Cofactor Requirements for Nuclear Export of Rev Response Element (RRE)– and Constitutive Transport Element (CTE)–containing Retroviral RNAs: An Unexpected Role for Actin

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Abstract. Nuclear export of proteins containing leucine-rich nuclear export signals (NESs) is mediated by the export receptor CRM1/exportin1. However, additional protein factors interacting with leucine-rich NESs have been described. Here, we investigate human immunodeficiency virus type 1 (HIV-1) Rev-mediated nuclear export and Mason-Pfizer monkey virus (MPMV) constitutive transport element (CTE)–mediated nuclear export in microinjected *Xenopus laevis* oocytes. We show that eukaryotic initiation factor 5A (eIF-5A) is essential for Rev and Rev-mediated viral RNA export, but not for nuclear export of CTE RNA. In vitro binding studies demonstrate that eIF-5A is required for efficient interaction of Rev–NES with CRM1/exportin1 and that eIF-5A interacts with the nucleoporins CAN/nup214, nup153, nup98, and nup62. Quite unexpectedly, nuclear actin was also identified as an eIF-5A binding protein. We show that actin is associated with the nucleoplasmic filaments of nuclear pore complexes and is critically involved in export processes. Finally, actin- and energy-dependent nuclear export of HIV-1 Rev is reconstituted by using a novel in vitro egg extract system. In summary, our data provide evidence that actin plays an important functional role in nuclear export not only of retroviral RNAs but also of host proteins such as protein kinase inhibitor (PKI).

Key words: eIF-5A • CRM1 • nuclear actin • nuclear export • HIV-1 Rev

Introduction

In eukaryotic cells, chromatin is enclosed by a double-layered membrane system, the nuclear envelope, which separates the nuclear genome and its transcriptional apparatus from the site of protein synthesis. Thus, a constant flow of macromolecules has to cross the nuclear membranes in both directions. This transport into and out of the nucleus is mediated by nuclear pore complexes (NPCs),[1] which are elaborate proteinaceous assemblies inserted into the nuclear envelope. Their overall structural features, in particular their striking eightfold rotational symmetry, are remarkably similar in all eukaryotic cells, and several constituent proteins, termed nucleoporins, have been characterized at the molecular level (for review see Stoffler et al., 1999). Extensive peripheral structures in the form of fine filaments extend from the structural core of the NPC into the cytoplasm and nuclear interior, respectively. The nucleoplasmic NPC-attached filaments form basket-like assemblies or long cylindrial arrays, which are especially clearly seen in amphibian oocytes (Franke and Scheer, 1974; Jarnik and Aebi, 1991; Stoffler et al., 1999). The filaments projecting away from the cytoplasmic face of the NPCs are much shorter. Both filamentous NPC-attached appendages are thought to provide docking sites and guiding structures for transport cargoes before their translocation through the central pore channel.

Competition experiments in *Xenopus laevis* oocytes suggested that different classes of RNA (e.g., mRNA, rRNA, U snRNA, tRNA) are exported from the nucleus by spe-

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[1]*Abbreviations used in this paper:* CTE, constitutive transport element; eIF-5A, eukaryotic initiation factor 5A; F-actin, actin filaments; FG, phenylalanine–glycine; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HIV-1, human immunodeficiency virus type 1; MPMV, Mason-Pfizer monkey virus; NES, nuclear export signals; NPC, nuclear pore complex; PKI, protein kinase inhibitor; RRE, Rev response element; wt, wild-type.

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cific export factors (Jarmolowski et al., 1994). In general, RNA export seems to be a multistep mechanism that is mediated by RNA-binding proteins that contain nuclear export signals (NESs) (for reviews see Izaurralde and Adam, 1998; Mattaj and Englmeier, 1998; Stutz and Rosbash, 1998; Görlich and Kutay, 1999; Nakielny and Dreyfuss, 1999). Typically, NESs are recognized by soluble export receptors that target the transport complexes to the NPCs. In addition, efficient export requires the action of several critical factors, of which the GTPase Ran/TC4 and associated components play a major functional role in determining the direction of nucleocytoplasmic transport (for reviews see Görlich and Kutay, 1999; Melchior and Gerace, 1998; Moore, 1998).

To date, the most extensively investigated specific mRNA export factor is the Rev trans-activator protein of human immunodeficiency virus type 1 (HIV-1) (for review see Pollard and Malim, 1998). Rev is a nucleocytoplasmic shuttle protein that directly binds to its Rev-response element (RRE) RNA target sequence, which is part of all unspliced and incompletely spliced viral mRNAs. Upon multimerization and subsequent interaction with multiple cellular cofactors, Rev promotes the translocation of these mRNAs across the nuclear envelope. The region of Rev that interacts with cellular cofactors that are required for nuclear export of Rev–RRE ribonucleoprotein particles has been defined as either the activation or effector domain. This domain contains a short stretch of hydrophobic, primarily leucine amino acids and constitutes a prototypic NES that, upon fusion to heterologous protein substrates, mediates the rapid and active nuclear export of these otherwise inert proteins (Fischer et al., 1995; Stauber et al., 1995; Wen et al., 1995; Meyer et al., 1996; Elfgang et al., 1999).

A series of studies has shown that the primary target of leucine-rich Rev-like NESs is the export receptor CRM1/exportin1 and, furthermore, that NES-CRM1/exportin1 interaction depends on the presence of RanGTP (Fornerod et al., 1997a; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997; Askjaer et al., 1998). Studies with leptomycin B, a specific inhibitor of CRM1/exportin1 (Kudo et al., 1998, 1999) that prevents the formation of stable NES-CRM1/exportin1 complexes, demonstrated that CRM1/exportin1 indeed mediates the translocation of all Rev-like NES-containing export cargoes through the NPC (Fornerod et al., 1997a; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Wolff et al., 1997; Engel et al., 1998; Freedman and Levine, 1998; Kudo et al., 1998; Toyoshima et al., 1998; Wada et al., 1998; Stommel et al., 1999). However, investigation of HIV-1 Rev function also led to the identification of another cellular protein, eukaryotic initiation factor 5A (eIF-5A), that binds to the Rev–NES (Ruhl et al., 1993). eIF-5A is unique because it is the only cellular protein known to date to contain the unusual amino acid hypusine (Park et al., 1993). The hypusine modification, which does occur in eukaryotes and archaebacteria but not in eubacteria, is a spermidine-dependent posttranslational modification that is essential for eIF-5A function, although its exact function is yet unknown. Distinct eIF-5A mutants have been described that block Rev activity and thereby HIV-1 replication in human T cells in trans (Bevec et al., 1996; Junker et al., 1996). Moreover, microinjection experiments in somatic cells have demonstrated that eIF-5A is an essential cofactor specifically required for the nuclear export of HIV-1 Rev and the functionally equivalent Rex protein of human T cell leukemia virus type I (HTLV-I) (Elfgang et al., 1999). Finally, studies using *Xenopus* oocytes have revealed that eIF-5A accumulates at NPC-associated intranuclear filaments, interacts with the export receptor CRM1/exportin1, and is by itself exported from the nucleus to the cytoplasm in a leptomycin B–sensitive manner (Rosorius et al., 1999b).

In this study, we investigated the cofactor requirements for nuclear export of the prototypic HIV-1 Rev RNA export factor in *Xenopus* oocytes. Microinjection experiments demonstrate that nuclear export of glutathione *S*-transferase (GST)–Rev–NES fusion proteins, as well as Rev-mediated RRE RNA export, depends in oocytes, as it does in mammalian cells, on the Rev cofactor eIF-5A. Furthermore, binding studies using recombinant GST–eIF-5A and nuclear envelope proteins from oocytes demonstrate that eIF-5A interacts specifically with nucleoporins that are known to participate in nuclear export. In the course of these experiments, we noted that eIF-5A interacts not only with bona fide nucleoporins but also with nuclear actin that was localized at the nucleoplasmic face of NPCs, particularly at the NPC-associated intranuclear filaments. We present evidence that nuclear actin is functionally involved in nuclear export processes. Finally, by using *Xenopus* egg extract, we establish an energy-dependent in vitro nuclear export system and demonstrate that actin also participates in Rev-NES–mediated nuclear export in this cell-free system.

Materials and Methods

Biological Materials

Xenopus laevis were purchased from Nasco. Salamanders of the species *Pleurodeles waltl* were provided by Peter Eichhorn (German Cancer Research Center, Heidelberg, Germany). Pieces of *Xenopus* or *Pleurodeles* ovary were removed from anesthetized animals and placed in modified Barth medium (Gurdon, 1991). For studies on the nuclear export of the protein kinase inhibitor (PKI)–derived NES, *Xenopus* were stimulated by injection of human chorionic gonadotropine hormone 2 d before removal of pieces of ovary. Nuclei, nuclear envelopes, and cytoplasmic portions were manually obtained from full-grown oocytes as described (Krohne and Franke, 1983). Vero cells were maintained in minimum essential medium (MEM), and HeLa cells constitutively expressing HIV-Rev protein (Wolff et al., 1995) were maintained in DME containing 5 or 10% FCS, respectively, at 37°C in a 5% CO_2 incubator.

Antibodies

Antibodies against the following antigens were used: eIF-5A and Rev (rabbit antibodies; Hammerschmid et al., 1994); CRM1 (rabbit serum; Kudo et al., 1997); actin (mAb 2G2; Gonsior et al., 1999); phenylalanine–glycine (FG) repeat nucleoporins (mAb 414; Hiss Diagnostics; Davis and Blobel, 1986). mAbs specific for GST and BSA or His were obtained from Sigma-Aldrich or QIAGEN, respectively. The peroxidase or gold-conjugated secondary antibodies were from Dianova.

Recombinant Proteins

GST–Rev, GST–Rev–NES, GST–PKI–NES, wild-type (wt) GST–eIF-5A, and GST–eIF-5A–M14 fusion proteins expressed in *Escherichia coli* BL21 as well as wt eIF-5A and eIF-5A–M14 were previously described (Bevec et al., 1996; Elfgang et al., 1999). GST–Rev–NES–green fluorescent protein (GFP) and GST–RanQ69L was expressed in *E. coli* BL21 and purified as described (Rosorius et al., 1999a). His-tagged CRM1 was expressed in *E. coli* TG1 and affinity purified from crude lysates using Ni beads (QIAGEN) as essentially described (Guan et al., 2000), with the exception that a further purification step was added to this protocol by using size S200 exclusion chromatography (Amersham Pharmacia Biotech).

Molecular Clones and In Vitro Synthesis of Radiolabeled RNA

To generate the expression vector pGEX–RanQ69L, the Ran coding region was amplified by polymerase chain reaction from pET3dRanQ69L (Klebe et al., 1993), using appropriate oligonucleotides, and inserted into the BamHI and EcoRI sites of pGEX–3X (Amersham Pharmacia Biotech). A sequence encoding the NES of the HIV–Rev protein (LQLPPLERLTL) was cloned between the BamHI and NheI site of the bacterial expression vector pGEX–GFP (Rosorius et al., 1999a) by using double-stranded synthetic oligonucleotides, resulting in the plasmid pGEX–Rev–NES–GFP.

The plasmid template pGEM–RRE, which is a derivative of the vector pGEM–3Zf(+) (Promega), was used for the synthesis of a 252-nucleotide wt RRE probe and has been described in detail previously (Malim et al., 1990). The variant vector pGEM–RREΔSLII was used to synthesize a nuclear export–deficient RRE probe in which stem loop II (ΔSLII, lacking nucleotides 41–105, as shown in Malim et al. [1990]), the high affinity Rev binding site within the RRE, is deleted. The template and the method used for the synthesis of the wt constitutive transport element (CTE) and the double mutant M2/M11 CTE RNA has been described (Ernst et al., 1997). U6 RNA was synthesized using the vector SP6-U6 (Jarmolowski et al., 1994). wt and mutant HIV-1 RRE RNA and wt and mutant Mason-Pfizer monkey virus (MPMV) CTE RNA were radiolabeled by in vitro transcription using [α-^{32}P]UTP and T7 RNA polymerase (Promega) as described previously (Daly et al., 1993a). Likewise, U6 RNA was synthesized using SP6 RNA polymerase (Promega).

Microinjection Experiments

Just before injection, the defolliculated *Xenopus* oocytes were placed with the animal hemisphere pointing up in a miniplate containing modified Barth's medium and were centrifuged at 1,200 *g* for 15 min at 22°C in order to visualize the site of the nucleus. For nuclear export analysis, 20 nl export substrates was injected into the nuclei of 20 full-grown *Xenopus* oocytes that had been injected 1 h before with either purified antibodies against eIF-5A (IgG; 0.5 mg/ml), purified antibodies against actin (IgM; 1 mg/ml), nonspecific rabbit IgG (0.5 mg/ml), nonspecific mouse IgM (1 mg/ml), the actin-binding drugs latrunculin B (3 mM; Calbiochem), or swinholide A (3 mM; Alexis). Injected export substrates were GST–Rev–NES or GST–PKI–NES (0.1 mg/ml), RRE RNA (0.6 μM wt or mutant RRE RNA, 0.8–1.0 $\times$ 10^6 cpm, incubated 30 min on ice with Rev recombinant protein as described by Fischer et al. [1995]) and CTE RNA (wt or mutant ^{32}P-labeled CTE RNA, 10^7 cpm). In some experiments, wt eIF-5A or eIF-5A–M14 proteins (using a 10-fold molar excess over the coinjected GST–Rev–NES or GST–Rev fusion proteins) were preincubated for 1 h at 4°C before microinjection into the oocyte nuclei. BSA (1 mg/ml; Sigma-Aldrich) or ^{32}P-labeled U6 RNA were coinjected as internal markers for monitoring the site of injection. For the microinjection experiments in Vero cells, GFP–Rev–NES–GST fusion protein (1 mg/ml) was coinjected into the nuclei with actin antibodies (IgM, 1 mg/ml) or with nonspecific mouse IgM (1 mg/ml).

Preparation and Analysis of Injected Samples

After an incubation time of 2 h, nuclei and cytoplasms from *Xenopus* oocytes were manually isolated, and the samples prepared for protein gel electrophoreseis as described (Dabauvalle and Franke, 1982). The proteins were separated by SDS-PAGE (Thomas and Kornberg, 1975), using 18% acrylamide. For immunoblots, polypeptides were transferred electrophoretically to nitrocellulose filters. Nonspecific protein binding sites were blocked by overnight incubation with TBST (10% nonfat dry milk in 150 mM NaCl, 0.05% Tween-20, 10 mM Tris-HCl, pH 8.0) at 4°C. After several washes in TBST, the nitrocellulose filters were incubated for 2 h at room temperature with GST antibodies (diluted 1:1,000 in TBST). After washing again in TBST, the nitrocellulose filters were then incubated for 1 h at room temperature with the appropriate secondary antibodies coupled to peroxidase (diluted 1:10,000 in TBST containing 10% dry milk). After several further washes in TBST, the nitrocellulose filters were incubated for 2 h at room temperature with BSA antibodies (diluted 1:500 in TBST). The nitrocellulose filters were then washed several times in TBST and incubated for 1 h at room temperature with appropriate secondary antibodies coupled to peroxidase (diluted 1:10,000 in TBST containing 10% dry milk). Bound antibodies were visualized using the enhanced chemical luminescence detection system (ECL).

For RNA analysis, nuclei and cytoplasms were manually isolated as described (Dabauvalle and Franke, 1982). RNA from each fraction was extracted separately by using peqGOLD TriFast™ according to the manufacturer's protocol (Peqlab). Labeled RNA molecules were separated by electrophoresis on denaturating polyacrylamide gels (6% acrylamide/bisacrylamide 29:1; 8 M urea) and visualized by autoradiography.

Overlay Blot Analyses and Pull-Down Experiments

Proteins obtained from 100 manually isolated nuclear envelopes were separated by SDS-PAGE (Thomas and Kornberg, 1975), using 12% acrylamide, and processed for overlay blot assays as described previously (Lounsbury et al., 1994), using GST–eIF-5A. Bound proteins were visualized by incubation with anti-GST antibodies (diluted 1:1,000). Then, the nitrocellulose filters were stripped of bound antibodies and reprobed as described in the manufacturer's protocol (Amersham Pharmacia Biotech) with mAb 414 (diluted 1:1,000), antiactin antibodies (diluted 1: 1,000), or anti-CRM1 antiserum (diluted 1:250).

Pull-down experiments were performed as described previously (Yang et al., 1998), by using GST, wt GST–eIF-5A, GST–eIF-5A–M14, or GST–Rev–NES fusion proteins (1 μg/μl) and 150 manually isolated oocyte nuclei or total oocyte extract (corresponding to 150 oocytes). In another set of experiments, GST or GST–Rev–NES fusion protein (1 μg/μl) coupled to glutathione–Sepharose beads was incubated with a 10-fold molar excess of wt eIF-5A or eIF-5A–M14 protein before incubation with oocyte extract or with His-tagged CRM1 fusion protein (0.5 μg/μl). For the pull-down experiments with His-tagged CRM1, the glutathione–Sepharose beads were saturated by using 5% BSA, and the binding was performed with or without addition of the GTPase-deficient RanQ69L mutant. The bound and unbound proteins were separated by 12% SDS-PAGE, followed by silver staining (Switzer et al., 1979) or by immunoblotting using anti-CRM1 antiserum (diluted 1:250), anti-His antibodies (diluted 1:1,000), anti–eIF-5A antibodies, mAB 414, or antiactin antibodies, diluted as described above.

Mass Spectrometry Analysis and Peptide Sequencing

Proteins from manually isolated oocyte nuclear envelopes were separated by electrophoresis through a 12% SDS-PAGE gel and stained with Coomassie brilliant blue, and the 45-kD band was excised. Tryptic digestion of the protein sample within the polyacrylamide matrix was performed as previously described (Eckerskorn and Lottspeich, 1989). After extensive washing in water, the gel pieces were lyophylized and resoaked with buffer containing endoproteinase LysC (Boehringer). The resulting peptide fragments were eluted with formic acid/acetonitrile/water (5:50:45). Peptide mass fingerprinting on a Reflex III MALDI mass spectrometer (Bruker) identified the sample protein as actin. This was further confirmed by peptide sequencing after separation of the peptide mixture by reversed phase HPLC on a Microcart 150-1 Purospher C18 5-μm column (Merck). The eluents used in the HPLC were solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in acetonitrile). The gradient was developed in 0–60% solvent B for 60 min at a flow rate of 60 μl/min. Eluted peptides were detected at 206 nm, collected manually, and sequenced in an amino acid sequencer (PE Biosystems) according to the manufacturer's instructions. The sequence obtained ([D]SYVGDEAQSK) fitted the amino acid sequence of *X. laevis* actin isoforms, perfectly.

Immunogold Electron Microscopy

Manually isolated and unfixed nuclear envelopes were incubated with either the antiactin antibody 2G2 (diluted 1:200) alone or in combination with anti–eIF-5A antibodies (diluted 1:250), followed by incubation for 2 h with secondary antibodies coupled to 6- or 12-nm gold particles (diluted 1:10). Procedures used for preembedding immunoelectron microscopy of isolated nuclear envelopes from *Xenopus* and *Pleurodeles* oocytes have been described previously.

Nuclear Export In Vitro

Nuclei from HeLa cells constitutively expressing HIV-1 Rev protein (Wolff et al., 1995) were isolated as described (Krohne et al., 1981). Extract was prepared from activated *Xenopus* eggs using standard protocols, which include the use of cytochalasin B (Dabauvalle et al., 1991; Newmeyer and Wilson, 1991) or, alternatively, without this actin-binding drug. The nuclei were incubated in egg extract for 1 h with or without the addition of an ATP regenerating system (2 mM ATP, 20 mM phosphocreatine, 50 μg/ml creatine kinase). After incubation, the probes were centrifuged onto coverslips and prepared for immunofluorescence microscopy as described (Dabauvalle et al., 1991).

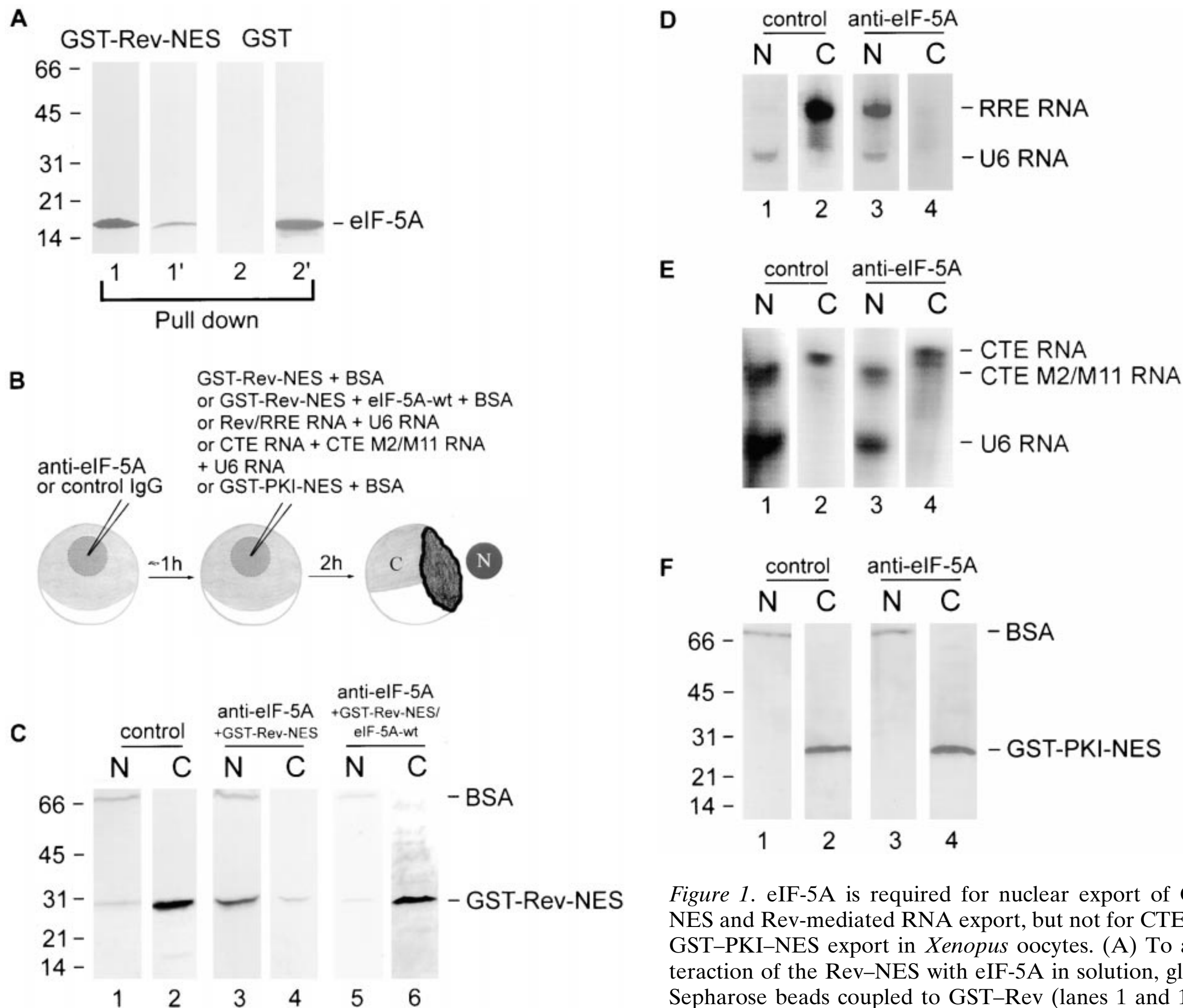

Figure 1. eIF-5A is required for nuclear export of GST–Rev–NES and Rev-mediated RNA export, but not for CTE RNA and GST–PKI–NES export in *Xenopus* oocytes. (A) To analyze interaction of the Rev–NES with eIF-5A in solution, glutathione–Sepharose beads coupled to GST–Rev (lanes 1 and 1′) or, as a control, GST (lanes 2 and 2′) were incubated with the extract of 150 *Xenopus* oocytes, pelleted, and then processed for immunoblotting with antibodies to eIF-5A. Bound proteins are shown in lanes 1 and 2, and the unbound material are shown in lanes 1′ and 2′. eIF-5A binds specifically to GST–Rev–NES (compare lanes 1 and 1′). Molecular mass standards are indicated in kD. (B) Oocyte injection protocol. After injection and incubation, oocytes were manually dissected in nuclear (N) and cytoplasmic (C) fractions. (C) Proteins were separated by 18% SDS-PAGE, and blots were probed with antibodies against GST and BSA. In the presence of control IgG, within 2 h, most GST–Rev–NES migrates from the nucleus to the cytoplasm (lanes 1 and 2). In contrast, in the presence of antibodies to eIF-5A, the nuclear export of GST–Rev–NES is largely inhibited (lanes 3 and 4). Coinjection of wt eIF-5A protein overcomes the blocking effect of anti–eIF-5A antibodies on GST–Rev–NES export (lanes 5 and 6). To monitor the site of injection, the export substrate was injected along with BSA, which is not exported. The exclusive presence of BSA in the nuclei confirms the specificity of the nuclear injections (lanes 1, 3, and 5). Molecular mass standards are indicated in kD. (D and E) RNA was analyzed by 8 M 6% acrylamide urea gel electrophoresis followed by autoradiography. (D) In the presence of Rev, the RRE RNA is exported (lane 2). In contrast, antibodies against eIF-5A inhibit this export (lane 4). (E) Antibodies against eIF-5A do not prevent the nuclear export of CTE RNA (lanes 2 and 4), indicating that eIF-5A is not involved in the CTE RNA export pathway. Nuclear injected CTE M2/M11 RNA mutant remains in the nuclei (lanes 1 and 3). The nuclear localization of U6-RNA confirms the accuracy of nuclear injections. (F) Nuclear export of the injected GST–PKI–NES fusion protein was analyzed as shown in B. The export of GST–PKI–NES was unaffected by the presence of anti–eIF-5A antibodies (lanes 2 and 4), confirming that eIF-5A is not involved in PKI-NES–mediated nuclear export.

Results

eIF-5A Is a Critical Factor Involved in Rev-dependent Nuclear Export in Xenopus Oocytes

Microinjection of export substrates into the nucleus of *Xenopus* oocytes and the subsequent monitoring of their nucleocytoplasmic distribution provides a powerful tool to investigate nuclear export. Here, we have used this technology to investigate the cofactor requirements for nuclear export of the HIV-1 Rev trans-activator protein. It has been shown that treatment of *Xenopus* oocytes with the drug leptomycin B, which directly binds to the general export receptor CRM1/exportin1 and prevents its interaction with leucine-rich NESs (Kudo et al., 1998, 1999), efficiently blocks nucleocytoplasmic translocation of Rev (Fornerod et al., 1997a). In contrast, however, eIF-5A, which is another cellular Rev cofactor, has so far not been investigated in detail with respect to Rev-mediated nuclear export in *Xenopus* oocytes. Some discrepancies exist

with respect to Rev function in oocytes. In particular, Rev function could not be detected in oocytes that were microinjected with expression plasmids encoding Rev and a Rev-responsive CAT reporter (Ruhl et al., 1993). In contrast, nucleocytoplasmic translocation of a radiolabeled RRE RNA probe, which was comicroinjected with high amounts of recombinant Rev protein, has been reported in other studies (Fischer et al., 1995; Stutz et al., 1996). Although the reason for this discrepancy is not known, the simplest explanation may be the significantly different sensitivities of the assay systems used (e.g., Rev trans-activation versus Rev-mediated nuclear RNA export; for a detailed discussion of Rev activities in oocytes also see Rosorius et al., 1999b).

The Rev–NES has been shown previously to interact with HeLa cell–derived and recombinant eIF-5A (Ruhl et al., 1993; Bevec et al., 1996). To verify the interaction of the Rev–NES with oocyte-derived eIF-5A, we first performed binding studies in solution. For this, purified GST–Rev–NES fusion protein was immobilized on glutathione–Sepharose beads and incubated with total protein extracts from *Xenopus* oocytes. The beads were then pelleted by centrifugation, and the bound and unbound material was analyzed by Western analysis using antibodies directed against eIF-5A (Elfgang et al., 1999). As shown, eIF-5A bound the GST–Rev–NES fusion protein (Fig. 1 A, compare lanes 1 and 1′) but not GST alone (Fig. 1 A, compare lanes 2 and 2′).

To characterize the role of eIF-5A in oocytes, we next tested anti–eIF-5A antibodies for their ability to interfere with Rev–NES-mediated nuclear export. For this, nonspecific immunoglobulin G (IgG) or anti–eIF-5A antibodies were microinjected into oocyte nuclei (Fig. 1 B). After 1 h of incubation, recombinant GST–Rev–NES export substrate, together with BSA (export-deficient injection control), was microinjected into the same nuclei. After another 2-h period of incubation, the injected oocytes were dissected into nuclear and cytoplasmic components. The proteins from each fraction were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-GST and anti-BSA antibodies. As expected and shown in Fig. 1 C, the BSA injection control was found exclusively in the nuclei (lanes 1, 3, and 5). In agreement with previous studies (Fornerod et al., 1997a; Ullman et al., 1999), nuclear injected GST–Rev–NES fusion protein was rapidly exported to the cytoplasm in presence of nonspecific IgG (Fig. 1 C, lanes 1 and 2). In sharp contrast, however, injection of anti–eIF-5A antibodies resulted in an effective block of the nuclear export of GST–Rev–NES fusion protein (Fig. 1 C, lanes 3 and 4). To demonstrate the specificity of the used anti–eIF-5A antibodies, we next tested the ability of wt eIF-5A to overcome this inhibitory effect. As shown (Fig. 1 C, lanes 5 and 6), comicroinjection of GST–Rev–NES together with wt eIF-5A protein clearly restored the nuclear export of the GST–Rev–NES fusion protein.

Next, we used the same experimental design to investigate Rev-mediated nuclear export of HIV-1–derived RNA (Fig. 1 B). For this, the RRE RNA target sequence was radiolabeled by in vitro transcription and microinjected together with recombinant Rev protein into oocyte nuclei. Coinjection of export-deficient radiolabeled U6 RNA served as the control for accurate nuclear injection in these experiments (Hamm and Mattaj, 1989). 2 h after microinjection of the RNA export substrate, the oocyte nuclear components and cytoplasmic components were separated, and localization of the various RNAs was analyzed by gel electrophoresis and autoradiography. In agreement with the data discussed above, efficient nuclear export of RRE RNA occurred in the presence of nonspecific IgG (Fig. 1 D, lanes 1 and 2), whereas its export was inhibited by anti–eIF-5A antibodies (Fig. 1 D, lanes 3 and 4). Notably, translocation of RRE RNA across the nuclear envelope was only observed in the presence of Rev transactivator protein (data not shown).

All retroviruses have to export unspliced intron-containing RNA from the nucleus to the cytoplasm. However, simple retroviruses such as the type D retrovirus MPMV do not encode Rev-like activities. In the RNA genome of these viruses is a cis-active structure, termed the CTE, which mediates efficient nuclear export of their unspliced RNAs (Bray et al., 1994; Ernst et al., 1997; Pasquinelli et al., 1997; Saavedra et al., 1997). CTE-mediated export depends exclusively on endogenous cellular factors. Indeed, the TAP protein, which is a human protein involved in mRNA transport, was recently found to bind to the CTE and stimulate nuclear export of CTE-containing viral RNAs (Grüter et al., 1998; Braun et al., 1999; Bachi et al., 2000). Importantly, the CTE-TAP–dependent nuclear RNA export pathway appears to be distinct from the specific RNA export pathway that is accessed by the HIV-1 Rev protein (Pasquinelli et al., 1997; Bogerd et al., 1998; Kang and Cullen, 1999; Zolotukhin and Felber, 1999). Thus, inhibition of Rev export should not negatively affect the translocation of CTE-containing RNA across the oocyte nuclear envelope.

To test this, we investigated the effect of anti–eIF-5A antibodies on nuclear export of the prototypic MPMV CTE (Ernst et al., 1997). As shown in Fig. 1 E, nuclear export of wt CTE RNA was easily detectable in the presence of nonspecific control IgG, whereas, in contrast, a coinjected export-deficient CTE deletion mutant (M2/M11; Pasquinelli et al., 1997) remained in the nuclear compartment of the injected oocytes (lanes 1 and 2). Coinjection of U6 RNA served as the injection control in these experiments. In the presence of anti–eIF-5A antibodies, nuclear export of CTE RNA continued (Fig. 1 E, lanes 3 and 4). Note that, under the same conditions, Rev-mediated RRE RNA export was completely blocked (Fig. 1 D, lanes 3 and 4). These results demonstrate that eIF-5A does not participate in the export of CTE RNA.

Previously, we have reported that, in somatic cells, eIF-5A is required for the nuclear export of the Rev–NES and Rex–NES, but not for the export of the PKI–NES (Elfgang et al., 1999). Therefore, we tested anti–eIF-5A antibodies for their ability to interfere with PKI-NES–mediated nuclear export in oocytes. To our initial surprise, the microinjected GST–PKI–NES fusion protein remained in the microinjected nuclei in absence of anti–eIF-5A antibodies, even after extended periods of incubation (data not shown). However, when we microinjected oocytes derived from hormone-induced animals, the GST–PKI–NES export cargo was rapidly exported from the nucleus to the cytoplasm (Fig. 1 F, lanes 1 and 2). These data already indicated that, in oocytes, significant differences exist with respect to the regulation of PKI-NES– versus Rev-NES–mediated nuclear export. Importantly, however, comicro-

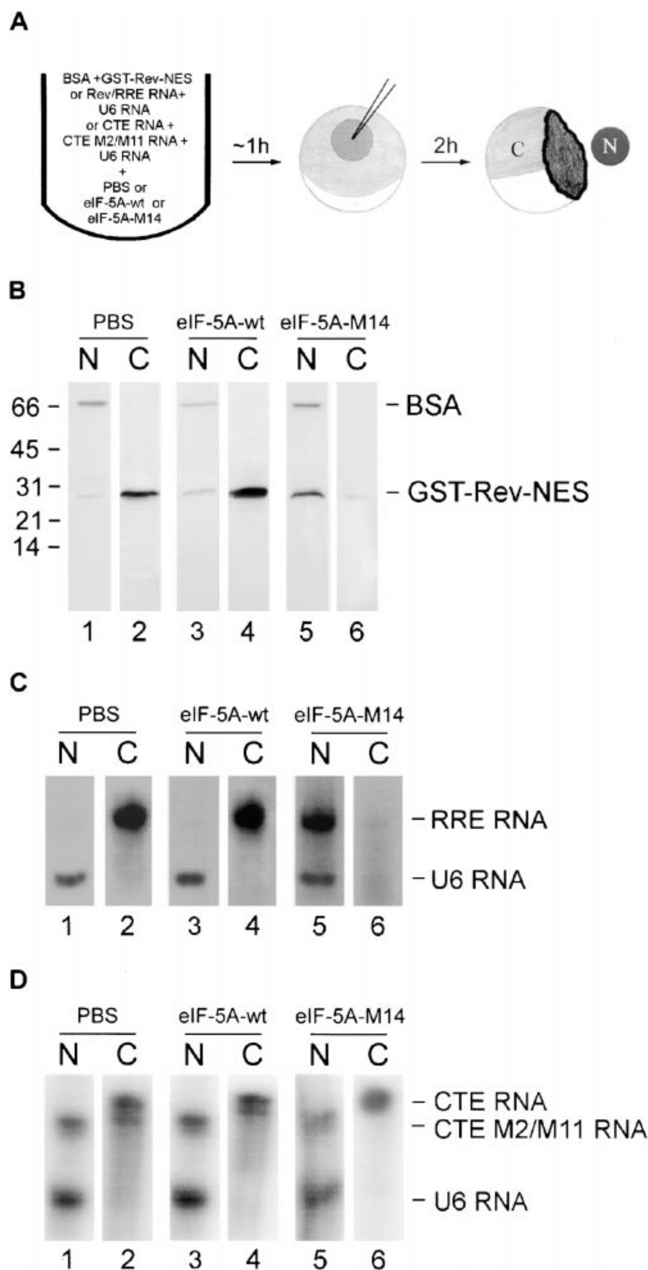

Figure 2. The eIF-5A mutant protein M14 inhibits the nuclear export of GST–Rev–NES and RRE RNA but not of CTE RNA. (A) Oocyte injection protocol. Before nuclear injection, the export substrate was incubated for 1 h with wt or mutant eIF-5A or, as a control, with PBS alone. (B) Nuclear (N) and cytoplasmic (C) proteins were separated by 18% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to GST and BSA. In the presence of PBS or wt eIF-5A protein, GST–Rev–NES migrates efficiently to the cytoplasm (lanes 2 and 4). In contrast, the export is blocked in the presence of the eIF-5A mutant M14 (lane 6). The nonexportable protein BSA was injected along with the export substrate to verify the specificity of nuclear injections. Molecular mass standards are indicated in kD. (C and D) RNA was analyzed by 8 M 6% acrylamide urea gel electrophoresis followed by autoradiography. (C) In the presence of PBS or wt eIF-5A, the Rev–RRE RNA is exported (lanes 2 and 4). In contrast, export is inhibited in presence of the mutant protein eIF-5A–M14 (lane 6). (D) Nuclear export of CTE RNA is not prevented by PBS (lane 2), wt eIF-5A (lane 4), or eIF-5A–M14 (lane 6), indicating that eIF-5A is not involved in the CTE RNA export pathway. Nuclear injected CTE M2/M11 mutant RNA remains in the nuclei (lanes 1, 3, and 5). The nuclear localization of U6-RNA confirms the accuracy of nuclear injections.

injection of anti–eIF-5A antibodies did not abrogate nuclear export of the GST–PKI–NES export substrate (Fig. 1 F, lanes 3 and 4), which is in full agreement with the data raised in somatic cells (Elfgang et al., 1999).

Constitutive expression of an eIF-5A mutant gene (eIF-5A–M14) in human T cells has been previously shown to efficiently inhibit HIV-1 replication in these cells (Bevec et al., 1996; Junker et al., 1996). Subsequently, microinjection studies in somatic cells revealed that eIF-5A–M14 interferes with the nuclear export activity of Rev (Bevec et al., 1996; Elfgang et al., 1999), explaining its pronounced inhibitory phenotype on virus replication. Therefore, we next included wt eIF-5A and eIF-5A–M14 mutant protein in our oocyte microinjection studies, as outlined in Fig. 2 A. The GST–Rev–NES export substrate was incubated either with PBS, wt eIF-5A, or eIF-5A–M14 protein for 1 h (using a 10-fold M excess over the GST–Rev–NES fusion protein), in order to allow binding of the Rev–NES to the recombinant eIF-5A proteins. Subsequent nuclear microinjection demonstrated that the eIF-5A–M14 mutant protein also inhibited GST–Rev–NES export in oocytes (Fig. 2 B, lanes 5 and 6). This inhibitory phenotype was not observed in the presence of PBS (Fig. 2 B, lanes 1 and 2, negative control) or wt eIF-5A protein (Fig. 2 B, lanes 3 and 4). Moreover, the presence of eIF-5A–M14 also blocked Rev-mediated RRE RNA export (Fig. 2 C, lanes 5 and 6). As expected, nuclear export of CTE RNA was not inhibited by eIF-5A–M14 (Fig. 2 D, lanes 5 and 6).

Together, these data show that eIF-5A plays an essential role in Rev and Rev-dependent RRE RNA export in *Xenopus* oocytes. To date, however, it is not clear which precise function eIF-5A executes during the nuclear export of Rev–RRE-containing ribonucleoproteins. In particular, it is not known at which stage during nucleocytoplasmic translocation eIF-5A mutant proteins (e.g., M14) block Rev export. Therefore, we next investigated whether or not eIF-5A–M14 by itself is exported from the nuclear to the cytoplasmic compartment. As depicted in Fig. 3 A, wt GST–eIF-5A or GST–eIF-5A–M14 fusion proteins were microinjected into oocyte nuclei, and localization of the injected proteins was analyzed using specific antibodies, 2 h after injection. In agreement with previous data using somatic cells (Rosorius et al., 1999b), a significant amount of the nuclear-injected wt GST–eIF-5A protein was transported to the cytoplasm in these experiments (Fig. 3 B, lanes 1 and 2). In sharp contrast, however, the GST–eIF-5A–M14 protein remained predominantly in the injected oocyte nuclei (lanes 3 and 4). It has recently been shown by overlay blot assays and binding studies in solution that unmodified eIF-5A is able to interact with CRM1/exportin1 (Rosorius et al., 1999b). In our next set of experiments, therefore, we analyzed the binding of wt eIF-5A and eIF-5A–M14 to CRM1/exportin1 using GST–eIF-5A fusion proteins and total protein extracts from stage VI oocytes, which are a rich source for CRM1/exportin1. The use of oocyte extracts in these experiments has the additional advantage that these extracts also contain the cofactors that are possibly required for the formation of nuclear export complexes (e.g., RanGTP). wt GST–eIF-5A or GST–eIF-5A–M14 fusion proteins were immobilized on glutathione–Sepharose beads and incubated with oocyte protein extracts. The beads were then pelleted by centrifugation, and the bound and unbound material was analyzed by Western blots using antiserum raised against CRM1/

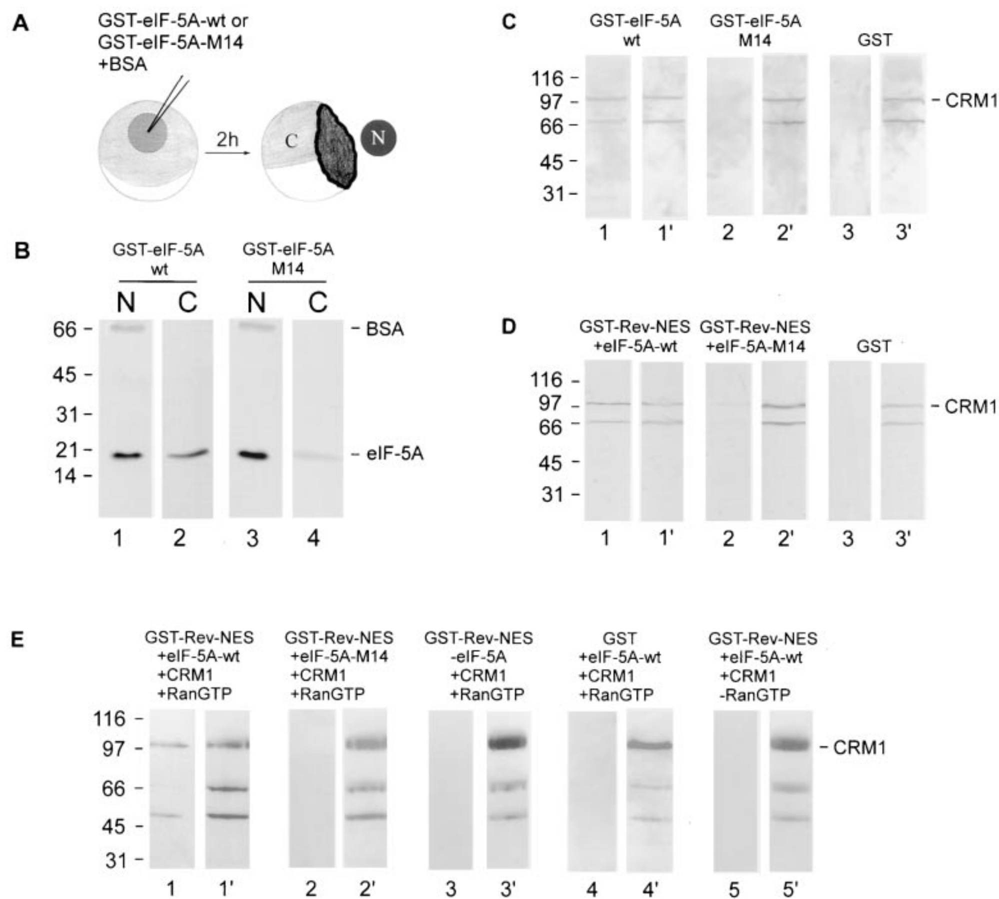

Figure 3. The eIF-5A mutant protein M14 is not exported and is unable to bind the export factor CRM1–exportin 1. (A) Oocyte injection protocol. 2 h after nuclear injection of wt eIF-5A or mutant protein M14, oocytes were manually dissected into nuclear (N) and cytoplasmic (C) fractions. (B) Proteins were separated by 18% SDS-PAGE, and blots were analyzed with antibodies to GST and BSA. A substantial fraction of the injected wt GST–eIF-5A migrates into the cytoplasm within 2 h (lane 2). In contrast, most of the eIF-5A mutant protein M14 remains in the nuclei (compare lanes 3 and 4). The exclusive presence of BSA in the nuclei confirms the specificity of the nuclear injections. (C) Interaction of wt eIF-5A and the mutant M14 with CRM1 as determined by pull-down assay. Glutathione–Sepharose beads coupled to the GST proteins indicated were incubated with oocyte extract. Bound and unbound proteins were resolved by SDS-PAGE and analyzed by immunoblotting experiments with anti-CRM1 antiserum. Bound proteins are shown in lanes 1–3, unbound proteins are shown in lanes 1′–3′. CRM1 binds to the wt form of eIF-5A (lane 1) but not to the M14 mutant (lane 2) and GST alone (lane 3). (D) Role of eIF-5A for interaction between CRM1 and Rev–NES was analyzed by pull-down assay. GST–Rev–NES coupled to glutathione–Sepharose beads was incubated with wt eIF-5A/eIF-5A–M14 before incubation with oocyte extract. Bound (lanes 1–3) and unbound (1′–3′) proteins were analyzed as described above. CRM1 binds to GST–Rev–NES only in the presence of the wt form of eIF-5A (lane 1) but not of the M14 mutant (lane 2). (E) Role of eIF-5A for interaction between recombinant CRM1 fusion protein and Rev–NES was analyzed by pull-down assay. GST–Rev–NES or GST alone coupled to glutathione-Sepharose beads were incubated in various combinations with RanGTP (GST-RanQ69L), wt eIF-5A, or eIF-5A–M14 before addition of recombinant His-tagged CRM1. Bound (lanes 1–5) and unbound (lanes 1′–5′) CRM1 was visualized by using His-specific antibodies. CRM1 fusion protein binds to GST–Rev–NES only in the presence of the wt form of eIF-5A and RanGTP (lane 1 versus lanes 3 and 5) but not in presence of the M14 mutant (lane 2) and not to GST (lane 4). The 66- and 45-kD components apparently represent CRM1 degradation products. Molecular mass standards are indicated in kD.

exportin1 (Kudo et al., 1997). As shown in Fig. 3 C, CRM1/exportin1 bound wt GST–eIF-5A protein (lane 1) but not GST alone (lane 3). Interestingly, the Rev inhibitory mutant eIF-5A–M14 clearly failed to interact with CRM1/exportin1 (lane 2). Thus, the inability of the eIF-5A–M14 protein to interact with CRM1/exportin1 (Fig. 3 C) correlates with its diminished nuclear export activity. Please also note that the ~66-kD cross-reacting protein appears to be a specific CRM1–exportin 1 degradation product (Rosorius et al., 1999b). To further clarify the mode of action of the inhibitory phenotype of eIF-5A–M14 on Rev export (Bevec et al., 1996; Elfgang et al., 1999), we investigated whether eIF-5A acts as a Rev–CRM1/exportin1 bridging factor (Fig. 3 D). GST–Rev–NES fusion protein was immobilized on glutathione–Sepharose beads and then incubated with a 10-fold M excess of either wt eIF-5A or eIF-5A–M14 to allow saturation of the Rev–NES. Next, oocyte extract was added, and the relative amount of bound and unbound CRM1/exportin1 was determined as before. As shown in Fig. 3 D, CRM1/exportin1 only bound efficiently to the Rev–NES in the presence of wt eIF-5A (lane 1) and almost completely failed to interact with the NES in the presence of eIF-5A–M14 (lane 2). Next, we substituted the oocyte extract by recombinant CRM1/exportin1 and RanGTP (Fig. 3 E). As shown, His-tagged CRM1/exportin1 only bound to GST–Rev–NES fusion protein in the presence of wt eIF-5A and the GTP-bound form of Ran (GST-RanQ69L; compare lanes 1, 3, and 5). Again, no interaction of CRM1/exportin1 with the Rev–NES was visible in presence of eIF-5A–M14 (lane 2). Finally, formation of CRM1/exportin1-containing nuclear export complexes did not occur in absence of the Rev–NES (lane 4). Note that the observed CRM1 degradation products of ~66 and ~49 kD were also detectable in these experiments using CRM1-specific antibodies (not shown).

The combined data demonstrate that eIF-5A participates specifically in nuclear export of the HIV-1 Rev RNA transport factor but not in MPMV CTE–mediated viral RNA export. Apparently, eIF-5A mediates efficient inter-

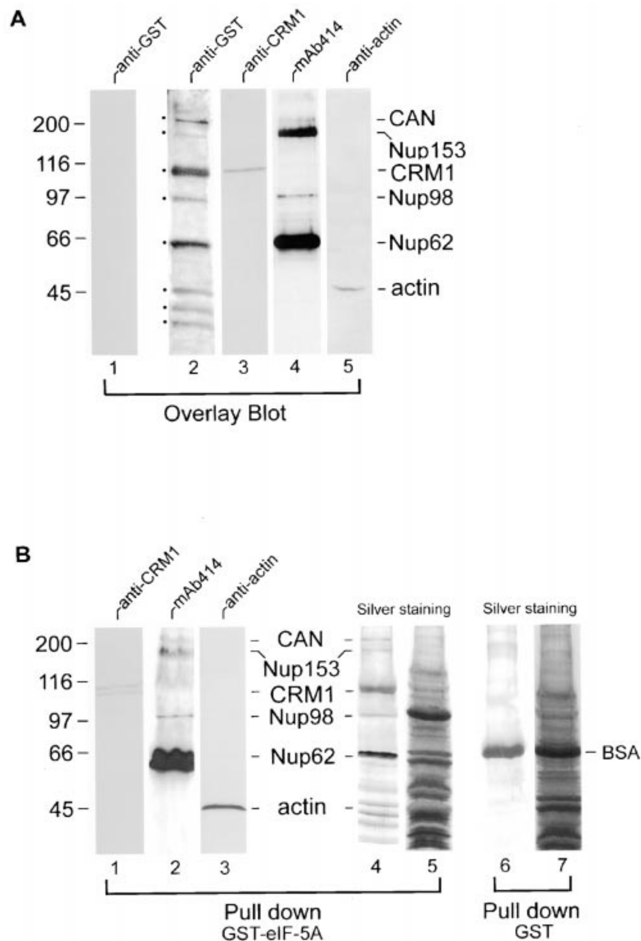

Figure 4. Interaction of eIF-5A with nuclear envelope proteins as determined by overlay assays and pull-down experiments. (A) Nuclear envelopes were manually isolated from *Xenopus* oocytes, and the proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose, and incubated with GST alone (lane 1) or GST–eIF-5A fusion protein (lanes 2–5). eIF-5A binds to eight polypeptide bands as revealed by anti-GST antibodies (indicated by dots, lane 2). Membranes were then stripped of bound antibodies and reprobed with antibodies to CRM1 (lane 3), to a family of nucleoporins (mAb 414, lane 4), and to actin (lane 5). The eIF-5A binding proteins identified are indicated at the right. (B) To analyze interactions of eIF-5A with nuclear proteins in solution, glutathione–Sepharose beads coupled to GST–eIF-5A (lanes 1–5) or to GST (lanes 6 and 7) were incubated with the extract of 150 manually isolated nuclei of *Xenopus* oocytes. The bound pelleted proteins were analyzed by immunoblotting (lanes 1–3) with antibodies to CRM1 (lane 1), nucleoporins (lane 2), and actin (lane 3), or by silver staining (lanes 4 and 6). The unbound proteins were visualized by silver staining (lanes 5 and 7). Molecular mass standards are indicated in kD.

action of the Rev–NES with CRM1/exportin1. Moreover, the previously reported eIF-5A–M14 mutant does not bind to CRM1/exportin1, thereby preventing efficient interaction of the Rev–NES with CRM1/exportin1. This binding defect of the eIF-5A mutant explains its previously observed ability to inhibit nuclear export of Rev.

Interaction of the Rev Cofactor eIF-5A with Nuclear Envelope Proteins

It has already been reported that eIF-5A accumulates at the nucleoplasmic periphery of the NPC, particularly at

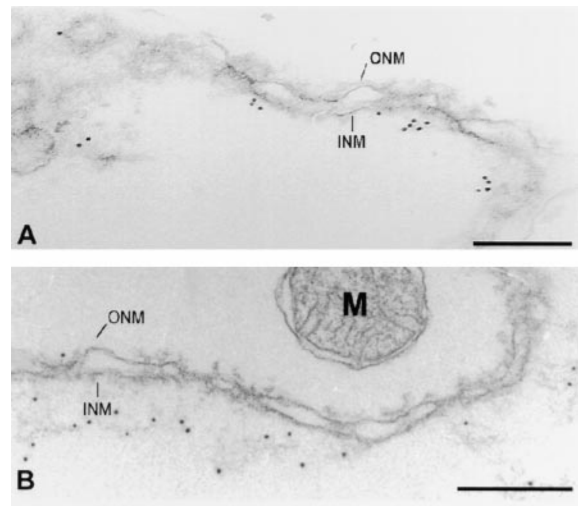

Figure 5. Actin is a constituent of the nucleoplasmic NPC-associated filaments as shown by immungold electron microscopy. Manually isolated nuclear envelopes from *Xenopus* (A) and *Pleurodeles* (B) oocytes were incubated with antiactin antibody 2G2 followed by secondary antibodies coupled to 12-nm gold particles. The nuclear filaments extending from the NPCs are particularly prominent in *Pleurodeles* oocytes (B). The occasional presence of mitochondria (M) clearly identifies the cytoplasmic side of the nuclear envelope. ONM, outer nuclear membrane; INM, inner nuclear membrane. Bars, 0.3 μm.

the nucleoplasmic filaments that emanate from the NPC surface into the nucleoplasm (Rosorius et al., 1999b). These filaments might serve as docking sites at which export cargoes initially interact with the NPC before their exit through the pore channel (e.g., Franke and Scheer, 1974; Cordes et al., 1993). After we had established that eIF-5A is required for Rev export in *Xenopus* oocytes, we used GST–eIF-5A fusion protein as a bait in order to identify components of the NPC that participate in this export pathway. Therefore, we manually isolated nuclear envelopes from oocytes. The nuclear envelope proteins were separated by SDS-PAGE, transferred onto nitrocellulose filters, renatured, and incubated with GST–eIF-5A fusion protein or GST alone as control. Bound proteins were detected with anti-GST antibodies. As shown in Fig. 4 A, GST–eIF-5A interacted with several distinct oocyte nuclear envelope proteins (lane 2, denoted by dots), whereas GST alone did not bind (lane 1). To identify these potential eIF-5A interaction partners, the nitrocellulose filters were stripped of the bound proteins and reprobed with specific antibodies. In agreement with the data presented above (Fig. 3 C), the signal detected at ~110 kD was identified as the export receptor CRM1/exportin1 (lane 3), which in fact has been reported to accumulate at both the nucleoplasmic and cytoplasmic face of the NPC, particularly associating with the nucleoporin CAN/nup214 (Fornerod et al., 1996, 1997b). The use of the mAb 414, which recognizes several nucleoporins containing multiple copies of FG repeats revealed that the nucleoporins CAN/nup214, nup153, nup98, and nup62 are potential eIF-5A binding partners (Fig. 4 A, lane 4). These interactions were reproduced in blot assays, using two-dimensional gels (data not shown), allowing us to unequivocally confirm that the abundant band observed at ~110 kD in the overlay blot (Fig. 4 A, lane 2) is in fact CRM1/exportin1

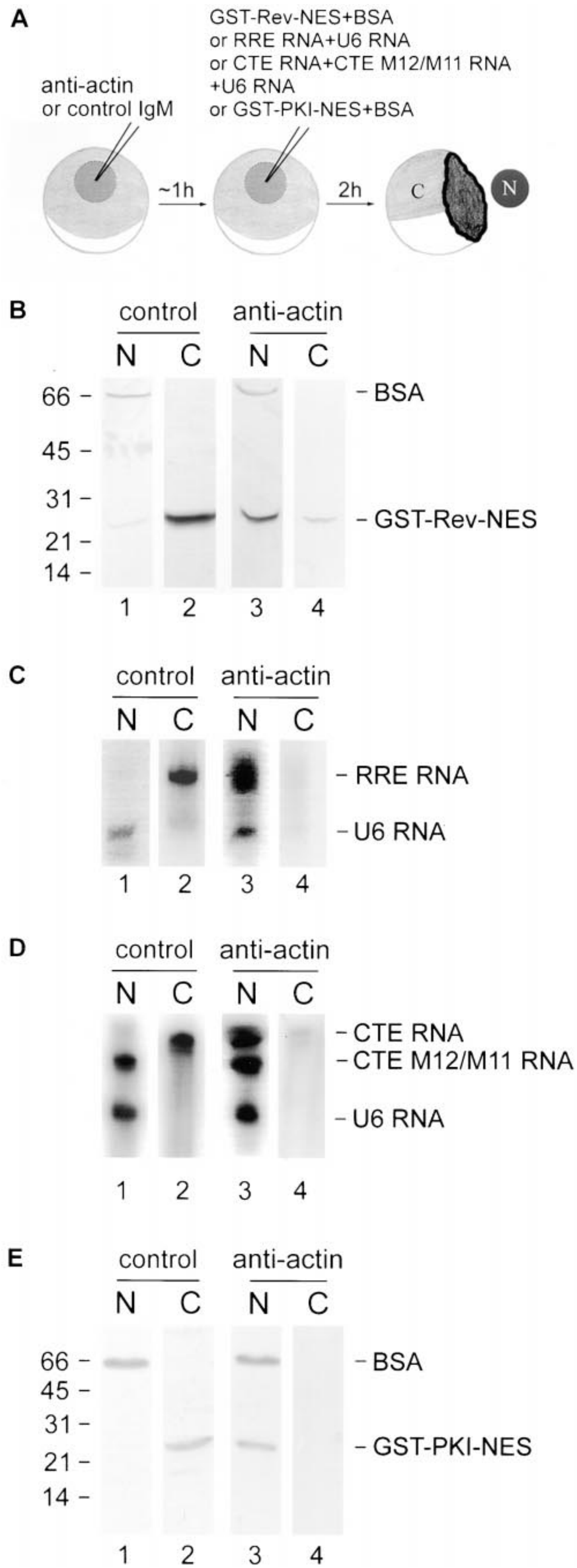

Figure 6. Antibodies against actin inhibit nuclear export of GST–Rev–NES, GST–PKI–NES, RRE RNA, and CTE RNA in *Xenopus* oocytes. (A) Oocyte injection protocol. Nuclear (N) and cytoplasmic (C) fractions were manually isolated. (B and E) Proteins were separated by 18% SDS-PAGE and analyzed by immunoblotting with antibodies to GST and BSA. The exclusive presence of the nonexportable protein BSA in the nuclear fractions confirms the accuracy of the injections. (B) The nuclear export of GST–Rev–NES is strongly inhibited by antibodies against actin (lane 4) but not by control IgM (lane 2). (E) Antiactin antibodies also block the nuclear export of GST–PKI–NES (lane 4), whereas, in contrast, it is exported in the presence of control IgM (lane 2). Molecular mass standards are indicated in kD. (C and D) RNA separated by 8 M 6% acrylamide urea gels and visualized by autoradiography. (C) In the presence of antiactin antibodies, the Rev-mediated export of RRE RNA is inhibited (compare lanes 2 and 4). (D) Antiactin antibodies also inhibit the export of CTE RNA (compare lanes 2 and 4). As expected, the mutant CTE M2/M11 is not exported (lanes 1 and 3). The nuclear localization of the nonexportable U6 RNA confirms the accuracy of the nuclear injections.

and not exportin4, which has recently been described as a novel importin-β family member with a similar molecular weight to CRM1/exportin1 (Lipowsky et al., 2000). Finally, these interactions were also confirmed in solution by pull-down experiments using wt GST–eIF-5A protein and extracts of manually isolated nuclei of *Xenopus* oocytes (Fig. 4 B, lanes 1–3). The corresponding silver-stained gel showing the pattern of bound (Fig. 4 B, lane 4) and unbound (Fig. 4 B, lane 5) proteins to GST–eIF-5A demonstrated the specificity of this binding assay. Only a very few specific binding proteins were visible (Fig. 4 B, compare lanes 4 and 5). Furthermore, an additional control experiment was performed using GST alone. The pattern of GST-bound (Fig 4 B, lane 6) and unbound (Fig. 4 B, lane 7) proteins are shown. As expected, no GST-bound nuclear envelope–associated proteins were detected. Of note, the predominant signal that was observed in this experiment is BSA, an ingredient of the used blocking buffer (Fig. 4 B, compare lanes 6 and 7).

Inspection of the overlay blot shown in Fig. 4 A (lane 2) also indicated an affinity of eIF-5A for three proteins of relatively low molecular mass (ranging from ~30 to ~45 kD). These eIF-5A interacting proteins were analyzed by mass spectrometry and peptide sequencing. The data obtained identified the ~45-kD protein as actin. This result was confirmed by reprobing the nitrocellulose filters with antiactin antibody 2G2 (Fig. 4 A, lane 5) as well as by pull-down experiments (Fig. 4 B, lane 3). Additional solution binding studies using purified actin monomers and nuclear extracts from *Xenopus* oocyte nuclei confirmed that actin indeed binds to eIF-5A. Under these conditions, actin also interacted with the nucleoporin nup62 (data not shown).

Then, we characterized the nuclear envelope association of actin at the ultrastuctural level by preembedding immunogold electron microscopy. To this end, nuclear envelopes were manually isolated from *Xenopus* or *Pleurodeles* oocytes and incubated with antiactin antibody 2G2 (Gonsior et al., 1999), followed by gold-coupled secondary antibodies. Interestingly, actin was found specifically at the nucleoplasmic face of the NPC and along the nucleoplasmic NPC-attached fibrils that are particularly prominent in *Pleurodeles* oocytes (Fig. 5, A and B). It should be stressed that the NPC-associated fibers are not actin filaments (F-actin). When isolated nuclear envelopes from *Xenopus* and *Pleurodeles* oocytes were incubated in the

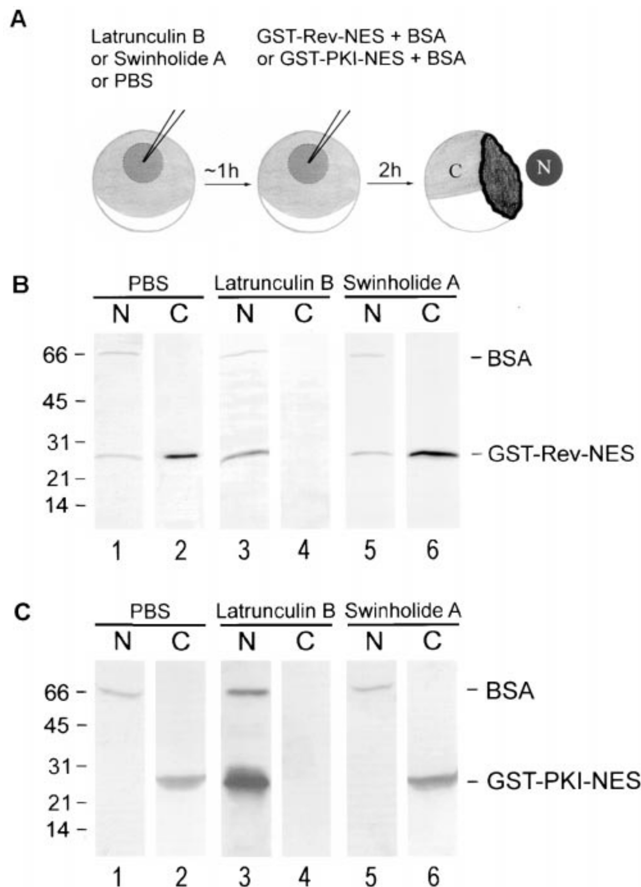

Figure 7. Different effects of actin-binding drugs on nuclear export of GST–Rev–NES and GST–PKI–NES. (A) Oocyte injection protocol. 1 h after nuclear injection of latrunculin B, swinholide A or PBS (control) and the export substrate GST–Rev–NES or GST–PKI–NES, together with BSA, were microinjected into oocyte nuclei. 2 h later, oocytes were manually dissected into nuclear (N) and cytoplasmic (C) fractions. (B and C) Proteins were separated by 18% SDS-PAGE, and blots were analyzed with antibodies to GST and BSA. A substantial fraction of the injected export substrate migrates into the cytoplasm in the presence of PBS (lane 2) or swinholide A (lane 6). In contrast, the export substrate remains exclusively in the nuclei in presence of latrunculin B (lane 3). Detection of BSA in the nuclei confirms the specificity of the nuclear injections.

presence of Ca^{2+} with gelsolin, an actin severing protein, the NPC-associated filaments remained intact (data not shown). Thus, although actin is clearly associated with these filaments, their integrity is not based on polymerized actin but must involve other proteins.

Actin Participates in Nuclear Export Processes

To examine whether or not nuclear actin is functionally involved in nuclear export, first, we tested the effect of antiactin antibodies on the nuclear export of GST–Rev–NES and GST–PKI–NES fusion proteins as well as on Rev-mediated RRE RNA export and Rev-independent MPMV CTE RNA export. For this, *Xenopus* oocyte nuclei were microinjected as outlined above and depicted in Fig. 6 A. These experiments demonstrated that antibodies directed against actin are efficient export inhibitors when delivered to the nuclear interior. In fact, Rev–NES and Rev-mediated RRE RNA export (Fig. 6, B and C, respectively) was as efficiently abrogated by these antibodies as the nuclear export of the MPMV CTE (Fig. 6 D) and of the PKI–NES (Fig. 6 E). Upon injection into the cytoplasm, the antibodies had no effect on nuclear export (data not shown).

Since nuclei of somatic cells and amphibian oocytes are usually not stained by fluorescent phalloidin, a specific marker of F-actin in immunofluorescence microscopy, it is possible that nuclear actin may exist in an unpolymerized or oligomeric form that is not recognized by phalloidin or organized in higher order structures different from F-actin (Gonsior et al., 1999). To further test the role of actin polymerization in nuclear export, we studied the effect of two actin-binding drugs with different specificities. The toxin latrunculin binds to G-actin and prevents its polymerization (Coue et al., 1987). Microinjection of latrunculin B into oocyte nuclei inhibited not only the export of Rev–NES (Fig. 7 B, lanes 3 and 4) and RRE RNA (data not shown) but also the export of PKI–NES (Fig. 7 C, lanes 3 and 4) and of the MPMV CTE (data not shown). In contrast, nuclear injection of swinholide A, a drug that severs F-actin and stabilizes actin dimers (Bubb et al., 1995), did not inhibit nuclear export (Fig. 7, B and C, lanes 5 and 6). These results support the idea that a soluble form of nuclear actin, rather than F-actin, participate in multiple nuclear export pathways in *Xenopus* oocytes.

Since these data provided evidence for a novel export function of actin, we also wanted to test whether these results could be confirmed in somatic cells. Therefore, we microinjected nuclei of Vero cells with recombinant nuclear export substrate in which Rev–NES–GST was fused to the GFP. This GFP–Rev–NES–GST fusion protein allows the monitoring of Rev–NES-mediated nuclear export in live cells (Rosorius et al., 1999a). GFP-specific fluorescence and phase contrast images were taken immediately and 30 min after injection. Coinjection of nonspecific IgM did not interfere with Rev–NES-mediated nuclear export (Fig. 8, A′ and B′). However, coinjection of antiactin antibodies completely abrogated the nucleocytoplasmic translocation of the export substrate (Fig. 8, C′ and D′), confirming the data raised in *Xenopus* oocytes. As expected, antiactin antibodies were able to effectively block the nuclear export of GST–PKI–NES (data not shown), again confirming the data obtained in *Xenopus* oocytes.

To analyze the actin requirement for nuclear export in more detail, we next wanted to establish an in vitro nuclear export system. It has previously been shown that extracts prepared from activated amphibian eggs are capable of assembling purified DNA or chromatin into synthetic nuclei, which accumulate soluble karyophilic proteins from the surrounding medium by signal- and energy-dependent nuclear import processes (for review see Newmeyer and Wilson, 1991). However, transport in the opposite direction, i.e., nuclear export of macromolecules, has not yet been observed, and it is therefore generally believed that these in vitro systems are export deficient. In fact, our initial attempts to establish such a Rev export in vitro system failed and confirmed this notion. However, after having identified actin as a factor involved in nuclear export, we realized that standard protocols for the preparation of egg extract include the drug cytochalasin B in or-

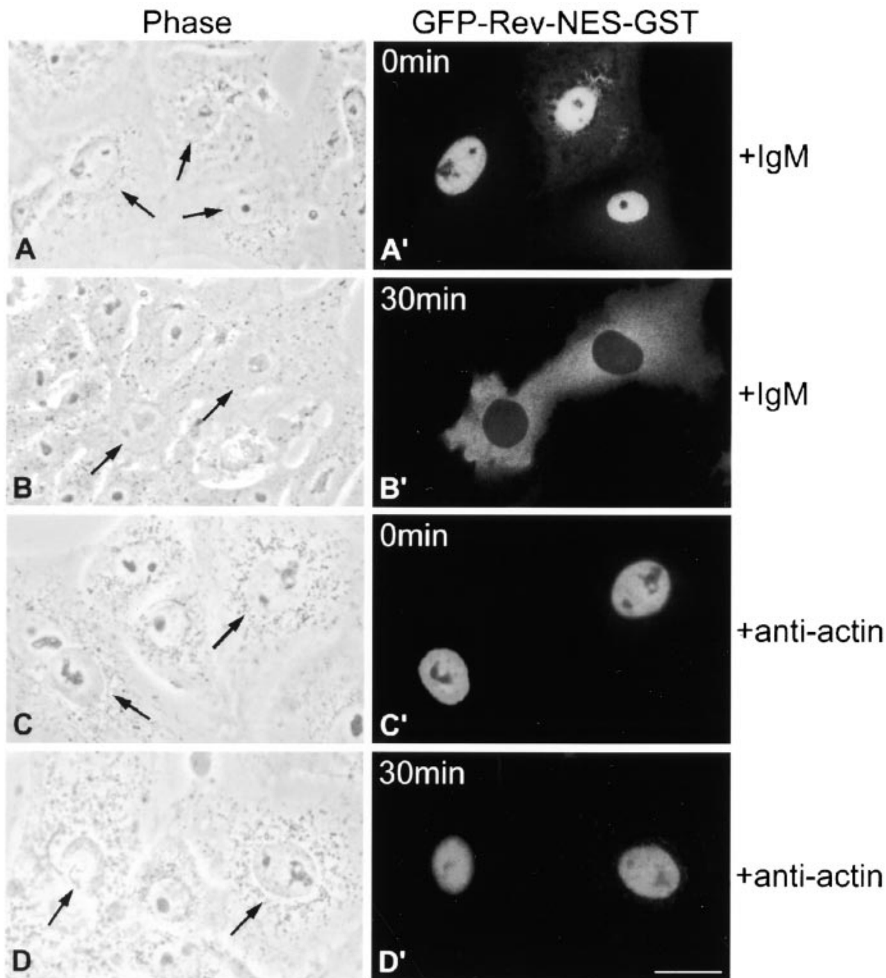

Figure 8. Inhibition of nuclear export of GFP–Rev–NES–GST fusion protein by antiactin antibodies in cultured Vero cells. A mixture of GFP–Rev–NES–GST fusion protein, together with either control IgM (A and B), or antiactin antibodies (C and D) was microinjected into nuclei of Vero cells. Phase–contrast images (left) and GFP fluorescence (right) were recorded at the indicated postinjection time points. The export substrate is efficiently translocated from the nucleus to the cytoplasm in the presence of control IgM (A′ and B′). In contrast, coinjected antiactin antibodies completely prevent nuclear export (C′ and D′). Microinjected cells are denoted by arrows. Bar, 20 μm.

der to prevent actin polymerization and gelation of the extract (Newmeyer and Wilson, 1991). The effect of cytochalasins on actin dynamics is quite complex and arises not only from an inhibition of the elongation of F-actin but also from an alteration of the properties of actin monomers (Sampath and Pollard, 1991). To clarify whether the presence of cytochalasin B might interfere with nuclear export processes in vitro, we performed the after experiments. First, we isolated nuclei from HeLa–Rev cells, which is a cell line that constitutively expresses high levels of nuclear HIV-1 Rev protein (Wolff et al., 1995). The isolated nuclei were then incubated for 1 h in *Xenopus* egg extracts, which were prepared with or without the addition of cytochalasin B. Subsequently, the nuclei were centrifuged onto coverslips, washed, and analyzed by immunofluorescence microscopy with antibodies against Rev. Egg extracts that contained cytochalasin B did not support the nuclear export of Rev, irrespective of whether or not an energy-regenerating system was present (Fig. 9, A and B). In striking contrast, however, nuclear export of Rev was easily detectable when egg extracts lacking cytochalasin B were used (Fig. 9 C). This transport appeared to be an active and CRM1–exportin 1-mediated process, since it depended critically on the presence of an energy-regenerating system (Fig. 9 D) and was blocked by leptomycin B (data not shown). The ability of the isolated nuclei to promote nuclear import was confirmed by analyzing the import of BSA–SV40T–FITC. This transport substrates was efficiently taken up by the isolated nuclei in egg extract prepared with or without cytochalasin B (data not shown). Together, our data demonstrate that nuclear export of Rev can be reproduced in a cell-free system provided that the egg extract is prepared without the actin-binding drug cytochalasin B.

Discussion

The bidirectional transport of macromolecules between the nucleus and cytoplasm across the NPC is an energy-dependent and highly selective process. A family of transporter proteins known as importins or exportins mediate this intracellular movement of proteins and RNA (Corbett and Silver, 1997; Izaurralde and Adam, 1998; Görlich and Kutay, 1999; Nakielny and Dreyfuss, 1999). In contrast to nuclear import processes, the export of macromolecules from the nucleus is less well understood. Although some general characteristics of nuclear export have been established and some components of the export machinery have already been identified, their exact mode of action is not clear.

It has previously been reported that eIF-5A is required in somatic cells specifically for the nucleocytoplasmic translocation of the Rev/Rex class of retroviral RNA nuclear export factors (Ruhl et al., 1993; Katahira et al., 1995; Bevec et al., 1996). Moreover, inhibition studies in somatic

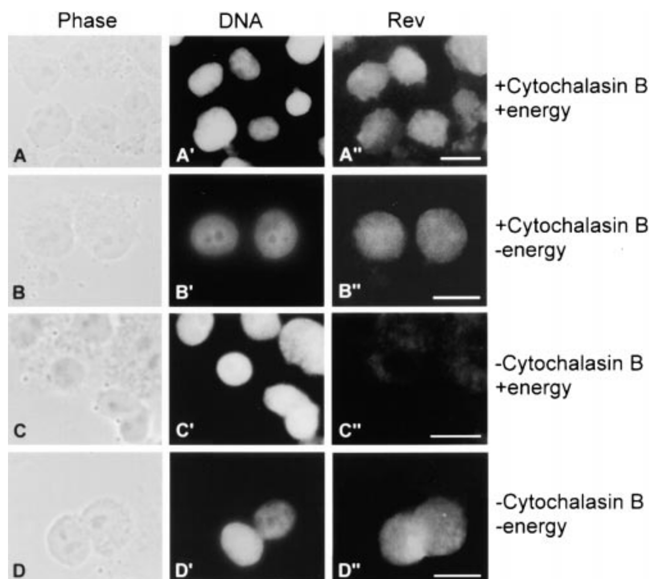

Figure 9. Nuclear export of Rev in a cell-free system. Nuclei were isolated from a HeLa cell line that constitutively expresses high levels of nuclear HIV-1-Rev and were incubated in *Xenopus* egg extract with or without cytochalasin B and an energy-regenerating system. After 1 h, nuclei were processed for immunofluorescence with antibodies to Rev (A″–D″) and counterstained with the DNA-specific dye Hoechst (A′–D′). In the absence of cytochalasin B and the presence of energy, Rev is exported from the nuclei (C″). The corresponding phase–contrast images are shown (A–D). Bars, 15 μm.

cells suggested that eIF-5A acts before or simultaneously with CRM1/exportin1 in the nuclear export of Rev and Rex (Elfgang et al., 1999). In agreement with the notion that eIF-5A is a protein that is part of this specific nuclear export pathway, we were able to demonstrate that eIF-5A accumulates in particular at the NPC-associated nucleoplasmic fibrils in *Xenopus* oocytes and somatic cells, interacts with the general export receptor CRM1/exportin1, and is by itself exported from the nucleus to the cytoplasm in a leptomycin B–sensitive manner (Rosorius et al., 1999b). In particular, the latter finding that eIF-5A is actively exported from the nucleus has been confirmed by a recent study (Lipowsky et al., 2000). In this work, it was demonstrated that eIF-5A also interacts with a novel export receptor, named exportin4. Importantly, binding of eIF-5A to exportin4 was shown to depend on the hypusine modification in eIF-5A, with the implication that hypusine is required for nuclear export of eIF-5A. On the other hand, however, unmodified eIF-5A has been shown to be efficiently exported from the nucleus to the cytoplasm in microinjected somatic cells (Rosorius et al., 1999b) and *Xenopus* oocytes (this study), as well as in permeabilized cells (Lipowsky et al., 2000). Moreover, human eIF-5A is fully functional in yeast strains that are disrupted for *HYP2* encoding the yeast homologue of eIF-5A (Schwelberger et al., 1993; Magdolen et al., 1994), despite the fact that exportin4 is apparently not present in this organism (Lipowsky et al., 2000). In summary, these data suggest that the unique hypusine modification is not essential for nuclear export of eIF-5A and that it is possible that eIF-5A interacts with multiple export receptors (e.g., CRM1/exportin1 and exportin4). It remains to be investigated in future studies whether or not the interaction of eIF-5A with different export receptors reflects a distinct regulation of a specific nuclear export pathways in response to specific physiological conditions.

The results of this study clearly demonstrate that nuclear export of Rev and RRE RNA in *Xenopus* oocytes is also dependent on eIF-5A. In contrast, however, eIF-5A is not essential for CTE-mediated RNA export, which is in perfect agreement with previously published data that reported that the CTE RNA export is distinctly regulated when compared with Rev/Rex–mediated RNA export (Pasquinelli et al., 1997; Bogerd et al., 1998; Kang and Cullen, 1999; Zolotukhin and Felber, 1999).

The finding that the eIF-5A–M14 mutant protein is unable to bind to the general export receptor CRM1/exportin1, and that this correlates with an export-deficient phenotype of this protein, suggested a mechanism in which eIF-5A may act as an adapter that targets the Rev–NES to the nucleoplasmic face of the NPC and mediates efficient binding to CRM1/exportin1. CRM1/exportin1 and associated components (e.g., Ran) then translocate the Rev-NES/eIF-5A–containing ribonucleoprotein particle through the pore channel. Indeed, our subsequent in vitro binding studies demonstrated that efficient Rev-NES–CRM1 complex formation was only mediated by wt eIF-5A but not by the eIF-5A–M14 mutant protein.

The eIF-5A protein was originally detected as a potential Rev cofactor in HeLa nuclei using reversible cross-linker molecules in combination with structurally flexible Rev–NES peptide mimics (Ruhl et al., 1993). The binding experiments in this study were performed with GST–Rev–NES fusion proteins in which a structurally flexible glycine linker element separates the GST from the NES moiety (Elfgang et al., 1999). It should be noted that when full-length Rev protein was used in in vitro binding studies, an interaction with eIF-5A was only observed when Rev was bound to the RRE RNA at concentrations that allowed the formation of a dimeric Rev–specific complex (Bevec et al., 1996). In contrast, no in vitro interaction of Rev with eIF-5A was detected in a study in which Rev–RRE ratios were used that favored the formation of monomeric or multimeric Rev–specific complexes on the RRE RNA (Henderson and Percipalle, 1997). More recent in vitro binding studies using full-length recombinant Rev protein and cytoplasmic HeLa cell extracts (as a source of eIF-5A) did also not detect binding of eIF-5A to Rev (Lipowsky et al., 2000). However, this experimental design is not suitable for the investigation of the interaction of full-length Rev protein with any nuclear export factor, since it neglects the well established fact that Rev homomultimer formation on the RRE RNA is essential for Rev function (Pollard and Malim, 1998). As shown previously (Elfgang et al., 1999) and in this study, the requirement of RRE RNA binding and subsequent multimerization can apparently be overcome by using structurally flexible Rev–NES mimics. The combined data may therefore suggest that dimerization of Rev protein on its RRE RNA target is required for its interaction with nuclear export factors. In particular, a study in which Rev in vitro multimerization was investigated in combination with functional cell-based assays indeed concluded that two monomeric Rev proteins

bound to the RRE RNA is sufficient for biological activity (Daly et al., 1993b). However, future studies are required to elucidate the exact protein composition of Rev-containing export complexes.

The concept that eIF-5A facilitates the interaction of the Rev–NES with CRM1/exportin1 is in full agreement with recent studies in which the binding affinities of Rev–NES to CRM1/exportin1 were investigated using recombinant proteins. When RRE RNA-bound Rev protein was incubated together with CRM1/exportin1 and RanGTP in RNA gel retardation assays, only an extremely poor level of complex formation was detected (Askjaer et al., 1998). It appears that, in these types of experiments, $<1\%$ of the preformed RRE–Rev ribonucleoprotein complexes interacted with recombinant CRM1/exportin1 and Ran-GTP. Moreover, in another study, efficient interaction of CRM1/exportin1 with RanGTP in the presence of Rev–NES peptides was only observed when CRM1/exportin1 derived from reticulocyte lysates was used (Fornerod et al., 1997a). Reticulocyte extracts contain high levels of eIF-5A (Schatz et al., 1998). Interestingly, when CRM1/exportin1 was produced by translation in an *E. coli*–derived lysate (which does not contain eIF-5A), binding was only observed using the PKI-derived NES (Fornerod et al., 1997a), again suggesting that factors in addition to RanGTP are required for efficient Rev-NES–CRM1 interaction. Finally, quantification of the Rev-NES–CRM1 affinity using an assay that measures the hydrolysis of Ran-bound GTP upon complex formation (RanGAP assay) clearly demonstrated that the Rev–NES displays an extremely low affinity for CRM1/exportin1, at a level that is almost indistinguishable from that of an export-deficient mutant NES (Askjaer et al., 1999). In sharp contrast, however, complex formation with CRM1/exportin1 was clearly observed when the PKI–NES was used in this assay (Askjaer et al., 1999). In this context it is also important to reemphasize that inhibition of eIF-5A has previously been shown to block Rev-NES–mediated but not PKI-NES–mediated nuclear export in somatic cells (Elfgang et al., 1999), a fact that was reproduced in *Xenopus* oocytes (this study). Clearly our data showing that eIF-5A mediates Rev-NES–CRM1 interaction provides an explanation for these findings. Moreover, our data reveal the mode of action of eIF-5A mutant proteins (e.g., M14) that have been shown to efficiently block HIV-1 replication in human T cells when constitutively expressed in trans (Bevec et al., 1996; Junker et al., 1996).

To analyze interactions of eIF-5A with components of the NPC, we performed in vitro binding studies using overlay blot assays and precipitation analyses. We were able to identify specific interactions between eIF-5A and the *Xenopus* oocyte nucleoporins nup153, nup98, nup62, and CAN/nup 214 (for review see Stoffler et al., 1999). Although the functional contribution of these interactions to eIF-5A export remains to be established, it is certainly intriguing that this assay only detected nucleoporins that have been implicated in nuclear export pathways (Bastos et al., 1995; Fornerod et al., 1997b; Powers et al., 1997; Askjaer et al., 1999; Kehlenbach et al., 1999; Nakielny et al., 1999; Pritchard et al., 1999; Ullman et al., 1999; Bachi et al., 2000), whereas other FG repeat–containing nucleoporins like RanBP2 are not detected. In this context it is also important to note that a series of recent studies indeed demonstrated the direct participation of CAN/nup214, nup153, and nup98 in the nuclear export of Rev and Rev-mediated viral mRNA export (Bogerd et al., 1998; Ullman et al., 1999; Zolotukhin and Felber, 1999). Thus, the identification of NPC-associated proteins that interact with the Rev cofactor eIF-5A appears to be a promising experimental approach to detect novel factors that participate in nuclear export of Rev. In fact, in overlay assays and in solution binding studies using isolated nuclei from *Xenopus* oocytes, eIF-5A interacted not only with a specific set of nucleoporins but also with actin. By immunogold electron microscopy, we could exclude that the nuclear envelope–associated actin represented cytoplasmic contamination since it localized specifically to the nucleoplasmic face of the NPCs and the attached pore filaments. Amphibian oocyte nuclei contain high concentrations of nonmuscle β- and γ-actin, mostly in a soluble, i.e., unpolymerized or short polymer, form (Scheer et al., 1984; Ankenbauer et al., 1989; Gonsior et al., 1999). Our finding that eIF-5A colocalizes together with nuclear actin (this study) and nup153 (Rosorius et al., 1999b) at the filamentous arrays extending from the nucleoplasmic side of NPCs is in perfect agreement with the in vitro binding studies and, furthermore, suggests that the nucleoplasmic NPC filaments serve as docking sites for RRE RNA containing viral export complexes before nuclear exit.

Even with the inherent problems of cytoplasmic contamination, it is now generally accepted that actin is present not only in oocyte nuclei but also in the interphase nuclei of somatic cells (Scheer et al., 1984; Sahlas et al., 1993; Gonsior et al., 1999; Rando et al., 2000). Given its abundance and widespread occurrence, it is quite likely that nuclear actin is involved in specific nuclear processes that are, however, not necessarily dependent on filament formation. Among other functions (Gonsior et al., 1999; Rando et al., 2000), nuclear actin has been implicated in transcription of cellular and viral genes (Egly et al., 1984; Scheer et al., 1984; Burke et al., 1998) and chromatin remodelling events (Zhao et al., 1998). Based on nuclear injection experiments of antiactin antibodies and actin-binding drugs, this study now shows that nuclear actin also may be involved in the later stages of gene expression, namely the NPC-mediated transport of RNA out of the nucleus. This view is further corroborated by a recent study in which the authors also provide evidence for a functional involvement of nuclear actin in the nuclear export of Rev-dependent HIV-1 pre-mRNA (Kimura et al., 2000). However, although these authors emphasize the importance of a filamentous form of nuclear actin, our data suggest that it is the unpolymerized or short polymer form of nuclear actin that functions in nuclear export processes (see below).

At the moment, we do not know whether or not actin is a general cofactor that also participates in the nuclear export pathways of cellular RNA species. Nonetheless, our data demonstrate that actin plays a functional role in at least two distinct export pathways accessed by viral RNAs, the Rev-dependent RRE RNA, and the TAP-dependent CTE RNA export. Since TAP also contributes to the export of cellular mRNAs (Grüter et al., 1998; Braun et al., 1999; Görlich and Kutay, 1999; Nakielny and Dreyfuss, 1999), it is conceivable that actin could likewise function in

this pathway. Furthermore, we show that nuclear actin participates in the nuclear export of the eIF-5A–independent, CRM1-dependent NES of PKI. Finally, it should be stressed that the inhibition of nuclear export by antibodies to actin was not restricted to oocytes but appears to be a general phenomenon as it was also observed in cultured mammalian cells.

Our findings that (a) actin is located at the inner pore fibrils, (b) actin interacts in vitro with the export factor eIF-5A and with the nucleoporin nup62, and (c) Rev and TAP-mediated nuclear export of viral RNAs as well as nuclear export of PKI can be inhibited by introducing either antiactin antibodies or the actin-binding drug latrunculin B into the cell nucleus, indicate a novel and unexpected role for nuclear actin. Since we observed the export-inhibitory effect with both agents, we conclude that it is caused by direct targeting and functional inactivation of nuclear actin rather than by more indirect effects such as precipitation of nuclear actin. Nuclear import was not inhibited under the same experimental conditions (not shown), indicating that the injected antibodies did not cause a nonspecific steric inhibition of transport complexes at the pore. Several lines of evidence suggest that it is the unpolymerized or short polymer form of nuclear actin that is involved in nuclear export rather than F-actin. First, most of the nuclear actin of *Xenopus* oocytes exists in a soluble, although polymerization-competent form (Scheer et al., 1984; Ankenbauer et al., 1989). Second, fluorescent phalloidin, which is selective for F-actin, does not stain F-actin in the nuclei of *Xenopus* oocytes and cultured vertebrate cells under normal conditions, i.e., without experimental manipulations (Sahlas et al., 1993; Gonsior et al., 1999). Third, the antiactin antibody we have used for the microinjection experiments (2G2) does not recognize native F-actin but is selective for a specific conformational state of actin that is predominantly found in nuclei (Gonsior et al., 1999). It is tempting to speculate that actin dimers, which have been reported to assume a conformation not present in polymerized F-actin (lower dimer or LD [Millonig et al., 1988]), are the major targets of this antibody. Fourth, Rev and PKI export are not affected by injection of swinholide A into nuclei. This actin-binding drug severs F-actin and stabilizes actin dimers (Bubb et al., 1995), demonstrating that neither the absence of F-actin nor a decrease in the G-actin level affects the nuclear export pathway. Finally, Rev and PKI export are inhibited by latrunculin B, which sequesters G-actin monomers and prevents its polymerization (Coue et al., 1987). The opposing effects of latrunculin B and swinholide A support the concept that actin dimers function in nuclear export.

The critical role of actin in the Rev export pathway was also demonstrated in a cell-free system using *Xenopus* egg extract. When we omitted cytochalasin B, which is normally added to the extract as a gelation inhibitor (Newmeyer and Wilson, 1991), Rev was efficiently exported in an energy-dependent manner from incubated isolated nuclei. Since the interactions of cytochalasin with actin are complex and involve capping of the barbed ends of F-actin and hydrolysis of the monomeric ATP–actin pool (Sampath and Pollard, 1991), the interpretation of the inhibitory mechanism of cytochalasin B in this experimental system is difficult. More importantly, the cell-free nuclear export assay described here should provide the means to further explore the export pathway of Rev–RRE and possibly also of cellular RNAs since the extract can be easily manipulated and nuclear export monitored by simple immunofluorescence microscopy. Furthermore, this model system should help to clarify whether or not the ability of soluble actin to shuttle between the nucleus and the cytoplasm (Wada et al., 1998) is linked to nuclear export processes. However, we consider this quite unlikely in view of the observation that the nuclear export of G-actin is inhibited by the drug leptomycin B (Wada et al., 1998), whereas the TAP–CTE export pathway is not affected (Otero et al., 1998). We have shown that nuclear actin participates in the export of CTE RNA. Obviously, the shuttling potential of actin is of no relevance in this context, since the CTE RNA export resists inhibition by leptomycin B.

In summary, our data demonstrate that actin accumulates at NPC-attached nucleoplasmic filaments and can contribute to the nuclear export of multiple export substrates. We propose that unpolymerized nuclear actin is critically involved in the assembly of multiprotein export complexes. Investigation of the exact functional role of actin as part of the NPC-associated nuclear export machinery is a major research goal for the future.

We thank Nicole Studtrucker, Grit Schneider, and Corinna Zünkler for excellent technical assistance and Dr. Sarah L. Thomas for critical comments on the manuscript. We are indebted to Dr. Larry Gerace for many critical and helpful discussions during the course of this study. We are grateful to Dr. Marie-Louise Hammarskjöld (University of Virginia, Charlottesville, VA) for MPMV CTE constructs, Dr. Frauke Melchior (Max-Planck-Institute for Biochemistry, Martinsried, Germany) for the RanQ69L expression plasmid, Dr. Minoru Yoshida (The University of Tokyo, Tokyo, Japan) for anti-CRM1 antiserum, Dr. Reinhard Lührmann (Max-Planck-Institute, Göttingen, Germany) for U6 snRNA clone, and Dr. Horst Hinssen (Biochemical Cell Biology, University of Bielefeld, Germany) for providing monomeric actin.

This work received financial support from the Deutsche Forschungsgemeinschaft (SFB 466, SFB 176, and grant DA 243/1-1).

Submitted: 3 August 2000
Revised: 16 January 2001
Accepted: 16 January 2001

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