additional independent experiments is summarized in Figure 3B. Preinjection of hCRM1 mRNA reproducibly stimulated the rate of export of both U1 and U5 snRNAs. Preinjection of mRNAs encoding either importin β or transportin had no effect on RNA export (data not shown).

An analogous experiment with Rev was carried out. The incubation time (65 min) was chosen such that only 60% of the microinjected Rev protein was exported (Figure 4, lanes 1–6). Rev export was not affected by control cytoplasmic preinjection (Figure 4, lanes 4–6) but was stimulated to 95% by the preinjection of hCRM1 mRNA (lanes 7–9). At this time point, Rev export was significantly inhibited when control oocytes were incubated

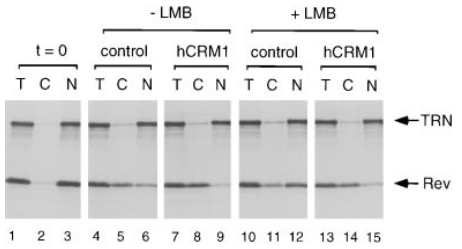

Figure 4. hCRM1 Accelerates Rev Export and Reverses the Inhibitory Effect of Leptomycin B

Either water (lanes 1–6 and 10–12) or hCRM1 mRNA (lanes 7–9 and 13–15) was injected into the cytoplasm of Xenopus oocytes. After 23 hr the oocytes were placed in control medium (lanes 1–9) or leptomycin B (40 nM, lanes 10–15). After 2 hr ^{35}S-labeled Rev and transportin (TRN) proteins were injected into the nucleus. Proteins were extracted immediately (lanes 1–3) or 65 min (lanes 4–15) after injection.

in 40 nM leptomycin B (Figure 4, lanes 10–12). This inhibitory effect was completely reversed in the oocytes that had been preinjected with hCRM1 mRNA (lanes 13–15). Transportin was coinjected with Rev as an internal control. Its export was not affected by any of the treatments (Figure 4). Thus, increasing the concentration of CRM1 in Xenopus oocytes by supplementing the endogenous protein with exogenously expressed human CRM1 stimulates the rate of export of both U snRNAs and Rev protein, and the inhibitory effect of leptomycin B on Rev export was reversed by hCRM1. This is direct evidence of a role for CRM1 in U snRNA and Rev export.

Cooperative Interactions Involving hCRM1, RanGTP, and NES Peptides

The above data stongly implicate CRM1 in NES protein export. If CRM1 is the "NES receptor" it should interact with NES peptides directly, perhaps (see Introduction) in a RanGTP-dependent way. To investigate binding of Ran and NES peptides to CRM1, we made use of a RanQ69L IgG-binding domain fusion protein (the gift of D. Görlich). RanQ69L is incapable of GTP hydrolysis (Bischoff et al., 1994; Klebe et al., 1995). This protein was immobilized on IgG-Sepharose beads. ^{35}S-labeled CRM1 protein made in reticulocyte lysate was incubated with the beads, but, unlike importin β (Figure 5A, lanes 15 and 16), did not bind immobilized RanQ69LGTP and remained in the supernatant (Figure 5A, lanes 1 and 2). In the presence of increasing concentrations of wild-type Rev NES peptide (CLPPLERLTR; Malim et al., 1989, 1991; Fischer et al., 1995), however, a greater and greater fraction of CRM1 bound to the RanQ69L column (Figure 5A, lanes 3–10). M10 mutant NES peptide (CLPPDLRLTR; Malim et al., 1989) did not allow efficient binding of CRM1 to RanGTP (Figure 5B, lanes 1–6). A second protein with an NES is the protein kinase A inhibitor PKI (Wen et al., 1995). We therefore tested whether a PKI NES peptide (CELALKLAGLDIN) would also promote complex assembly on RanGTP. The wild-type PKI peptide caused CRM1 binding (Figure 5B, lanes 7 and 8), while three inactive mutant versions that change two (P10) or one (P12, P13) amino acid in the

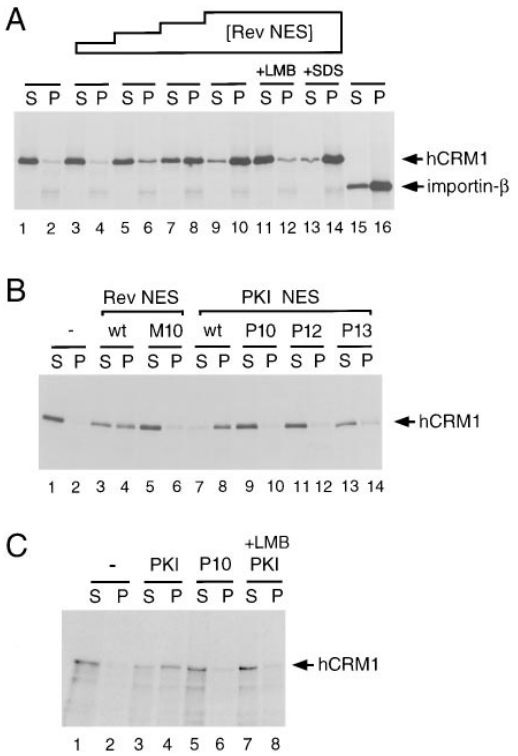

Figure 5. Interaction of hCRM1 with Immobilized RanQ69LGTP in the Presence of NES Peptide

(A) ^{35}S-labeled rabbit reticulocyte-derived CRM1 (lanes 1–14) or importin-β (lanes 15–16) was incubated with RanQ69LGTP beads in the absence (lanes 1–2 and 15–16) or presence of Rev NES peptide at 1 μM (lanes 3–4), 10 μM (lanes 5–6), 100 μM (lanes 7–8) or 1 mM (lanes 9–10). Separate reactions with the highest concentration NES peptide were supplemented with 400 nM leptomycin B (lanes 11–12) or SDS (lanes 13–14). Samples were subsequently fractionated into supernatant (S) and bound pellet (P) fractions, and analyzed by SDS-PAGE.

(B) A comparison of wild-type (wt) and mutant versions of either the Rev NES peptide (lanes 3–6) or the PKI NES peptide (lanes 7–14) on the interaction of hCRM1 with RanQ69LGTP. Reactions were performed as in A and contained no peptide (lanes 1 and 2) or 100 μM of Rev NES peptide (lanes 3 and 4), M10 mutant Rev NES peptide (lanes 5 and 6), PKI NES peptide (lanes 7 and 8), and three inactive mutants thereof (P10, P12, and P13, respectively, lanes 9–14).

(C) E. coli lysate-derived ^{35}S-labeled hCRM1 was incubated with RanQ69LGTP beads in the absence (lanes 1–2) or presence of 200 μM PKI NES (lanes 3–4 and 7–8) or PKI P10 mutant (lanes 5–6) peptide, and in the absence or presence (lanes 7–8) of 400 nM leptomycin B (LMB), and analyzed as in (A).

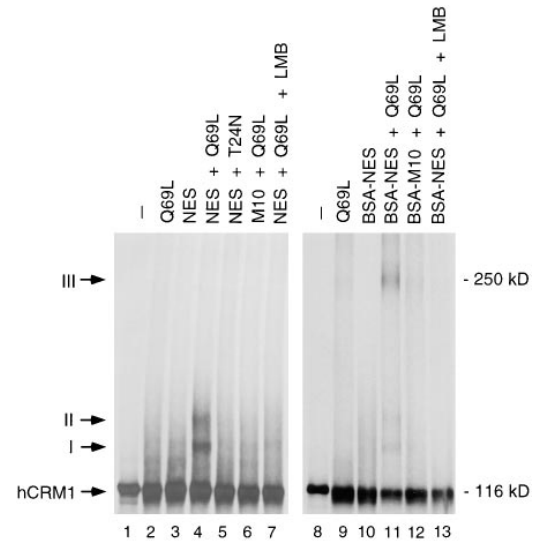

Figure 6. Complexes Containing CRM1

^{35}S-labeled hCRM1 protein was incubated as indicated above the lanes in the presence or absence of 200 μM NES peptide (NES), M10 mutant peptide (M10), 1 μg of BSA-NES or BSA-M10 conjugate, 1.6 μM RanQ69LGTP (Q69L), RanT24NGDP (T24N), and/or 400 nM leptomycin B (LMB). Proteins were subsequently cross-linked with 0.02% glutaraldehyde (lanes 2–7 and 9–13). Positions of hCRM1 and complexes I, II, and III as well as of molecular weight markers are indicated.

peptide (Wen et al., 1995) did not (Figure 5B, lanes 9–14). Finally, leptomycin B inhibited CRM1-RanQ69L interaction even in the presence of the highest concentration of NES peptide (Figure 5A, lanes 11 and 12). Leptomycin B is a lipophilic molecule with several polar groups, somewhat resembling a weak detergent. However, no inhibitory effect on CRM1 binding was seen when the same concentration of SDS or of Triton X-100 was used (Figure 5A, lanes 13 and 14, and data not shown). These binding studies showed that CRM1 binds to RanGTP in a wild-type NES–dependent way but used CRM1 protein made in reticulocyte lysate, making it possible that other components of the lysate were required for complex formation. To rule this out, we made hCRM1 by translation in an E. coli–derived lysate. Again, binding of hCRM1 to RanQ69L was seen only in the presence of wild-type NES peptide (Figure 5C, lanes 1–6) and was abolished by leptomycin B (lanes 7 and 8).

To further establish that the NES is part of the RanGTP-CRM1 complex, a cross-linking approach was used. CRM1 produced in reticulocyte lysate was employed because the many incomplete translation products in the bacterially produced protein (Figure 5C) gave rise to a smear of products in cross-linking experiments. Glutaraldehyde treatment of CRM1 protein in the presence of RanQ69L or Rev NES peptide did not affect its mobility (Figure 6, lanes 1–3). However, two new complexes (I and II) were seen when both RanQ69LGTP and Rev NES peptide were added simultaneously (lane 4). These complexes contain Ran, as they can be affinity-selected on NTA-nickel resin when Histidine-tagged Ran is used (data not shown), but may also contain other components (see Discussion). Their formation depends on wild-type NES peptide (lane 6), is inhibited by leptomycin B (lane 7), and does not occur when RanT24NGDP (Klebe et al., 1995), that cannot stably form the GTP-bound state, is substituted for RanQ69LGTP (lane 5). Thus, like the RanGTP-CRM1-NES peptide interactions described in Figure 5, formation of the cross-linked complexes involves cooperative binding.

The small size of the NES peptides in relation to complexes I and II, and the unlikelihood that such small targets would be efficiently cross-linked to CRM1, prevents the definitive conclusion that the NES is part of complexes I and II. We therefore repeated the cross-linking experiment making use of BSA-NES or BSA-M10

peptide conjugates, whose apparent size is 70–80 kDa (data not shown). In the simultaneous presence of the wild-type NES conjugate and of RanQ69LGTP, we now see a new complex whose apparent molecular weight is 70 kDa larger than complex II (complex III, Figure 6, lanes 8–11). Formation of complex III is only seen with the wild-type NES conjugate (lanes 11 and 12) and is inhibited by leptomycin B (lane 13). If larger BSA conjugates containing more peptides are used in a cross-linking experiment, the corresponding complex III is also larger (data not shown). In conclusion, the results in this section show that CRM1 binds directly to both NES peptide and to RanGTP in a cooperative way.

Discussion

The data presented suggest that CRM1 is a nuclear export receptor for both U snRNAs and proteins like Rev that carry a leucine-rich NES. First, leptomycin B, a cytotoxin that we show interacts with human or S. pombe CRM1, inhibits both Rev and U snRNA export. Second, expression of human CRM1 in Xenopus oocytes leads to an increase in the rate of Rev and U snRNA export and can reverse the export inhibition induced by leptomycin B. Third, CRM1 forms a complex in vitro that contains RanGTP and the NES and is dependent on both of these for its formation. Complex formation is inhibited by leptomycin B.

Earlier work had revealed that CRM1 is distantly related to nuclear protein import receptors like importin β and transportin (Fornerod et al., 1997; Görlich et al., 1997), and that, similar to those molecules, it is located in part at nuclear pore complexes (NPCs) and in part in soluble form, in the case of CRM1 in the nucleoplasm (Adachi and Yanagida, 1989; Fornerod et al., 1997). At least one nucleoporin with which CRM1 interacts has been identified, CAN/Nup214, and this association appears to be a dynamic one (Fornerod et al., 1996, 1997). The role of this interaction in export will be an interesting area of future study.

Association of CRM1 and Export Substrate

CRM1 forms a complex that is shown by a combination of cross-linking and affinity purification to include both the NES-containing substrate and RanGTP. The cross-linked complexes (I and II), formed in the presence of a 1.2 kDa Rev NES peptide and whose apparent molecular weight would thus be little affected by the substrate, are 28 and 52 kDa larger than CRM1. Since Ran has an apparent molecular weight close to 28 kDa and is also shown to bind CRM1, the smaller complex is likely to contain CRM1 and Ran. Its formation also requires NES peptide, which is likely to be an integral component as shown by the fact that NES peptides are readily cross-linked to the complex when conjugated to BSA.

It is less certain what, in addition to Ran and CRM1, gives rise to complex II. Reasonable possibilities are that it is complex I plus either a second Ran molecule or Ran BP1 from the reticulocyte lysate. Although Ran usually behaves as a monomer, it was shown to interact with itself in a two-hybrid assay (Dingwall et al., 1995). RanBP1 is a candidate as importin β, which interacts with Ran through a domain similar to that found in CRM1

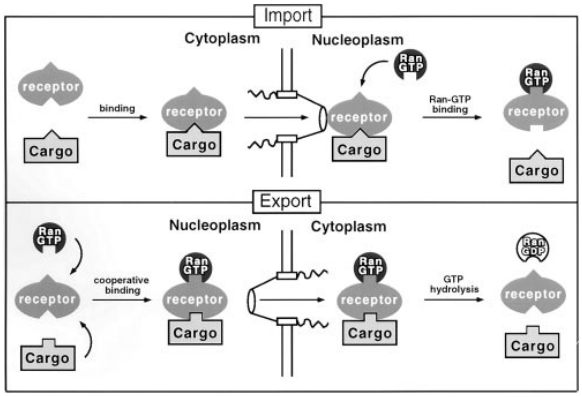

Figure 7. A Model for Directional Nucleocytoplasmic Transport

Import or export of receptor-cargo complexes through nuclear pore complexes (center of each panel) is shown schematically. Although we show both receptors and cargos as single entities, as exemplified by the import complex consisting of transportin and hnRNP A1, both the receptor and the cargo can be di- or oligomeric. RanGTP binding to an import receptor through the conserved Ran binding domain dissociates the cargo-receptor complex in the nucleus. Conversely, RanGTP binding to the same domain of export receptors (exportins) enables cargo-receptor interaction in the nucleus. Note that it is not yet known whether an intact RanGTP-receptor-cargo export complex would ever leave the NPC intact. It is possible that Ran and/or cargo dissociation could occur on the NPC.

and can form a stable complex that contains both Ran and Ran-bound RanBP1 (Chi et al., 1996; Görlich et al., 1996a; Kutay et al., 1997; Lounsbury and Macara, 1997). Further work is required to characterize complex II.

Leptomycin B inhibits the formation of the complex that contains the NES, RanGTP and CRM1. Since both ligands are required to stably form this complex, their binding to CRM1 must involve cooperativity. There is no evidence that the NES can directly interact with Ran. We therefore consider it likely that RanGTP and the NES peptide bind to distinct sites on CRM1 and cause a conformational change that stabilizes the complex (as illustrated schematically in Figure 7). Leptomycin B could therefore act in several ways to prevent complex formation. It could inhibit either RanGTP binding, inhibit NES binding, or prevent the change in conformation necessary to stabilize the CRM1-export cargo complex.

CRM1 also acts in U snRNA export. This process is inhibited by injection of saturating levels of NES peptide conjugate (Fischer et al., 1995), suggesting the existence of a mediator of U snRNA export that has a Rev-like NES. The only U snRNA-associated proteins known to be involved in export from the nucleus are CBP80 and CBP20, the components of the nuclear cap-binding complex (CBC; Izaurralde et al., 1994, 1995b). At present, it is unclear whether CBC might form a complex directly with CRM1 or whether other proteins are involved that form bridging interactions between CBC and CRM1. It is possible that the interacting protein in this case could be identified using the RanQ69LGTP-binding assay (Figure 5). Indeed, this could potentially be a general assay for active NES sequences.

CRM1 or Rip/Rab or Both as the NES Receptor

Previous studies, mainly using the two-hybrid assay, have suggested that the Rev NES binds specifically to

the yeast Rip protein or to the related human Rip/Rab protein (Bogerd et al., 1995; Fritz et al., 1995; Stutz et al., 1995). These studies also provided evidence of a role for Rip/Rab in Rev-mediated pre-mRNA export. The two-hybrid interactions with Rip/Rab are specific for proteins containing an active NES, and a good correlation exists between the activity of a protein in export and its strength of interaction with either yeast or human Rip in this assay (Bogerd et al., 1995; Fritz et al., 1995; Stutz et al., 1995). One group has also reported interaction between a GST-Rev fusion protein and human Rab produced in reticulocyte lysate (Bogerd et al., 1995).

The Rip proteins resemble a family of nucleoporins that contain irregular repeats ending in FG dipeptides (Doye and Hurt, 1997). Several of these proteins, including the vertebrate CAN/Nup214, contain FG repeats that produce positive results with the Rev NES in the two-hybrid interaction assay (Fritz and Green, 1996; Stutz et al., 1996). The functional relevance of interaction with these repeats is supported by the observation that microinjection of some repeat-containing nucleoporin fragments into Xenopus oocyte nuclei inhibits nuclear export of Rev and U snRNAs (Stutz et al., 1996), presumably by titrating a factor required for their export. This factor could be CRM1 (see below).

Of particular interest here is the fact that FG repeats from CAN/Nup214 were among those that showed positive interaction with the Rev NES in the two-hybrid assay. A significant fraction of hCRM1 exists in a complex with the nucleoporin CAN/Nup214, and this interaction involves the FG repeat region (Fornerod et al., 1996). It seems possible that human Rip/Rab is a nucleoporin, and there is good evidence that yeast Rip is one (Fritz et al., 1995; Stutz et al., 1995; F. Stutz and M. Rosbash, personal communication). One possibility is therefore that the two-hybrid interaction seen, and conceivably also the interaction observed between Rab and Rev in reticulocyte lysate, may have been indirect and mediated by CRM1.

If we model the function of CRM1 on what is known about importin β and transportin, it is likely that CRM1 translocates through the NPC bound to the NES. This might preclude direct NES-nucleoporin interaction, but it is conceivable that CRM1 hands off the NES to one or more FG repeat-containing nucleoporins, for example, at the end of the translocation process, and that both the interaction with CRM1 and with the nucleoporin are direct. It is also possible that Rip/Rab and CRM1 would cooperatively interact with the NES. Our attempts to detect an influence of the FG repeats on formation of the CRM1/NES-containing complex have, however, been unsuccessful thus far.

The Ran Asymmetry and Models for Directional Transport

In order to see NES- and CRM1-containing complexes, it was necessary to add RanGTP, which itself was incorporated into the complex. Conversely, RanGTP binding to CRM1 was detected only in the presence of NES peptides. This complex therefore assembles cooperatively (Figure 7, bottom left). RanGTP-dependent assembly is likely to have general significance for nuclear export, based on the following arguments.

As described in the introduction, the components of the Ran system (RanGAP1, RanBP1, and RCC1) are organized within the cell in a way that predicts the existence of a steep RanGTP concentration gradient across the nuclear pore complex. The significance of this distribution relates to the effect of RanGTP on complexes between transport cargo and either import or export receptors, as summarized schematically in Figure 7.

Import receptors, like importin β and transportin, are separated from their cargos by interaction with RanGTP (Rexach and Blobel, 1995; Chi et al., 1996; Görlich et al., 1996a; E. Izaurralde et al., submitted). As noted previously (Görlich and Mattaj, 1996; Görlich et al., 1996a, 1996b), interaction between RanGTP and importin β occurring in the nucleus, but not in the cytoplasm, would provide asymmetry across the nuclear envelope in import complex formation and dissociation (Figure 7) and thus impart directionality to the import process. In the case of transportin, the cargo is an M9-containing protein, whereas here we define the importin β cargo as the importin α-NLS protein complex. Note that this is not the only function of RanGTP in import. RanGTP binding also dissociates importin β from the NPC and in vitro from individual nucleoporins (Rexach and Blobel, 1995; Görlich et al., 1996a; Weis et al., 1996). In addition, GTP hydrolysis by Ran is required for NLS protein import (Melchior et al., 1993; Moore and Blobel, 1993; Görlich et al., 1996a; Palacios et al., 1996; Weis et al., 1996).

Ignoring these latter additional functions of RanGTP, two possible roles for the Ran asymmetry in export could have been proposed. In the first, RanGDP in the cytoplasm might have bound to an export receptor and caused cargo release. In the second (Figure 7, bottom), RanGTP would again be the active form of the GTPase but in this case would act to enable complex formation between export cargo and an export receptor in the nucleus. The complex would be dissociated by cytoplasmic GTP hydrolysis. Our data strongly suggest that CRM1-mediated NES protein export reflects aspects of this latter model, with complex formation occurring only in the presence of cargo and RanGTP. The model presented in Figure 7 may be generally applicable to nuclear export processes that require Ran and involve cargo. In support of this, reducing the RanGTP concentration in the nucleus has recently been shown to block export of both NES proteins (Richards et al., 1997) and of multiple other export substrates (E. Izaurralde et al., submitted). A variant of this model is represented by the recycling of import receptors like importin β (Görlich et al., 1997; Kutay et al., 1997). In this case, RanGTP binding to the receptor would form an export complex and would not require the simultaneous presence of export cargo.

CRM1 is a member of a family of proteins related to importin β (Fornerod et al., 1997; Görlich et al., 1997) of which several had been shown to be mediators of nuclear protein import (see Introduction). CRM1 in contrast appears to have a specific role in nuclear export, and a second family member has been identified as the mediator of recycling (reexport) of importin α from the nucleus (Kutay et al., [this issue of *Cell*]). As the names of these family members are mainly descriptive reflections of the phenotypes they caused when first studied, a systematic nomenclature might be appropriate, and we propose the name "exportins" for those involved in nuclear

export. In the future, we will therefore refer to CRM1 as exportin 1.

Experimental Procedures

In Vitro Transcription and Translation

[^{35}S] methionine-labeled proteins were synthesized in rabbit reticulocyte lysate or E. coli S30 extract for linear templates (Promega) according to the manufacturer's instructions, except that for E. coli in vitro translation, the reaction was supplemented with 20 U T7 RNA polymerase and performed at 30°C. Reaction mixtures were diluted 20× in PBS/8.7% glycerol and reconcentrated in centrifugal concentrators (Nanosep, Pall Filtron Corporation). Templates for in vitro transcription/translation were as follows: T7-hCRM1 (Fornerod et al., 1997), pRSET-Rev (Stutz et al., 1996), T7-importin β (Görlich et al., 1997), T7-transportin (E. Izaurralde et al., submitted), pRSET-importin α (Weis et al., 1996), pRSET-hnRNPA1 (Izaurralde et al., 1997), and pT7–7TT-CBP80 (Izaurralde et al., 1995b). S. cerevisiae and S. pombe CRM1 proteins and hCRM1 produced in E. coli S30 extract were synthesised from PCR-amplified DNA using as templates pHY51 (Toda et al., 1992), pYA284 (Adachi and Yanagida, 1989), and T7-hCRM1, respectively. Forward primers contained T7 promoter and Kozak (Cestari et al., 1993) or Shine and Delgarno consensus sequences. PCR was for 30 cycles using *Pfu* DNA polymerase (Stratagene). Amplified DNAs were phenol-extracted and 2-propanol–precipitated before use.

Cold mRNA for oocyte injection was prepared according to Jarmolowski et al. (1994), using plasmids T7-hCRM1 and T7-DHFR (Kambach and Mattaj, 1992; Jarmolowski et al., 1994), except that 3 mM m^{7}GpppG (New England BioLabs) was used.

Oocyte Injection

Microinjection of RNAs and proteins into oocytes, incubations, extractions, and analysis were as described (Kambach and Mattaj, 1992; Jarmolowski et al., 1994). Cytoplasmic injection of DHFR or hCRM1 mRNA used 200 ng.

Gel Mobility Shift Assay

In vitro–translated CRM1 protein (0.5 μl) was incubated for 45 min at 30°C with leptomycin B in a buffer containing 20 mM HEPES-KOH (pH 7.9), 60 mM KCl, 3 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 5% glycerol (G buffer) in a volume of 5 μl. Samples were analyzed on a 6% polyacrylamide gel in 0.5× TBE (8 V/cm for 16 hr at 4°C) and subjected to fluorography.

Cross-Linking

hCRM1 (0.1 μl in 5 μl) was incubated for 30 min in G buffer with or without 1.6 μM RanQ69L (Palacios et al., 1996) or RanT24N (E. Izaurralde et al., submitted), 200 μM wild-type or M10 mutant NES peptides (Fischer et al., 1995), 1 μg BSA-NES or BSA-M10 conjugate (Fischer et al., 1995), equivalent to approximately 20 μM free peptide, or 400 nM leptomycin B. This buffer was supplemented with 5 μM GTP with RanQ69L and with 5 μM GDP for RanT24N. Cross-linking was subsequently carried out by addition of 0.02% glutaraldehyde and incubation for 30 min at 0°C. Proteins were analyzed by 6% SDS-PAGE and fluorography.

Pull-Down Assay

Approximately 500 pmol of bacterially expressed z-tagged RanQ69L, a fusion between the IgG-binding z domain of Staphylococcus aureus protein A and RanQ69L (a gift of D. Görlich), was prebound to 10 μl of IgG sepharose 6 FF (Pharmacia) by incubation of the affinity matrix with 35 μl of postribosomal supernatant of the expressing E. coli strain in 60 μl 50 mM HEPES-KOH (pH 7.9), 200 mM NaCl, 5 mM $MgCl_2$, and 5 μM GTP (B buffer) for 1 hr at 20°C. Beads were washed twice with B buffer and incubated for 2 hr with 0.3 μl (reticulocyte lysate) or 2 μl (S30 extract) of in vitro–translated hCRM1 in the presence or absence of peptides representing the wild-type Rev NES, Rev NES M10 mutant, wild-type PKI-NES, or PKI-NES P10, P12, and P13 mutant peptides (Fischer et al., 1995; Wen et al., 1995) in 50 μl B buffer. In some reactions 400 nM leptomycin B or sodium dodecyl sulphate (SDS) was added. After binding, supernatants were collected, and beads were washed once with 100 μl B buffer containing the same supplements as during binding. Equal fractions of supernatants and beads were analyzed by 6% SDS-PAGE and fluorography.

Acknowledgments

We wish to thank Bertrand Séraphin and the members of our laboratory for critical comments on the manuscript, and I. Palacios, P. Fortes, Dirk Görlich, and Elisa Izaurralde for numerous useful discussions. We particularly acknowledge Ludwig Englmeier for stimulating the initiation and progress of this study. Drs. T. Toda, Y. Adachi, M. Yanagida, G. Grosveld, and G.-J. Arts are thanked for gifts of materials. In particular, we acknowledge Dirk Görlich for the gift of the RanQ69L fusion protein before its publication and for his generosity with other reagents. The Deutsche Forschungsgemeinschaft (RNA Biochimie Schwerpunktprogramm) and EMBO supported M. O. and M. F., respectively.

Received July 3, 1997; revised August 8, 1997.

References

Adachi, Y., and Yanagida, M. (1989). Higher order chromosome structure is affected by cold-sensitive mutations in a Schizosaccharomyces pombe gene CRM1+, which encodes a 115-kD protein preferentially localized in the nucleus and its periphery. J. Cell Biol. *108*, 1195–1207.

Aitchison, J.D., Blobel, G., and Rout, M.P. (1996). Kap104: a karyopherin involved in the nuclear transport of messenger RNA–binding proteins. Science *274*, 624–627.

Bischoff, F.R., Klebe, C., Kretschmer, J., Wittinghofer, A., and Ponstingl, H. (1994). RanGAP1 induces GTPase activity of nuclear ras-related Ran. Proc. Natl. Acad. Sci. USA *91*, 2587–2591.

Bogerd, H.P., Fridell, R.A., Madore, S., and Cullen, B.R. (1995). Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. Cell *82*, 485–494.

Cestari, I.N., Albuquerque, E.X., and Burt, D.R. (1993). A PCR shortcut to oocyte expression. BioTechniques *14*, 404–407.

Chi, N.C., Adam, E.J.H., Visser, G.D., and Adam, S.A. (1996). RanBP1 stabilizes the interaction of Ran with p97 in nuclear protein import. J. Cell Biol. *135*, 559–569.

Dingwall, C., and Laskey, R.A. (1991). Nuclear targeting sequences—a consensus? Trends Biochem. Sci. *16*, 478–481.

Dingwall, C., Kandels-Lewis, S., and Séraphin, B. (1995). A family of Ran-binding proteins that includes nucleoporins. Proc. Natl. Acad. Sci. USA *92*, 7525–7529.

Doye, V., and Hurt, E. (1997). From nucleoporins to nuclear pore complexes. Curr. Opin. Cell Biol. *9*, 401–411.

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

Fischer, U., Huber, J., Boelens, W.C., Mattaj, I.W., and Lührmann, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell *82*, 475–483.

Fornerod, M., Boer, J., van Baal, S., Morreau, H., and Grosveld, G. (1996). Interaction of cellular proteins with the leukemia specific fusion proteins DEK-CAN and SET-CAN and their normal counterpart, the nucleoporin CAN. Oncogene *13*, 1801–1808.

Fornerod, M., van Deursen, J., van Baal, S., Reynolds, A., Davis, D., Murti, K.G., Fransen, J., and Grosveld, G. (1997). The human homolog of yeast Crm1 is in a dynamic subcomplex with Can/Nup214 and a novel nuclear pore component Nup 88. EMBO J. *16*, 807–816.

Fridell, R.A., Truant, R., Thorne, L., Benson, R.E., and Cullen, B.R. (1997). Nuclear import of hnRNP A1 is mediated by a novel cellular cofactor related to karyopherin-β. J. Cell Sci. *110*.

Fritz, C.C., and Green, M.R. (1996). HIV Rev uses a conserved cellular protein export pathway for the nucleocytoplasmic transport of viral RNAs. Curr. Biol. *6*, 848–854.

Fritz, C.C., Zapp, M.L., and Green, M.R. (1995). A human nucleoporin-like protein that specifically interacts with HIV Rev. Nature *376*, 530–533.

Goldfarb, D.S., Gariepy, J., Schoolnik, G., and Kornberg, R.D. (1986). Synthetic peptides as nuclear localization signals. Nature *322*, 641–644.

Görlich, D., and Mattaj, I.W. (1996). Nucleocytoplasmic transport. Science *271*, 1513–1518.

Görlich, D., Panté, N., Kutay, U., Aebi, U., and Bischoff, F.R. (1996a). Identification of different roles for RanGDP and RanGTP in nuclear protein import. EMBO J. *15*, 5584–5594.

Görlich, D., Kraft, R., Kostka, S., Vogel, F., Hartmann, E., Laskey, R.A., Mattaj, I.W., and Izaurralde, E. (1996b). Importin provides a link between nuclear protein import and U snRNA export. Cell *87*, 21–32.

Görlich, D., Dabrowski, M., Bischoff, F.R., Kutay, U., Bork, P., Hartmann, E., Prehn, S., and Izaurralde, E. (1997). A novel class of RanGTP-binding proteins. J. Cell Biol. *138*, 65–80.

Hamamoto, T., Gunji, S., Tsuji, H., and Beppu, T. (1983). Leptomycins A and B, new antifungal antibiotics I. Taxonomy of the producing strain and their fermentation, purification, and characterization. J. Antibiot. *36*, 639–645.

Hamm, J., and Mattaj, I.W. (1990). Monomethylated cap structures facilitate RNA export from the nucleus. Cell *63*, 109–118.

Izaurralde, E., Lewis, J., McGuigan, C., Jankowska, M., Darzynkiewicz, E., and Mattaj, I.W. (1994). A nuclear cap-binding protein complex involved in pre-mRNA splicing. Cell *78*, 657–668.

Izaurralde, E., McGuigan, C., and Mattaj, I.W. (1995a). Nuclear localization of a cap-binding protein complex. Cold Spring Harb. Symp. Quant. Biol., *60*, 669–675.

Izaurralde, E., Lewis, J., Gamberi, C., Jarmolowski, A., McGuigan, C., and Mattaj, I.W. (1995b). A cap-binding protein complex mediating U snRNA export. Nature *376*, 709–712.

Izaurralde, E., Jarmolowski, A., Beisel, C., Mattaj, I.W., Dreyfuss, G., and Fischer, U. (1997). A role for the M9 transport signal of hnRNP A1 in mRNA nuclear export. J. Cell Biol. *137*, 27–35.

Jarmolowski, A., Boelens, W.C., Izaurralde, E., and Mattaj, I.W. (1994). Nuclear export of different classes of RNA is mediated by specific factors. J. Cell Biol. *124*, 627–635.

Kambach, C., and Mattaj, I.W. (1992). Intracellular distribution of the U1A protein depends on active transport and nuclear binding to U1 snRNA. J. Cell Biol. *118*, 11–21.

Klebe, C., Bischoff, F.R., Ponstingl, H., and Wittinghofer, A. (1995). Interaction of the nuclear GTP-binding protein Ran with its regulatory proteins RCC1 and RanGAP1. Biochemistry *34*, 639–647.

Koepp, D.M., and Silver, P.A. (1996). A GTPase controlling nuclear trafficking: running the right way or walking randomly? Cell *87*, 1–4.

Kutay, U., Bischoff, F.R., Kostka, S., Kraft, R., and Görlich, D. (1997). Export of importinα from the nucleus is mediated by a specific nuclear transport factor. Cell *90*, this issue, 1061–1071.

Kutay, U., Izaurralde, E., Bischoff, F.R., Mattaj, I.W., and Görlich, D. (1997). Dominant-negative mutants of importin-β block multiple pathways of import and export through the nuclear pore complex. EMBO J. *16*, 1153–1163.

Lounsbury, K.M., and Macara, I.G. (1997). Ran-binding protein 1 (RanBP1) forms a ternary complex with Ran and Karyopherin β and reduces GTPase-activating protein (RanGAP) inhibition by karyopherin β. J. Biol. Chem. *272*, 551–555.

Malim, M.H., Böhnlein, S., Hauber, J., and Cullen, B.R. (1989). Functional dissection of the HIV-1 Rev transactivator: derivation of a transdominant repressor of Rev function. Cell *58*, 205–214.

Malim, M.H., McCarn, D.F., Tiley, L.S., and Cullen, B.R. (1991). Mutational definition of the human immunodeficiency virus type 1 Rev activation domain. J. Virol. *65*, 4248–4254.

Mandell, R.B., and Feldherr, C.M. (1990). Identification of two HSP70-related Xenopus oocyte proteins that are capable of recycling across the nuclear envelope. J. Cell Biol. *111*, 1775–1783.

Melchior, F., Paschal, B., Evans, E., and Gerace, L. (1993). Inhibition of nuclear protein import by nonhydrolyzable analogs of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. J. Cell Biol. *123*, 1649–1659.

Michael, W.M., Choi, M., and Dreyfuss, G. (1995). A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. Cell *83*, 415–422.

Michael, W.M., Eder, P.S., and Dreyfuss, G. (1997). The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein. EMBO J. *12*, 3587–3598.

Moore, M.S., and Blobel, G. (1993). The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature *365*, 661–663.

Moore, M.S., and Blobel, G. (1994). A G protein involved in nucleocytoplasmic transport: the role of Ran. Trends Biochem. Sci. *19*, 211–216.

Nakielny, S., Siomi, M.C., Siomi, H., Michael, W.M., Pollard, V., and Dreyfuss, G. (1996). Transportin: nuclear transport receptor of a novel nuclear protein import pathway. Exp. Cell. Res. *229*, 261–266.

Nigg, E.A. (1997). Nucleocytoplasmic transport: signals, mechanism and regulation. Nature *386*, 779–787.

Nishi, K., Yoshida, M., Fujiwara, D., Nishikawa, M., Horinouchi, S., and Beppu, T. (1994). Leptomycin B targets a regulatory cascade of CRM1, a fission yeast nuclear protein, involved in control of higher order chromosome structure and gene expression. J. Biol. Chem. *269*, 6320–6324.

Palacios, I., Weis, K., Klebe, C., Mattaj, I.W., and Dingwall, C. (1996). Ran/TC4 mutants identify a common requirement for snRNP and protein import into the nucleus. J. Cell Biol. *133*, 485–494.

Pokrywka, N.J., and Goldfarb, D.S. (1995). Nuclear export pathways of tRNA and 40S ribosomes include both common and specific intermediates. J. Biol. Chem. *270*, 3619–3624.

Pollard, V.W., Michael, W.M., Nakielny, S., Siomi, M.C., Wang, P., and Dreyfuss, G. (1996). A novel receptor-mediated nuclear protein import pathway. Cell *88*, 985–994.

Powers, M.A., and Forbes, D.J. (1994). Cytosolic factors in nuclear transport: what's importin? Cell *79*, 931–934.

Powers, M.A., Forbes, D.J., Dahlberg, J.E., and Lund E. (1997). The vertebrate GLFG nucleoporin, Nup98, is an essential component of multiple RNA export pathways. J. Cell Biol. *136*, 241–250.

Rexach, M., and Blobel, G. (1995). Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. Cell *83*, 683–692.

Richards, S.A., Carey, K.L., and Macara I.G. (1997). Requirement of guanosine triphosphate-bound Ran for signal-mediated nuclear protein export. Science *276*, 1842–1844.

Rout, M.P., Blobel, G., and Aitchison, J.D. (1997). A distinct nuclear import pathway used by ribosomal proteins. Cell *89*, 715–725.

Siomi, H., and Dreyfuss, G. (1995). A nuclear localization domain in the hnRNP A1 protein. J. Cell Biol. *129*, 551–560.

Stutz, F., Neville, M., and Rosbash, M. (1995). Identification of a novel nuclear pore-associated protein as a functional target of the HIV-1 Rev protein in yeast. Cell *82*, 495–506.

Stutz, F., Izaurralde, E., Mattaj, I.W., and Rosbash, M. (1996). A role for nucleoporin FG repeat domains in export of human immunodeficiency virus type 1 Rev protein and RNA from the nucleus. Mol. Cell Biol. *16*, 7144–7150.

Sweet, D.J., and Gerace, L. (1996). A GTPase distinct from Ran is involved in nuclear protein import. J. Cell Biol. *133*, 971–983.

Toda, T., Shimanuki, M., Saka, Y., Yamano, H., Adachi, Y., Shirakawa, M., Kyogoku, Y., and Yanagida, M. (1992). Fission yeast pap1-dependent transcription is negatively regulated by an essential nuclear protein, CRM1. Mol. Cell Biol. *12*, 5474–5484.

Weighardt, F., Biamonti, G., and Riva, S. (1995). Nucleo-cytoplasmic distribution of human hnRNP proteins: a search for the targeting domains of hnRNP A1. J. Cell Sci. *108*, 545–555.

Weis, K., Dingwall, C., and Lamond, A.I. (1996). Characterization of the nuclear protein import mechanism using Ran mutants with altered nucleotide binding specificities. EMBO J. *15*, 7120–7128.

Wen, W., Meinkoth, J.L., Tsien, R.Y., and Taylor, S.S. (1995). Identification of a signal for rapid export of proteins from the nucleus. Cell *82*, 463–473.

Wolff, B., Sanglier, J.-J., and Wang, Y. (1997). Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. Chem. Biol. *4*, 139–147.

Zasloff, M. (1983). tRNA transport from the nucleus in a eukaryotic cell: carrier-mediated translocation process. Proc. Natl. Acad. Sci. USA *80*, 6436–6440.

Notes Added in Proof

The observation referred to as F. Stutz and M. Rosbash, personal communication, is now in press: Stutz, F., Kantor, J., Zhang, D., McCarthy, T., and Rosbash, M. (1997). The yeast nucleoporin Rip1p contributes to multiple export pathways with no essential role for its FG-repeat region. Genes Dev.

The data and observations referred to throughout as E. Izaurralde et al., submitted, is now in press: Izaurralde, E., Kutay, U., von Kobbe, C., Mattaj, I.W., Görlich, D. (1997). The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. EMBO J.