membrane-anchored syntaxin 1A directly modulates N-type calcium-channel activity in brain[9–11]. It will be important to determine whether syntaxin 1A directly binds to CFTR and if it regulates epithelial CFTR chloride channels and neuronal calcium channels by similar mechanisms. We speculate that the CFTR–syntaxin–Munc18 interactions play a role in fine-tuning CFTR activity in response to certain physiological cues, such as the activation of second messenger pathways that regulate the physical interactions between these proteins[23,24]. Understanding the mechanisms by which these molecules regulate CFTR activity may be relevant to the design of strategies for augmenting epithelial CFTR function in cystic fibrosis. □

Methods

***In vitro* binding and co-immunoprecipitation.** Qualitative 'pull down' assays were done by adding immobilized GST, GST–syn1AΔC or GST–syn3ΔC (50 μg) to a 1% NP-40 lysate of HT29-CL19A cells for 12 h at 4 °C. Beads were pelleted, washed extensively in lysis buffer and analysed for CFTR by immunoblotting. Inhibition of CFTR-syntaxin 1A binding by Munc18a (Fig. 3d) was verified by preincubating GST–syn1AΔC (15 μg) with or without excess Munc18a before assaying CFTR binding. Quantitative solution binding assays were done by adding soluble eluted GST fusion protein to an HT29-CL19A cell lysate for 3 h at 4 °C. GST fusion protein was then precipitated with excess glutathione–agarose and CFTR binding quantified by immunoblotting and densitometry. Co-immunoprecipitation was performed by passing HT29-CL19A cell lysates (0.8% Triton X-100 in HEPES-buffered saline (pH 7.4)) through a hydrazide-derivatized disc (Actidisc, FMC Corp) to which anti-CFTR IgG (anti NBD1; residues 426–588; ref. 20) or non-immune IgG was covalently bound. Bound proteins were eluted and analysed (Fig. 2 legend).

Electrophysiology. *Xenopus* oocytes were injected with the cRNAs encoding CFTR (1 ng), full-length syntaxin 1A (5 ng, or as indicated in Fig. 3) and Munc18a (as indicated in Fig. 3) and assayed 48–72 h later for CFTR currents[25]. Currents were typically activated with a cocktail containing 20 μM forskolin, 100 μM dibutryl cyclic AMP and 100 μM IBMX. A similar inhibition of CFTR currents by syntaxin 1A was also observed using a supermaximal CFTR activation cocktail (20 μM forskolin, 200 μM dibutryl cAMP and 5 mM IBMX; data not shown). Whole-cell patch-clamping of T84 cells was done as described[19,22], except that 5 mM instead of 1 mM Mg-ATP was used in the pipette solution. Cell capacitance was measured by integrating the current during a 10-mV voltage step and subtracting a baseline established ~15 ms after the step. Currents were activated with 500 μM cpt-cAMP in the pipette or with an extracellular cocktail (100 μM cpt-cAMP, 50 μM forskolin and 50 μM IBMX) for those experiments involving pre-equilibration of the cell interior with CFTR-blocking antibody[19].

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Correspondence and requests for materials should be addressed to K.L.K. (e-mail: kirk@phybio.bhs.uab.edu).

Caenorhabditis elegans CED-9 protein is a bifunctional cell-death inhibitor

Ding Xue* & H. Robert Horvitz

Howard Hughes Medical Institute, Department of Biology, 68-425, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA

The *Caenorhabditis elegans* gene *ced-9* prevents cells from undergoing programmed cell death and encodes a protein similar to the mammalian cell-death inhibitor Bcl-2 (refs 1–7). We show here that the CED-9 protein is a substrate for the *C. elegans* cell-death protease CED-3 (refs 8, 9), which is a member of a family of cysteine proteases first defined by CED-3 and human interleukin-1β converting enzyme (ICE)[10–12]. CED-9 can be cleaved by CED-3 at two sites near its amino terminus, and the presence of at least one of these sites is important for complete protection by CED-9 against cell death. Cleavage of CED-9 by CED-3 generates a carboxy-terminal product that resembles Bcl-2 in sequence and in function. Bcl-2 and the baculovirus protein p35, which inhibits cell death in different species through a mechanism that depends on the presence of its cleavage site for the CED-3/ICE family of proteases[9,13–17], inhibit cell death additively in *C. elegans*. Our results indicate that CED-9 prevents programmed cell death in *C. elegans* through two distinct mechanisms: first, CED-9 may, by analogy with p35 (refs 9, 17), directly inhibit the CED-3 protease by an interaction involving the CED-3 cleavage sites in CED-9; second, CED-9 may directly or indirectly inhibit CED-3 by means of a protective mechanism similar to that used by mammalian Bcl-2.

Baculovirus p35 protein, a general inhibitor of programmed cell death[9,13–17], is a substrate for the *C. elegans* cell-death protease CED-3 and may act as a competitive inhibitor of CED-3 (ref. 9). We tested whether CED-9 protein, an endogenous *C. elegans* cell-death inhibitor[1,2], might act similarly. We found that ^{35}S-methionine-labelled CED-9 synthesized *in vitro* was cleaved by purified CED-3 protease[18] to generate two products of relative molecular masses

* Present address: Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309, USA.

(M_r) 7K and 24K (Fig. 1a, lanes 1 and 2). A C-terminal truncated CED-9 fusion protein, His_{10}CED-9(1–251), in which the CED-9 putative membrane-anchoring region (amino acids 252 to 280)[2] was deleted, was also cleaved by CED-3 protease, yielding two major products of 22K and 9K and one minor product of 24K (Fig. 1b). The N-terminal sequences of the 22K and 24K His_{10}CED-9(1–251) cleavage products (LPSPS and GKINDW in single-letter code) indicated that CED-3 cleaved CED-9 at two different sites, between aspartate 47 and leucine 48, and between aspartate 67 and glycine 68, respectively (data not shown). The amino-acid sequences N-terminal to these cleavage sites (P_4 to P_1; DAQD and ESID) are similar to the consensus CED-3 cleavage site D/EXXD, in which the P_4 residue is aspartate or glutamate and X may be any amino acid (D.X. and H.R.H., unpublished observations)[18]. The P_4 and P_1 residues are the most important sites for substrate recognition and for the specificity of the CED-3/ICE-family proteases[19].

We introduced mutations at either the P_4 (D44A) or P_1 (D67E) residue of the CED-3 cleavage sites and tested the effects of these mutations on the cleavage of CED-9 by the CED-3 protease *in vitro*. CED-9 proteins carrying mutations at either CED-3 cleavage site (D44A or D67E) were still cleaved by the CED-3 protease (Fig. 1a, lanes 3–6). CED-9 proteins with mutations at both CED-3 cleavage sites were cleaved less effectively (D44A, D67E) or hardly at all (D47E, D67E) by the CED-3 protease (Fig. 1a, lanes 7–10). The cleavage of CED-9(D67E) by CED-3 generated a 27K product instead of the 24K product seen with wild-type CED-9 and with CED-9(D44A); this 27K protein is presumably the C-terminal product generated by cleavage at the first CED-3 site, D47 (Fig. 1a).

We next investigated whether the presence of the two CED-3 cleavage sites in CED-9 is important for the ability of CED-9 to inhibit cell death in *C. elegans* by using an *in vivo* cell-death protection assay[2,9]. Briefly, we generated transgenic nematodes expressing either wild-type or mutant CED-9 proteins under the control of the *C. elegans* heat-shock promoters, and then determined the extent of protection from cell death conferred by the expression of these proteins by counting the number of extra or 'undead' cells in the anterior pharynx of the transgenic worms after heat-shock treatment. We found that mutations that disrupted either the first CED-3 cleavage site (D44A) or the second CED-3 cleavage site (D67E) in CED-9 seemed slightly to reduce the cell-death protective function of CED-9 (Table 1). By contrast, mutations that disrupted both CED-3 cleavage sites ((D44A, D67E) or (D47E, D67E)) reduced the protective activity of CED-9 by about two-thirds. This reduction in CED-9 activity was not caused by instability of CED-9 protein resulting from the amino-acid substitutions, because these CED-9 mutant proteins were present in amounts similar to that of the wild-type CED-9 protein, as judged by western-blot analysis (data not shown). These results indicate that the presence of at least one CED-3 cleavage site in CED-9 is important for full CED-9 function in cell-death protection in *C. elegans*. We also found that the two CED-3 cleavage sites are necessary for the *ced-9* gene to rescue the *ced-9* loss-of-function mutant phenotype[2] (data not shown).

These experiments indicate that the mutant proteins CED-9(D44A, D67E) and CED-9(D47E, D67E) both retained significant CED-9 activity (Table 1), which was probably a consequence of the C-terminal region of CED-9, as expression of the CED-9 C-terminal cleavage product CED-9(68–280) inhibited cell death to an extent comparable to that caused by CED-9(D47E, D67E). By contrast, expression of the CED-9 N-terminal region CED-9(1–67) did not inhibit cell death (Table 1): this failure correlated with its lack of cleavage by CED-3 *in vitro* (data not shown). Co-expression of CED-9(1–67) and CED-9(68–280) protected against cell death to the same extent as did expression of CED-9(68–280) on its own (Table 1), indicating that co-expression of these polypeptides could not restore full CED-9 activity.

CED-9(68–280) is similar in sequence to the mammalian cell-survival factor Bcl-2, especially in the two Bcl-2 homology domains known as BH1 and BH2 (Fig. 1)[2]. Bcl-2 inhibits cell death in *C. elegans* to the same extent as does CED-9(68–280) (Table 2)[2,20]; also, co-expression of Bcl-2 and CED-9(68–280) did not protect against cell death in *C. elegans* any better than either protein expressed by itself (Table 2), indicating that CED-9(68–280) and Bcl-2 may inhibit cell death by a similar mechanism. Thus, CED-9(68–280) and Bcl-2 may have similarity in function as well as in sequence.

To assess the contribution of the BH1 and BH2 domains to the protective effect of CED-9(68–280) against cell death, we introduced mutations into these regions. A single mutant (W214A) altered in the highly conserved Trp 214 residue of the BH2 domain of CED-9 partially reduced CED-9(68–280) activity (Table 2). A double mutant (G169L, W214A) that was additionally altered at a highly conserved glycine 169 residue in the BH1 domain abolished the protective activity of CED-9(68–280) (Table 2). Expression of both CED-9(68–280) mutant proteins was comparable to that of the original CED-9(68–280) (data not shown). These results indicate that both the BH1 and BH2 domains are important for CED-9(68–280) function. Substitution of the corresponding residues in the BH1 and BH2 domains of Bcl-2 abolished its death-protective effect and prevented it from forming a heterodimer with Bax, an interaction proposed to be important for protection against cell death by Bcl-2 (ref. 21).

A full-length CED-9(G169L, W214A) double mutant could still act as a substrate for CED-3 protease and gave partial protection against cell death (Fig. 1a, lanes 11 and 12; Table 2). Two further mutations (D44A, D67E) that disrupted both CED-3 cleavage sites abolished the remaining protective activity of CED-9(G169, W214A) (Fig. 1a, lanes 13 and 14; Table 2). These results support the hypothesis that the two CED-3 cleavage sites in CED-9 confer protection against programmed cell death, just like the CED-3 cleavage site in the p35 protein[9], indicating that CED-9 contains two distinct domains that can each inhibit cell death and which are both required for complete protection by CED-9.

We further tested this hypothesis by fusing the CED-9 N-terminal

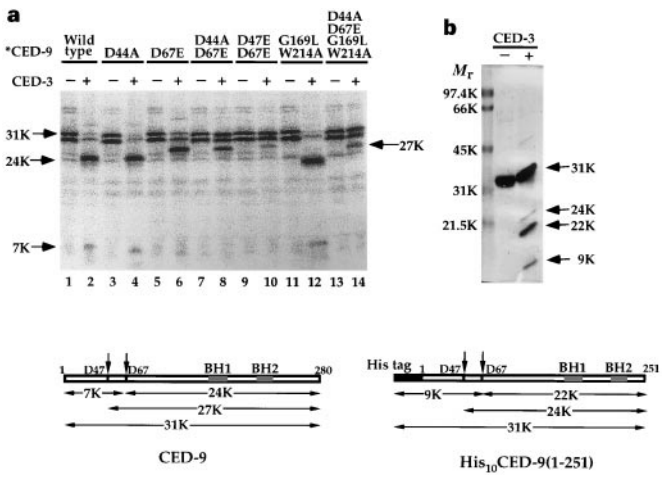

Figure 1 The *C. elegans* CED-9 protein is a substrate of the CED-3 protease. **a**, ^{35}S-methionine-labelled CED-9 protein (*CED-9) was incubated with (+) or without (–) 20 ng of purified CED-3 protease for 20 min at 30 °C. The band seen below the full-length 31K CED-9 band may be an internal initiation product starting at methionine 20 of CED-9. **b**, Purified His_{10}CED-9(1–251) protein (10 μg) was incubated in CED-3 buffer without (–) or with (+) 160 ng purified CED-3 protease for 2 h at 30 °C, resolved by 12.5% SDS–PAGE and stained with Coomassie brilliant blue. Diagrams underneath show the origins deduced for the detected protein bands: in **b**, the diagram is based on microsequencing analysis. Black box indicates the N-terminal tag, which includes ten histidines. Grey boxes (BH1 and BH2) indicate Bcl-2-homology domains[21]. Vertical arrows indicate proteolytic cleavage sites. Protein sizes are indicated. M_r, relative molecular masses of prestained protein markers (Bio-Rad).

Table 1 CED-3 cleavage sites are important for full CED-9 cell-death protective function

hsp construct*	Array*	No. of animals	Extra cells in anterior pharynx†	Range of extra cells†
Vector	1	27	0.2 ± 0.4	0–1
	2	21	0.1 ± 0.3	0–1
CED-9	1	20	10.9 ± 1.3	8–13
	2	24	10.5 ± 1.8	7–14
	3	14	9.8 ± 3.2	4–14
CED-9(D44A)	1	27	7.6 ± 3.0	1–14
	2	21	8.2 ± 2.6	3–11
CED-9(D67E)	1	25	9.4 ± 2.8	3–14
	2	22	9.1 ± 2.8	3–14
	3	12	9.6 ± 1.9	7–13
CED-9(D44A, D67E)	1	23	2.5 ± 2.4	0–7
	2	35	4.1 ± 3.0	0–11
	3	23	4.0 ± 2.7	0–9
CED-9(D47E, D67E)	1	19	2.8 ± 2.6	0–8
	2	15	3.6 ± 2.4	0–8
CED-9(68–280)	1	17	4.2 ± 3.4	0–10
	2	23	4.5 ± 3.0	0–10
	3	21	4.9 ± 2.9	0–10
CED-9(1–67)	1	8	0.8 ± 1.2	0–3
	2	20	0.4 ± 0.6	0–2
	3	20	0.7 ± 1.0	0–3
CED-9(1–67) + CED-9(68–280)	1	14	4.2 ± 2.4	0–8
	2	14	4.4 ± 3.0	0–8
	3	10	3.3 ± 2.7	1–6

* The *hsp* constructs were co-injected at 100 μg ml^{-1} each into wild-type N2 animals with pRF4 (at 50 μg ml^{-1}), which carries the dominant *rol-6* marker, as described[9]. Array indicates an extrachromosomal transgene carried by a given transgenic line.
† Heat-shock experiments were performed and scored as described[2]. Data shown are means ± s.e.m. All data depict results obtained after heat-shock treatment. *ced-3* mutants, presumably completely defective in cell death, have an average of 14 extra cells[2].

region (amino acids 1 to 80), which includes the two CED-3 cleavage sites, to the N terminus of human Bcl-2. This CED-9(1–80)–Bcl-2 chimaeric protein resulted in significantly better protection against cell death than did Bcl-2 protein alone (Table 2). In addition, co-expression in *C. elegans* of baculovirus p35 protein (which contains a CED-3 cleavage site necessary for its cell-death protective function)[9] and human Bcl-2 gave better protection than the separate proteins, indicating that p35, like CED-9(1–80) but unlike CED-9(68–280), can act additively with Bcl-2 (Table 2).

The *ced-9* gain-of-function mutation (G169E) enhances cell-death protection by *ced-9* (refs 1, 22). We found that CED-9(68–280) carrying the G169E mutation resulted in more effective protection against cell death than did CED-9(68–280) on its own, and was as effective as wild-type CED-9 (Table 2). This experiment indicates that the G169E mutation increases the potency of CED-9(68–280) in protecting against cell death.

We propose that CED-9 is a bifunctional cell-death inhibitor, protecting against programmed cell death in *C. elegans* by two distinct mechanisms. First, CED-9 inhibits cell death by interacting directly with the CED-3 death protease, possibly through a competitive mechanism like that proposed for baculovirus p35 protein[9]. Second, CED-9 also inhibits cell death through its C-terminal region by a mechanism similar to that used by mammalian Bcl-2. It has been proposed that CED-9 protects against CED-3 killing by acting at least in part via CED-4 (ref. 23), which like CED-3 is essential for programmed cell death in *C. elegans*[24,25]. Furthermore, the CED-4 protein can interact with CED-3 and CED-9 *in vitro*[26–28]. We have found that CED-9(68–280) is sufficient for interaction with CED-4 (D.X. and H.R.H., unpublished results). Our findings suggest that CED-9(68–280) inhibits programmed cell death in *C. elegans* as a result of a physical interaction with CED-4 and, furthermore, that Bcl-2 may inhibit cell death in mammals by means of a mammalian CED-4 homologue. □

Table 2 CED-9 may inhibit cell death through two different mechanisms

hsp construct*	Array*	No. of animals	Extra cells in* anterior pharynx	Range of extra cells*
CED-9	1	20	10.9 ± 1.3	8–13
	2	24	10.5 ± 1.8	7–14
	3	14	9.8 ± 3.2	4–14
CED-9(68–280)	1	17	4.2 ± 3.4	0–10
	2	23	4.5 ± 3.0	0–10
	3	21	4.9 ± 2.9	0–10
Human Bcl-2	1	24	4.7 ± 28	1–12
	2	10	4.5 ± 1.8	1–7
	3	24	4.1 ± 2.3	0–8
CED-9(68–280) + hBcl-2	1	24	4.3 ± 2.6	1–9
	2	20	4.8 ± 2.7	0–9
	3	11	5.4 ± 1.8	2–8
CED-9(68–280; W214A)	1	16	2.4 ± 1.9	0–5
	2	18	2.3 ± 2.2	0–7
CED-9(68–280; G169L, W214A)	1	26	0.8 ± 1.2	0–5
	2	21	0.8 ± 1.0	0–4
	3	16	0.6 ± 1.0	0–4
CED-9(G169L, W214A)	1	20	2.4 ± 1.2	0–6
	2	20	2.2 ± 1.6	0–5
CED-9(D44A, D67E, G169L, W214A)	1	14	0.6 ± 0.8	0–2
	2	30	0.8 ± 0.9	0–3
CED-9(1–80)-hBcl-2	1	9	8.7 ± 2.7	4–12
	2	21	7.0 ± 2.7	3–12
	3	11	8.2 ± 2.6	3–12
p35†	1	17	8.9 ± 2.6	5–13
	2	16	8.6 ± 3.5	2–13
	3	21	8.8 ± 3.1	3–16
p35 + hBcl – 2	1	13	11.2 ± 3.2	5–16
	2	21	12.8 ± 3.3	5–16
	3	30	12.1 ± 1.7	9–15
CED-9(68–280; G169E)	1	25	10.5 ± 3.3	3–16
	2	27	11.2 ± 2.4	6–15
	3	12	10.9 ± 1.9	7–13

* See descriptions in Table 1.
† Data from ref. 9.

Methods

Plasmid construction. We used standard methods of molecular biology[29] to generate various CED-9 constructs and their mutant derivatives.

Protease assays. Protease assays have been described[18]. Using purified His_{10}CED-9(1–251) protein and various CED-9 proteins synthesized in *E. coli* (N-terminal-tagged or C-terminal-tagged and purified, or untagged and unpurified), we were unable to demonstrate an inhibition of CED-3 protease activity (data not shown), perhaps because our *in vitro* conditions were unsuitable (such as the ionic conditions or protein concentrations, absent protein or non-protein cofactors, or inappropriate subcellular localization—other CED-9/Bcl-2 family members are associated with membranes[30]).

Microsequencing analysis. Purification of recombinant His_{10}CED-9(1–251) protein from *E. coli* and microsequencing of its 22K and 24K CED-3 cleavage products were performed using methods previously described[18].

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Correspondence and requests for materials should be addressed to H.R.H. (e-mail: Horvitz@mit.edu).

CRM1 is responsible for intracellular transport mediated by the nuclear export signal

Makoto Fukuda*, Shiro Asano*, Takahiro Nakamura*, Makoto Adachi*, Minoru Yoshida†, Mitsuhiro Yanagida* & Eisuke Nishida*

** Department of Biophysics, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan*
† Department of Biotechnology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

The discovery of nuclear export signals (NESs) in a number of proteins revealed the occurrence of signal-dependent transport of proteins from the nucleus to the cytoplasm[1–14]. Although the consensus motif of the NESs has been shown to be a leucine-rich, short amino-acid sequence[2,6,7], its receptor has not been identified. A cytotoxin leptomycin B (LMB) has recently been suggested to inhibit the NES-mediated transport of Rev protein[15]. Here we show that LMB is a potent and specific inhibitor of the NES-dependent nuclear export of proteins. Moreover, we have found a protein of relative molecular mass 110K (p110) in *Xenopus* oocyte extracts that binds to the intact NES but not to the mutated, non-functional NES. The binding of p110 to NES is inhibited by LMB. We show that p110 is CRM1, which is an evolutionarily conserved protein[16–18] originally found as an essential nuclear protein in fission yeast[16] and known as a likely target of LMB[19]. We also show that nuclear export of a fission yeast protein, Dsk1, which has a leucine-rich NES, is disrupted in wild-type yeast treated with LMB or in the *crm1* mutant. These results indicate that CRM1 is an essential mediator of the NES-dependent nuclear export of proteins in eukaryotic cells.

A peptide corresponding to the NES sequence of mitogen-activated protein kinase kinase (MAPKK)[5] was crosslinked to ovalbumin (OVA), and the resultant conjugate (KK–NES–OVA) was injected with rhodamine-labelled bovine serum albumin (RITC–BSA) into the nuclei of fibroblastic cells. Ten minutes after the injection, almost all the KK–NES–OVA was excluded from the nucleus, whereas RITC–BSA remained in the nucleus[5] (Fig. 1a). When cells were pretreated with 0.2 ng ml^{-1} LMB, both KK–NES–OVA and RITC–BSA were present exclusively in the nucleus after the injection (Fig. 1). In more than 95% of the injected cells, the nuclear export of KK–NES–OVA was inhibited completely by 0.2 ng ml^{-1} LMB. This strong inhibition of the nuclear export was observed at concentrations of 0.2–20 ng ml^{-1} LMB (data not shown). A conjugate between OVA and the NES peptide of Rev protein (Rev–NES–OVA) moved to the cytoplasm within 10 min when injected into the nucleus, and this nuclear export was also strongly inhibited by LMB (Fig. 1b). In contrast, the nuclear import of nuclear localization signal (NLS)-conjugated BSA (NLS–BSA) was not inhibited by LMB, even at a concentration of 20 ng ml^{-1} (Fig. 1c). The NES of MAPKK was found to compete with the NES of PKI-α[5] and the NES of Rev (M.F., S.A. & E.N., unpublished data). The above data, together with the previous observation that LMB disrupted the NES-directed cytoplasmic localization of Rev[15], therefore indicate that LMB specifically inhibits the nuclear export of proteins, which is mediated by the NES rich in hydrophobic residues (usually leucine). We therefore examined the effect of LMB on the subcellular distribution of MAPKK, the cytoplasmic localization of which is determined by its NES[5]. Within 5 min of LMB treatment, MAPKK began to appear in the nucleus, and by 20–30 min MAPKK was distributed evenly throughout the cell (Fig. 1d). In about 30% of the cells, MAPKK appeared to be accumulated in the nucleus after 60 min. This pattern of intracellular distribution of MAPKK is similar to that of the NES-disrupted MAPKK in cells without LMB[5]. Thus LMB is shown to inhibit the NES-dependent nuclear export of proteins.

To determine which protein(s) binds specifically to the NES, we searched for a protein that binds to the intact NES sequence of MAPKK, but not to the non-functional, mutated (leucine to alanine) NES sequence. The fusion proteins between glutathione *S*-transferase (GST) and the amino-terminal region of MAPKK (residues 1–60) that had either an intact wild-type NES (WT–NES, residues 33–44) or a leucine-to-alanine mutated NES (LA–NES) were immobilized on glutathione Sepharose and incubated with extracts obtained from *Xenopus* oocytes. After washing, the bound proteins were eluted with glutathione. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of eluted fractions showed that a protein with an apparent relative molecular mass of 110,000 (M_r 110K), p110, binds to the WT–NES, but not to LA–NES (Fig. 2a). The binding of p110 to WT–NES beads was inhibited by free NES peptide (corresponding to residues 27–44 of MAPKK), but not by free, mutated NES (LA–NES) peptide (Fig. 2c, left). Furthermore, the binding was completely blocked in the presence of LMB (Fig. 2d, left). These results suggest that p110 is a candidate for a transport factor for NES-bearing proteins.

A fission yeast *Schizosaccharomyces pombe* gene, *crm1*$^+$, which encodes a nuclear protein of M_r 115K, was originally isolated by complementation of a cold-sensitive mutant causing deformed chromosome structure[16], and has been suggested to be a target of LMB[19]. A human homologue of CRM1 has recently been identified[18] and shown to bind to the oncogenic nucleoporin CAN/Nup214 (ref. 20) and to move between the nuclear pore and the nucleoplasm. It has therefore been proposed that CRM1 may be a soluble nuclear transport factor[18]. Human CRM1 was reported to have an M_r of 112K on SDS–PAGE[18,20], very similar to p110. We therefore hypothesized that p110 might be *Xenopus* CRM1. To test this