Biogenesis and nuclear export of ribosomal subunits in higher eukaryotes depend on the CRM1 export pathway

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Summary

The production of ribosomes constitutes a major biosynthetic task for cells. Eukaryotic small and large ribosomal subunits are assembled in the nucleolus and independently exported to the cytoplasm. Most nuclear export pathways require RanGTP-binding export receptors. We analyzed the role of CRM1, the export receptor for leucine-rich nuclear export signals (NES), in the biogenesis of ribosomal subunits in vertebrate cells. Inhibition of the CRM1 export pathway led to a defect in nuclear export of both 40S and 60S subunits in HeLa cells. Moreover, the export of newly made ribosomal subunits in *Xenopus* oocytes was efficiently and specifically competed by BSA-NES conjugates. The CRM1 dependence of 60S subunit export suggested a conserved function for NMD3, a factor proposed to be a 60S subunit export adaptor in yeast. Indeed, we observed that nuclear export of human NMD3 (hNMD3) is sensitive to leptomycin B (LMB), which inactivates CRM1. It had, however, not yet been demonstrated that Nmd3 can interact with CRM1. Using purified recombinant proteins we have shown here that hNMD3 binds to CRM1 directly, in a RanGTP-dependent manner, by way of a C-terminal NES sequence. Our results suggest that the functions of CRM1 and NMD3 in ribosomal subunit export are conserved from yeast to higher eukaryotes.

Key words: Nucleo-cytoplasmic transport, Ribosome, Export, CRM1, NMD3, LMB

Introduction

In vertebrates, the mature cytoplasmic 60S subunit consists of three rRNA chains (28S, 5.8S, 5S) and ~46 ribosomal proteins whereas the 40S subunit contains a single 18S rRNA species and ~32 ribosomal proteins. Biogenesis of ribosomal subunits starts with the synthesis of a large ribosomal RNA (rRNA) precursor by RNA polymerase I (pre-rRNA) in the nucleolus and 5S rRNA by polymerase III. The precursor rRNA is then rapidly processed in a series of endo- and exonucleolytic steps and modified by, for example, methylation and pseudouridylation. At the same time, ribosomal proteins assemble with the rRNAs into the pre-ribosomal subunits. Processing and assembly are well coordinated and require the assistance of a great number of trans-acting factors (for a review, see Kressler et al., 1999; Warner, 2001; Fatica and Tollervey, 2002). As ribosomal particles mature further, they leave the nucleolus independently of each other and finally exit the nucleus as separate entities through nuclear pore complexes (NPCs).

Nuclear export of ribosomal subunits is a receptor-mediated process (Bataillé et al., 1990). Over the past few years, it has been intensively studied in *Saccharomyces cerevisiae* (for a review, see Johnson et al., 2002). In yeast, nuclear export of both ribosomal subunits has been shown to require the nuclear export receptor Crm1p and a functional RanGTPase system (Hurt et al., 1999; Moy and Silver, 1999; Ho et al., 2000b; Stage-Zimmermann et al., 2000; Gadal et al., 2001; Moy and Silver, 2002). The exportin Crm1p/exportin 1 belongs to a conserved superfamily of RanGTP-binding nuclear transport receptors (Fornerod et al., 1997; Görlich et al., 1997), which facilitate the nuclear pore passage of proteins, RNAs and ribonucleoproteins (RNPs) in all eukaryotes. The binding of RanGTP to these transport receptors controls the compartment-specific association with their transport substrates (Mattaj and Englmeier, 1998; Görlich and Kutay, 1999). Export complexes between exportins (such as CRM1) and their respective export cargos form in the presence of RanGTP in the nucleus, where RanGTP is highly concentrated. After translocation through the NPC these export complexes are disassembled in the cytoplasm, where GTP hydrolysis on Ran is activated by the RanGTPase activating protein (RanGAP) aided by cytoplasmic Ran-binding proteins.

The exportin CRM1 recognizes leucine-rich nuclear export signals (NES) on most of its export substrates (Fornerod et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997). CRM1 not only mediates the nuclear export of a great variety of different proteins, but is also involved in the export of different classes of RNAs. In contrast to other exportins (Arts et al., 1998; Kutay et al., 1998; Calado et al., 2002), CRM1 does not recognize these RNAs directly. Rather, these RNAs associate with specific NES-containing adaptor proteins, which funnel these RNAs into the CRM1-mediated export pathway (Fischer et al., 1995; Ohno et al., 2000).

Although Crm1p has been implicated in ribosomal subunit export in yeast, it is not yet fully understood how it serves this process. So far, an interaction of Crm1p with either of the two

subunits has not been demonstrated. The binding of Crm1p to pre-ribosomal subunits might require bridging by adaptor proteins. Although no such candidate adaptor protein has been identified for the pre-40S particles, yeast Nmd3p has been suggested to serve as an NES-containing export adaptor for the 60S subunit (Ho et al., 2000b; Gadal et al., 2001). Strikingly, yeast strains harboring *NMD3* ts alleles show defects in 60S subunit export, whereas 40S subunit export is unaffected. Furthermore, Nmd3p is a shuttling protein that contains a nuclear localization signal (NLS) and a leucine-rich NES in its C-terminal domain (Ho et al., 2000b; Gadal et al., 2001). At steady state, Nmd3p localizes predominantly to the cytoplasm where it is bound to free 60S subunits (Ho and Johnson, 1999; Ho et al., 2000a). An NES deletion mutant of Nmd3p accumulates in the nucleus and, if overexpressed, interferes with nuclear export of pre-60S particles. Nmd3p interacts physically and genetically with ribosomal protein L10 (Gadal et al., 2001). Interestingly, RPL10 was found in a genetic screen for ribosomal export mutants (rix mutants) (Gadal et al., 2001). It has been proposed that Rpl10p serves as a landing pad for Nmd3p on nucleoplasmic pre-60S subunits. Proteomic analysis of distinct nucleolar and nucleoplasmic pre-60S particles showed that Nmd3p joins these subunits late during biogenesis and is associated with pre-60S particles believed to represent export-competent species (Nissan et al., 2002). These data are consistent with a model in which Nmd3p associates with export-competent 60S subunits in the nucleus, recruits Crm1p into an export complex, which then facilitates the translocation of the subunit through the NPC (Ho et al., 2000b; Gadal et al., 2001). However, so far it has not been demonstrated that Nmd3p binds to Crm1p directly nor have Crm1p and Ran been found on any of the analyzed 60S or pre-60S particles (Bassler et al., 2001; Harnpicharnchai et al., 2001; Saveanu et al., 2001; Fatica et al., 2002; Nissan et al., 2002).

Neither ribosomal export adaptors nor export receptors have been identified in higher eukaryotes. Thus, we have investigated the potential involvement of the exportin CRM1 in ribosomal subunit export in higher eukaryotes. Our results show that the export of both subunits is dependent on functional CRM1. Further, we cloned the mammalian homologue of Nmd3p (hNMD3) and demonstrate that it contains a conserved NES in its C-terminal region. Importantly, we also show that hNMD3 binds CRM1 in a RanGTP-dependent manner and that the conserved NES in hNMD3 is required for CRM1 binding. Although hNMD3 can associate both with 60S ribosomal subunits and with CRM1, it was unable to recruit CRM1 to purified, cytoplasmic 60S, suggesting that the association of hNMD3 with cytoplasmic and nucleoplasmic 60S subunits might differ mechanistically so as to recruit CRM1 to only the nuclear form of the subunit.

Materials and Methods

Antibodies

A rabbit anti-rpS6 antibody was a kind gift of S. Pinol-Roma (Mount Sinai School of Medicine, New York, USA) and a rabbit anti-rpL23a (Pool et al., 2002) was a gift from M. Pool (ZMBH, Heidelberg, Germany). The rabbit anti-rpL10 antibody was purchased from Santa Cruz (QM C-17) and the mouse anti-RGS•His^{TM} from Qiagen. The mouse anti-digoxigenin antibody was from Jackson Laboratories. Fluorescently labeled secondary antibodies were from Molecular Probes.

Molecular cloning, protein expression and purification

The complete coding regions of human rpS5, rpL29 and human NMD3 (CGI-07 protein, NP_057022), or the various deletion derivatives of hNMD3, were amplified by PCR using HeLa cell cDNA as a template. To express GFP fusions of hNMD3 or C-terminal hNMD3 deletion mutants in HeLa cells, the corresponding PCR fragments were cloned into the *Hin*dIII/*Bam*HI sites of pEGFP-C1 (Clontech) generating pEGFP-hNMD3, pEGFP-hNMD3ΔC27 and pEGFP-hNMD3ΔC71. Site-directed mutagenesis on pEGFP-hNMD3 was performed using the QuikChange® Site-Directed Mutagenesis Kit from Stratagene, generating pEGFP-hNMD3-NESmut containing changes L487A and I489A. The rpS5 and rpL29 PCR fragments were inserted into the *Eco*RI/*Bam*HI sites of pEGFP-N3. For expression of hNMD3 in *E. coli*, PCR fragments were cloned into the *Nco*I/*Bam*HI sites of pQE602z or into the *Bam*HI/*Xma*I sites of pQE30, yielding pQE602z-hNMD3, pQE602z-hNMD3ΔC27 and pQE30-hNMD3.

2z-hNMD3 and 2z-hNMD3ΔC27 were expressed in *E. coli* BLR(pRep4) at 24°C. Cells were lysed by sonication in 50 mM Tris pH 7.5, 500 mM NaCl, 5 mM $MgCl_2$, 5% glycerol and 2 mM β-mercaptoethanol (β-ME). The lysate was cleared by ultracentrifugation, then passed over Ni-NTA agarose (Qiagen) and eluted with 400 mM imidazole in lysis buffer. Peak fractions were pooled and the buffer exchanged to 50 mM Tris pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$. Expression and purification of CRM1 and RanQ69L was performed as previously described (Englmeier et al., 2001).

Transfection, immunolocalization and FISH

HeLa cells were transiently transfected using the FuGene transfection reagent (Roche). For fixation, cells were washed twice with PBS and then incubated in 3.7% paraformaldehyde for 10 minutes. To visualize GFP fusion proteins, coverslips were washed in PBS and mounted. Indirect immunolocalization was performed as previously described (Calado and Carmo-Fonseca, 2000).

For in situ hybridization, cells were fixed/permeabilized with 3.7% formaldehyde and 0.1% TX-100 in 1× PBS. Cells were washed twice in 2× SSC. In case RNase treatment was performed, cells were incubated with 100 μg/ml RNase A for 1 hour at 37°C and subsequently washed three times with 2× SSC. Digoxigenin-labeled probes to rRNAs, generated by nick translation from plasmids containing parts of the mature regions of 18S and 28S rRNA, respectively (Rothblum et al., 1982; Subrahmanyam et al., 1982), were a kind gift of C. Carvalho and M. Carmo-Fonseca (University of Lisbon, Portugal) (Carvalho et al., 2001). Prior to hybridization, cells were washed with 50% deionized formamide, 2× SSC. Hybridization was performed at 37°C for 4 hours, using either 10 ng of DIG-labeled 28S rRNA probe or 100 ng of DIG-labeled 18S rRNA probe in hybridization buffer (50% deionized formamide, 2× SSC, 10% dextran sulfate, 50 mM sodium phosphate buffer, pH 7.0 and 1 μg/μl *E. coli* tRNA). Note that the probes were denatured prior to use for 5 minutes at 70°C. After hybridization, samples were washed three times with 53% deionized formamide, 2× SSC at 45°C, twice with 0.1× SSC at 60°C, twice with 4× SSC at 25°C and finally with 4× SSC, 0.05% Tween. Detection of the DIG probes was done using a mouse anti-DIG antibody followed by a fluorescently labeled anti-mouse antibody. LMB was used at a concentration of 10 ng/ml medium.

In vitro binding to 2z-hNMD3

Purified recombinant 2z-hNMD3 or 2z-hNMD3ΔC27 (40 pmol), CRM1 (50 pmol) and RanQ69L (125 pmol) were incubated together

in a final volume of 100 μl in 50 mM Tris pH 7.5, 150 mM potassium acetate, 2 mM $MgCl_2$, 0.4 mg/ml bovine serum albumin (BSA) for 30 minutes on ice. Then, 20 μl IgG-Sepharose beads (Pharmacia) were added and the samples occasionally mixed. After 20 minutes, beads were washed three times with 1.5 ml of binding buffer and once with buffer 50 mM Tris/HCl pH 7.5, 0.001% TX-100. Bound proteins were eluted from the resin with sample buffer containing 4% sodium dodecyl sulfate (SDS).

Xenopus oocyte injections and rRNA analysis

For labeling of endogenous RNAs, *Xenopus* oocytes were injected with 30 kBq of [α-^{32}P]-GTP. To compete with CRM1-mediated protein export, 10 nl of BSA-peptide conjugates (200 ng of BSA-peptide conjugate per oocyte) were injected into oocyte nuclei using dextran blue as a marker for nuclear injection. The peptides had been coupled to BSA by virtue of an N-terminal cysteine residue and comprised the wild-type NES of PKI (CELALKLAGLDIN) or a mutant form (CELALKAAGADIN).

RNAs were extracted from nuclear and cytoplasmic fractions of 5 oocytes per time point. To ensure efficient recovery of RNA from the nuclear fractions, an uninjected oocyte was added to these samples. After proteinase K treatment, RNA was isolated by phenol extraction and ethanol precipitation. RNAs were dissolved in water, mixed with equal amounts of glyoxal gel sample buffer (Ambion) and denatured for 30 minutes at 50°C. RNAs were separated in a 1% agarose gel in 10 mM sodium phosphate buffer pH 7.0. To control for equal recovery of RNA in each sample, the rRNAs were first visualized by ethidium bromide staining. Gels were then dried onto Zeta Probe membranes (BioRad) and analyzed by phosphoimaging.

Purification of 60S and 40S subunits from HeLa cells

Ribosomal subunits were prepared from pellets of a high-speed centrifugation used for the preparation of HeLa cell extracts (Kutay et al., 2000). The pellets were resuspended in 50 mM Hepes pH 7.5, 300 mM KCl, 5 mM $MgCl_2$ and 6 mM β-ME and spun through a 1.25 M sucrose cushion prepared with the same buffer. The ribosomal pellet was resuspended in high salt buffer (HSB) containing 50 mM Hepes pH 7.5, 600 mM KCl, 10 mM $MgCl_2$ and 5 mM β-ME. The ribosomal subunits were then separated on a linear 12.5-27.5% sucrose gradient in high salt buffer for 19 hours at 80,000 ***g*** in a Beckman SW41 rotor. The gradient was fractionated, the peak fractions containing 40S or 60S subunits were pooled, diluted with equal volume of HSB and sedimented overnight at 540,000 ***g*** in a Beckman TLA 100.4 rotor. The pellets were resuspended in 50 mM Hepes pH 7.5, 100 mM potassium acetate, 5 mM $MgCl_2$ and 250 mM sucrose. The integrity of ribosomal RNA was analyzed and the ratio of absorption at 260 and 280 nm measured to control for the quality of the preparation.

Sedimentation assay

Cytoplasmic 60S ribosomal subunits were incubated for 15 minutes with purified recombinant factors in 140 μl of 50 mM Hepes pH 7.5, 100 mM potassium acetate, 5 mM magnesium acetate and then pelleted by centrifugation at 436,000 ***g*** for 15 minutes in a Beckman TLA 100 rotor. Proteins in the supernatants and ribosomal pellets (resuspended in water) were trichloroacetic acid (TCA) precipitated, washed with acetone and dissolved in 50 μl SDS sample buffer.

Sucrose density gradient

Ribosomal subunits were incubated with 6His-hNMD3 in 40 μl of 50 mM Hepes pH 7.5, 100 mM potassium acetate, 5 mM magnesium acetate**,** 0.4 mg/ml BSA for 15 minutes on ice, diluted to 150 μl and then loaded on top of a 10-40% sucrose gradient. Centrifugation was for 2 hours at 260,000 ***g*** in a Beckman TLS 55 rotor. The gradient was fractionated into 16 samples, protein precipitated with 15% TCA and resuspended in SDS sample buffer. Because of the BSA in the top fractions of the gradient, from fractions 1 to 5 only half the amount of protein relative to fraction 6 to 16 was loaded on a 12% SDS gel.

RanGTPase assay

The RanGTPase assay was performed as described previously (Bischoff et al., 1995; Kutay et al., 1997; Kutay et al., 2000).

Results

Leptomycin B affects the localization of ribosomal subunits in HeLa cells

To test whether mammalian CRM1/exportin 1 plays a role in the biogenesis of ribosomal subunits in higher eukaryotes, we treated HeLa cells with leptomycin B (LMB). LMB is a *Streptomyces* metabolite that inactivates CRM1 by covalent attachment to a cysteine residue in the central region of CRM1 (Kudo et al., 1999). If CRM1 is involved in ribosomal subunit biogenesis and export, then treatment with LMB should affect the steady state localization of 40S and 60S subunits in the cell and lead to a nuclear accumulation of the subunits.

As a reporter for the intracellular distribution of ribosomal subunits, we first expressed GFP fusions of ribosomal proteins. Most of these GFP-ribosomal protein chimeras were not suited for this analysis because they were probably not incorporated into newly made subunits. They gave rise to strong nucleolar signals but did not localize to the cytoplasm even after long periods of expression, indicating a failure in their assembly into ribosomal subunits in the nucleolus or a dominant negative effect on ribosomal biogenesis (data not shown). These proteins included GFP-rpS6, GFP-rpS20 and GFP-rpS23 of the small subunit and GFP-rpL7 and GFP-rpL30a of the large subunit. Two other reporter constructs, rpS5-GFP and rpL29-GFP, were mainly nuclear 16 hours after transfection, but displayed a cytoplasmic staining in addition to a nuclear/nucleolar localization over prolonged expression times of up to 38 hours (Fig. 1A). This time-dependent change in localization suggested that these two proteins were able to incorporate into ribosomal subunits and to leave the nucleolus as part of ribosomal subunits. To investigate the involvement of CRM1 in this relocalization process and potentially ribosomal subunit export, HeLa cells were transferred into LMB containing medium 16 hours after transfection. Five or 18 hours later, cells were fixed and analyzed by fluorescence microscopy (Fig. 1B). LMB treatment resulted in a significant loss of the cytoplasmic GFP signal when compared to control cells, suggesting that the CRM1 pathway might play a role in the export of both reporter proteins and hence in the export of 40S and the 60S ribosomal subunits.

We wished to verify this result by analyzing the effect of LMB on the intracellular distribution of two endogenous ribosomal proteins by indirect immunofluorescence using specific antibodies. RpL23a and rpS6 were chosen as markers for the large and small subunit, respectively, because antibodies directed against the two individual proteins were available and highly specific when tested by immunoblotting (Fig. 2A). Immunolocalization of rpL23a and rpS6 revealed the expected cytoplasmic staining for both proteins. When the cells were

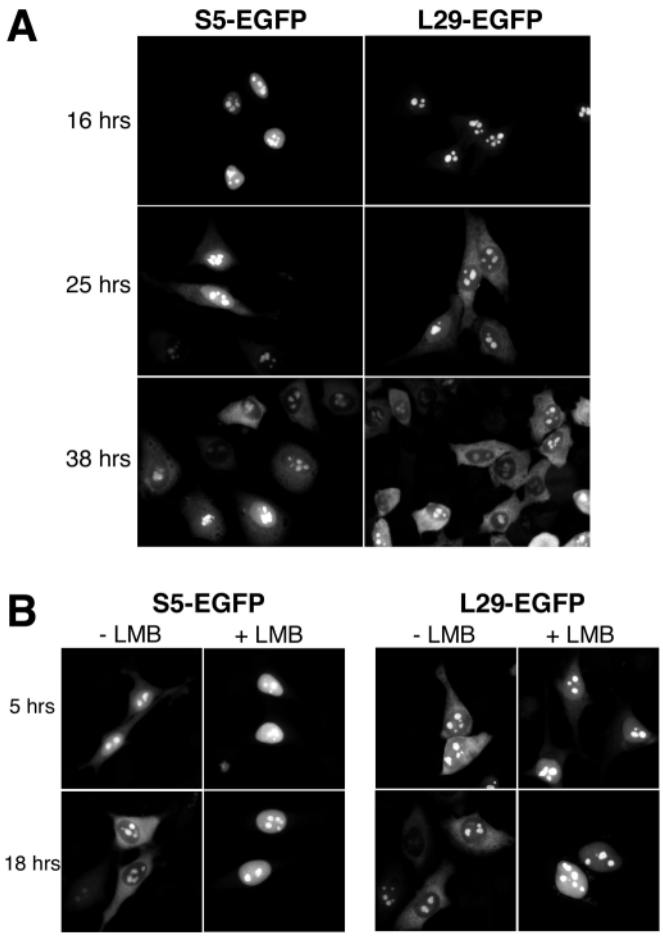

Fig. 1. Leptomycin B (LMB) affects the intracellular localization of rpS5-EGFP and rpL29-EGFP. (A) Localization of rpS5-EGFP and rpL29-EGFP at different times after transfection. HeLa cells were grown on coverslips and transiently transfected with constructs coding for EGFP fusions of rpS5 or rpL29. Cells were fixed with 3.7% paraformaldehyde and further processed for analysis by fluorescence microscopy at 16 hours, 25 hours and 38 hours after transfection, respectively. (B) LMB-sensitive localization of rpS5-EGFP and rpL29-EGFP. HeLa cells were transiently transfected with constructs coding for EGFP fusions of rpS5 and rpL29, respectively. After 16 hours, cells were transferred into fresh medium with or without LMB (10 ng/ml). Five or 18 hours later, cells were fixed and analyzed by fluorescence microscopy.

extracted under harsher conditions using a mixture of acetone/methanol during the fixation/permeabilization step, a faint nucleolar signal was also observed. Upon treatment of HeLa cells with LMB, the intranuclear signal increased significantly for both proteins (Fig. 2B). RpL23a mainly accumulated in the nucleoli of the LMB-treated cells. The staining pattern of rpS6 differed in that the antibody detected the protein mainly in the nucleoplasm and only to some degree in the nucleoli, as revealed upon extraction of the cells with acetone/methanol. The nucleoplasmic staining of rpS6 might either be because of a defect of rpS6 incorporation into pre-40S subunits or reflect a nucleoplasmic accumulation of pre-40S subunits upon inactivation of CRM1. In general, LMB-induced nuclear accumulation of the endogenous ribosomal proteins confirms the foregoing observations made with the GFP-tagged ribosomal proteins.

For both approaches presented so far, we could formally not exclude the possibility that the ribosomal proteins detected in the nucleus may not be part of ribosomal subunits. To directly access the location of both subunits, we next studied the effect of LMB on the intracellular distribution of rRNAs by fluorescent in situ hybridization (FISH) using digoxigenin-labeled oligonucleotides. These probes were directed towards the mature 18S and 28S rRNAs, respectively, and hence suited to detect the ribosomal RNAs during all stages of their biosynthesis. In untreated cells, both ribosomal subunits were detected in the cytoplasmic compartment and to different extent in the nucleoli (Fig. 3). When cells were exposed to LMB, the nucleoplasmic signal detected with the 18S rRNA probe increased, whereas 28S rRNA accumulated mainly in the nucleoli. Again, as for the detection of the endogenous ribosomal proteins, LMB treatment induced a more nucleoplasmic accumulation of the small subunit whereas the large subunit appeared to be deficient in leaving the nucleolus. Taken together, we have demonstrated using three different experimental approaches that the inhibition of the CRM1 export pathway by LMB treatment induces defects in the biogenesis and nuclear export of both ribosomal subunits.

NES peptides compete for the nuclear export of ribosomal subunits

Although our LMB experiments strongly indicated a role for CRM1 in ribosomal subunit export, we wanted to gain more supportive evidence, because we had to treat HeLa cells with LMB for long periods (5 to 18 hours) to observe the nuclear accumulation of ribosomal subunit markers. To investigate nuclear export of newly synthesized ribosomal RNAs, we used an experimental system ideally suited to follow the fate of freshly synthesized ribosomal subunits. In *Xenopus* oocytes, ribosomal RNAs are actively transcribed from a cluster of amplified rRNA genes. These precursor rRNAs can be metabolically labeled by injection of the oocytes with [α-^{32}P]-GTP and their processing to the mature rRNAs analyzed upon extraction of the rRNAs. Specific rRNA intermediates are characteristic for these processing reactions (Fig. 4A). Ribosomal subunit export can be monitored upon manual dissection of the oocytes into a cytoplasmic and nuclear fraction and subsequent analysis of the distribution of newly synthesized 18S and 28S rRNAs (Fig. 4B). 18S rRNA started to accumulate in the cytoplasm three hours after injection of the label. 28S rRNA appeared in the cytoplasmic fraction approximately four hours later, indicating that the maturation of the large subunit takes longer than that of the small subunit. In addition, several processing intermediates could be observed. Only the mature rRNAs were exported to the cytoplasm.

To analyze the involvement of CRM1 in the nuclear export of the ribosomal subunits in the oocytes, we tested the effect of competing concentrations of NES peptides on the cytoplasmic delivery of the newly synthesized ribosomal subunits. Peptides comprising the NES of protein kinase A inhibitor (PKI), known as potent competitors of CRM1-mediated NES protein export (Fischer et al., 1995; Fornerod et al., 1997; Pasquinelli et al., 1997), were coupled to BSA. These

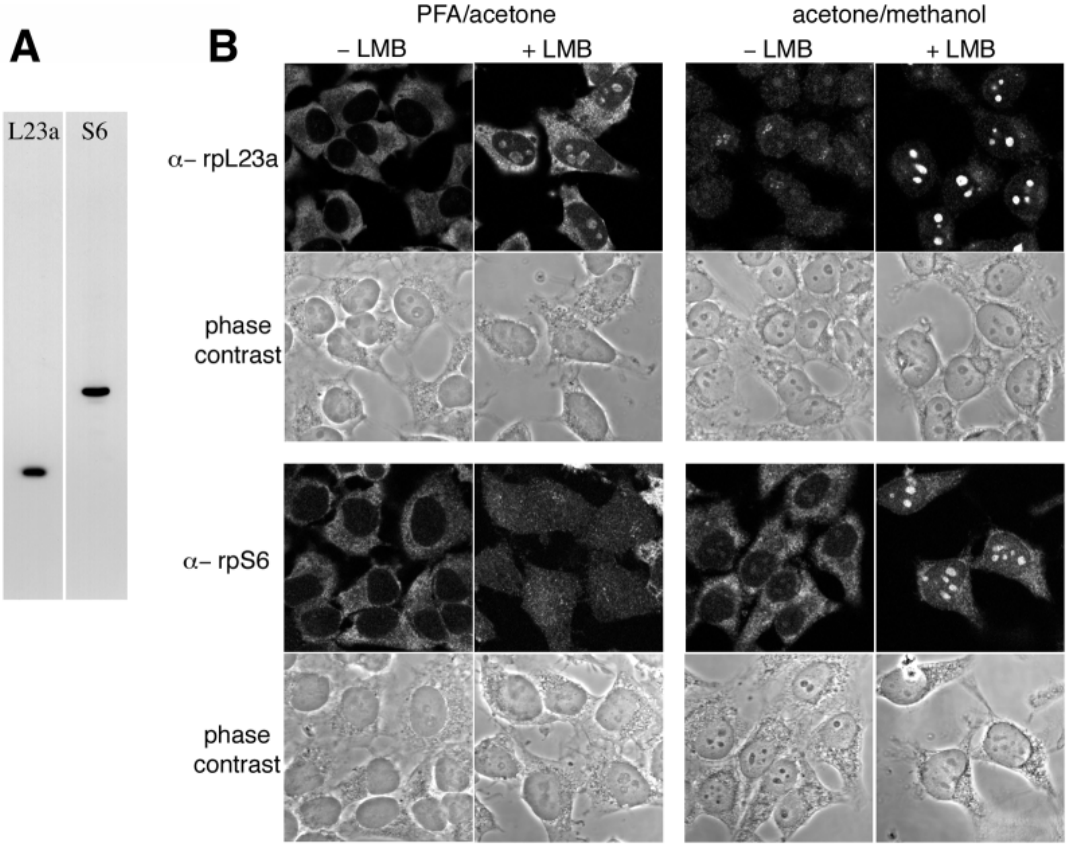

Fig. 2. Leptomycin B (LMB)-sensitive localization of endogenous rpS6 and rpL23a. (A) Specificity of the anti-L23a and anti-S6 antibodies. Total HeLa cell proteins were separated by 8% SDS-PAGE followed by immunoblotting using anti-L23a and anti-S6 antibodies. Note that both antibodies are highly specific and detect a single protein band. (B) HeLa cells were either left untreated or treated with LMB for 18 hours. Then, cells were fixed/permeabilized with either 3.7% paraformaldehyde (PFA)/acetone or with acetone/methanol. The localization of rpL23a or rpS6 was analyzed by indirect immunofluorescence using anti-L23a and anti-S6 antibodies, respectively. Images were taken by confocal fluorescence microscopy.

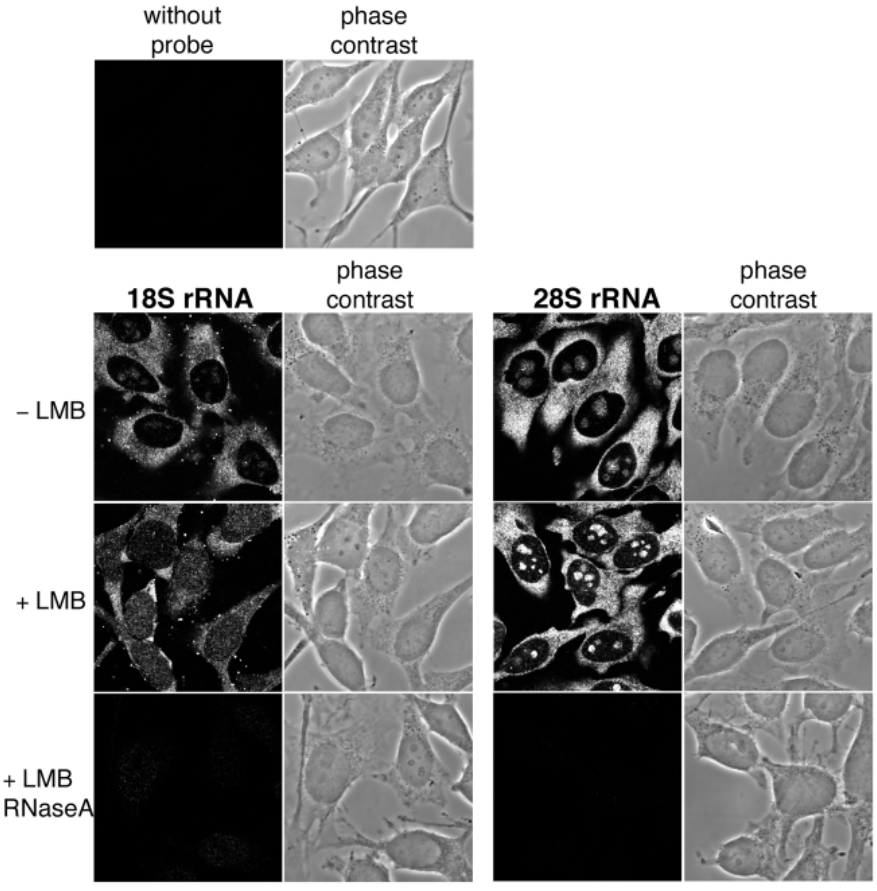

Fig. 3. Nuclear accumulation of rRNAs upon leptomycin B (LMB) treatment of HeLa cells. Localization of rRNAs was analyzed in untreated HeLa cells or cells that had been treated with LMB for 18 hours by fluorescent in situ hybridization (FISH) using digoxigenin-labeled oligonucleotides directed to 18S and 28S rRNA, respectively. To control for the specificity of the detection, cells were either treated with RNaseA before FISH analysis or the probes were omitted (upper panels). Images were taken by confocal fluorescence microscopy.

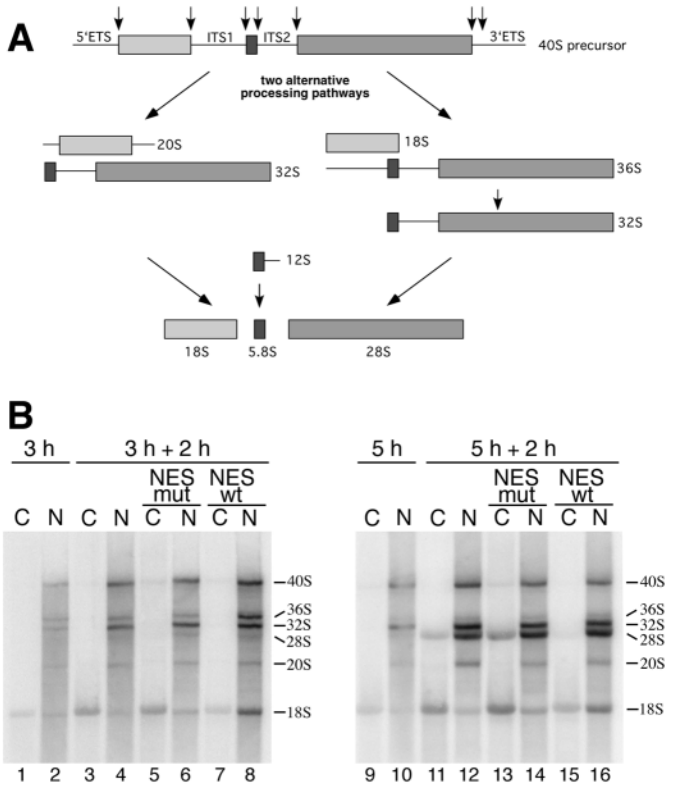

Fig. 4. Nuclear export of ribosomal subunits is competed by NES peptides. (A) Scheme illustrating the processing of pre-rRNA along two co-existing pathways in *Xenopus* oocytes (modified according to Peculis, 1997) (Peculis, 1997). Cleavages occur in an orderly fashion at numerous sites indicated by arrows. This leads to the removal of externally and internally transcribed spacers (ETS, ITS) to yield mature 5.8S, 18S and 28S rRNA. (B) Competition of the CRM1-mediated NES export pathway interferes with nuclear exit of both ribosomal subunits. *Xenopus* oocytes were injected with [α-^{32}P]GTP. After 3 or 5 hours, PKI-NES wild-type or mutant peptides conjugated to BSA were injected into the nucleus. At the indicated times, RNA was isolated from nuclear (N) or cytoplasmic (C) fractions, separated by agarose gel electrophoresis and analyzed first by phosphoimaging for quantification and thereafter by autoradiography.

conjugates were injected into the oocyte nuclei 3 or 5 hours after application of the radioactive label. Two hours later, rRNA was isolated from nuclear and cytoplasmic fractions. The nuclear export of both 18S rRNA and 28S rRNA was efficiently competed by nuclear injection of BSA-NES (NESwt, Fig. 4B, lanes 7, 8, 15 and 16). Injection of a peptide, in which two of the critical leucine residues of the NES were mutated to alanines (NESmut), did not affect export of the ribosomal subunits, demonstrating the specificity of the competition with the wild-type NES peptides (compare lanes 3 to 6 with 7 and 8 as well as lanes 11 to 14 with 15 and 16). In the presence of the BSA-NESwt competitor, the nuclear export of the rRNAs was drastically reduced such that there was no significant increase in the cytoplasmic signal whereas the nuclear signal of the mature rRNAs was enhanced in comparison to the control injections. Quantification of the data revealed that after 5 hours in the absence of competitor, approximately 70% of the newly synthesized 18S rRNA had been exported. In the presence of competitor during the last two hours of the experiment, export was reduced to 28%. If export was analyzed 7 hours after labeling, 85% of 18S rRNA and 32% of 28S rRNA had reached the cytoplasm. The two hours competition with NES peptides reduced export of 18S rRNA and 28S rRNA to 38% and 4%, respectively. Taken together, our results show that nuclear export of 40S and 60S subunits requires the CRM1 export pathway.

To exclude a toxic, non-specific effect of the injected peptides on nuclear export, we performed an additional control experiment. The NES competitor should not affect export of a substrate that is exported by a different transport receptor. We selected importin α (Imp α) as a model protein, which is exported by the exportin CAS (Kutay et al., 1997). A mixture of in vitro translated, radiolabeled Imp α, GST-NES and GST-GST, which served as an injection control, was injected into the nuclei of *Xenopus* oocytes (see supplementary Fig. 1: http://jcs.biologists.org/supplemental). After 2 hours, the GST-NES fusion protein had been exported out of the nucleus and also approximately 50% of Imp α was found in the cytoplasmic fraction, probably reflecting its steady state distribution in the oocytes (Izaurralde et al., 1997). Coinjection of the NES competitor reduced GST-NES export but did not affect export of Imp α.

Human NMD3 possesses a functional leucine-rich NES

As is the case for CRM1-mediated export of other RNPs, the CRM1 dependence of ribosomal subunit export suggested the involvement of one or more NES-containing adaptor proteins in this export pathway. Yeast Nmd3p had been proposed to serve such a function for pre-60S subunits (Ho et al., 2000b; Gadal et al., 2001). Proteins homologous to *S.c.*Nmd3p exist not only in other fungi and metazoans but also in archaebacteria, suggesting that Nmd3p might play a primary role in ribosomal function unrelated to nuclear export. However, Nmd3p in yeast is a shuttling protein that has acquired an additional C-terminal domain containing a conserved leucine-rich NES and a potential nuclear localization signal (NLS) (Fig. 5A). This domain is found in NMD3 from all eukaryotes.

To analyze whether the C-terminal NES-like sequence present in human NMD3 (hNMD3) is a functional export signal recognized by CRM1, we first determined whether the intracellular localization of hNMD3 is affected by LMB. A GFP-hNMD3 fusion protein was expressed in transiently transfected HeLa cells and found to be localized primarily to the cytoplasm (Fig. 5B). In addition, some nuclear signal excluded from the nucleoli could be detected. LMB treatment of HeLa cells for 2 hours (data not shown) or 4 hours caused relocalization of the chimeric protein such that it was almost exclusively in the nucleoplasm. Mutant forms of hNMD3 lacking the last 27 or 71 amino acids containing the NES sequence, GFP-hNMD3ΔC27 and GFP-hNMD3ΔC71, were already mainly nuclear in the absence of LMB and did not change localization upon LMB treatment. Interestingly, GFP-hNMD3ΔC71 was also found in the nucleoli. When two leucine residues of the potential C-terminal NES in the full length protein were changed to alanines, the GFP fusion protein was predominantly nuclear and insensitive to LMB

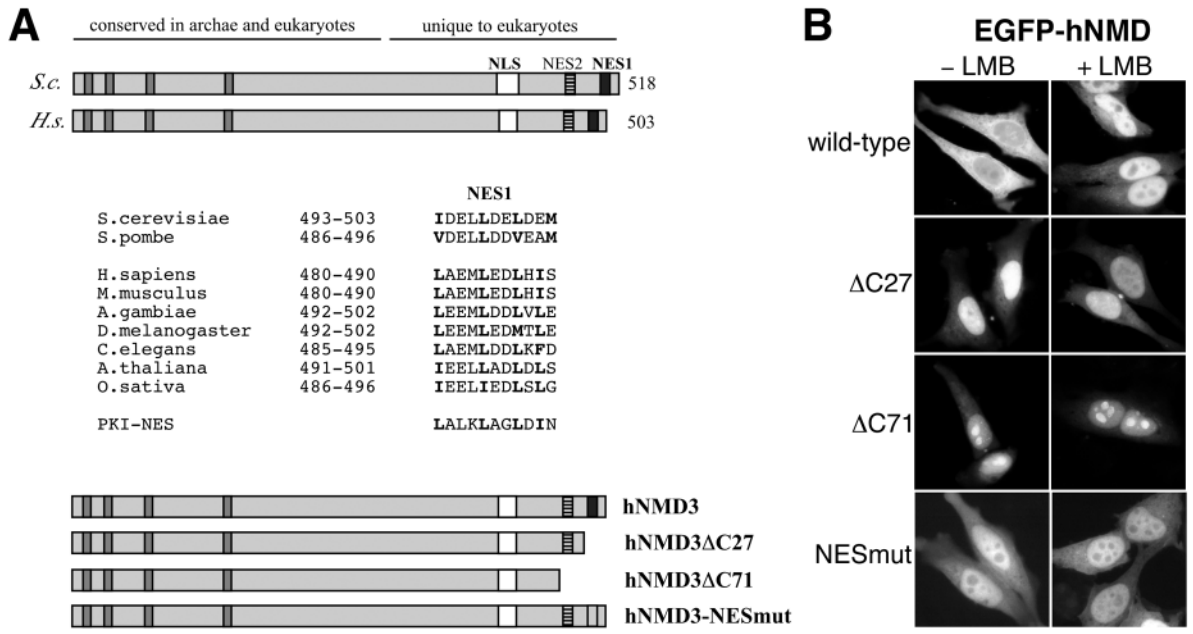

Fig. 5. hNMD3 possesses a conserved, C-terminal NES sequence. (A) Scheme illustrating the position of sequence motifs in NMD3 from different species and constructs used for transient expression. NMD3 proteins from all species possess an N-terminal conserved domain containing 4 putative zinc fingers (gray boxes). NMD3s in eukaryotes have acquired a C-terminal domain harboring the NLS (white box) and two potential C-terminal NES sequences, according to Ho et al. and Gadal et al. (Ho et al., 2000b; Gadal et al., 2001). Deletion of NES1 (black box) in *S.c.Nmd3p* dramatically affects cell growth and leads to a nuclear localization of the protein, whereas the potential NES2 (striped box) does show some deviation from the NES consensus sequence but in conjunction with NES2 is needed for viability of yeast (Gadal et al., 2001). The NES1 sequence is conserved throughout higher eukaryotes. Conserved residues are highlighted in bold. For comparison, the PKI NES is shown. Accession numbers of the different NMD3 proteins are the following: *S.c.* P38861, *S.p.* Q09817, *H.s.* NP_057022, *M.m.* NP_598548, *A.g.* EAA11292, *D.m.* CAB42049, *C.e.* CAA96689, *A.t.* AAL07089 and *O.s.* AAK00432. Full-length wild-type hNMD3, C-terminal deletion mutants lacking NES1 (hNMD3ΔC27), or both NESs (hNMD3ΔC71), and an NES mutant (hNMD3-NESmut), in which L487 and I489 were changed to alanines, are depicted. (B) hNMD3 contains an NES sequence in the last 20 amino acids. GFP fusions of the different constructs as presented in A were expressed in transiently transfected HeLa cells. Eighteen hours post-transfection, the cells were transferred into fresh medium without or with leptomycin B (LMB) and fixed after 4 hours. The intracellular distribution of the individual proteins was determined by fluorescence microscopy.

exposure of the cells. These results suggested that hNMD3 is exported from the nucleus via interaction of CRM1 with the C-terminal NES in hNMD3.

Recombinant hNMD3 binds to 60S ribosomal subunits and can directly interact with CRM1

To investigate the biochemical properties of the potential export adaptor hNMD3, we expressed 6His-hNMD3 in *E. coli*. The purified protein was first tested for binding to 60S subunits, using a co-sedimentation assay. Increasing amounts of 6His-hNMD3 were incubated with purified cytoplasmic 60S subunits. Then, ribosomal subunits and bound hNMD3 were separated from unbound protein by a short ultracentrifugation. In the absence of 60S subunits, all of the 6His-hNMD3 was soluble and retrieved in the supernatant (Fig. 6A, lanes 1 and 2). In the presence of 60S ribosomal subunits, hNMD3 co-sedimented and this association was saturable suggesting that binding might occur at specific interaction site(s). Control experiments showed that there was no binding of unrelated factors, such as snurportin (Huber et al., 1998), to 60S subunits (data not shown). When we tested hNMD3 binding to 40S subunits, as expected, the majority of hNMD3 stayed in the supernatant although a minor portion was recovered in the 40S pellet (data not shown). However, in vivo, yeast Nmd3p exclusively binds to 60S subunits (Ho and Johnson, 1999; Ho et al., 2000a). To confirm that recombinant hNMD3 preferentially binds to 60S subunits in vitro, we incubated hNMD3 with a mixture of equal amounts of 40S and 60S subunits. After separation of the subunits on a sucrose density gradient, hNMD3 was found on top of the gradient representing the pool of unbound hNMD3 and, in addition, in the fractions containing 60S subunits but not in the part of the gradient containing 40S subunits (Fig. 6B). Taken together, similar to what has been reported for yeast Nmd3p, human recombinant NMD3 binds to cytoplasmic 60S subunits. A differentially tagged version of hNMD3, 2z-hNMD3 containing two N-terminal IgG-binding domains (z-tag) derived from protein A, also bound to 60S subunits in a saturable manner although with slightly reduced efficiency (data not shown and supplementary Fig. 2 at jcs.biologists.org/supplemental). In summary, recombinant hNMD3 fulfilled one expectation of an export adaptor, namely binding to 60S ribosomal subunits.

*S.c.*Nmd3p has been suggested to provide a link between the 60S preribosomal subunits and the exportin CRM1. It has, however, never been demonstrated that NMD3 and CRM1 interact directly. Thus, we next performed in vitro binding experiments using purified 2z-hNMD3 and CRM1. As presented in Fig. 7A, 2z-hNMD3 could directly bind CRM1

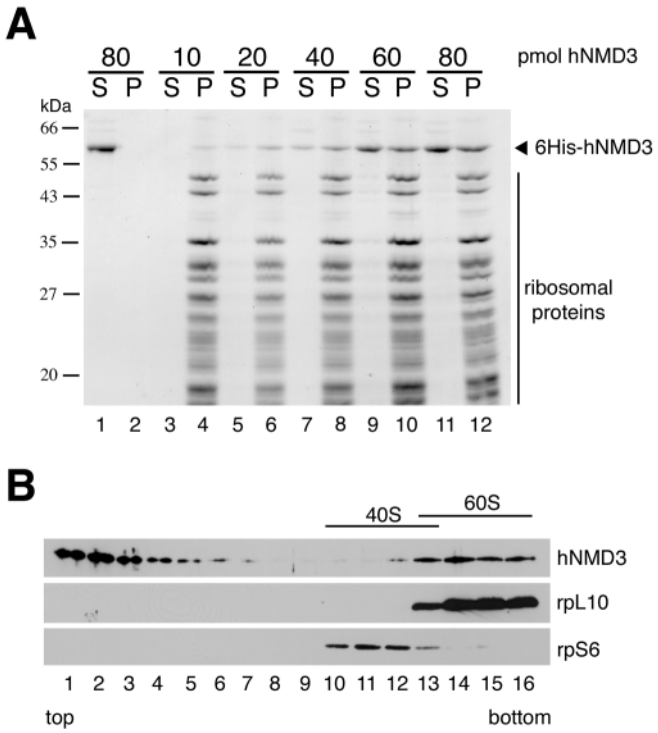

Fig. 6. hNMD3 binds to 60S subunits in vitro. (A) Saturable binding of recombinant hNMD3 to 60S ribosomal subunits. Twenty-five pmol of purified HeLa cell 60S subunits (lanes 3 to 12) were incubated with increasing amounts of 6His-hNMD3 (10, 20, 40, 60 or 80 pmol). Ribosomal subunits and associated hNMD3 were pelleted by sedimentation. The proteins in the pellet (P) and supernatant (S) were separated by 10% SDS-PAGE followed by Coomassie blue staining. Load of proteins in the unbound fractions (supernatants) equals the load of the ribosomal pellet fractions. Note that there is no sedimentation of 6His-hNMD3 in the absence of 60S subunits (lanes 1 and 2). (B) hNMD3 cosediments with 60S subunits. A mixture of each 25 pmol purified 40S and 60S subunits was incubated with 60 pmol of 6His-hNMD3 and then fractionated on a 10-40% sucrose density gradient. Fractions from the gradient were analyzed by Western blotting using antibodies directed to rpL10 and rpS6 as markers for the migration of 60S and 40S subunits, respectively. Detection of hNMD3 was by an antibody that recognizes the N-terminal 6His-tag of recombinant hNMD3. Note that hNMD3 comigrates with 60S subunits and that, in addition, it is found on the top of the gradient as free protein but not in association with 40S subunits.

and recruit the exportin into the IgG-bound fraction in a pull-down assay. Complex formation was dependent on the presence of RanGTP (compare lanes 3 and 4), as expected for a bona fide exportin-cargo interaction. Significantly, the 2z-hNMD3ΔC27 mutant protein, lacking the C-terminal leucine-rich NES sequence, bound CRM1 at background levels and binding was not further stimulated by the presence of RanGTP (compare lanes 6 and 7 with lane 1).

The binding of RanGTP and export substrates to exportins is often co-operative (Fornerod et al., 1997; Kutay et al., 1997). Hence, RanGTP should stimulate the binding of hNMD3 to CRM1 and hNMD3 should in turn enhance the interaction between CRM1 and RanGTP. Quantitative measurement of complex formation between RanGTP-binding transport receptors and RanGTP can be performed using the RanGTPase assay. The assay measures protection of GTP on Ran from RanGAP-induced GTP hydrolysis when RanGTP is bound to a nuclear transport receptor (Floer and Blobel, 1996; Görlich et al., 1996). Most exportins have a low affinity for RanGTP and can only efficiently bind and protect RanGTP against RanGAP-induced GTP hydrolysis when the export substrate is also present (Kutay et al., 1997; Kutay et al., 1998; Paraskeva et al., 1999). We observed the same effect for 2z-hNMD3 on the CRM1-RanGTP interaction. Incubation of RanGTP with high concentrations of CRM1 alone did not protect Ran-bound GTP from RanGAP-induced GTP hydrolysis (Fig. 7B, open triangles). However, in the presence of 2 μM 2z-hNMD3, increasing concentrations of CRM1 led to a progressive inhibition of RanGAP-induced GTP hydrolysis (Fig. 7B, stars). Half-maximal inhibition was observed at a CRM1 concentration of approximately 30 nM. Thus, 2z-hNMD3 is able to form a stable complex with RanGTP and CRM1. In agreement with the data from the pull-down experiments, 2z-hNMD3ΔC27 was unable to bind to CRM1 and could not force CRM1 into a protective complex with RanGTP (Fig. 7B, squares). Surprisingly, we found that CRM1 bound to 6His-hNMD3 only weakly, showing a half-maximal inhibition at approximately 600 nM CRM1. The association of 6His-hNMD3 with CRM1 was approximately a factor of 20 weaker compared to the 2z-tagged version of the protein. This difference might be explained by distinct folding properties of the differentially tagged proteins and account for the previous failure of others to demonstrate a Crm1p-Nmd3p interaction. In summary, hNMD3 contains a functional C-terminal NES that directly interacts with CRM1 in a manner consistent with other exportin-cargo interactions.

Discussion

We have demonstrated using three different experimental approaches that the inactivation of the exportin CRM1 in HeLa cells leads to a nuclear accumulation of ribosomal subunit markers. Both, ribosomal proteins, as revealed by localization of transiently expressed GFP-fusion proteins and immunostaining of endogenous ribosomal proteins, and rRNA, as judged from FISH experiments, were affected in their steady-state localization upon treatment of cells with leptomycin B (Figs 1-3). The analysis of ribosomal marker proteins alone might not directly reflect the localization of ribosomal subunits, because the signal could also be derived from a pool of unassembled protein. However, in conjunction with the rRNA localization, it is possible to draw conclusions for the fate of the preribosomal subunits. Extrapolating from the localization of endogenous rRNAs and ribosomal proteins to ribosomal subunits, LMB treatment led to an accumulation of pre-40S subunits both in the nucleoplasm and the nucleoli of HeLa cells, whereas precursors to the 60S subunits accumulated mainly in the nucleoli. Apparently, inactivation of CRM1 influences the biogenesis and nuclear export of 40S and 60S subunits differentially, because production of 60S subunits was more affected at the nucleolar stage than was production of 40S subunits. It is presently unclear, whether the exit of pre-60S subunits from the nucleolus requires CRM1 directly. Alternatively, the interference with CRM1-mediated nuclear export may cause indirect effects on earlier events in the 60S biogenesis pathway.

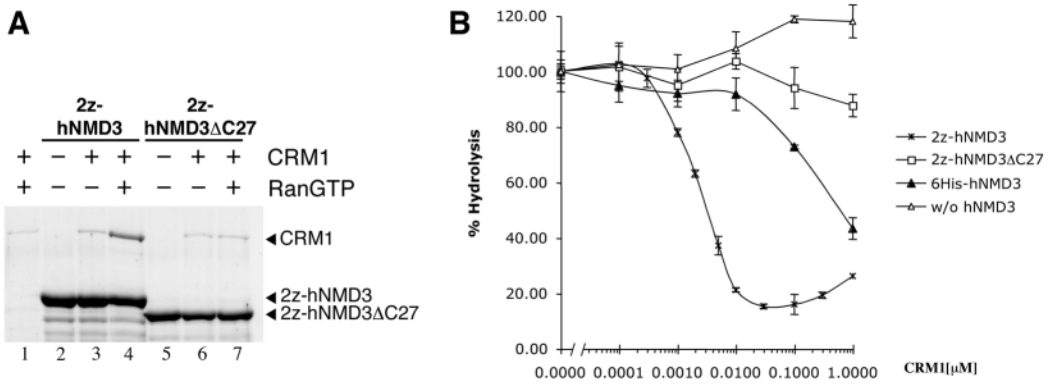

Fig. 7. hNMD3 can engage into a complex with CRM1/RanGTP. (A) RanGTP stimulates the direct binding of CRM1 to NMD3. Recombinant 2z-hNMD3 FL (lanes 2 to 4) or 2z-hNMD3ΔC27 (lanes 5 to 7) were incubated with recombinant CRM1 in the absence or presence of RanQ69L(GTP). 2z-hNMD3 associated factors were retrieved from the reaction mixtures by IgG-Sepharose. Bound proteins were separated by SDS-PAGE and detected by Coomassie blue staining. The amount of unspecific binding of CRM1 in the presence of RanGTP to IgG-Sepharose was also determined (lane 1). (B) 2z-hNMD3 forces CRM1 into a complex with RanGTP. Apparent dissociation constants of complexes between RanGTP and CRM1 were estimated in the presence of either 2 μM 2z-hNMD3, 2z-hNMD3ΔC27 or 6His-hNMD3 using the RanGTPase assay. Note that only 2z-hNMD3 and only to some extent 6His-hNMD3, but not 2z-hNMD3ΔC27, are able to force CRM1 into complex with Ran[γ-^{32}P]GTP. The complex formation of RanGTP and CRM1 protects the GTP on Ran from RanGAP-induced GTP hydrolysis.

More direct evidence that CRM1 is needed for nuclear export of pre-40S and pre-60S subunits comes from our studies on ribosomal biogenesis in *Xenopus* oocytes (Fig. 4). Competition experiments with NES peptides allowed us to monitor the fate of mature nuclear rRNAs and to directly investigate the role of CRM1 in their export. The nuclear injection of the NES peptide-conjugates affected rRNA processing only slightly but at the same time efficiently reduced the export of the mature rRNAs, demonstrating that CRM1 is most probably required at a step following assembly of the subunits, namely for the cytoplasmic delivery of the subunits. Our data indicate that ribosomal subunit biogenesis and export in vertebrates are dependent on a functional CRM1 export pathway similar to what has been observed in *S. cerevisiae*. Yeast cells bearing a LMB-sensitive version of Crm1p accumulate pre-40S in the nucleoplasm rapidly upon treatment with the drug. Furthermore, nuclear export of pre-40S subunits is impaired in a yeast strain harboring a deletion of YRB2 or by Yrb2p overexpression (Moy and Silver, 2002). Yrb2p is a co-factor required for the efficient export of NES substrates. Although the export of pre-60S subunits is dependent on the CRM1 export pathway, it is not affected by a YRB2 deletion (Ho et al., 2000b; Gadal et al., 2001; Moy and Silver, 2002).

The crucial, yet unresolved question is whether CRM1 mediates nuclear export of pre-ribosomal subunits by associating directly with the subunits or with ribosomal export adaptor proteins. Currently, no information is available as to how, or if, Crm1p binds to pre-40S. However, based on several findings, it has been proposed that yeast Nmd3p serves as an export adaptor for CRM1 in 60S subunit export (see Introduction). As demonstrated here, human NMD3 also possesses features of a 60S export adaptor. hNMD3 behaves like a nucleo-cytoplasmic shuttling protein. It is localized to both the cytoplasm and the nucleus in HeLa cells and cytoplasmic residence depends on a conserved C-terminal leucine-rich export signal. Inactivation of CRM1 leads to a rapid nuclear accumulation of GFP-hNMD3 (Fig. 5). In addition, expression of mutant forms of NMD3 lacking the C-terminal NES causes a dominant negative inhibition of 60S subunit export in *Xenopus* oocytes (Trotta et al., 2003).

However, several points must be addressed to unambiguously prove the suggested role of hNMD3 or *S.c.*Nmd3p as export adaptor for 60S subunits. First, a direct interaction between CRM1 and NMD3 needs to be demonstrated. Here, we have shown for the first time, using pull-down and RanGAP protection assays, that human NMD3 can form a stable complex with CRM1 in the presence of RanGTP (Fig. 7). The C-terminal NES of hNMD3 is required for this interaction. Surprisingly, we found that differentially tagged hNMD3 proteins showed differences in the strength of their interaction with CRM1. Although 2z-hNMD3 bound CRM1 strongly, 6His-hNMD3 displayed only a weak interaction. This difference might be because of differences in the folding and, hence, accessibility of the NES in the differentially tagged proteins. The NES in 6His-hNMD3 appears to be hidden. An interesting possibility would be that 6His-hNMD3 mimics the conformation of endogenous hNMD3. It is tempting to speculate that endogenous hNMD3 undergoes a conformational change to expose its NES upon binding to a partner in the nucleus. This switch would prevent futile cycles of hNMD3 shuttling. According to the simplest model, this nuclear binding partner would be pre-60S ribosomal subunits.

Second, it has not been demonstrated that Nmd3p can recruit Crm1p onto pre-60S subunits. As a first step in this direction, we tested the hNMD3-dependent binding of CRM1 to cytoplasmic 60S subunits (see supplementary data: http://jcs.biologists.org/supplemental). However, neither 6His-hNMD3 nor 2z-hNMD3 supported the association of CRM1 with the subunits. The complex might be too unstable to detect this interaction, although the timespan of the experiment did

not exceed the one used to show CRM1/2z-hNMD3 complex formation in the pull-down experiment (Fig. 7A). Alternatively, although *S.c.*Nmd3p is mainly found associated with cytoplasmic 60S subunits, these subunits might differ in some way from export competent, nuclear pre-60S subunits, so that NMD3 can recruit CRM1 only to the nuclear form of the subunit. We have been unable to isolate nuclear pre-60S subunits from vertebrate cells in sufficient quantities and homogeneity to use them as a tool for testing NMD3-dependent recruitment of CRM1. It should be noted that also in yeast, neither Crm1p nor Ran has been detected on supposedly nucleoplasmic pre-60S subunits whereas Nmd3p was present (Nissan et al., 2002). This may indicate that the association of CRM1 with these subunits is too transient to detect or that the complex does not resist the particular purification procedure that was used.

Finally, the formal possibility exists that NMD3 does not directly contribute to 60S subunit export. For example, NMD3 could be an inhibitor of 60S subunit export, which might have to be kept out of the nucleus to allow the efficient pre-60S export. It has been argued that Nmd3p is unlikely to be such an inhibitor of 60S subunit export, because overexpression of wild-type Nmd3p does not impair 60S subunit export in yeast (Johnson et al., 2002). Alternatively, Nmd3p might mediate the export of an unidentified inhibitor of 60S biogenesis. If Nmd3p would contribute to the export of an inhibitory factor, then overexpression of Nmd3p would not be expected to be inhibitory. But still, the most compelling argument in favor of a direct role of Nmd3p in 60S subunit export is its association with nuclear pre-60S subunits in yeast (Nissan et al., 2002).

In summary, we have demonstrated that nuclear export of both the small and large ribosomal subunit in vertebrate cells requires a functional CRM1 export pathway. It remains to be investigated in future, how CRM1 is recruited to pre-ribosomal subunits in the nucleus. For pre-60S subunits, a candidate export adaptor is hNMD3. Both in yeast and vertebrates, the identity of a potential export adaptor for 40S pre-ribosomal subunits is still entirely open.

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