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The Specificity of the CRM1-Rev Nuclear Export Signal Interaction Is Mediated by RanGTP*

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Nuclear export of intron-containing human immunodeficiency virus type 1 (HIV-1) RNA is mediated by the viral Rev protein that contains both an RNA binding domain specific for the viral Rev response element (RRE) and a nuclear export signal (NES). The cellular CRM1 (Exportin1) protein functions as a nuclear export receptor for proteins carrying a Rev-like NES in a process that also requires the GTP bound form of the Ran GTPase. Using purified recombinant factors, we show by co-precipitation, gel mobility shift and protein footprinting assays that full-length Rev protein interacts directly with CRM1 *in vitro* independently of both the integrity of the characteristic leucine residues of the NES and the presence of the cytotoxin leptomycin B (LMB). Addition of RanGTP induces the formation of an RRE-Rev-CRM1-RanGTP complex that is sensitive to LMB, NES mutations, and Ran being charged with GTP. Within this complex, CRM1 is readily cross-linked to Cys^{89} near the NES of Rev. By protein footprinting, we demonstrate that the NES of Rev and two regions in CRM1 become inaccessible to endoproteinases upon binding suggesting that these regions are involved in protein-protein interactions. Our data are consistent with a model in which CRM1 is the nuclear export receptor for the Rev-RRE ribonucleoprotein complex and that RanGTP binds to a preformed Rev-CRM1 complex and specifies a functional interaction with the NES.

Human immunodeficiency virus type 1 (HIV-1)[1] encodes the regulatory protein Rev, which is absolutely required for viral replication. Rev promotes the nuclear export of incompletely spliced HIV-1 mRNA species, which are specified by the presence of a complex RNA structure, the Rev response element (RRE). Rev interacts directly with a purine-rich stem-loop called IIB within the RRE, and through oligomerization additional Rev molecules bind to lower affinity sites throughout the RRE (reviewed in Ref. 1). Rev is a 116-amino acids protein that has been shown to continuously shuttle between the nucleus and the cytoplasm (2, 3). Nuclear import of Rev is mediated by an arginine-rich nuclear localization signal (NLS) embedded in the RNA binding domain of the Rev protein (amino acids 34–50) (4–6). The NLS of Rev has been reported to associate with importin β, the large subunit of the conventional importin α/importin β NLS receptor heterodimer, as well as with B23, a mammalian protein involved in the nuclear import of ribosomal proteins (7–9). Nuclear export of Rev is mediated by the nuclear export signal (NES), which contains a conserved stretch of characteristically spaced leucine residues (10, 11). Mutation of any of three leucines (Leu^{78}, Leu^{81}, or Leu^{83}) in the NES-core domain generates nonfunctional Rev proteins that loose their ability to exit the nucleus (2–4,12–16). The fact that these mutants remain active for specific binding and oligomerization on the RRE *in vitro,* has prompted the suggestion that they are unable to interact with a functionally important cellular export factor(s) (4). This model has gained support by the observation that peptides comprising a wild type Rev NES allow export of larger reporter proteins otherwise restricted to the nucleus, whereas nuclear proteins conjugated to peptides containing appropriate NES mutations remain in the nucleus (10, 11). The list of proteins containing Rev NES-like sequences is rapidly growing and includes other viral proteins with functions similar to Rev as well as several yeast and vertebrate proteins putatively involved in nucleocytoplasmic trafficking of RNA and protein (for recent reviews see Refs. 17 and 18).

Most import and export processes studied so far require the Ran GTPase protein (for review see Ref. 17 and 18). Like other GTPases, Ran cycles between two forms, RanGTP, and RanGDP, and requires effectors that modulate its nucleotide bound state. The low intrinsic GTPase activity of Ran is stimulated by the Ran GTPase-activating protein, RanGAP1, an effect further potentiated by the Ran-binding protein 1, RanBP1 (19–22). Conversely, the replacement of GDP with GTP is stimulated by the nucleotide exchange factor RCC1 (23, 24). Because the bulk of RanGAP1 in the cell is cytoplasmic, whereas RCC1 is predominantly nuclear, it is predicted that cytoplasmic Ran exists primarily in the GDP bound state, and nuclear Ran is mainly on the GTP form. This asymmetric distribution of the two forms of Ran has been shown to be essential for import and export of several classes of RNA and proteins across the nuclear membrane (25–27). The protein import process is so far best characterized. In the cytoplasm, the cargo to be imported is recognized either directly by the corresponding import receptor (28, 29) or via importin α as an adaptor (30). After translocation through the nuclear pore complex into the nucleus, RanGTP triggers the dissociation of the

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus type-1; NES, nuclear export signal; LMB, leptomycin B; RRE, Rev response element; NLS, nuclear localization signal; HMK, heart muscle kinase; MAPKK, mitogen-activated protein kinase kinase; PCR, polymerase chain reaction; BSA, bovine serum albumin.

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import complex and releases the NLS cargo to the nucleoplasm (25, 26). It has been suggested that an opposite situation exists in export where nuclear RanGTP promotes assembly and docking of the export complex to the nuclear pore complex, and concomitant hydrolysis of RanGTP to RanGDP on the cytoplasmic side would cause release of the export substrate (17, 18).

Recently, several papers identified the highly conserved CRM1 (chromosome region maintenance 1) protein as a functional nuclear receptor for Rev-like NESs and hence the protein was also renamed Exportin1 (31–35). One line of evidence came from yeast genetic data. When grown at the nonpermissive temperature, nuclear export of NES-containing marker proteins was disrupted in yeast strains containing conditional *crm1* mutants (32, 34). Independent experiments in *Xenopus* oocytes showed that overexpression of human CRM1 increased the export of nuclear-injected Rev protein (31). Moreover, the cytotoxin leptomycin B (LMB) inhibited export of Rev protein in both mammalian cells (36) and in *Xenopus* oocytes (31). This effect by LMB is probably direct because LMB binds to *in vitro* translated CRM1 (31), and, in *Schizosaccharomyces pombe*, resistance to LMB maps to the *crm1* gene (37). Further support for CRM1 being an export receptor for NES-containing proteins is based on the observation that the N-terminal region of CRM1 is homologous to the RanGTP binding domain of importin β, placing CRM1 in the family of RanGTP-binding proteins that includes other known and putative import and export receptors (38, 39). CRM1 has also been found in complex with at least two proteins associated with the human nuclear pore complex, namely the nucleoporins CAN/Nup214 and Nup88 (39), and two hybrid assays in *Saccharomyces cerevisiae* have demonstrated interactions between CRM1 and several nucleoporins as well as Rev and Ran (34, 35).

The molecular details behind CRM1-mediated Rev export are still largely unknown. It has been reported that NES peptides originating either from the IkappaBα protein (IκBα), or the mitogen-activated protein kinase kinase (MAPKK), interact with CRM1 prepared either from a reticulocyte lysate or *Xenopus* oocyte extracts, respectively (32, 33). Moreover, direct binding of the MAPKK NES peptide to recombinant CRM1 produced in *Escherichia coli* has been demonstrated (32). In contrast to this observation, Fornerod *et al.* (31) found that *in vitro* translated CRM1 binds a functional NES peptide from either Rev or PKI only in the presence of RanGTP.

Using recombinant factors made in *E. coli,* we have studied the assembly of the RRE-Rev-CRM1-RanGTP complex in greater detail *in vitro*. We show that CRM1 interacts directly with full-length Rev independently of the conserved leucine residues within the core of the NES and the presence of RanGTP. This Ran-independent interaction is LMB insensitive. In contrast, formation of the ternary Rev-CRM1-RanGTP complex is highly sensitive to NES mutations and is readily dissociated *in vitro* by LMB or by hydrolysis of RanGTP. We have mapped the sites within CRM1 and Rev involved in this interaction by site-specific UV cross-linking and protein footprinting.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The pET-His-H and pET-H-His vectors, for production of fusion proteins containing a His-tag at one terminus and a heart muscle kinase (HMK)-radiolabeling site at the other, have been described, as have pET-His-Rev-H and pET-H-Rev-His (40). To construct pET-H-RevM10-His, overlapping fragments of Rev, introducing the relevant nucleotide changes, were made. The 5′-fragment was made by PCR using pGEX-GTH-Rev (41) as template and the M10 mutation primer: 5′-CCTCTTCAGCTACCACCGGATGCTAGAGCTACTGCTGATTGTAACGAGGATTG and 3′-Rev primer (41). The 3′-fragment was made using the same template but with the reverse M10 mutation primer: 5′-CAATCCTCGTTACAATCAGCAGTAGCTCTAGCATCCGGTGGTAGCTGAAGAGG and 5′-Rev primer (41). The overlapping PCR fragments were "sewn" together by PCR using the 5′- and 3′-Rev primers introducing a 5′ *Bcl*I and a 3′ *Eco*RI site, respectively. The resulting fragment was cut with *Bcl*I and *Eco*RI and ligated into the *Bam*HI and *Eco*RI sites of pET-H-His, creating pET-H-RevM10-His. pET-H-RevM32-His was created using the same strategy but with the two M32 mutation primers: 5′-CCTCTTCAGCTACCACCGGCTGAGAGAGCTACTGCTGATTGTAACGAGGATTG and 5′-CAATCCTCGTTACAATCAGCAGTAGCTCTCTCAGCCGGTGGTAGCTGAAGAGG for the 5′- and 3′- half, respectively. The plasmid pET-His-Rev-H/C85S was constructed by whole plasmid PCR amplification using pET-His-Rev-H as template and the primers 5′-TTGATTCTAACGAGGATTGTGGAAC and 5′-GAGTAAGTCTCTCAAGCGGTG (ExSite™ kit from Stratagene). The pET-His-Rev-H/C85S/C89S was made using the same strategy. The plasmid pDS-H6rev for expression of His_6-Rev (without the HMK site) is described elsewhere (42). From this plasmid a construct encoding His_6-RevM10 was synthesized using the same strategy as for the construction of the pET-H-RevM10-His plasmid, except that the *Bam*HI and *Hin*dIII cloning sites were used.

The plasmid pET-His-CRM1-H encoding His_6-CRM1-HMK (hereafter simply CRM1) was made by PCR amplification of human CRM1 cDNA using T7-hCRM1 (31) as template and primers 5′-GAGGATCCATGCCAGCAATTATGACAATGT and 5′-GACAATTGAATCACACATTTCTTCTGGAAT. The PCR product was digested with *Bam*HI and *Mun*I and ligated into the *Bam*HI and *Eco*RI sites of pET-His-H. The plasmid pRRE-260 encoding a 260-nucleotide-long RRE was made by PCR amplification using pgTat-CMV (43) as template and primers 5′-CGGAATTCGCAGTGGGAATAGGAG-3′ and 5′-CAACCCAAGCTTGCAACCCCAAATCCCC-3′. The PCR fragment was digested with *Eco*RI and *Hin*dIII and inserted into the corresponding sites in the polylinker of pBS(+) (Stratagene). The plasmids pQE-32 Ran, pQE-60 Rna1p, and pQE-60 zzRBP1 encoding His_6-Ran, His_6-Rna1p, and Z-RanBP1 1-160-His_6 (the 160 N-terminal amino acids of RanBP1 fused to a Z-tag at the N terminus and a His-tag at the C terminus), respectively, were a gift from Dirk Görlich and are described elsewhere (26, 44). These proteins are hereafter referred to as Ran, Rna1p, and RanBP1 in the text.

Expression and Purification of Protein—The various His-tagged Rev proteins were expressed and purified as described earlier for His-Rev-HMK (40) and His_6-Rev (42, 45). Purified Rev proteins were stored in Rev buffer (50 mM Tris/HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA) at −20 °C. Preparation of nontagged wild type Rev has been described previously (46). CRM1, Ran, Rna1p, and RanBP1 were expressed and purified under native conditions essentially as outlined by the QIAGEN manual. Bacterial cultures were induced in early log phase with 1 mM isopropyl-1-thio-β-D-galactopyranoside and grown for 2–5 h further. Bacteria were recovered by centrifugation and resuspended in ice cold buffer N (200 mM Tris/HCl, pH 7.5, 500 mM NaCl, 5 mM β-mercaptoethanol), containing proteinase inhibitors. Moreover, 0.5 mM of either GDP (ICN) or GTP (Amersham Pharmacia Biotech) was added to Ran preparations. The suspensions were gently sonicated and cleared by centrifugation. For preparation of Ran, an ammonium sulfate precipitation step was included to improve purity (44). Imidazole was added to the supernatants to a final concentration of 10 mM followed by incubation with Ni-NTA agarose (Qiagen) at 4 °C for 1 h. The samples were loaded on columns and washed extensively with buffer N containing 10 mM imidazole. Finally, the proteins were eluted with a stepwise gradient of buffer N containing 50–500 mM imidazole and 0.5 mM GDP or GTP for Ran preparations. Eluates were analyzed by SDS-polyacrylamide gel electrophoresis and relevant fractions were pooled. CRM1 was dialyzed against CRM1 buffer (20 mM HEPES/KOH, pH 7.5, 80 mM CH_3COOK, 4 mM $(CH_3COO)_2Mg$, 250 mM sucrose, 1 mM dithiothreitol) and stored at −80 °C. Ran, Rna1p, and RanBP1 were stored at −80 °C in storage buffer (buffer N containing 10% glycerol and 0.5 mM GDP or 0.5 mM GTP for Ran preparations). Before use, aliquots of Ran were recharged with nucleotides. Purified Ran in storage buffer was incubated with 10 mM EDTA and 2 mM GDP or GTP on ice for 4 h, followed by the addition of $MgCl_2$ to a final concentration of 10 mM. Finally, the samples were applied to Bio-Spin™ 6 columns (Bio-Rad), and Ran was eluted in Ran buffer (50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$, 2 mM dithiothreitol, 10% glycerol) containing 2 mM of either GDP or GTP. Radiolabeling of purified proteins containing a HMK phosphorylation site was performed in solution with HMK enzyme (Sigma) as described earlier (40). Unincorporated nucleotides were removed by affinity purification of His-tagged Rev derivatives on Ni-NTA agarose or by gel filtration of CRM1 using Bio-Spin™ 6 columns (Bio-Rad). LMB was a gift from Iain W. Mattaj and stored at −80 °C in 96% ethanol.

RNA Preparation—RRE RNA was produced by run-off transcription using *Hin*dIII linearized pRRE-260 and IIB′ RNA, which contain helix

IIB of the RRE (47), and a tail of 5 uridines to enhance biotinylation efficiency was prepared from *Dra*I linearized pT7-IIB-*Dra*I.[2] IIB RNA was produced as described previously (47). Randomly radiolabeled RNA was synthesized using T7-MEGAshortscript™ (Ambion) and [α-^{32}P]UTP (Amersham Pharmacia Biotech, 800 Ci/mmol). Likewise, randomly biotinylated RNA was synthesized using T7-MEGAshortscript™ (Ambion) including 1 mM biotin-16-UTP (Boehringer Mannheim) and 5 mM UTP. All RNAs were quantified by the specific activity of ^{32}P incorporation or by UV absorption at 260 nm.

Coprecipitation Assay—For each reaction, 5 pmol of biotinylated RNA was renatured by incubation at 80 °C for 5 min in 2 μl of renaturation buffer (10 mM HEPES/KOH, pH 7.6, 100 mM KCl) followed by incubation at 37 °C for 20 min. Each sample was subsequently incubated on ice with 2 μl of Rev buffer containing 0–30 pmol of Rev, 4 μl of 2× Rev/RRE binding buffer (20 mM HEPES/KOH, pH 7.6, 200 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol, 20% glycerol, 0.1 μg/μl *E. coli* tRNA), and 2 μl of H_2O. After 10 min, the RNA was bound to Dynabeads™ M-280 Streptavidin (Dynal™) equilibrated with 1× Rev/RRE binding buffer and washed with 1× Rev/RRE binding buffer containing 0.1% bovine serum albumin (BSA) and 0.1% Nonidet P-40 to remove unbound RNA and protein. Approximately 0.5 pmol of radiolabeled CRM1 in 2 μl of CRM1 buffer was then added to each sample together with 2 μl of Ran buffer containing 0–10 pmol of RanGTP. Following incubation on ice for 30 min, 1 μl of Ran buffer containing 0–4 pmol of Rna1p and 0–4 pmol of RanBP1 was added, and the samples were incubated at 25 °C for 2 min and put back on ice. The beads were washed with 1× Rev/RRE binding buffer containing 0.1% BSA and 0.1% Nonidet P-40, and the amount of CRM1 retained on the beads was measured by scintillation counting.

Electrophoretic Mobility Shift Assay—One μl of Rev buffer containing 0–7 pmol of His_6-Rev, His_6-RevM10, or H-RevM32-His protein was preincubated on ice for 10 min with 2 μl of 5× Rev/RRE binding buffer containing 1% Triton X-100, 5 μl of Ran buffer containing 0–2 pmol of RanGDP or RanGTP, and 1 μl of CRM1 buffer containing either 3 pmol of CRM1 or 3 pmol of BSA before addition of 1 μl of renaturation buffer containing 50 fmol of renatured radiolabeled RNA. After incubation at 0 °C for 30 min the reactions were analyzed in 5% nondenaturing polyacrylamide gels containing 100 mM Tris borate, pH 8.3, and 1 mM EDTA, followed by autoradiography.

Protein Footprinting Analysis—For protein footprinting experiments using radiolabeled Rev protein, binding reactions were assembled at room temperature by mixing 4 μl 2× Rev/RRE binding buffer containing 250 ng/μl BSA and 0.2% Nonidet P-40, 1 μl H_2O, ~0.01 pmol (~100.000 cpm) radiolabeled Rev or Rev mutant protein diluted 1:25 in 1 μl of Rev storage buffer, 0–10 pmol CRM1 (or BSA as a control) in 1 μl CRM1 buffer, and 0–7 pmol RanGTP (or BSA as a control) in 1 μl of RanGTP buffer. For reactions containing LMB, 10 pmol of CRM1 protein in 2 μl of CRM1 buffer was preincubated with 1 μl of the drug (diluted in H_2O to variable concentrations) for 5 min before adding it to the other components. For footprinting experiments on radiolabeled CRM1, ~0.1 pmol (~100.000 cpm) of the protein was used per reaction. Nontagged wild type Rev protein was used as ligand, and the binding reactions were performed as described above. Binding was carried out for 30 min at room temperature, whereafter 2 μl of the respective endoproteinase (diluted in water) was added, and this mixture was incubated for 15 min at 37 °C. Reactions were put on ice and stopped by the addition of SDS loading buffer followed by incubation at 80 °C for 3 min. Cleavage products were resolved on 12% or 20% SDS-polyacrylamide gels. The concentrations in the final reaction mixture of the proteinases were as follows: 10^{-4} unit/μl thrombin (Amersham Pharmacia Biotech unit definition), 1–10 ng/μl Lys-C (Sigma), 5–50 pg/μl trypsin, tosyl-phenyl-alanine chloromethylketone-treated (Cooper Biomedicals), 0.2–2 ng/μl Glu-C (Boehringer Mannheim), 0.1–1 ng/μl Asp-N (Sigma), 5–50 pg/μl proteinase K (Boehringer Mannheim), 0.5–5 ng/μl Pronase (Boehringer Mannheim), 50–500 pg/μl bromelain (Boehringer Mannheim), 0.5–5 ng/μl Arg-C (Boehringer Mannheim), 20–200 pg/μl chymotrypsin (Boehringer Mannheim).

Site-specific UV Cross-linking—Twenty μg of Rev or Rev C85S was modified overnight at room temperature with 1 mM *p*-azidophenacyl bromide (Sigma) in 100 μl of buffer containing 50 mM Tris/HCl, pH 7.8, 500 mM NaCl, 1 mM EDTA, and 5% Me_2SO, giving rise to Rev* and Rev C85S*, respectively. In a control reaction, double-mutated Rev C85S/C89S was used as protein source. The reactions were stopped by adding 5 μl of 0.1 M β-mercaptoethanol. Modified protein was diluted 1:1 with 24 mM $MgCl_2$ before radiolabeling with HMK enzyme (Sigma). A Sephadex G-50 column (Amersham Pharmacia Biotech) equilibrated with cross-link buffer (25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$) was used to remove nucleotides and the cross-linking reagent. In each cross-linking reaction, 5 μl of radiolabeled Rev* or Rev C85S* (approximately 2.5 pmol/μl in cross-link buffer) was incubated for 20 min on ice with 2 μl CRM1 buffer containing 0–20 pmol of CRM1 and 4 μl of Ran buffer containing 0–20 pmol of RanGDP or RanGTP. The samples were irradiated with a 6 watt UV lamp (365 nm) at a distance of 1.5 cm for 5 min. Two μl of 5× SDS loading buffer (50% glycerol, 10% SDS, 250 mM Tris/HCl, pH 6.8, 5% 2-mercaptoethanol, 0.025% (w/v) bromphenol blue) were added, and cross-linked species were identified by analysis on a 6% SDS-polyacrylamide gel followed by autoradiography.

RESULTS

Rev Protein Interacts Directly with CRM1—To facilitate an investigation of the Rev-CRM1 interaction *in vitro*, we produced the relevant proteins in *E. coli*. CRM1 was produced as a fusion protein, His_6-CRM1-H (hereafter simply CRM1) containing an N-terminal His-tag and a C-terminal HMK recognition site. Purified CRM1 could subsequently be ^{32}P-labeled at the HMK site using [γ-^{32}P]ATP and HMK enzyme. RanGDP, RanGTP, Rna1p (the *S. pombe* homologue of mammalian RanGAP1) (21) and Ran binding protein 1, RanBP1, which potentiates the activity of RanGAP1 (20) were also produced as His-tagged proteins. Although the intrinsic GTPase activity of Ran is very low (24), aliquots of purified Ran were always reloaded with either GDP or GTP before use.

To establish whether CRM1 can interact with RRE-bound Rev protein, biotinylated RRE was coupled to Streptavidin-coated magnetic beads and incubated with or without recombinant His_6-Rev (hereafter simply Rev). After removal of unbound Rev, the beads were incubated with radiolabeled CRM1 in the absence and presence of RanGTP, Rna1p, and RanBP1, and finally washed. The level of retained CRM1 was measured by scintillation counting and plotted (Fig. 1). The amount of retained CRM1 increased by approximately 10-fold over background in the presence of Rev (Fig. 1, compare *columns 1* and *3*), implying a direct Rev-CRM1 interaction, also in the presence of RRE. A very similar result was obtained when using a biotinylated high affinity Rev target site IIB′ instead of the RRE (data not shown). This RNA accommodates a 39-nucleotide-long high affinity Rev binding site of the RRE (IIB) (47) followed by a tail of 5 uridines to enhance the biotinylation efficiency. IIB′ is able to bind 1–2 molecules of Rev as judged by gel shift analysis (See Fig. 2), thus indicating that extensive multimerization of Rev on the RRE is not required for CRM1 interaction *in vitro*.

Addition of RanGTP did not alter the level of CRM1 retained by Rev (Fig. 1, compare *columns 3* and *4*), suggesting that under these conditions CRM1 and RanGTP do not bind to Rev synergistically. However, it is likely that RanGTP becomes part of the RNA-protein complex for the following reason; addition of either Rna1p or RanBP1 alone to RanGTP-containing samples reduced CRM1 retention only slightly (Fig. 1, *columns 5* and *6*), but when Rna1p and RanBP1 were added together the amount of retained CRM1 was significantly decreased (Fig. 1, *column 7*). This indicates that hydrolysis of RanGTP can be activated by the concerted action of RanGAP1 and RanBP1 *in vitro*, causing a partial release of CRM1 from Rev.

Addition of RanGTP to Rev/CRM1 Results in a LMB-sensitive Ternary Complex—To further analyze the Rev-CRM1 interaction, we applied a gel mobility shift assay using the radiolabeled IIB′ RNA as a probe. The RNA was incubated with Rev and different combinations of CRM1, RanGDP, RanGTP, and LMB. Adding Rev alone resulted in 1–2 complexes suggesting that IIB′ can accommodate 1–2 Rev molecules. When purified CRM1 protein was also added to this binding reaction, an additional slower migrating complex, denoted A, appeared in the native polyacrylamide gel (Fig. 2, *lanes 3* and *6–10*). When

[2] Allan Jensen and J. Kjems, unpublished result.

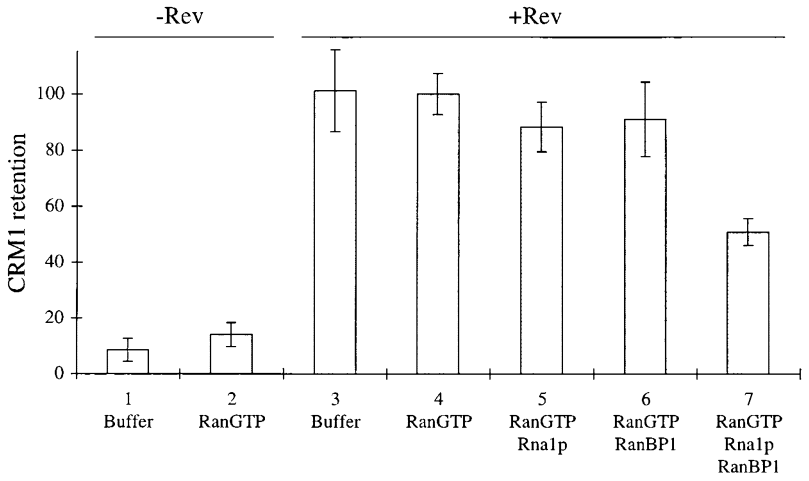

FIG. 1. **CRM1 binds Rev-RRE complexes in a coprecipitation assay.** The columns denote the relative amount of radiolabeled CRM1 protein retained by immobilized RRE RNA in the presence of various proteins as indicated. Five pmol of biotinylated RRE RNA was incubated with either 30 pmol of Rev (*columns 3–7*) or Rev buffer (*columns 1* and *2*) and subsequently bound to streptavidin-coated beads. The beads were washed and ~0.5 pmol of radiolabeled CRM1 was added to each reaction with either Ran buffer (*columns 1* and *3*), or 10 pmol of RanGTP (*columns 2, 4–7*). After incubation and washing steps, the retained CRM1 was measured by scintillation counting. In the hydrolysis experiments, Rna1p (*columns 5* and *7*) and RanBP1 (*columns 6* and *7*) were added before the washing step, and the incubation was continued at 25 °C for 2 min. The retention values represent an average of four independent experiments, and the bars indicate the standard deviation. The efficiency of retention is measured relative to the sample containing Rev and Ran GTP (*column 4*, index 100).

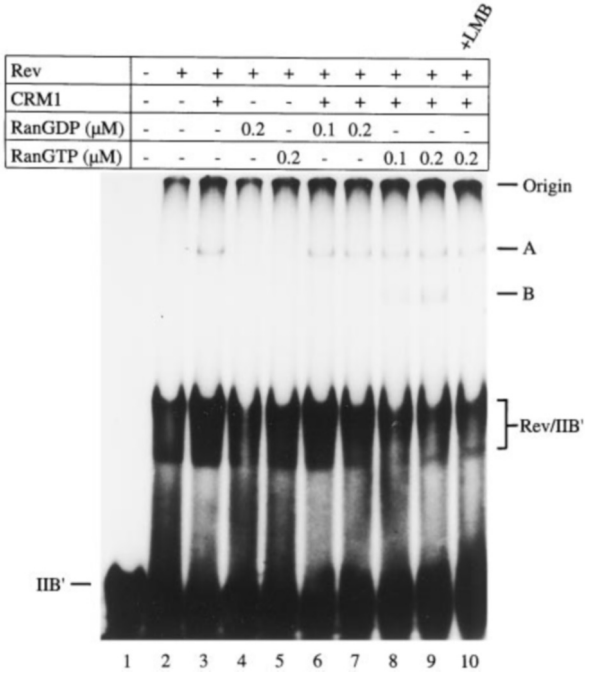

FIG. 2. **Assembly of RNA-Rev-CRM1-Ran export complex in a gel mobility shift assay.** Complexes were formed by incubating radioactively labeled IIB′ RNA (5 nM) in the absence and presence of Rev (0.7 μM), CRM1 (0.3 μM), and LMB (0.5 μM) with variable amounts of RanGDP or RanGTP as indicated. The complexes were subsequently separated on a native 5% polyacrylamide gel and visualized by autoradiography. Free IIB′ RNA, IIB′-Rev complexes and the origin of the gel are indicated. *A* and *B* mark the putative IIB′-Rev-CRM1 and IIB′-Rev-CRM1-RanGTP complexes, respectively. In contrast to complex A, complex B was sensitive to LMB and formed only in the presence of Ran charged with GTP.

RanGTP was included in the binding reaction, a faster migrating complex B was formed that was sensitive to Ran being charged with GTP (Fig. 2, compare *lanes 6* and *7* to *lanes 8* and *9*). In the absence of Rev no RNA binding activity was observed for CRM1 or RanGTP (data not shown). Complex A most likely corresponds to a complex containing IIB′, Rev, and CRM1, thus supporting that Rev can interact directly with CRM1 as inferred from our co-precipitation experiment. Complex B most likely also contains CRM1, as the complex is not formed when IIB′ and Rev is incubated with a large excess of RanGTP (Fig. 2, *lane 5*, and data not shown). Moreover, complex B also contains RanGTP, based on the observation that radiolabeled RanGTP added to the reaction comigrates with complex B in the gel (data not shown). A single Rev molecule is probably sufficient to form the RNA-Rev-CRM1-RanGTP complex because a similar pattern of complexes were seen when a slightly shorter RNA substrate, IIB, containing just a single high affinity Rev binding site, was utilized in gel shift analysis (data not shown). Interestingly, when the two complexes A and B were challenged by the CRM1 binding drug LMB only complex B disappeared (Fig. 2, *lane 10*), indicating that the direct RanGTP-independent Rev-CRM1 interaction is insensitive to this cytotoxin.

The structural aspects of the complex formed between Rev, CRM1, and RanGTP were further investigated by protein footprinting analysis. We have previously used this assay successfully to map the binding sites of the RRE, monoclonal antibodies, and short peptides on Rev (40, 48, 49). In this approach radioactively end-labeled protein is digested partially by a variety of endoproteinases in the absence and presence of a particular ligand. The ligand will sterically hinder endoproteinase cleavages at the site of interaction and produce a "footprint." C-terminally radiolabeled Rev protein was initially incubated with or without purified CRM1 protein and digested with the endoproteinases chymotrypsin, proteinase K, trypsin, bromelain, pronase, Lys-C, Glu-C, and Arg-C (Fig. 3; data not shown). To favor single hit kinetics, the conditions were chosen so that at least 50% of the labeled protein remained uncleaved. The relative migration of the bands produced by endoproteinases of different specificities enabled a putative identification of most of the cleavage sites. For instance, chymotrypsin has two major cleavage sites in the Rev protein (Fig. 3*A*, *lane 3*): one just N-terminal to the Arg^{66} marker in Rev putatively assigned to be after Leu^{64} and a second cut immediately N-terminal to the NES core domain assigned to be either Leu^{73} or Leu^{75} (hereafter called $Leu^{73/75}$). Addition of CRM1 resulted in strong protection of the chymotrypsin cleavage after $Leu^{73/75}$ (Fig. 3*A*, compare *lanes 3* and *4*), the trypsin cleavage after Arg^{66} (compare *lanes 7* and *8*), and a bromelain cleavage around position

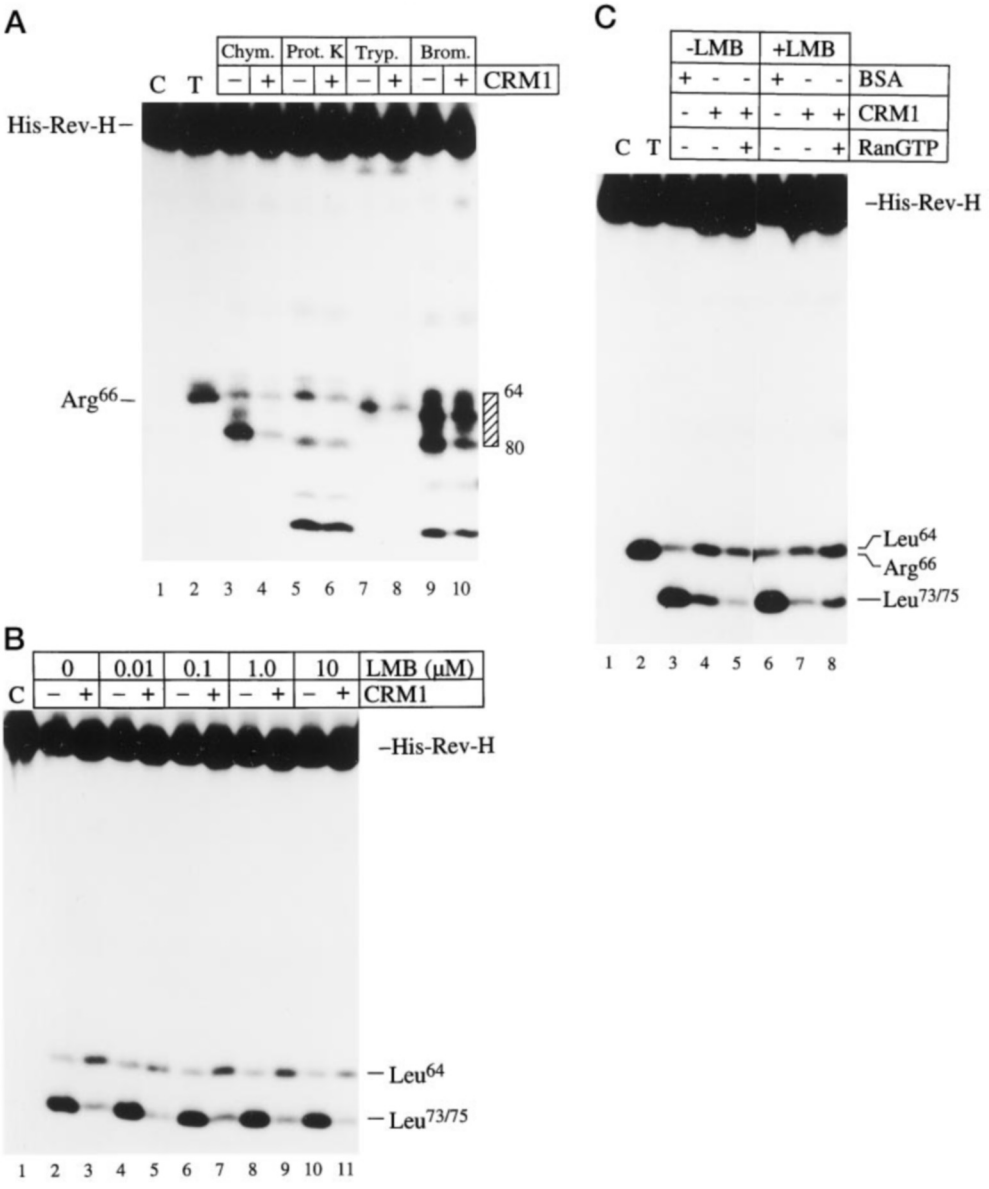

FIG. 3. **Protein footprinting of Rev alone and complexed with CRM1 and CRM1-RanGTP.** C-terminally radiolabeled Rev protein was probed with various endoproteinases in the absence or presence of CRM1 (*panel A*), CRM1 and LMB (*panel B*) or a more complex set of factors (*panel C*) as indicated above the lanes. The cleavage pattern was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. *C* denotes control lanes containing undigested radiolabeled Rev protein (marked His-Rev-H). *T* denotes an Arg^{66} marker obtained by thrombin cleavage (48). *Panel A*, Rev protein was incubated with 1 μg BSA (−) or 1 μg CRM1 (+) and probed with the endoproteinases chymotrypsin (*Chym.*), proteinase K (*Prot. K*), trypsin (*Tryp.*), or bromelain (*Brom.*) as indicated. The region of Rev protected by the CRM1 protein is indicated with a *cross-hatched bar* to the right. In addition, footprinting was performed using Lys-C, Glu-C, Arg-C, and pronase, but no CRM1 protections were observed using these proteinases (results not shown). *Panel B*, Rev protein was incubated with 1 μg BSA (−) or 1 μg CRM1 (+) and probed with chymotrypsin in the absence and presence of increasing amounts of LMB as indicated. The migration of the radioactive C-terminal Rev peptides originating from putative cleavages after Leu^{64} and $Leu^{73/75}$ are indicated on the *right*. *Panel C*, Rev protein was incubated with 1 μg BSA or 1 μg CRM1 and probed with chymotrypsin in the absence and presence of RanGTP and LMB as indicated.

80 (compare *lanes 9* and *10*). The accessibility of Leu^{64} to chymotrypsin was variable in the presence of CRM1. Although a weak protection was seen in this particular experiment, the band was often enhanced upon CRM1 addition (*e.g.* in Fig. 3*B*). On longer exposures many additional bands became visible but these were all unaffected by the presence of CRM1 (data not shown). The footprinting experiment confirms that CRM1 is able to interact directly with Rev and further maps the site of interaction to a region that overlaps with the NES of Rev.

Next, we investigated the LMB sensitivity of the CRM1 footprint on Rev using chymotrypsin as a probe (Fig. 3*B*). As above, a pronounced decrease in endoproteinase accessibility is observed at $Leu^{73/75}$ of Rev when CRM1 is present (Fig. 3*B*, compare *lanes 2* and *3*). Titrating the LMB concentration to as much as 10 μM in the footprinting reaction did not affect the CRM1-specific protection of $Leu^{73/75}$ (Fig. 3*B*, *lanes 4–11*). This result corresponds well with the resistance of complex A to LMB in gel mobility shift assays. We then added RanGTP together with CRM1 to the protein footprinting reaction. Interestingly, this gave rise to a significant enhancement of the CRM1-induced protection of $Leu^{73/75}$ in Rev (Fig. 3*C*, compare *lanes 4* and *5*), indicating that RanGTP stabilizes the interaction of CRM1 with the NES of Rev, or alternatively, that RanGTP by itself is able to bind the NES of Rev. Moreover, the presence of LMB in this reaction reversed the RanGTP-induced enhancement of the footprint (Fig. 3*C*, *lane 8*). Thus, the protein footprinting results are consistent with the results from the gel mobility shift analysis, showing that the interaction

between Rev and CRM1 is LMB-insensitive, whereas the entry of RanGTP into the complex constitutes the LMB-sensitive part of the assembly process.

RanGTP Specifies the Interaction of Rev, CRM1, and RanGTP—Mutating any of the leucines within the core of the Rev NES is known to affect Rev function *in vivo* (4, 12–16) and to prevent complex formation between a Rev NES peptide, RanGTP and *in vitro* translated CRM1 (31). To examine the molecular constituents that mediate this NES specificity in more detail, we employed two NES-mutated Rev proteins, RevM10 and RevM32, in our studies. RevM10 has a substitution of the Leu^{78}-Glu^{79} dipeptide to Asp-Leu, whereas in RevM32, the leucine residues at positions 78, 81, and 83 are all replaced by alanines (4, 15). Incubation of the Rev mutants with radiolabeled IIB′ RNA and CRM1 resulted in formation of complex A in a native gel similar to the complex A formed by wild type Rev (Fig. 4*A*, *lanes 3*, *6,* and *9*, complex denoted A as in Fig. 2). When adding RanGTP, a complex B is observed only with wild type Rev and not with RevM10 or RevM32 (Fig. 4*A*, compare *lanes 4*, *7,* and *10*, complex denoted B as in Fig. 2). Thus, this shows that the association of CRM1 with Rev is independent of the three leucine residues known to be critical to Rev function, whereas formation of the Rev-CRM1-RanGTP ternary protein complex on the other hand requires a wild type Rev-NES. Employing protein footprinting, we confirmed the direct interaction of RevM10 and RevM32 with CRM1 (Fig. 4*B*). Using the two N-terminally radiolabeled Rev mutants and chymotrypsin as probe, we found that CRM1 reduces the cleavage after $Leu^{73/75}$ to approximately the same degree as seen for wild type Rev (Fig. 4*B*, compare *lanes 2* and *3*, *5* and *6,* and *8* and *9*).

Site-specific UV Cross-linking of CRM1 to Rev—In addition to protein footprinting, we have used UV cross-linking to characterize the region of Rev involved in CRM1 binding. Rev protein, which naturally contains two cysteine residues C-terminal to the NES at position 85 and 89, was modified with *p*-azidophenacyl bromide that covalently binds to cysteine sulfhydryl groups and functions as a long wave UV light-inducible cross-linking agent. Modified Rev, denoted Rev*, was radiolabeled and subsequently incubated with combinations of CRM1, RanGDP, RanGTP, and LMB. The samples were then UV-irradiated and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 5*A*). Addition of CRM1 alone to Rev* did not lead to formation of any detectable cross-linked product (Fig. 5*A, lane 2*), whereas the presence of both CRM1 and RanGTP gave rise to a complex that migrated slightly slower than radiolabeled CRM1 alone (Fig. 5*A, lane 4 and* Fig. 5*B, lane 6*) suggesting that this cross-linked complex consists of at least CRM1 and Rev*. Without UV-irradiation or cross-linking reagent or when using a Rev mutant in which both Cys^{85} and Cys^{89} had been deleted, no complex formation could be detected (data not shown) ensuring that cross-linking occurs via the cysteine-coupled cross-linking agent. The complex formation was LMB-sensitive and dependent on Ran being in the GTP bound form (Fig. 5*A, lanes 3* and *5*). These observations correlate with our results obtained for complex B in the gel mobility shift assay (Fig. 2). However, even at prolonged exposures we were unable to detect a complex in the absence of RanGTP. Thus, it is possible that RanGTP induces a conformational change in the Rev-CRM1 complex bringing CRM1 closer to the cysteines located C-terminally to the NES. To determine whether CRM1 was cross-linked to Cys^{85} or Cys^{89} or both, we made a Rev mutant in which Cys^{85} is replaced by a serine residue (RevC85S). In the modified mutant, RevC85S*, the cross-linking agent is, consequently, only attached to Cys^{89}. Using the same conditions as above, we again detected efficient

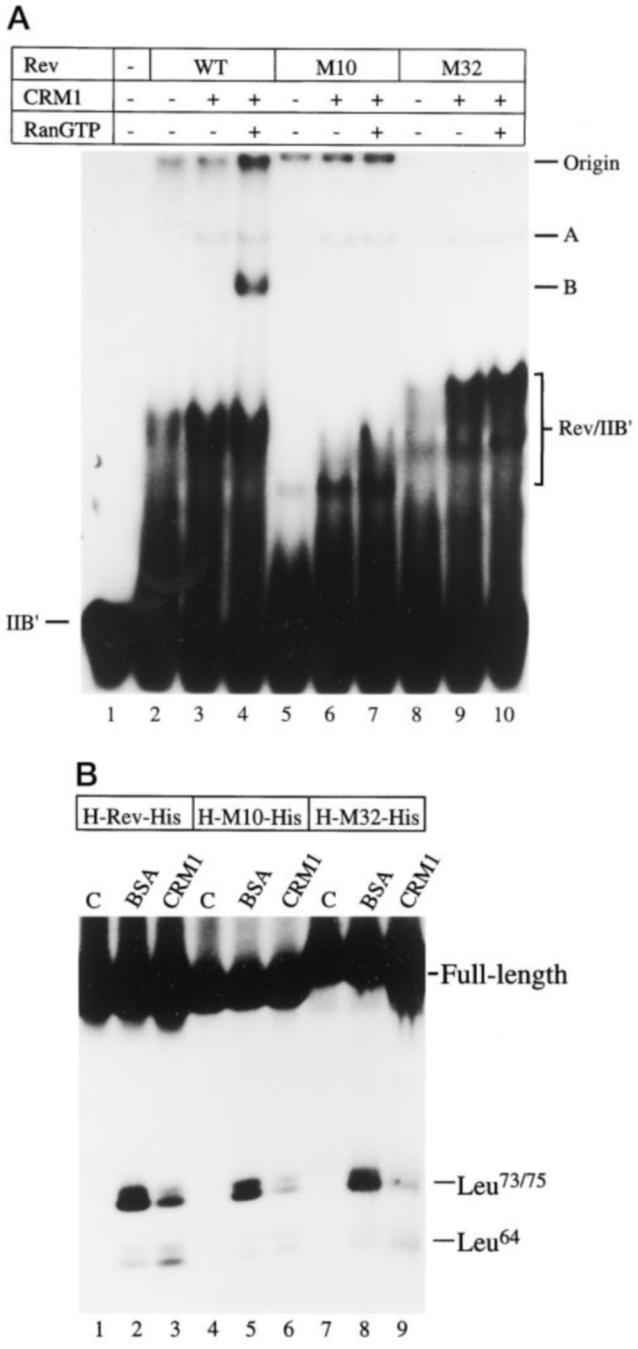

FIG. 4. **RanGTP is required for leucine-specific interaction between CRM1 and NES of Rev.** The ability of complexes to form on Rev NES mutants was investigated by gel mobility shift analysis (*panel A*) and by protein footprinting assay (*panel B*). *Panel A*, radioactively labeled IIB′ RNA (5 nM) was incubated alone (*lane 1*) or in the presence of wild type Rev (*lanes 2–4*), Rev mutants M10 (*lanes 5–7*), or M32 (*lanes 8–10*) (all at 0.7 μM) with CRM1 (0.3 μM) and RanGTP (0.2 μM) as indicated. Only formation of complex B is sensitive to the NES mutations. See Fig. 2 for details and denotations. *Panel B*, protein footprinting assay of N-terminally labeled wild type Rev or Rev mutant M10 (H-RevM10-His) and M32 (H-RevM32-His) in the presence of 1 μg BSA or 1 μg CRM1 using chymotrypsin as probe. The migration of full-length Rev, RevM10, and RevM32 and the N-terminal peptides originating from putative cleavages after Leu^{64} and $Leu^{73/75}$ are indicated on the *right*. See Fig. 3 for details and denotations. CRM1 specifically protects the NES region independently of the NES mutations. Note that the positions of the two major chymotrypsin specific cleavages in the gel are reversed because of the opposite positioning of the radioactive label as compared with Fig. 3. Because of partial cleavage of the HMK tag by chymotrypsin at a position immediately N-terminal to the phosphorylation site, the cleavage products of N-terminal HMK-fused Rev appear as double bands.

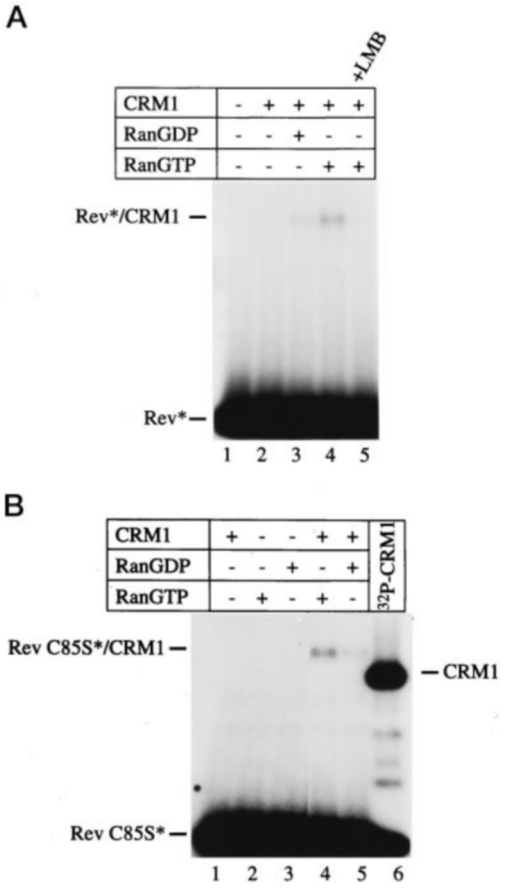

FIG. 5. **CRM1 cross-links to Cys[89] in Rev in the presence of RanGTP.** Radioactive wild type Rev (*panel A*) or C85S mutant Rev (*panel B*) were modified with a UV cross-linking agent (denoted by *) and incubated in the absence and presence of CRM1 (2 μM), RanGDP (2 μM), RanGTP (2 μM), and LMB (1.25 μM). Rev C85S contains only a single cysteine at position 89 that can be modified. The reactions were irradiated with UV light and analyzed by 6% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The migration of the modified Rev species (Rev* and Rev C85S*) and the corresponding CRM1-associated complexes (Rev*-CRM1 and Rev C85S*-CRM1) are indicated on the *left*. No complexes were observed using a cysteine-less double-mutated Rev C85S/C89S or unmodified wild type Rev proteins (data not shown). Radiolabeled CRM1 was included in *lane 6* of *panel B* to illustrate the migration of free CRM1 protein.

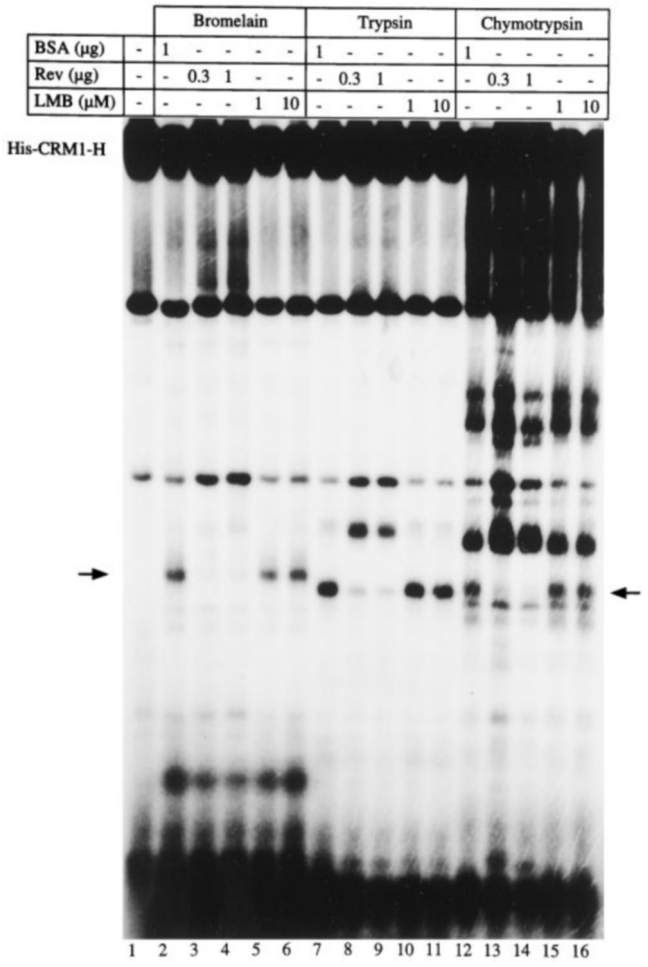

FIG. 6. **Mapping the binding site of Rev on CRM1 by protein footprinting.** C-terminally labeled CRM1 protein was incubated with BSA or increasing amounts of either Rev or LMB as indicated. The accessibility of CRM1 was probed with the endoproteinases bromelain, trypsin, and chymotrypsin as denoted above. Protein fragments were resolved on a 12% SDS-polyacrylamide gel and subjected to autoradiography. *Lane 1* shows untreated radiolabeled CRM1 protein, and the migration of full-length CRM1 is indicated on the *left*. The *arrow* on the *right* indicates the trypsin-specific cleavage site in CRM1 that most likely corresponds to Lys[810] and which is strongly affected by the presence of Rev. Major protections are also seen in the same region when using bromelain (*arrow* on the *left*) and chymotrypsin.

cross-linking to CRM1 only in the presence of RanGTP (Fig. 5*B, lane 4*). Adding RanGDP or RanGTP alone did not reveal any slower migrating complexes (Fig. 5*B, lanes 2* and *3*).

Protein Footprinting of Rev on CRM1—To investigate the region of CRM1 involved in the direct association with Rev, we performed protein footprinting on C-terminally radiolabeled CRM1. Fig. 6 shows the result obtained when probing with the endoproteinases bromelain, trypsin, and chymotrypsin. Cleavage by all three endoproteinases was specifically inhibited by Rev within a localized region of CRM1 as compared with the BSA controls (Fig. 6, compare *lanes 2, 7,* and *12* with *3* and *4, 8* and *9,* and *13* and *14*, respectively). By comparing the migration of the various cleavage products from this experiment and from experiments using other proteinases with specificities for specific amino acids (Fig. 6 and data not shown), the protected region was assigned to a region encompassing amino acids 800–820, including the strongly protected Lys[810] cleavage site (Fig. 6, *lanes 7–9*). In addition to the protections shown in Fig. 6, we also observed a specific protection against an Asp-N cleavage putatively assigned to Asp[716] (data not shown). No Rev-specific protections were observed when using proteinase K and Arg-C proteinases as probes (data not shown). Because LMB has been reported to bind directly to CRM1 (31), we attempted to map the LMB binding site on CRM1 by protein footprinting. However, we were not able to detect any changes in proteinase cleavage pattern of CRM1 upon addition of LMB (Fig. 6, compare *lanes 2, 7,* and *12* with *5* and *6, 10* and *11,* and *15* and *16*, respectively, and data not shown).

DISCUSSION

We have applied four different *in vitro* approaches to study the assembly of the nuclear export complex on the Rev protein at the molecular level. Our results have led us to conclude that full-length Rev can interact directly with the nuclear export receptor CRM1. Surprisingly, this association occurs independently of mutations of the conserved leucines in the Rev NES, which renders Rev functionally inactive in transport. On the contrary, formation of the ternary Rev/CRM1/RanGTP complex is highly sensitive to LMB and the integrity of the NES region, suggesting that RanGTP is the key regulator in specifying the transport signal.

To mimic the export complex as closely as possible, we investigated the binding of CRM1 and nucleotide bound Ran to

Rev when positioned on the RRE RNA using a co-precipitation assay and by gel mobility shift analysis. Rev and CRM1 were coprecipitated in the absence of Ran and under physiological ionic conditions, implying a direct association between the two proteins. Addition of RanGTP did not influence the retention of CRM1. However, RanGTP is likely to be a part of the complex formed, based on the observation that induction of RanGTP hydrolysis by the concerted action of the Ran effectors Rna1p and RanBP1 led to a significant reduction in CRM1 retention. Importantly, the RanBP1 used in this study lacks its Rev-like NES and therefore cannot compete with Rev for (re-)association with CRM1 after RanGTP hydrolysis (26). The observed dissociation of the Rev-CRM1 complex upon RanGTP hydrolysis most likely reflects the release process at the cytoplasmic side of the nuclear envelope after the export complex has traversed the nuclear pore complex, which is consistent with the current model (17, 18).

The direct binding between Rev and CRM1 was also evident in gel mobility shift assays using RNA substrates harboring one or multiple Rev binding sites. However, in this assay addition of RanGTP induced formation of a LMB-sensitive complex probably consisting of RNA-Rev-CRM1-RanGTP, which migrates faster in a native gel than the complex constituted by RNA-Rev-CRM1. The increased mobility is not likely to be caused by differences in the charge of the complex because His_6-tagged RanGTP is almost neutral at the assay conditions. A more likely explanation is that the association of RanGTP with RNA-Rev-CRM1 induces a conformational change (see below) that may stabilize the complex, thereby changing its migrational behavior in a gel. A similar explanation has been proposed for the observation that the CRM1-related tRNA export receptor, exportin-t, migrates faster in a native gel when RanGTP is added (50). We observed no ternary complex formation when using RanGDP instead of RanGTP, nor any reduction in formation of the RNA-Rev-CRM1 complex.

The footprinting experiments revealed a more detailed picture of the regions of Rev and CRM1 involved in complex formation. Within Rev, cleavages after several residues in the region from Leu^{64} to Arg^{80} were inhibited by CRM1, whereas residues in other parts of Rev remained unaffected. The most pronounced effects were the protections of the chymotrypsin cleavage after Leu^{73} or Leu^{75} and a bromelain cleavage around position 80, which is within or near the core domain of the NES (region 78–83). The positions of the footprints did not change upon addition of RanGTP, but the level of protection increased suggesting that RanGTP further stabilizes the Rev-CRM1 complex. LMB reverses this effect creating a footprint similar to that observed for Rev-CRM1 alone. Because the footprinting experiments were carried out in the absence of RNA, we can conclude that RRE binding by Rev is not a prerequisite for the Rev-CRM1 association.

The direct interaction between Rev and CRM1 was also evident from the reverse protein footprinting experiment using CRM1 as target and Rev as ligand. Rev specifically protected residues assigned to be Asp^{716} and Lys^{810} as well as other residues in the neighborhood of Lys^{810}. These regions of CRM1 have not yet been assigned any function, whereas RanGTP binding is expected to be mediated by a region near the N terminus based on alignment studies with other Ran-binding proteins (38, 39). The molecular mechanism for LMB inhibition is largely unknown. The most obvious target is CRM1 based on the observation that LMB interacts with *in vitro* translated CRM1 (31). Moreover, a potential target region within CRM1 has been identified based on the observation that a LMB-resistant strain of *S. pombe* has been characterized, which contains amino acid substitutions at positions 503 (Gly → Asp) and 546 (Met → Ile) (37). We also used LMB as ligand in the footprinting of CRM1, but were unable to detect any protections of endoproteolytic cleavages. However, the finding that LMB abrogates the ternary Rev-CRM1-RanGTP complex, although having no effect on the direct binding of Rev to CRM1 neither in gel mobility shift assays nor in protein footprinting, suggests that LMB interferes with the CRM1-RanGTP association step rather than the Rev-CRM1 interaction.

Another important issue addressed by our investigations is the molecular basis for NES recognition. For this purpose, we used two previously characterized export-deficient Rev mutants, RevM10 and RevM32, which are mutated within the string of characteristically spaced leucines of the NES (4, 15). We found that both of the mutated Rev proteins formed the RNA-Rev-CRM1 complex (complex A) in a gel mobility shift assay with an efficiency comparable to wild type Rev, and in the protein footprinting experiment the CRM1-induced protection of $Leu^{73/75}$ in the mutants was indistinguishable from that of wild type Rev. However, NES mutations in Rev completely abrogated the formation of the ternary Rev-CRM1-RanGTP complex (complex B). Hence, recognition of the conserved leucines of the NES seems to occur at the level of RanGTP entry to the Rev-CRM1 complex.

The direct association of CRM1 with Rev, independent of a functional NES, raises the possibility of an alternative explanation for the dominant-negative effect NES mutants have over wild type Rev function. It has previously been suggested that this phenotype was caused by competition of the Rev NES mutant with wild type Rev for binding to the RRE target site (4). Our observations are compatible with the possibility that the NES-mutated Rev proteins may sequester endogenous CRM1, thereby inhibiting wild type Rev function. It is likely that these NES mutant-CRM1 complexes would be retained in the nucleus because of their inability to be recognized by RanGTP for export. This notion may help explain why injection of a BSA M10 NES-peptide conjugate in *Xenopus* oocytes has a negative effect on the export of some classes of cellular RNAs (51) and that constitutive RevM10 expression is toxic in CMT3/COS cells.[3]

Some discrepancy exists in previous studies about the requirement for a cofactor (RanGTP) to support CRM1-NES interactions (31–33). At least three explanations, which are not mutually exclusive, can account for this. First, in some studies *in vitro* translated CRM1 was used as a protein source raising the possibility that contaminating RanGTP from the lysate supplements the binding reaction. Second, the natural differences in amino acid sequence of the NESs investigated might result in different binding affinities and specificities to CRM1. Third, although several core-NES peptides have proven to contain enough information to confer export of various fusion partners, optimal recognition by CRM1 might require residues outside the core of the NES. Our observations that CRM1 also footprints to residues N-terminally to the NES and that CRM1 can be cross-linked to Cys^{89} C-terminally to the NES is compatible with the latter suggestion.

How may RanGTP increase the specificity of the complex formation? One model is that RanGTP binds both to CRM1 and the NES sequence directly and thereby stabilizes the complex. Another possibility, not mutually exclusive with the first model, is that binding of RanGTP to CRM1 induces a conformational change in the complex that increases the specificity of the CRM1 for the NES. The latter model is supported by our site-specific cross-linking data, which demonstrated that CRM1 was only cross-linked to Cys^{89} in the presence of

[3] Marie-Louise Hammarskjold, personal communication.

RanGTP. The lack of CRM1-Rev cross-links in the absence of RanGTP, even after prolonged exposure times, suggests that CRM1 is positioned differently on the NES in the absence and presence of RanGTP. This physical movement may potentially be triggered by a RanGTP-induced conformational change.

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