Anti-idiotype RNAs that mimic the leucine-rich nuclear export signal and specifically bind to CRM1/exportin 1

Jörg Hamm[1] and Maarten Fornerod[2]

Background: Anti-idiotype approaches are based on the assumption that an antibody recognising a ligand can be structurally related to the receptor. Recently we have generated anti-idiotype RNA aptamers designed to mimic the human immunodeficiency virus-1 (HIV-1) Rev nuclear export signal (NES). Nuclear injection of either NES–peptide conjugates or aptamer causes the inhibition of Rev-mediated export. This implied that NES mimics and export substrate might compete for binding to the NES receptor. The mechanism of inhibition, however, is unknown.

Results: The interaction between the export aptamer and CRM1 was characterised *in vitro*. The aptamer binds specifically to CRM1 and this interaction is sensitive to competition by Rev NES–peptide conjugates. The recognition domain of CRM1 has been mapped and includes residues found previously to affect binding of leptomycin B, a fungicide interfering with nuclear export.

Conclusions: Inhibition of Rev-mediated export *in vivo* by export aptamers appears to result from the binding of the aptamers to the NES-recognition domain of CRM1. This observation demonstrates that anti-idiotype RNA can mimic faithfully structural and functional properties of a protein and can be used to map ligand-binding domains of receptors.

Addresses: [1]Department of Biochemistry, University of Dundee, Wellcome Trust Building, Dow Street, Dundee DD1 5EH, Scotland, UK. [2]European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117 Heidelberg, Germany.

Correspondence: Jörg Hamm
E-mail: J.HAMM@dundee.ac.uk

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The transport of macromolecules across the nuclear envelope is a saturable temperature- and energy-dependent process. Cargo molecules contain targeting sequences, which are recognised by soluble receptors either directly or via adaptors. The receptors interact with nucleoporins, which are part of nuclear pore complexes (NPCs), large proteinaceous regulated channels that span the nuclear membrane [1–3].

The majority of nuclear transport receptors belong to a protein super family that is prototyped by importin β, the nuclear import receptor of basic nuclear localisation signal (NLS) proteins [4,5]. Importin-β-like transport receptors have binding sites for their different cargoes, nucleoporins and RanGTP (the nuclear form of the small GTPase Ran). RanGTP dissociates importin–cargo complexes and stabilises exportin–cargo complexes, thus imposing directionality on this type of receptor-mediated nucleocytoplasmic transport [3,6]. Among the first exportins described was CRM1 (also known as exportin 1 [7–10]) the export receptor for leucine-rich nuclear export signals (NESs).

Most of what is known about nuclear export is the result of studies of the human immunodeficiency virus-1 (HIV-1) Rev protein. Rev is required for the export of unspliced HIV-1 mRNA from the nucleus to the cytoplasm [11]. The across species, as Rev-mediated export has been observed in yeast, *Xenopus* oocytes and mammalian cells [7–10]. Furthermore, all of the species examined contain highly related CRM1 proteins, which include a central portion of roughly 200 amino acids that is almost identical in all proteins.

Rev contains a leucine-rich NES and mutation of the NES interferes with Rev-mediated export [12–14]. It was, therefore, proposed that the inability of NES mutants of Rev to facilitate export could be due to their lack of binding to essential transport factors, such as CRM1. This model is in agreement with data showing that the formation of a stable trimeric complex comprising Rev, CRM1 and RanGTP requires an intact NES [7]. Furthermore, the fungicide leptomycin B (LMB), which inhibits Rev-mediated export, can bind directly to CRM1 and interferes with the formation of a stable trimeric export complex [7,15]. These observations, however, do not exclude the existence of intermediate dimeric complexes. Although no stable CRM1–Ran dimer could be detected [7], a stable CRM1–Rev complex has been characterised recently [16], but the observed dimeric complexes are unlikely to be productive export complexes.

Anti-idiotype RNA aptamers were previously isolated from combinatorial RNA libraries [17–22] with an antibody

that recognised the Rev NES–peptide [23]. These aptamers specifically inhibited CRM1-mediated nuclear export when co-injected with export substrates into the nucleus of *Xenopus* oocytes, exactly as observed with NES–peptide conjugates [24], and were therefore named export aptamers (XAPs). It appeared likely that the inhibitory effect of the XAPs was due to competition with Rev for binding to the NES receptor. At that time, however, this model could not be tested directly because CRM1 had not been identified as the NES receptor. In this work, we describe the characterisation of the interaction between the RNA aptamers and purified, recombinant transport factors *in vitro*. The export aptamers bind specifically to CRM1 and the strength of this interaction facilitated the mapping of a putative NES-recognition domain of CRM1, which appears to co-localise with the LMB binding site. These results provide strong evidence that the anti-idiotype RNA method functions as predicted and suggest that it might be generally useful both in the analysis of protein–protein interactions and in generating inhibitors that target these interactions.

Results

Export aptamers bind specifically to CRM1

To investigate whether the putative RNA mimics of Rev NES were able to interact with the NES-receptor CRM1 *in vitro*, we performed binding studies with purified, recombinant transport factors produced in bacteria. We used one of the previously described export aptamers, XAP1 [23], for the experiments reported here. This RNA is predicted to form a perfect 18 basepair (bp) stem closed by a three-layer G-quartet structure and is representative of the family of sequences isolated with an anti-NES–peptide antibody. Other export aptamers behaved identically to XAP1, both in the export competition studies performed previously and in the binding studies with purified transport factors [23] (and data not shown).

Two mutant RNAs derived from XAP1 served as highly specific control RNAs for the experiments. These RNAs, V_{GG} and V_{TL}, share some structural similarities with XAP1 but failed to interact with the anti-NES antibody and did not inhibit Rev-dependent export when injected into the nucleus of *Xenopus* oocytes [23]. Both of these variant RNAs have the 18 bp stem element that is present in XAP1, but differ in the sequences closing the stem. V_{TL} carries a stable tetraloop sequence (UUCG), whereas in V_{GG} only two of the three G residues are present in each of the four groups of G residues found in XAP1. Furthermore, tRNA and 5S RNA were included as additional controls.

The interaction between radioactively labelled XAP1 and transport factors was studied by native gel analysis of RNA–protein complexes. A stable complex was easily detectable after a short incubation of CRM1 with XAP1 (Figure 1a). Quantification of this CRM1 titration experiment indicates that the apparent K_d value of the XAP1–CRM1 interaction is about 100 nM (Figure 1b). No complexes were observed when the specific control RNAs V_{GG} and V_{TL} (Figure 1c, lanes 5 and 7) or unrelated RNAs (Figure 1c, lanes 9 and 11) were analysed under identical conditions. Furthermore, no interaction between export aptamers and the transport receptors importin β (Figure 1c, lane 1) or exportin (tRNA) (Figure 1c, lane 13) were observed. We therefore conclude that export aptamers bind specifically to the NES receptor CRM1 *in vitro*. In addition, there is a perfect correlation between the ability of RNAs to bind to CRM1 *in vitro* and the ability to inhibit CRM1-dependent transport *in vivo*.

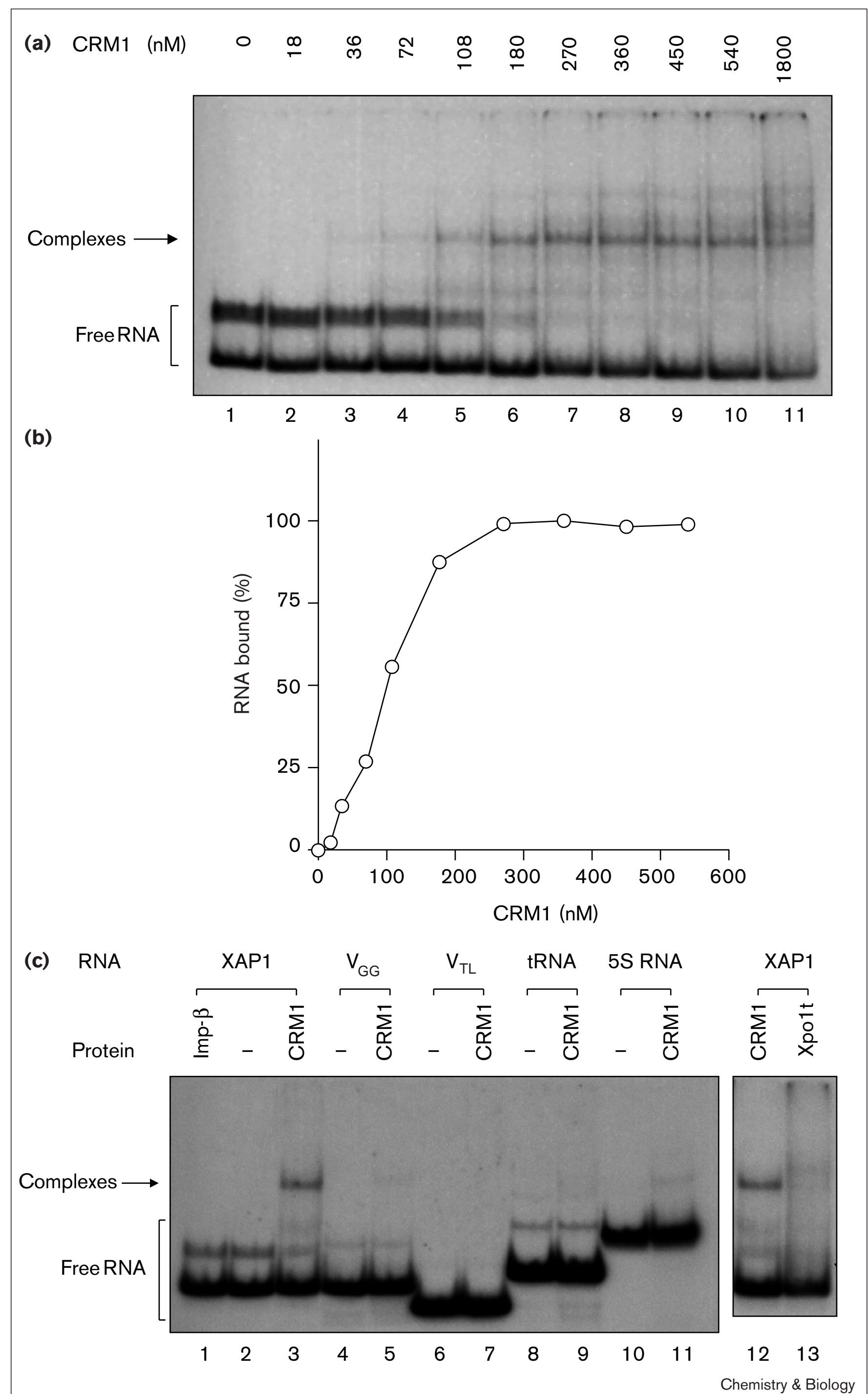
The interaction of export aptamers and CRM1 was not sensitive to the presence or absence of RanGTP or RanGDP in our experiments. RanGTP did not stimulate complex formation and we did not detect a complex containing CRM1, Ran and XAP1. This is in contrast to what is observed with Rev–CRM1 complexes. Although Rev can bind to CRM1 in the absence of RanGTP, binding is stimulated greatly by the presence of RanGTP [7,14,16]. The lack of stimulation of XAP1 binding by RanGTP might be due to a number of factors: steric hindrance when XAP1 and RanGTP are both in complex with CRM1, differences in the conformation of CRM1 induced upon binding, or RanGTP–NES interactions that are not mimicked by XAP1.

Structural requirements of the export aptamers

Based on the sequence of the selected export aptamers, we had suggested that the RNA would form an 18 bp stem closed by a three-layered G-quartet structure. The binding assay with recombinant CRM1 allowed us to test this prediction experimentally.

Folding of G-quartet structures is known to require potassium ions which intercalate between individual quartet layers [25]. We therefore anticipated that XAP1 would not fold properly in the absence of potassium ions and consequently would fail to bind to CRM1 if a G-quartet structure was part of the binding site. XAP1 was renatured in the presence of either sodium or potassium ions and incubated with recombinant CRM1 in a potassium-free buffer, prior to native gel analysis. Complexes were observed when XAP1 had been renatured in the presence of potassium ions (Figure 2a, lane 4) but not in the presence of sodium ions (Figure 2a, lane 2). Furthermore, we had noted previously that XAP1 that is not bound to CRM1 migrates at two distinct positions in the native gel system (Figure 2a, lane 3). Based on the results of the renaturation test it appears that only the slower migrating band corresponds to correctly folded XAP1, because this band was not detected when RNA was renatured in the absence of sodium ions (Figure 2a, compare lanes 1 and 3). This

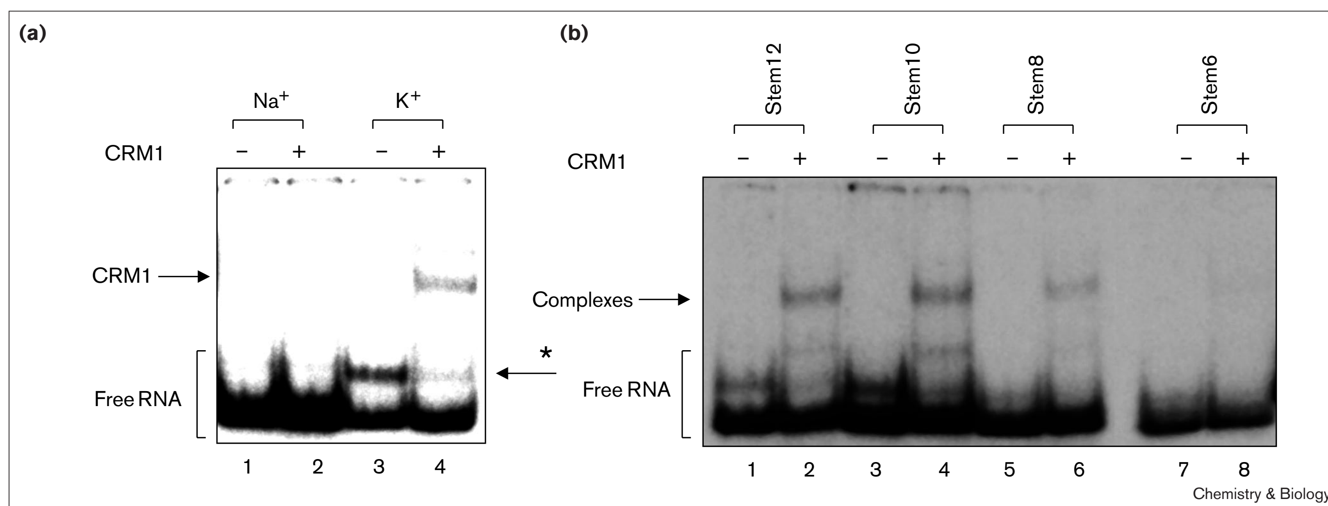
Figure 1



XAP1 binds to CRM1. **(a)** Titration of CRM1. Radioactively labelled export aptamer XAP1 (1 nM) was incubated with increasing amounts of purified, recombinant CRM1 (0–1800 nM) and CRM1–XAP1 complexes were analysed on native acrylamide gels. **(b)** Quantification of XAP1–CRM1 interaction. The relative amount of RNA (%) present in CRM1–XAP1 complexes from the titration shown in (a) was quantified and plotted against the CRM1 concentration. **(c)** Specificity of XAP1 binding to CRM1. Radioactively labelled (XAP1), mutant RNAs (V_{TL} and V_{GG}) unable to bind an anti-NES antibody *in vitro* or to inhibit export *in vivo*, and unrelated RNAs (tRNA and 5S RNA) were incubated (all RNAs 1 nM) with purified, recombinant proteins (CRM1, importin β [imp-β], exportin (tRNA) [xpo1t]; 200 nM) and complexes analysed on native gels. The positions of free RNA and of RNA–protein complexes are indicated on the left-hand side of the figure.

interpretation would be in agreement with the observation that only the upper band of the XAP1 doublet disappears upon addition of CRM1 (Figure 1a, compare lanes 2 and 11; Figure 2a, compare lanes 3 and 4). In addition, the proportion of the RNA migrating at the upper position was much lower for the variant V_{GG} (Figure 1c, compare lanes 2 and 4). This variant could, at best, form only a two-layered quartet structure, which would have reduced stability compared to a three-layered structure. The observation that V_{GG} shows only weak binding to CRM1 could, therefore, be due to the inefficient formation of the G-quartet structure.

Figure 2



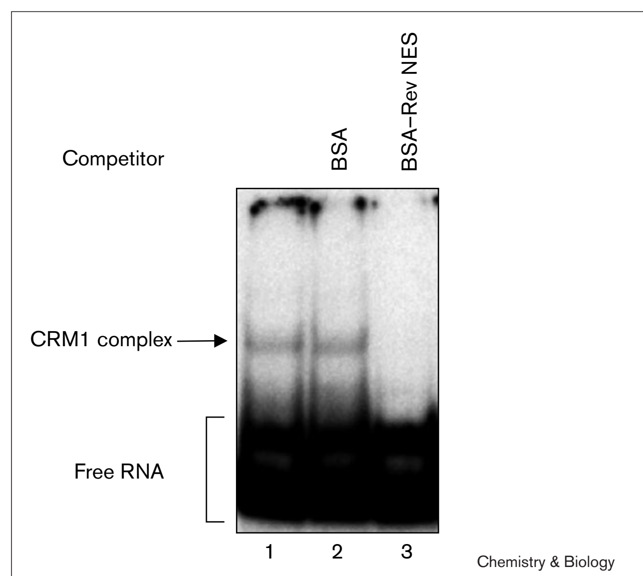
Structural requirements for the binding of XAP1 to CRM1. **(a)** CRM1 binding of the RNA mimics requires potassium ions. RNA (XAP1) was renatured in the presence of either potassium or sodium ions (100 mM each, +1 mM $MgCl_2$, 10 mM Tris pH 7.5) prior to incubation with recombinant CRM1 in potassium-free buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$). Complexes formed were analysed on native acrylamide gels. The active conformation of the free RNA is indicated by an asterisk (*) on the right-hand side of the figure. **(b)** A minimum stem of 8 bp is required to form the functional RNA. The length of the predicted stem of XAP1 (18 bp) was reduced gradually from 12 bp to 6 bp and the stem variants were analysed for their ability to bind recombinant CRM1 as in (a).

The 18 bp stem present in XAP1 was part of the structural constraint incorporated into the RNA library, and was not due to a selective pressure applied during the selection process. We therefore wanted to establish whether a stem was necessary for formation of the active XAP1 structure. The stem was reduced in steps of two basepairs from 18 bp to 6 bp and the stem variants of XAP1 were then tested for their ability to bind CRM1 (Figure 2b). XAP1 with a 8 bp stem was still able to bind to CRM1 (Figure 2b, lane 6), whereas a further reduction of the stem length resulted in loss of a detectable interaction. We conclude that functional XAP1 appears to form a three-layered G-quartet structure that requires a stem of at least 8 bp, possibly for coaxial stacking of the G-quartet and the stem structure.

NES–peptide conjugates and leptomycin B block binding of the export aptamers to CRM1

CRM1-dependent transport *in vivo* is blocked by NES–peptide conjugates and RNA mimics of NES. Furthermore, it has been shown that Rev can interact specifically with CRM1 in the absence of RanGTP [16]. Competition experiments were therefore performed to investigate whether peptides and RNA would target the same CRM1 domain *in vitro*. As anticipated, pre-incubation of CRM1 with bovine serum albumin (BSA)–Rev NES–peptides conjugates interfered with the formation of XAP1–CRM1 complexes (Figure 3, lane 3), whereas BSA alone had no effect (Figure 3, lane 2). The results of this competition experiment indicate that the NES peptide and XAP1 compete for the same CRM1 domain. A quantification of the NES-competition experiment is not possible due to the low affinity of NES peptides or Rev protein

Figure 3

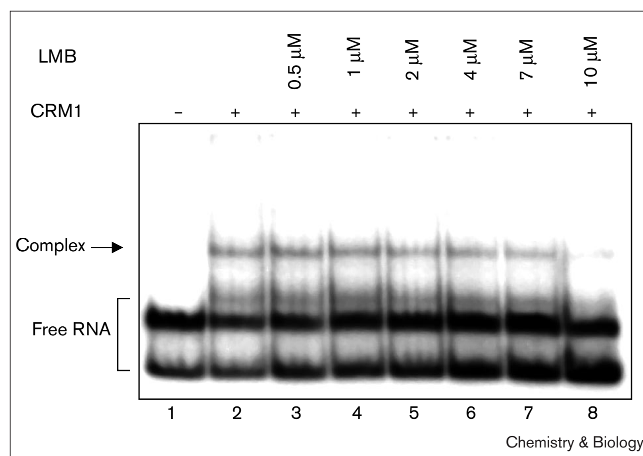


NES–peptide conjugates compete with XAP1 for binding to recombinant CRM1. Radioactively labelled export aptamer (XAP1, 0.2 nM) was incubated with recombinant CRM1 (200 nM) in the presence of 10 μg BSA or BSA–peptide conjugate (BSA–Rev NES; 10 μg = 1.8–2.3 nmol NES, NES concentration = 90–110 μM) and analysed on native gels.

for CRM1 in the absence of RanGTP [16]. Furthermore, as is generally true for unconstrained peptides, only a subfraction of the NES peptides will be in the proper conformation compatible with binding to CRM1. A detailed numerical quantification would, therefore, be likely to claim a precision that is impossible to achieve and has consequently not been attempted.

LMB is an inhibitor of CRM1-dependent export *in vivo* and it has been shown that LMB interferes with the formation of NES–RanGTP–CRM1 complexes *in vitro* [7,26]. Although it has been demonstrated that LMB can bind directly to CRM1 *in vitro*, it is not known whether LMB competes with the NES or with RanGTP for binding to CRM1. To address this issue we pre-incubated CRM1 in the presence or absence of LMB and analysed binding of XAP1. Complex formation was severely reduced in the presence of 10 μM LMB (Figure 4, lane 8), whereas lower LMB concentrations had little effect (Figure 4, lanes 3–6 and data not shown). These experiments suggest that the export aptamers are capable of binding to CRM1 in a NES-like fashion.

Figure 4



Binding of XAP1 is leptomycin B (LMB) sensitive. Radioactively labelled export aptamer (XAP1, 1 nM) was incubated with recombinant CRM1 (200 nM) in the presence of LMB (0–10 μM) and analysed on native gels.

Mapping of recognition domains of CRM1

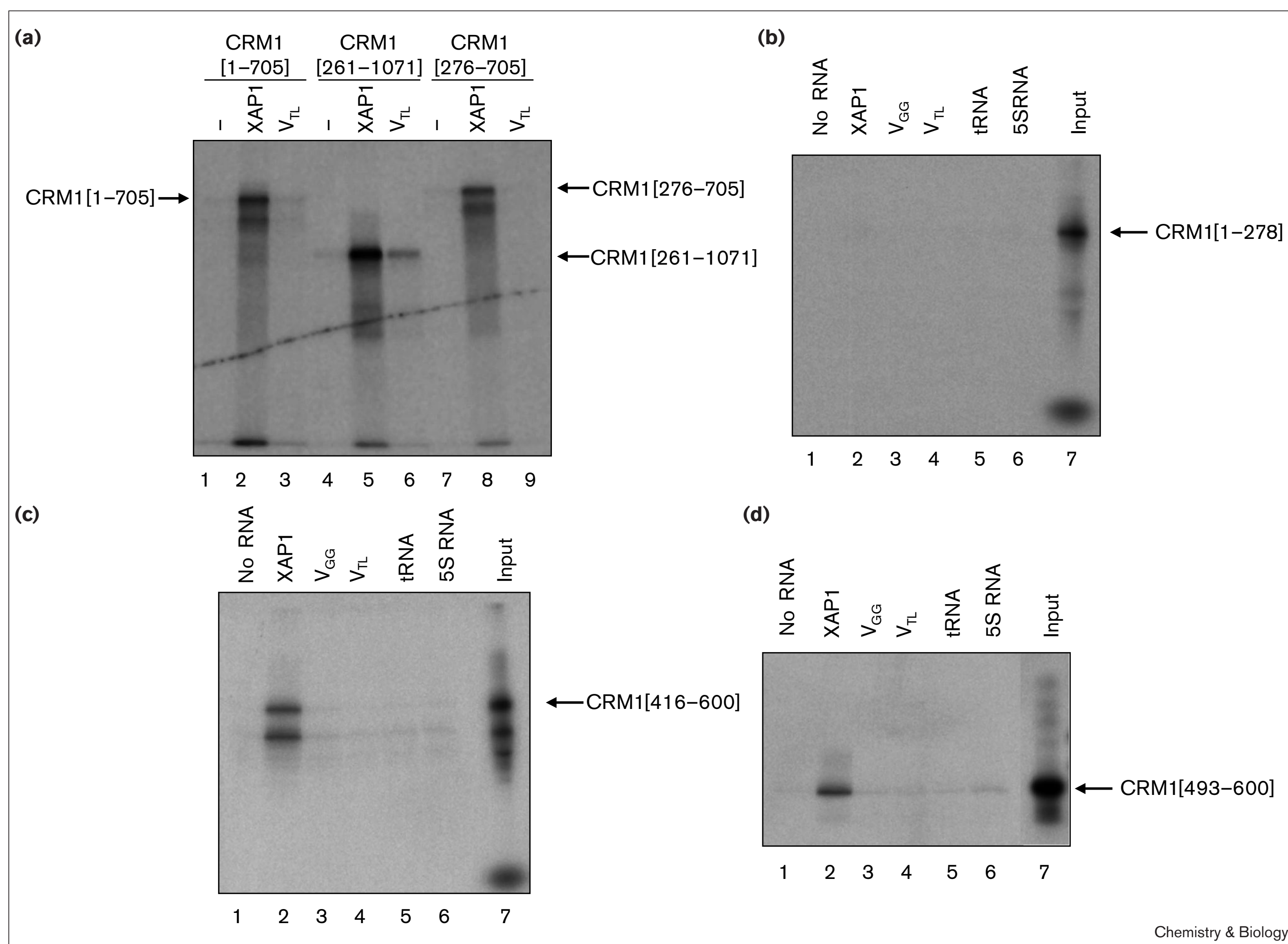
As with other transport receptors of the importin-β superfamily, CRM1 contains a putative Ran-binding domain, which is located at the amino terminus of the protein. In addition, the comparison of human, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* CRM1 sequences revealed a stretch of ~200 amino acids located in the centre of the protein that is absolutely conserved between species. Point mutations that were found to affect the sensitivity of CRM1 to LMB in *S. pombe* are also located in this central area of CRM1 [27].

It was therefore anticipated that CRM1 might contain a Ran-binding domain located at the amino terminus, an LMB-binding domain in the central region and a substrate-binding domain at the carboxyl terminus. Initial attempts to separate functional domains using binding studies with NES peptides and CRM1 deletion mutants with some of these domains missing were unsuccessful due to the cooperativity of the binding of RanGTP and substrate to CRM1 (M.F., unpublished observations). The ability of XAP1 to bind strongly and specifically to CRM1 in the absence of Ran, however, led us to test the deletion mutants for their ability to bind to the export aptamer.

Truncated CRM1 proteins were translated *in vitro* in the presence of radioactively labelled amino acids and precipitated with biotinylated RNAs coupled to streptavidin beads. XAP1 was the experimental RNA used and mutant RNA variants were used as negative controls. The putative Ran-binding domain was deleted either alone or in combination with a carboxy-terminal domain to evaluate the feasibility of this approach for mapping the NES-recognition domain of CRM1. Both of the deletion mutants lacking the amino terminus of CRM1 (CRM1[276–705] and CRM1[261–1071]) can be precipitated specifically by XAP1, indicating that the putative Ran-binding domain is not required for interaction with the NES mimic (Figure 5a, lanes 5 and 8). In addition, the deletion of carboxy-terminal amino acids between positions 706 and 1071 did not affect binding to XAP1 (Figure 5a, lanes 2 and 5). We therefore reasoned that the putative NES-recognition domain of CRM1 might be located in the central part of the protein and a second set of deletion mutants was tested to map this domain more precisely.

We focused our attention on the most highly conserved central portion of CRM1, residues 416–600 (the numbering corresponds to the human CRM1 sequence), for the second part of the analysis. An even smaller fragment was constructed that comprises only amino acids 493–600 but still contains the amino acids known to affect LMB sensitivity of CRM1 in *S. pombe* when CRM1 is mutated (corresponding to Gly502 and Met545 in human CRM1 [27–29]). As an additional control for specific precipitation of labelled proteins, we generated a fragment from the Ran-binding region (CRM1[1–278]) alone. The Ran-binding domain fragment was not precipitated by any of the biotinylated RNAs (Figure 5b, lanes 1–6), as would have been expected from the results of the experiment shown in Figure 5a. In contrast, both CRM1[416–600] and CRM1[493–600] bound specifically to export aptamers (Figure 5c,d, lane 2) but not to any of the control RNAs (Figure 5c,d, lanes 3–6). The results of the binding studies with partial CRM1 proteins suggest that the XAP1-binding region, and therefore the likely NES-recognition domain of CRM1, is part of the highly conserved, central

Figure 5



The NES-recognition domain of CRM1 is located in the evolutionarily highly conserved central part of the protein. CRM1 deletion mutants (the amino acids of CRM1 present in the partial CRM1 proteins are indicated) were translated *in vitro* in the presence of [^{35}S]-methionine and incubated together with competitor RNA (tRNA) and biotinylated RNA bound to streptavidin beads (no RNA: –, RNA mimic: XAP1, control RNA: V_{GG}, V_{TL}, tRNA or 5S RNA). Beads were washed extensively with buffer (50 mM Tris-HCl pH 7.5,100 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 0.1% NP-40), proteins were eluted with sodium dodecyl sulphate (SDS)gel sample buffer and analysed on SDS gels. An aliquot of the untreated translation reaction was loaded in (b–d) for comparison (input). **(a)** Mapping of the XAP1-binding domain of CRM1. Large deletions were introduced either separately or in combination at the amino terminus (CRM1[261–1071] and CRM1[276–705], to remove the putative Ran-binding domain) and at the carboxyl terminus of CRM1 (CRM1[1–705], CRM1[276–705]). **(b)** The amino-terminal, putative Ran-binding domain (CRM1[1–278]) is not recognised by RNA mimics of NES. **(c)** The XAP1-binding domain is localised in the evolutionarily highly conserved central 200 amino acids (CRM1[416–600]). The faster migrating protein, which is still precipitated specifically by XAP1, is likely to be due to initiation of translation at a site downstream of position 416. We did not attempt to localise the precise position of this aberrant initiation site (located only slightly downstream of position 416, but far upstream of position 493) further due to the results shown in (d). **(d)** A fragment of 108 amino acids (CRM1[493–600]) comprising amino acids known to affect LMB sensitivity in *S. pombe* binds specifically to XAP1.

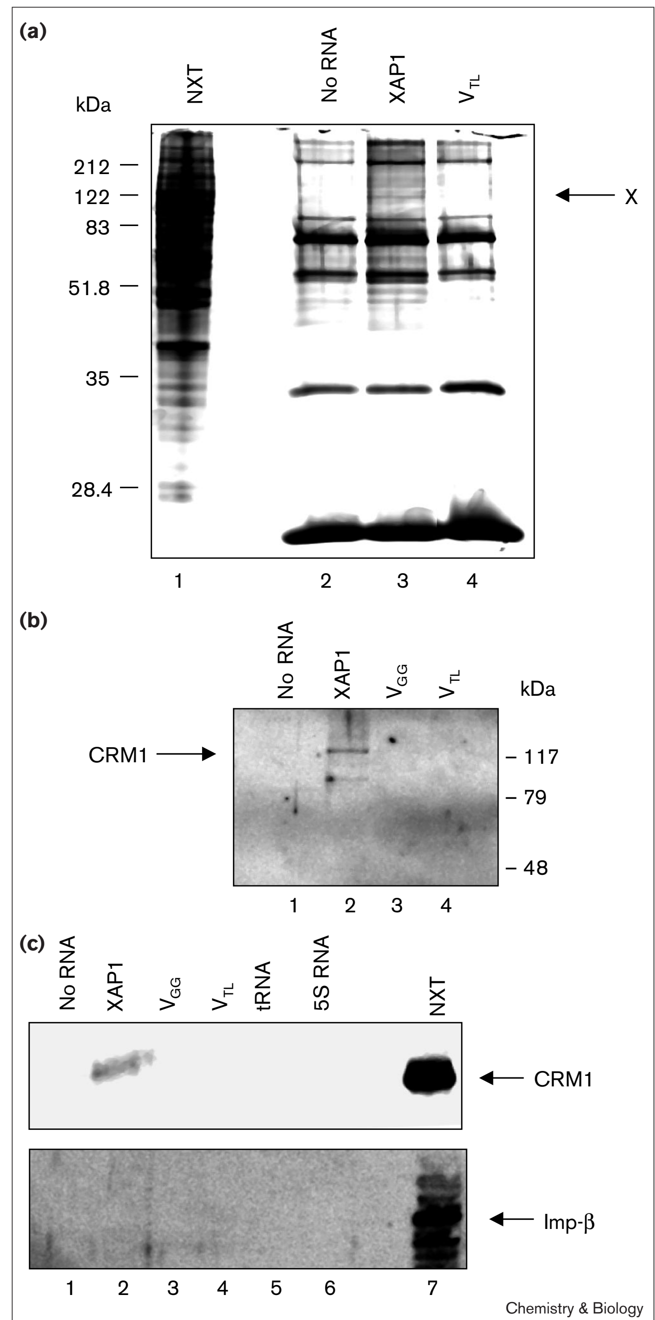
part of the protein. Furthermore, the NES- and LMB-recognition domains of CRM1 appear to colocalise [28,29].

Export aptamers bind CRM1 present in nuclear extracts

The experiments described above demonstrate that RNA mimics of the Rev NES–peptide interact specifically with the NES receptor CRM1. Furthermore, the export inhibition studies performed in oocytes suggest that RNA mimics could interact with CRM1 *in vivo*. It would therefore appear possible to isolate an unidentified receptor for a known ligand using an anti-idiotype approach similar to the one which resulted in the isolation of the export aptamers [23]. It might be sufficient to use the RNA mimics of a ligand domain to generate an affinity purification column to enrich the receptor for identification from nuclear extracts.

We performed a simple test experiment to verify this hypothesis by incubating immobilised RNA mimics or

Figure 6



XAP1 precipitates CRM1 from HeLa cell nuclear extracts. **(a)** Biotinylated XAP1 and a mutant form of XAP1 (V_{TL}) or streptavidin magnetic beads alone (no RNA) were incubated in HeLa nuclear extract and RNA–protein complexes were precipitated and washed with buffer (50 mM Tris-HCl (pH 7.5), 100 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 0.1% NP-40). Proteins were eluted from the beads, separated on SDS gels and analysed by silver staining. HeLa nuclear extract (NXT, 1 μg) was loaded on the same gel for comparison. A protein migrating at the position expected for CRM1 is indicated on the right (labelled X). **(b)** Biotinylated XAP1 and mutant forms of XAP1 (V_{GG} and V_{TL}) or unrelated RNAs (tRNA or 5S RNA) bound to streptavidin magnetic beads, or streptavidin magnetic beads alone (no RNA), were incubated in HeLa nuclear extract and RNA–protein complexes were precipitated and washed as described in (a). Proteins were eluted from the beads, separated on SDS gels and analysed by western blotting with anti-CRM1 antibodies. The position of intact CRM1 is indicated by the arrow, the weaker signal around 80 kDa corresponds to a degradation product of CRM1. **(c)** Western blot of precipitated proteins probed with anti-CRM1 antibodies (CRM1) or with anti-importin-β antibodies (imp-β). For comparison, 5 μg HeLa nuclear extract was loaded on the same gel (NXT).

control RNAs with HeLa cell nuclear extracts. The affinity beads were washed several times with buffer, bound proteins were eluted with denaturing buffer, separated on SDS-acrylamide gels and analysed by silver staining. Despite the simple experimental design, most of the proteins present in HeLa nuclear extract were not precipitated by the beads (Figure 6a, compare lane 1 with lanes 2–4). Furthermore, beads coated with XAP1 precipitated specifically a limited number of proteins, one of them migrating at the position corresponding to that expected for CRM1 (Figure 6a, lane 3, labelled ‘X’).

Proteins precipitated by the RNA-coated beads were analysed by probing western blots with various antibodies. CRM1 was precipitated from nuclear extracts only by the mimic XAP1 (Figure 6b, lane 2; Figure 6c, lane 2 [CRM1]) and not by the control RNAs (Figure 6b, lanes 3 and 4; Figure 6c, lanes 3–6 [CRM1]). Furthermore, neither importin-β (Figure 6c [imp-β]) nor exportin (tRNA) (data not shown) were precipitated by XAP1.

Although the enrichment for CRM1 was only moderate in our one-step pull-down experiments, it does appear possible to use an affinity enrichment with ligand-mimics subsequent to using conventional chromatographic purification steps to identify unknown receptors.

Discussion

The anti-idiotype approach

RNA aptamers that might resemble the HIV-1 Rev NES peptide were produced by an anti-idiotype approach [23]. Transport inhibition studies performed in *Xenopus* oocytes had shown that the so-called ‘export aptamers’ were capable of specifically blocking nuclear-export pathways [23]. This led to the prediction that the export aptamers were binding to the NES receptor CRM1 *in vivo* and interfering with export by competing with genuine export substrates for the NES-recognition domain. This model was based on a number of indirect experimental observations. Export aptamers and NES peptides competed for binding to the anti-NES antibody that had been used to generate the anti-idiotype RNAs, thus indicating that the export aptamers and NES peptides might share some structural similarity. Furthermore, co-injection of export substrates and export aptamers showed that only Rev- and cap-dependent export were inhibited, whereas tRNA export, which is known to use a different export pathway [3], was unaffected. In addition, the export aptamers were

actively transported into the cytoplasm after nuclear injection. Finally, RNA mutants unable to bind to the anti-NES antibody did not inhibit nuclear export nor were they transported actively after nuclear injection. Before we can conclude confidently that an anti-idiotype approach could be useful to study receptor–ligand interactions it was essential, however, to demonstrate directly that the export aptamers did recognise CRM1 specifically.

The results of our present study show that the export aptamers interact specifically with CRM1 but not with the related transport receptors importin-β or exportin (tRNA). Furthermore, only functional export aptamers that were both actively transported in oocytes and capable of inhibiting CRM1-dependent export bind to purified CRM1 *in vitro*. The lack of detection of CRM1–Ran–export aptamer complexes *in vitro* demonstrates that there are differences in export aptamer and NES peptide binding to CRM1. These differences could be due to the incompatibility of the presence of both Ran and an export aptamer in a single complex. Incompatibility could be caused by steric hindrance, or by electrostatic charges present in the RNA mimic, or its inability to make direct contacts with Ran. In any case, it appears that the RNA mimics of the NES reproduce faithfully at least some of the structural and functional features of an NES. We feel that these results represent a nontrivial outcome for an anti-idiotype approach, considering that the aptamers characterised here were isolated from combinatorial libraries simply by selecting ligands with a polyclonal anti-peptide antibody.

The work described in this manuscript complements and enhances the conclusions drawn from previously performed selection experiments using antibodies against proteins and peptides [30–34]. The selected RNA was in all cases able to compete with the protein antigen for binding to the antibody. Furthermore, the RNA mimics interacted, in some cases, with heterogeneous antibodies directed against the same protein epitope as the antibody used for the selection of the RNA. These results strengthen significantly the proposed models of structural mimicry [30,34]. As pointed out previously [23,30,31,34], in the absence of structural data that would allow the direct comparison of antibody–RNA complexes with the equivalent antibody–protein complexes, it is not possible to exclude the possibility that the RNA–protein pairs, including the Rev–XAP pair, would have only overlapping binding sites rather than being true structural mimics.

Strong support for models proposing the ability of RNA to mimic protein domains comes from the work of Nyborg and co-workers [35]. They have compared the crystal structure of the elongation factor EF-G with the structure of the trimeric tRNA–GDP–EF-Tu complex. The structures appear to be extremely similar and it was suggested that the ability of EF-G to induce translocation of the tRNA–GDP–EF-Tu complex on the ribosome might be due to structural mimicry [35,36].

The NES-recognition domain

CRM1 is able to interact with RanGTP, LMB and the NES. The Ran-binding domain could be localised to the amino-terminal region of the protein due to its homology with the Ran-binding domain of importin β [4,5]. The LMB-binding site is expected to be located in the central part of the protein based on the results of genetic screens in *S. pombe* that identified point mutations in CRM1 (corresponding to Gly502 and Met545 in human CRM1) leading to a reduced sensitivity to LMB [27]. Furthermore, it has been demonstrated that a single amino acid difference between the *S. cerevisiae* and *S. pombe*/human CRM1 (corresponding to position 528 of hCRM1, threonine in *S. cerevisiae* and cysteine in human and *S. pombe*) is the reason why the *S. cerevisiae* CRM1 does not bind to LMB [28,29]. It therefore appears likely that at least part of the LMB-binding site is located in the region between amino acids 502 and 545, although formally the possibility that the mutations affect the folding of CRM1 and that the LMB-binding site is in a different part of the protein cannot be excluded.

Very little is known about the NES-recognition domain of CRM1. Protein footprinting experiments indicated that residues 716 and 810 of CRM1 are protected in the presence of Rev protein against proteinase cleavage [16]. The resolution of the protection assay, however, is relatively low and the protected regions do not necessarily correspond to the interaction surface between CRM1 and Rev.

The ability of the export aptamers to bind tightly to CRM1 in the absence of Ran allowed us to separate the requirements for their binding from the CRM1 domains necessary for Ran binding or for the cooperative binding of Ran and NES peptides or NES proteins. For the reasons discussed above, it seems very probable that export aptamer and NES–peptide binding to CRM1 will involve overlapping regions of the protein. We have identified a region of 108 amino acids corresponding to CRM1[493–600] that interacts specifically with the export aptamer. This region is evolutionarily the most highly conserved CRM1 domain across species, as might be expected of the NES-recognition domain. As the amino acids known to affect LMB-binding in yeast co-localise with this putative NES-recognition domain and because binding of export aptamers to CRM1 is LMB sensitive, it is tempting to speculate that LMB inhibits CRM1-dependent export by interfering with substrate binding.

Significance

The export of protein and RNA from the nucleus to the cytoplasm is an active process that is dependent on

nuclear export signals (NESs). A functional NES is required for the formation of productive export complexes, which contain substrate, the NES receptor CRM1/exportin1 and RanGTP [1–3,7–10]. CRM1 was expected to contain three separate binding domains: one for interaction with RanGTP, one for interaction with the fungicide leptomycin B (LMB), which inhibits CRM1 function by binding directly to the protein [7,15], and the NES. The Ran-binding domain had been identified due to its homology with the known Ran-binding domain of importin β [4,5]. The LMB domain was expected to be located in the central domain of CRM1, which contained the amino acids that resulted in altered LMB sensitivity in genetic screens in *Schizosaccharomyces pombe* [25]. The substrate recognition domain had not yet been identified.

We have used a novel approach to identify ligand-binding domains of receptors. Anti-idiotype RNAs (export aptamers) that were isolated with an anti-NES antibody from combinatorial RNA libraries [17–22] and were designed to mimic the Rev NES have been shown to inhibit specifically Rev-mediated export in *Xenopus* oocytes [23]. In this study we describe the characterisation of the interaction of CRM1 and export aptamer RNA *in vitro* and demonstrate that the RNA is binding tightly and specifically to CRM1. Furthermore, we have used the export aptamer to map the putative NES-recognition domain of CRM1 to a region of 108 amino acids. This domain appears to co-localise with the previously identified LMB-binding domain of CRM1, which suggests that LMB might inhibit CRM1 function by blocking NES–CRM1 interactions.

In general, anti-idiotype RNA aptamers isolated with an anti-ligand antibody can apparently mimic faithfully some of the structural and functional properties of a protein domain *in vivo* and *in vitro* [23,30,34]. This indicates that anti-idiotype RNAs might be a valuable tool to identify or to characterise protein–protein interactions, and to disrupt them specifically.

Materials and methods

Native gels

Radioactively labelled RNA was prepared as described previously [21]. About 100 cps of [α-^{32}P]-GTP-labelled, gel-purified RNA was incubated with purified, recombinant protein (concentration 200 nM) in a volume of 20 μl in reaction buffer (20 mM HEPES (pH 7.5), 110 mM KOAc, 6 mM MgOAc, 2 mM DTT) for 10 min at room temperature. 87% glycerol (2 μl) were added and complexes analysed on nondenaturing 6% acrylamide gels (6% acrylamide, 0.1% Triton X-100, 1× TBE) as described [23]. For the competition experiments, proteins were incubated in the presence of competitor for 20 min on ice. Labelled RNA was added, the incubation was continued for 10 min at room temperature and samples analysed as described. For the stem mutants, deletions were introduced starting from position +4 (this leaves G1–G3 which are required for efficient transcription by T7 RNA polymerase) by deleting bases on both sides of the predicted stem structure (stem12: Δ U4–C9 + bases on the opposite stem side; stem10: Δ U4–C11 + opposite bases; stem8: Δ U4–G13 + opposite bases; stem6: Δ U4–C15 + opposite bases).

Protein production

Recombinant proteins contained His_6 tags and were prepared as described [37]. CRM1 deletion mutants for translation *in vitro* were generated by attaching a T7 RNA polymerase promoter sequence and a translational initiation sequence at the 5′-end (TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT G), and translational stop codons to the 3′-end of the CRM1 region to be transcribed and translated by PCR using a CRM1 expression plasmid as a template. PCR fragments were purified on 2% agarose gels and RNA transcribed with Promega Ribomax kits. RNA was purified by phenol extraction, Sephadex G25 columns and finally ethanol precipitation and was then resuspended in H_2O at a concentration of 1 μg/μl. Proteins were translated in rabbit reticulocyte lysate systems (Amersham) in the presence of ^{35}S-methionine.

Precipitation of labelled CRM1 proteins

Templates for the transcription of XAP1, V_{GG}, V_{TL}, tRNA and 5S RNA were prepared by PCR as described [21]. Purified fragments were transcribed for 90 min in a volume of 100 μl with standard transcription buffer (Stratagene) in the presence of 1.2 mM rNTPs, 0.12 mM bio-11 UTP, 50 u of T7RNA polymerase and 1 μl of RNase inhibitor. RNA was purified by phenol extraction, sephadex G25 columns and finally ethanol precipitation and resuspended in 30 μl H_2O. Biotinylated RNA (6 μl) was renatured for 10 min at 50°C in a volume of 25 μl (10 mM Tris (pH 7.5), 100 mM KCl; 1 mM $MgCl_2$). Dynal M280 streptavidin beads (50 μl) were washed with 3 × 250 μl NaK_{150} (50 mM Tris-HCl (pH 7.5), 100 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$), 0.1%NP-40 and RNA was coupled to the beads by incubating washed beads in 25 μl of renatured, biotinylated RNA and 200 μl NaK_{150}/NP-40 for 20 min in a rotating wheel (Dynal) at room temperature (RT). Beads were washed 3 × 3 min with 250 μl NaK_{150}/NP-40 in the wheel. Translation lysate (4 μl) containing the labelled proteins, 1.5 μl 1 mM yeast tRNA and 50 μl NaK_{150}/NP-40 were incubated with the beads bound RNA for 15 min at RT in the rotating wheel and were washed 3 × 3 min with 250 μl NaK_{150}/NP-40. Beads were suspended in 100 μl NaK_{150}/NP-40 and transferred to a fresh tube, the supernatant was removed and beads boiled in 30 μl SDS-loading buffer. The supernatant (20 μl) was analysed on 15% SDS-acrylamide gels (10 × 7.5 cm) that were run at 200 V for 60 min. Gels were treated with amplify (Amersham), dried and exposed at −80°C for 6–12 h.

Precipitation of proteins from nuclear extracts

Biotinylated RNA was coupled to M280 beads and beads were washed as described above. HeLa nuclear extract (16 μl; CCCC, Belgium, 20 μg/μl), 32 μl 1 mM ytRNA and 52 μl H_2O were incubated for 30 min at room temperature in a rotating wheel and beads were washed with NaK_{150} and analysed as described for the precipitation of labelled proteins. Proteins were transferred to nitro-cellulose membranes by semi-dry blotting and proteins detected with ECL-kits (Amersham). Antibodies anti-CRM1 [4] anti-importin-β (kind gift of O. Gruss) and anti-exportin (tRNA) [38] were diluted 1:1000.

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