Subcellular localization of CrmA: identification of a novel leucine-rich nuclear export signal conserved in anti-apoptotic serpins

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The cowpox virus-encoded anti-apoptotic protein cytokine response modifier A (CrmA) is a member of the serpin family that specifically inhibits the cellular proteins caspase 1, caspase 8 and granzyme B. In this study, we have used Flag- and yellow fluorescent protein (YFP)-tagged versions of CrmA to investigate the mechanisms that regulate its subcellular localization. We show that CrmA can actively enter and exit the nucleus and we demonstrate the role of the nuclear export receptor CRM1 in this shuttling process. CrmA contains a novel leucine-rich nuclear export signal (NES) that is functionally conserved in the anti-apoptotic cellular serpin PI-9. Besides this leucine-rich export signal, additional sequences mapping to a 103-amino-acid region flanking the NES contribute to the CRM1-dependent nuclear export of CrmA. Although YFP-tagged CrmA is primarily located in the cytoplasm, shifting its localization to be predominantly nuclear by fusion of a heterologous nuclear localization signal did not impair its ability to prevent Fas-induced apoptosis. We propose that nucleocytoplasmic shuttling would allow CrmA to efficiently target cellular pro-apoptotic proteins not only in the cytoplasm, but also in the nucleus, and thus to carry out its anti-apoptotic function in both compartments.

Key words: apoptosis, nuclear transport, CRM1.

INTRODUCTION

The regulated self-destruction of a cell by apoptosis constitutes a complex process that involves the co-ordinated activity of proteins localized to different subcellular compartments, such as the cell membrane, cytoplasm, mitochondria and nucleus. The apoptotic pathway triggered by the interaction between the death receptor Fas (CD95/APO-1) and its ligand on the cell membrane clearly illustrates this complexity (reviewed in [1]). Ligand binding leads to oligomerization of Fas and recruitment of cytoplasmic pro-caspase 8 to the intracellular domain of the receptor. Recruitment to the receptor, mediated by the adaptor protein FADD (Fas-associated death domain), results in the self-processing and activation of caspase 8 which, in turn, initiates a cascade of caspase-mediated proteolytic events that eventually leads to DNA fragmentation and degradation of nuclear proteins. Because activation of the caspase cascade in the cytoplasm ultimately leads to a series of nuclear changes that constitute classical hallmarks of apoptosis, the relay of the apoptotic signal to the nucleus is thought to represent an important step in the apoptotic process [2].

Communication between the nucleus and cytoplasm takes place through the nuclear pore complexes, gated channels that penetrate the double membrane of the nuclear envelope [3]. The diameter of these channels allows free diffusion of small molecules, but translocation of many proteins across the nuclear envelope involves active transport mechanisms mediated by specific import or export receptors [4]. Selective transport in and out of the nucleus depends on the presence of nuclear localization signals (NLSs) or nuclear export signals (NESs) in the cargo protein, that are recognized by the import and export receptors respectively [5,6]. How the nucleocytoplasmic transport machinery is involved in conveying the apoptotic signal to the nucleus remains to be clearly established. Elucidating the mechanisms and identifying the signals that regulate the nucleocytoplasmic localization of the proteins that are involved in the execution or regulation of apoptosis may contribute to our understanding of this process.

Several viruses have evolved proteins that interfere with the cellular apoptotic machinery as a mechanism to counteract the host immune response to infection. One of the best-characterized viral anti-apoptotic proteins is the cowpox virus-encoded CrmA (cytokine response modifier A), a 38 kDa protein member of the serine protease inhibitor (serpin) superfamily [7]. CrmA plays a critical role in viral pathogenesis by inactivating three cellular proteases: caspase 1, caspase 8 and granzyme B [7–11]. By targeting caspase 8 and granzyme B, CrmA blocks cytotoxic T lymphocyte-induced suicide of infected cells mediated by the Fas or the perforin/granzyme apoptotic pathways [9–11]. On the other hand, inhibition of caspase 1 prevents the proteolytic activation of pro-interleukin-1β [7,8], compromising the host inflammatory response. Besides its physiological role, CrmA is widely used as a tool to determine the role of particular pathways in the cellular response to different pro-apoptotic stimuli due to its target specificity [12–14].

The subcellular localization of the three cellular proteases targeted by CrmA has been addressed in several reports [15–21]. They have been shown to enter the nucleus during apoptosis [15–17,21], and a classical NLS has been identified in the pro-domain of caspase 1 [18]. CrmA has been shown previously to localize to either the cytoplasm [7] or both to the nucleus and cytoplasm [22], but the transport mechanisms and signals that regulate its subcellular localization have not been defined. The current view is that its small size allows CrmA to freely diffuse through the nuclear pores into the nucleus [22]. We show here that, besides passive diffusion, active cellular mechanisms of nucleocytoplasmic transport, including CRM1-dependent nuclear export, determine the subcellular localization of CrmA. We have identified and characterized in detail a novel leucine-rich NES in

Abbreviations: CHX, cycloheximide; CrmA, cytokine response modifier A; GFP, green fluorescent protein; LMB, leptomycin B; NES, nuclear export signal; NLS, nuclear localization signal; PARP, poly(ADP-ribose) polymerase; SV40, simian virus 40; YFP, yellow fluorescent protein.

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CrmA that is functionally conserved in the cellular anti-apoptotic serpin PI-9. Interestingly, CRM1-dependent nuclear export of CrmA is mediated not only by its leucine-rich export signal, but also by additional sequences that we mapped to a 103-amino-acid segment flanking the NES. The ability of CrmA to shuttle between the two cellular compartments suggests that it may function as an anti-apoptotic protein not only in the cytoplasm, but also in the nucleus. In support of this possibility, we show that the ability of yellow fluorescent protein (YFP)-CrmA, a primarily cytoplasmic protein, to prevent Fas-induced apoptosis is fully preserved when its localization is artificially shifted to be predominantly nuclear by the fusion of a heterologous NLS.

MATERIALS AND METHODS

Cell culture, transfection and leptomycin B (LMB) treatment

Human tumour-derived cell lines MCF-7 (breast carcinoma), HeLa (cervical carcinoma), A431 (vulval carcinoma), SW1573 (lung carcinoma) and HepG2 (hepatic carcinoma) were grown in RPMI or Dulbecco's modified Eagle's medium (BioWhittaker), supplemented with 10 % (v/v) fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco BRL). Cells were seeded on to sterile glass coverslips in 6- or 12-well trays, and transfected with 0.5–2 μg of plasmid DNA using the FuGene6 transfection reagent (Roche Molecular Biochemicals), following the manufacturer's protocol. LMB (generously provided by Dr Minoru Yoshida, University of Tokyo, Tokyo, Japan) was added to the culture medium to a final concentration of 6 ng/ml and the cells were incubated at 37 °C for the indicated period of time.

Cloning procedures and plasmid construction

The mammalian expression vectors pEF-FlagpGKpuro-CrmAwt and pEF-FlagpGKpuro-CrmAmt, encoding Flag-tagged wild-type CrmA or an inactive point mutant ($Thr^{291} \rightarrow Arg$) variant respectively [14], were generously provided by Dr David L. Vaux (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). To generate the plasmid encoding Flag-CrmA + NLS, the NLS of the simian virus 40 (SV40) large T antigen (PKKKRKV) [23] was cloned at the C-terminal end of wild-type CrmA using PCR with primers JAR34 and JAR61 (Table 1). The PCR product, digested with *Nde*I and *Xba*I restriction enzymes (Roche Molecular Biochemicals), was used to replace an *Nde*I/*Xba*I-excised fragment at the 3′-end of CrmA cDNA. To create the YFP-tagged versions of CrmA, CrmAT291R and CrmA + NLS, the corresponding cDNAs were amplified by PCR using primer set JAR51/JAR56 (CrmA wt and T291R) or JAR51/JAR83 (CrmA + NLS) and cloned as *Hin*dIII/*Kpn*I fragments into the pEYFP-C1 expression plasmid (Clontech, Palo Alto, CA, U.S.A.). PCR was also used to generate the deletion mutants of CrmA fused to YFP. The primer sets used to amplify each CrmA fragment were JAR51/JAR52 (residues 1–149), JAR53/JAR56 (100–341), JAR55/JAR56 (200–341), JAR53/JAR54 (100–270), JAR100/JAR102 (150–200), JAR55/JAR54 (200–270) and JAR101/JAR72 (263–303).

The DNA segments encoding the NES-like sequences of CrmA and a short flanking region were amplified by PCR using CrmA cDNA as template and cloned as *Bam*HI/*Age*I fragments into the previously described Rev(1.4)-GFP plasmid [24] (where GFP is green fluorescent protein). This plasmid, as well as the positive control for the export assay containing the wild-type Rev NES, were kindly provided by Dr Beric R. Henderson (The Westmead Institute for Cancer Research, Sydney, Australia). The primer sets used were JAR10/JAR11 [CrmA-(242–259)] and JAR65/JAR66 [CrmA-(223–244)]. Similarly, the DNA segments encoding the amino acid sequences of α1-antitrypsin, PI-9, and Maspin similar to CrmA NES were amplified by PCR using genomic or cDNA from Jurkat cells as template, and cloned as *Bam*HI/*Age*I fragments into Rev(1.4)-GFP. The primer sets used in this case were JAR75/JAR76 (α1-antitrypsin), JAR77/JAR78 (PI-9) and JAR79/JAR80 (Maspin). The single amino acid substitutions of CrmA NES in the export assay construct were generated using mutagenic PCR primers JAR24 ($Leu^{255} \rightarrow Ala$) and JAR25 ($Leu^{253} \rightarrow Ala$) in combination with JAR10. In all cases, the high fidelity DNA polymerase Pfu (Stratagene, La Jolla, CA, U.S.A.) was used in the PCR reactions, and the sequence of the inserts was verified by DNA sequencing.

Table 1 Sequences of the oligonucleotides used in cloning, plasmid construction and site-directed mutagenesis

Oligonucleotide	Sequence 5′ → 3′
JAR10	CGG GCC CGG GAT CCA GGC TCG TAT AAT CTG
JAR11	GTG GCG ACC GGT GGG AAC ACC TCT GTC AG
JAR24	GTG GCG ACC GGT GGG AAC ACC TCT GTC GCT CCC AA
JAR25	GTG GCG ACC GGT GGG AAC ACC TCT GTC AGT CCC GCC TTT AC
JAR32	GTA AAG GCG GGA GCG ACA GAG GTG TTC GGT TCA AC
JAR33	CTC TGT CGC TCC CGC CTT TAC TAG CGC ATC CAC CAG
JAR34	AGG CAT TTA ATC ACG CAT CTG
JAR35	ACG TGG GAG ACC TGA TAC TCT C
JAR51	TTA GTA CAA GCT TCG ATG GAT ATC TTC AGG G
JAR52	AAT GGA GGT ACC TCA ATA ATC ACT GGT AAA TTC C
JAR53	TTA GTA CAA GCT TCG AAG TGT GTT GAT ATC TTC
JAR54	AAT GGA GGT ACC TCA ACA CAT ATT GCT ATA ATC
JAR55	TTA GTA CAA GCT TCG CCA GAC AAT ATT GAT GG
JAR56	AAT GGA GGT ACC TTA ATT AGT TGT TGG AG
JAR61	GAC TAG TCT AGA TTA GAC TTT GCG CTT CTT CTT AGG ATT AGT TGT TGG AGA GCA ATA TC
JAR65	CGG GCC CGG GAT CCA TGT GAC TCT ATG GAT G
JAR66	GTG GCG ACC GGT GGA TAC GAG CCT GTT AC
JAR72	AAT GGA GGT ACC TTA GTC TGC CAC CAG CGC AC
JAR75	CGG GCC CGG GAT CCA GGA ACC TAT GAT CTG
JAR76	GTG GCG ACC GGT GGG AAG ACC TTA GTG ATG
JAR77	CGG GCC CGG GAT CCA GAG GAT TAT GAC ATG
JAR78	GTG GCG ACC GGT GGG AAG GCA TCA ACA ATT
JAR79	CGG GCC CGG GAT CCA AAG ATG ATT GAT CCC
JAR80	GTG GCG ACC GGT GGG AAG ATA TGT TTC AG
JAR83	AAT GGA GGT ACC TTA GAC TTT GCG CTT CTT C
JAR100	TTA GTA CAA GCT TCG CCC TTT TAC GTA TCT CC
JAR102	AAT GGA GGT ACC TCA AAG AAT TAC CAC CAT AC

Site-directed mutagenesis of CrmA NES

A two-step PCR-based site-directed mutagenesis approach was used to generate the NES-defective $CrmA^{NES}$, introducing two point mutations ($Leu^{253} \rightarrow Ala$ and $Leu^{255} \rightarrow Ala$) in the NES of CrmA. Two overlapping PCR fragments were amplified with primer combinations JAR34/JAR33 and JAR32/JAR35. Following gel-purification, the PCR products were mixed, annealed, and used as template for a second round of amplification with primers JAR34 and JAR35. The product of this second reaction was digested with *Nde*I and *Xba*I and used to replace the *Nde*I/*Xba*I-excised 3′-end of CrmA. After confirming the presence of the mutation by DNA sequencing, this plasmid was used as template in PCR reactions to generate YFP-$CrmA^{NESm}$ (primer set JAR51/JAR56), and the different YFP-fusion fragments of CrmA containing the NES mutation: 100–341^{NESm} (primer set JAR53/JAR56), 200–341^{NESm} (primer set JAR55/JAR56), 200–303^{NESm} (primer set JAR 55/JAR72), and 200–270^{NESm} (primer set

JAR55/JAR54). In all cases, the PCR products were inserted as *Hin*dIII/*Kpn*I fragments into pEYFP-C1.

Immunocytochemistry, fluorescence microscopy analysis and quantification of subcellular distribution

Cells transfected with plasmids encoding YFP-tagged proteins were fixed with 3.7 % formaldehyde in PBS for 30 min at room temperature, stained with the chromosome dye Hoechst 33285 (Sigma) for 30 min to counterstain the nuclei, and mounted on to microscope slides using Vectashield (Vector). Immunocytochemical detection of Flag-tagged CrmA was performed with the anti-Flag M2 monoclonal antibody (Stratagene), and a fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Santa Cruz), using a previously described immunostaining method [25]. Fluorescence microscopy analysis was carried out using an inverted Leica DMIRB/E fluorescence microscope (Leica Heidelberg, Heidelberg, Germany). Images were collected using the Q500MC Quantimet software V01.01 (Leica Cambridge, Cambridge, U.K.). To quantitatively determine their subcellular distribution, the localization of the ectopically expressed proteins was assessed in at least 200 transfected cells per sample. The degree of LMB-induced nuclear accumulation of proteins (LMB effect) was calculated as described previously [25].

In vivo Rev(1.4)-GFP nuclear export assay

The NES-like motifs in CrmA and other serpins were tested for export activity using the Rev(1.4)-GFP *in vivo* nuclear export assay [24]. Duplicate samples of MCF-7 cells were transfected with pRev(1.4)-GFP (negative control) and its derivative plasmids containing each of the candidate export sequences or the wild-type Rev NES (as positive control). At 24 h post-transfection, cells were treated for 3 h with either 10 μg/ml cycloheximide (CHX) plus 5 μg/ml actinomycin D or with CHX alone. CHX treatment ensures that cytoplasmic GFP arises from nuclear export and not from new protein synthesis, whereas actinomycin D prevents, by an undefined mechanism, the nuclear import of Rev and thus allows detection of weaker NESs. The subcellular localization of the GFP-fusion proteins was determined in at least 200 cells per sample and the activity of the functional NESs was rated according to the published scoring system [24].

The CRM1-dependence of the functional NESs identified in the nuclear export assay was further confirmed by LMB treatment of the transfected cells (6 ng/ml for 3 h).

Sequence alignment analysis

The amino acid sequence comparison between CrmA and cellular serpins was carried out using the BLASTP 2.2.3 program available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Induction and evaluation of Fas-dependent apoptosis

Duplicate samples of HeLa cells were transfected with expression plasmids encoding three YFP-tagged variants of CrmA: the wild-type protein, CrmA + NLS and, as control, the previously described loss-of-function mutant T291R [14]. At 24 h post-transfection, cells were treated with 100 ng/ml anti-Fas activating human monoclonal antibody CH11 (Upstate Biotechnology) plus 2 μg/ml CHX. After 20 h of incubation under control conditions or in the presence of anti-Fas/CHX, cells were examined using an inverted Leica DMIRB/E fluorescence microscope under UV and visible light. Viable cells were scored as those that remained attached to the plate and did not show morphological signs of apoptosis. The percentage of viability of transfected cells was determined by examining at least five independent microscopy fields and counting more than 600 YFP-positive cells. As a marker for apoptotic cell death, the cleavage of poly(ADP-ribose) polymerase (PARP) was determined, as previously described [26], in protein extracts generated from the detached and attached cell sub-populations of samples transfected with YFP-CrmA and YFP-CrmA + NLS. We also used an anti-GFP antibody (Sigma) to determine the expression of YFP-CrmA and YFP-CrmA + NLS in these cell sub-populations, and anti-actin antibody (Sigma) to control for equal protein loading.

RESULTS

The nucleocytoplasmic localization of CrmA is regulated by cellular mechanisms of active transport, including CRM1-dependent nuclear export

To investigate its subcellular localization, we transfected HeLa human cervical carcinoma cells with expression vectors encoding CrmA tagged with either a Flag epitope (Flag-CrmA) or YFP (YFP-CrmA). Fluorescence microscopy analysis (Figure 1A) of CrmA-expressing cells revealed a complex pattern of nucleocytoplasmic distribution, with CrmA being present only in the cytoplasm of some cells, but localizing to either the nucleus or both the nucleus and cytoplasm of other cells. The subcellular distribution of CrmA was influenced by the size of the tag used: the 65 kDa non-diffusing YFP-CrmA fusion protein was more often excluded from the nucleus than the smaller Flag-CrmA. We extended the analysis of YFP-CrmA localization to a broader panel of human cells derived from different types of tumour (Table 2). In every cell line tested, approx. 20–30 % of the transfected cells showed at least partial nuclear accumulation of YFP-CrmA. This indicates that nuclear import of CrmA occurs, at least in part, by an active mechanism.

These observations, and a recent report that the localization of the cellular serpin PI-9 is regulated by the export receptor CRM1 [22], prompted us to investigate the possibility that CrmA undergoes CRM1-dependent nuclear export. Treatment of the cells with the specific CRM1 inhibitor LMB [27] resulted in a consistent relocation of CrmA to the nucleus of MCF-7 and HeLa cells (Figure 1B), indicating a role for CRM1 as a mediator of CrmA nuclear export. The limited LMB effect observed could be due either to a slow rate of nuclear import or to the selective retention of CrmA in the cytoplasm caused by interaction with other proteins. Fusion of the NLS of the SV40 large T antigen [23] to the C-terminus of CrmA readily induced the nuclear accumulation of CrmA (see below and Figure 6A), indicating that a slow rate of nuclear import is largely responsible for the limited nuclear accumulation of CrmA upon LMB treatment, whereas cytoplasmic retention plays only a minor role. Altogether, these findings indicate that, in addition to the previously described diffusion through the nuclear pore, a slow rate of active nuclear import and CRM1-dependent nuclear export are major determinants of the subcellular localization of CrmA.

CrmA contains a functional leucine-rich NES

To map the regions involved in regulating CrmA nucleocytoplasmic transport, we generated a series of deletion mutants of CrmA

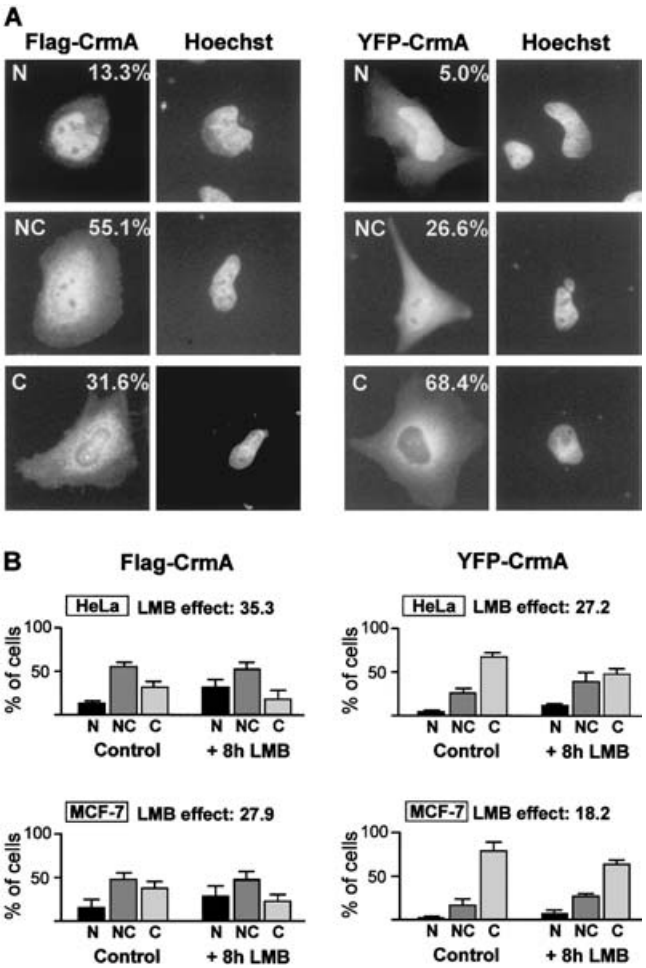

Figure 1 Subcellular localization and response to LMB of epitope-tagged CrmA

(**A**) Representative examples of transfected HeLa cells expressing Flag-CrmA (left panels) or YFP-CrmA (right panels). In some transfected cells, the localization of CrmA was predominantly nuclear (N), whereas it was both nuclear and cytoplasmic (NC), or predominantly cytoplasmic (C) in other cells. The proportion of cells showing each type of subcellular localization, indicated in each panel, was determined by counting more than 500 cells. Cells were counterstained with Hoechst to visualize the nuclei. (**B**) Incubation of HeLa or MCF-7 cells expressing Flag- or YFP-tagged CrmA in the presence of 6 ng/ml of the specific inhibitor LMB (+ 8 h LMB) increases the proportion of cells showing either nuclear (N) or nuclear and cytoplasmic (NC) CrmA with respect to untreated cells (control). Graphs show the results (mean ± S.D.) of at least three independent experiments in which the subcellular localization of CrmA was scored in more than 200 individual cells per experiment. The LMB effect was calculated as previously described [25].

Table 2 Nucleocytoplasmic distribution of YFP-tagged CrmA in human tumour cell lines

Cells were transfected with a plasmid encoding YFP-CrmA and the proportion of cells expressing the ectopic protein in the nucleus (N), cytoplasm (C) or both (NC) was determined by counting the indicated number of cells (*n*).

Cell line	Origin	*n*	N (%)	NC (%)	C (%)
MCF-7	Breast carcinoma	944	3.2	17.5	79.3
SW1573	Lung carcinoma	633	4.2	22	73.8
A431	Vulval carcinoma	540	3.5	25	71.5
HepG2	Hepatic carcinoma	590	3.4	27.5	69.1
HeLa	Cervical carcinoma	1856	5.0	26.6	68.4

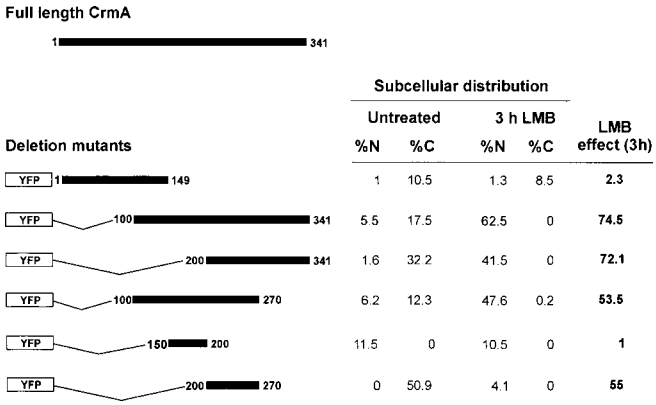

Figure 2 Deletion analysis to map the CrmA region responsible for nuclear export

A series of YFP-tagged truncated fragments of CrmA, schematically represented on the left, were expressed in MCF-7 cells. The subcellular distribution of each fragment in the absence (untreated) or presence (3 h LMB) of LMB is shown in the right. Only the percentage of cells expressing the YFP-fusion proteins predominantly in the nucleus (%N) or the cytoplasm (%C) is indicated. The remaining cells (results not shown) expressed similar levels in both compartments. Results are the mean of two independent experiments with less than 10 % variation. More than 200 cells per sample were counted in each experiment. The clear LMB effect on CrmA-(200–270) suggests the presence of sequences mediating CRM1-dependent nuclear export in this fragment.

fused to YFP. These constructs were transfected into MCF-7 cells and the subcellular distribution, as well as the response to LMB treatment of each region, was determined. As shown in Figure 2, the localization of CrmA fragments encompassing amino acids 1–149 and 150–200 did not change following incubation with LMB for 3 h. In contrast, LMB treatment induced a dramatic relocation of the other four CrmA fragments to the nucleus, which was already evident at 30 min after addition of the drug (results not shown). The results of this analysis clearly indicated the presence of sequences mediating CRM1-dependent nuclear export in the segment encompassing CrmA amino acids 200–270. In addition, the intense and rapid nuclear accumulation of the deletion mutant YFP-CrmA-(100–341) upon LMB treatment provides further evidence that CrmA is actively imported into the nucleus and, importantly, shows that sequences in the N-terminal region of CrmA may negatively regulate this process.

Visual examination of the amino acid sequence of CrmA segment 200–270 revealed the presence of three motifs that resemble the NES of HIV Rev protein (Figure 3A). These motifs consist of a cluster of large hydrophobic amino acids with a characteristic spacing. To determine if these NES-like motifs function as CRM1-dependent export signals, we used a recently developed *in vivo* nuclear export assay [24]. In this assay, the ability of a potential NES to complement the inactivated NES of the Rev(1.4) mutant protein is assessed. The export-deficient fluorescent protein Rev(1.4)-GFP is trapped in the nucleus, and active NESs are identified by their ability to induce its relocation to the cytoplasm when inserted between the Rev(1.4) and the GFP moieties. The extent of this relocation reflects the strength of the NES tested and thus the activity of different NESs can be compared and classified according to the proportion of cells with cytoplasmic GFP.

Two short fragments of CrmA encompassing the NES-like motifs and a few flanking residues were cloned into Rev(1.4)-GFP and transfected into MCF-7 cells, along with negative [Rev(1.4)-GFP with no inserted NES sequence] and positive [with the wild-type Rev NES re-inserted between Rev(1.4) and GFP] controls.

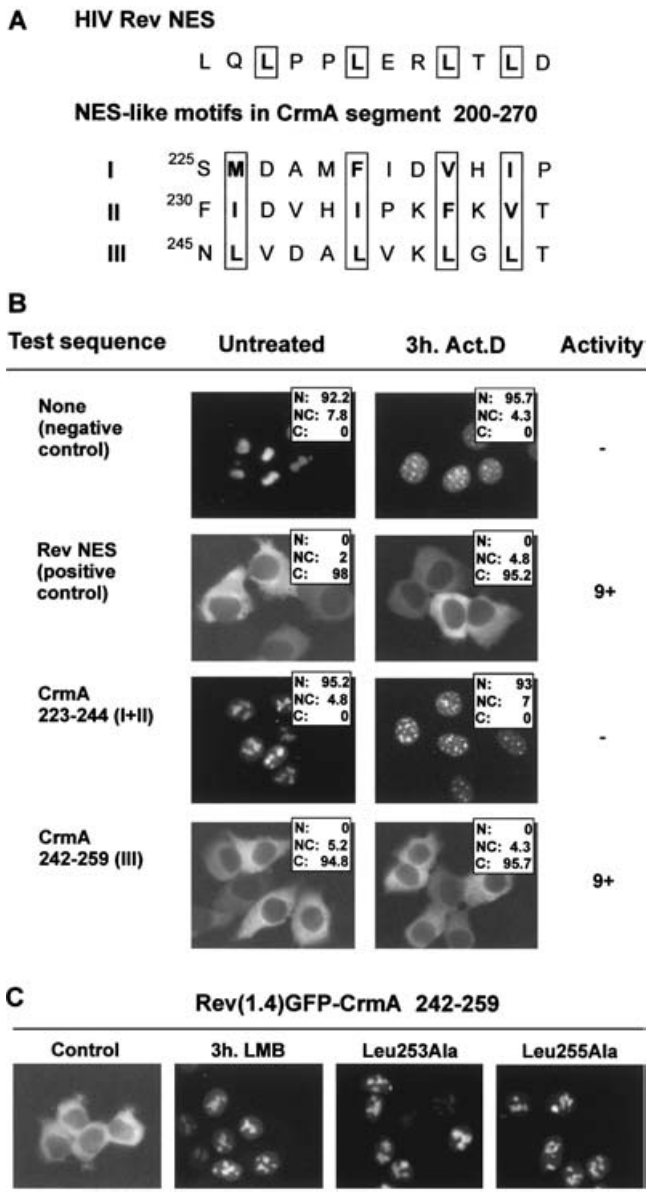

Figure 3 Identification and characterization of a functional leucine-rich NES in CrmA

(**A**) Sequence comparison between the NES of HIV Rev protein and three NES-like motifs in CrmA segment 200–270. The large hydrophobic residues that conform to the consensus NES spacing are boxed. (**B**) *In vivo* nuclear export assay in MCF-7 cells demonstrating that the CrmA fragment 242–259, containing the NES-like motif III, constitutes an active transferable NES, which induced the cytoplasmic accumulation of the nuclear Rev(1.4)-GFP protein (negative control). In contrast, CrmA fragment 223–244 showed no export activity in this assay. The subcellular distribution of each protein is indicated in the insets: N, nuclear; NC, nuclear and cytoplasmic; C, cytoplasmic. Results are the mean of two independent experiments with less than 10% variation. More than 200 cells per sample were counted in each experiment. According to the scoring system used in this assay [24], the activity of CrmA NES was classified as 9+, the same as that of Rev NES (positive control). Act.D, actinomycin D. (**C**) LMB treatment (6 ng/ml for 3 h) or mutagenesis of leucine residues Leu^{253} or Leu^{255} to alanine (Leu253Ala and Leu 255Ala respectively) abrogates CrmA NES-mediated nuclear export.

The overlapping NES-like motifs I and II were tested using a single fragment (223–244). As shown in Figure 3(B), only the CrmA fragment 242–259, which includes NES-like motif III, was able to induce the relocation of Rev(1.4)-GFP to the cytoplasm, indicating that this motif constitutes a functional, transferable NES. Under the conditions of this assay, the CrmA NES was as active as the Rev NES, resulting in >80% cytoplasmic localization of GFP, even in the presence of active Rev-mediated import (untreated samples in Figure 3B). The export activity of CrmA NES was therefore classified as 9+, the highest score in the NES scoring system used with this export assay [24].

Incubation with LMB or with mutants containing leucine-to-alanine substitutions of two of the residues that conform to the consensus NES sequence (Leu^{253} and Leu^{255}) (Figure 3C) efficiently abrogated CrmA NES-induced relocation of Rev(1.4)-GFP to the cytoplasm. These results therefore confirmed that the CrmA NES mediates nuclear export via the CRM1-dependent pathway, and identified critical residues for its activity.

The NES is functionally conserved in the cellular anti-apoptotic serpin PI-9

An amino acid sequence comparison between CrmA and a number of cellular serpins revealed that the four leucine residues that conform to the consensus NES motif are commonly conserved or changed to another hydrophobic residue such as methionine or isoleucine (Figure 4A). Thus the functionality of the signal might *a priori* be preserved, as such residues are often found in functional export signals, such as the NESs of PKI (protein kinase inhibitor) [28] or Ran-BP1 [29]. On the other hand, the neighbouring residues may also affect the activity of an NES [24] and therefore the functional status of this sequence in the different serpins cannot be directly inferred from sequence similarity. Using the nuclear export assay, we tested the region of identity with CrmA NES corresponding to the cellular serpins PI-9, α1-antitrypsin and Maspin. Of the three sequences tested, which show a variable degree of similarity to CrmA NES, only the PI-9 motif was found to be a functional NES (Figure 4B). The export activity of PI-9 NES was classified as 2+, considerably weaker than CrmA NES in this assay.

Additional sequences flanking the NES contribute to CrmA nuclear export

To analyse the activity of the NES in the context of the full-length protein, we used site-directed mutagenesis to generate a NES-defective CrmA variant ($CrmA^{NESm}$) containing two leucine-to-alanine point mutations that abrogate the activity of the NES in the nuclear export assay ($Leu^{253} \rightarrow$ Ala and $Leu^{255} \rightarrow$ Ala, Figure 5A). Although the localization of Flag-$CrmA^{NESm}$ in HeLa cells was less cytoplasmic than that of the wild-type protein, the mutational inactivation of the NES did not fully account for the LMB response (Figure 5B, upper panel). Similar results were observed for YFP-$CrmA^{NESm}$ (results not shown). Since the CrmA N-terminal region negatively regulates its nuclear import (see Figure 2), we introduced the NES-inactivating substitutions into the CrmA deletion mutant lacking the first 100 amino acids. Inactivation of the NES had a more dramatic effect in the context of CrmA-(100–341) (Figure 5B, lower panel). The proportion of cells showing nuclear localization of this protein increased markedly and its cytoplasmic localization was almost completely abrogated. However, as observed for its full-length counterpart, inactivation of the NES did not fully account for the LMB response, and the nuclear relocation of CrmA-$(100–341)^{NESm}$ was further enhanced by treatment with LMB. Surprisingly, $CrmA^{NESm}$ was more cytoplasmic than the wild-type protein in MCF-7 cells (results not shown), and its nuclear accumulation in reponse to LMB cells was decreased, suggesting that the $Leu^{253} \rightarrow$ Ala and $Leu^{255} \rightarrow$ Ala mutations may interfere with the nuclear import of CrmA in MCF-7 cells.

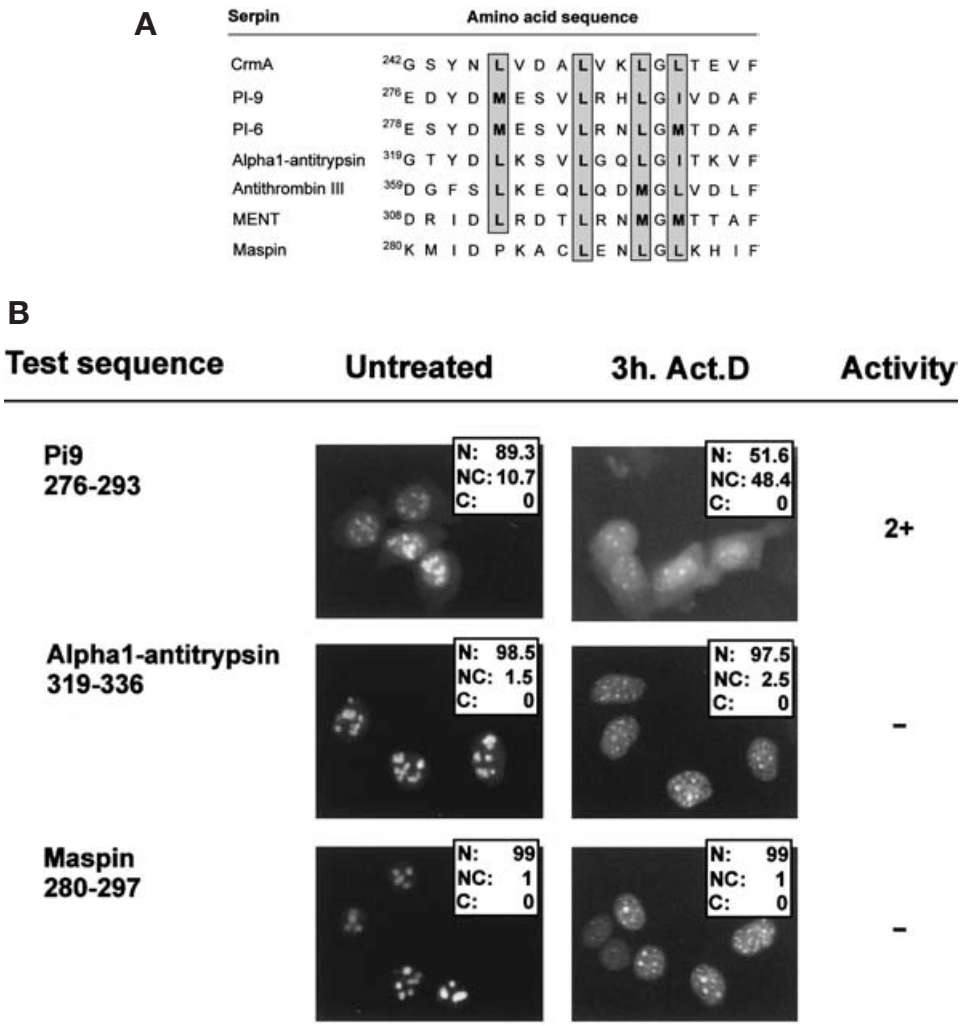

Figure 4 Functional conservation of the NES in the anti-apoptotic cellular serpin PI-9

(**A**) Amino acid sequence comparison between CrmA NES and similar regions in other serpins. The large hydrophobic amino acids that conform to the consensus NES are boxed. MENT, myeloid and erythroid nuclear termination stage-specific protein. (**B**) *In vivo* nuclear export assay showing that PI-9 fragment 276–293, similar to CrmA 242–259, is a functional NES, able to relocate Rev(1.4)-GFP to the cytoplasm of MCF-7 cells when Rev-mediated nuclear import is blocked by actinomycin D (Act. D) incubation. On the basis of the subcellular distribution of GFP (insets), the activity of PI-9 NES was classified as 2+. The regions of identity with CrmA NES in α1-antitrypsin and Maspin were inactive as export signals when tested in this assay.

These results therefore clearly indicate that the NES is only partially responsible for CrmA nuclear export in HeLa cells. According to the deletion analysis shown in Figure 2, other sequence motifs that might contribute to CRM1-dependent nuclear export of CrmA should be located within the CrmA region 200–341. To more precisely map these elements, we first tested two NES-like sequences located in this region (S^{195}MVVI **L**PDN**I**DG**L**ES**I**EQNL and ^{316}HPFIY**V**IRH**V**DGK**IL**F**V**GRYC; one-letter amino acid symbols, with bold letters highlighting residues of interest). Both sequences were negative in the nuclear export assay (results not shown). Next, we introduced the NES-inactivating mutations into YFP-tagged CrmA fragments spanning the 200–341 segment (Figure 5C). LMB treatment increased the nuclear localization of YFP-CrmA-$(200–341)^{NESm}$ and YFP–CrmA-$(200–303)^{NESm}$. However, further N- or C-terminal deletions abrogated the response to LMB. Combined, the results of this analysis enabled us to map the sequences responsible for NES-independent nuclear export of CrmA to an amino acid segment flanking the NES, comprising residues 200–303. Importantly, this region does not appear to contain other functional leucine-rich export signals.

Enhanced nuclear localization does not alter the ability of CrmA to prevent Fas-induced apoptosis

The ability of CrmA to undergo active nucleocytoplasmic transport suggests that it might carry out its anti-apoptotic function not only in the cytoplasm, but also in the nucleus. To optimally address this possibility, the anti-apoptotic activity of CrmA when exclusively located to the nucleus should be tested. This was hampered by the slow rate of nuclear import of CrmA and the fact that inactivating the NES does not fully prevent its nuclear export. One would expect, however, that enhancing the accumulation of CrmA in the nucleus would decrease its activity, if nuclear CrmA were unable to prevent apoptosis. Therefore we fused the strong NLS of the SV40 large T antigen to the C-terminal end of CrmA to generate a chimaeric protein (CrmA + NLS),

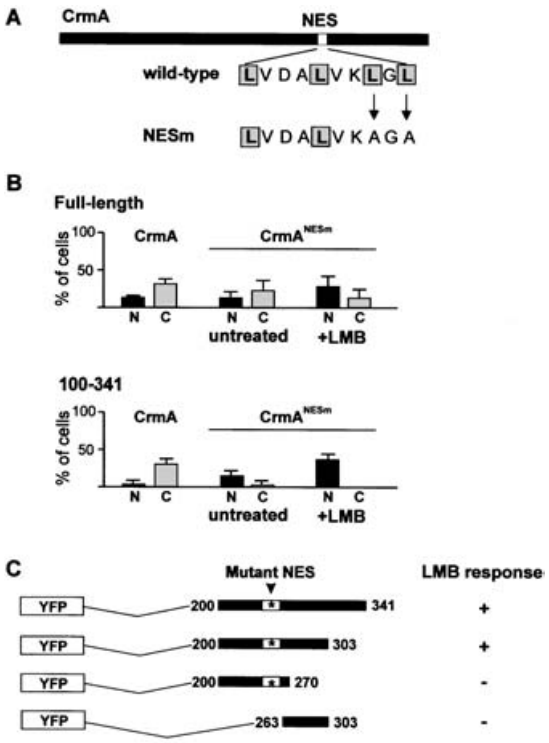

Figure 5 Additional CrmA sequences flanking the NES contribute to CrmA nuclear export

(**A**) Schematic representation of CrmA showing the location of the NES and the amino-acid substitutions ($Leu^{253} \rightarrow Ala$ and $Leu^{255} \rightarrow Ala$) introduced by site-directed mutagenesis to generate NES-defective $CrmA^{NESm}$. (**B**) Effect of mutational inactivation of the NES in the context of full-length Flag-CrmA (upper panel) and YFP-CrmA-(100–341) (lower panel). The NES mutation had a more dramatic effect on the localization of the N-terminally truncated protein. In both cases, however, the nuclear relocation of $CrmA^{NESm}$ was further enhanced by LMB treatment, suggesting the presence of additional export-mediating sequences. Bars represent the proportion of HeLa cells showing predominantly nuclear (N) or cytoplasmic (C) CrmA in the absence or presence of LMB (6 ng/ml for 6 h), and are the mean ± S.D. of three independent experiments. More than 200 transfected cells per sample were counted in each experiment. (**C**) Four YFP-tagged C-terminal fragments of CrmA lacking an active NES (depicted on the left) were transiently expressed in HeLa cells. The ability of each fragment to undergo LMB-induced nuclear relocation (LMB response) is indicated on the right.

whose nuclear accumulation is dramatically enhanced with respect to that of CrmA (Figure 6A). We next compared the ability of YFP-CrmA and YFP-CrmA + NLS to prevent Fas-induced apoptosis of HeLa cells. The use of YFP-tagged proteins enabled us to correlate the viability of transfected cells with the localization of CrmA on a single-cell basis. As a negative control, cells were transfected with YFP-CrmAT291R, a loss-of-function mutant of CrmA described previously [14]. At 24 h post transfection, cells were treated with anti-Fas agonistic antibody CH11 plus CHX for 20 h and the viability of transfected cells was assessed by fluorescence microscopy. After treatment, virtually all non-transfected cells (results not shown) and cells expressing YFP-CrmAT291R (Figure 6B) had detached from the bottom of the tissue culture tray and exhibited nuclear shrinkage and membrane blebbing characteristic of apoptotic cell death. In contrast, cells expressing wild-type YFP-CrmA (predominantly cytoplasmic) or YFP-CrmA + NLS (predominantly nuclear) remained attached and did not show any morphological evidence of apoptosis. The cleavage of PARP, determined by Western blot analysis (Figure 6C), confirmed that the detached, non-transfected cells had undergone apoptosis. These results therefore indicate that a dramatic nuclear relocation does not impair

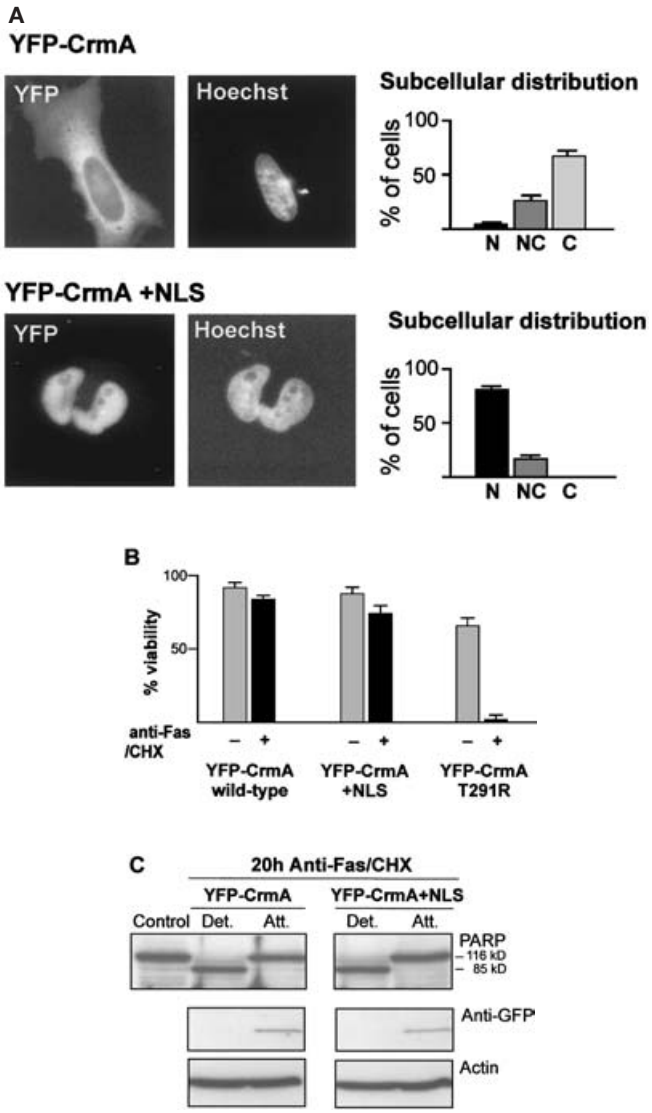

Figure 6 Enhanced nuclear localization does not alter the ability of CrmA to prevent Fas-induced apoptosis

(**A**) C-terminal fusion of the SV40 large T antigen NLS induces accumulation of CrmA into the nucleus. Photographs show representative examples of HeLa cells expressing YFP-CrmA (predominantly cytoplasmic) or YFP-CrmA + NLS (predominantly nuclear). Middle panels show the cell nucleus stained with Hoechst. Graphs show the subcellular distribution of each protein. (**B**) Graphs show the proportion of transfected HeLa cells showing no morphological evidence of apoptosis in untreated samples (–) or after a 20 h incubation with 100 ng/ml anti-Fas agonistic antibody CH11 plus 2 μg/ml CHX (+). Bars represent the mean ± S.D. of five independent microscopic fields. The viability was assessed in a single-cell basis and at least 100 YFP-positive cells per field were counted. (**C**) Western blot analysis of protein samples from the cells that had detached (Det) or that remained attached (Att) to the bottom of the six-well tray after 20 h incubation with anti-Fas agonistic antibody plus CHX. Upper panel shows that PARP cleavage, a marker of apoptosis, had occurred in detached cells but not in the attached cells. Middle panel shows that only the attached cells express ectopic YFP-CrmA or YFP-CrmA + NLS. An anti-actin antibody was used as a control for equal protein loading (bottom panel).

the ability of CrmA to protect HeLa cells from Fas-induced apoptosis.

DISCUSSION

It has recently become apparent that several members of the serpin family localize, to a different extent, to the cell nucleus [22,30–32]. Although it is predominantly localized in the

cytoplasm, the viral anti-apoptotic serpin CrmA has been proposed to enter the nucleus by diffusion through the nuclear pores [22]. Our results, comparing the localization of Flag- and YFP-tagged forms of CrmA, indicate that, in addition to passive diffusion, active transport mechanisms can regulate the nucleocytoplasmic localization of CrmA. The role of passive diffusion is evident from the different subcellular distribution of Flag- and YFP-tagged CrmA. As expected, the smaller Flag-CrmA was more frequently detected in the nucleus. The involvement of active nuclear import was first suggested by our observation that YFP-CrmA partially accumulates in the nucleus of human cells of different origin. YFP-CrmA is a 65 kDa protein whose size would prevent passive diffusion through the nuclear pores, thus requiring an active import process to enter the nucleus. Our findings contrast with the reported inability of GFP-CrmA to enter the nucleus of COS-1 cells [22], raising the possibility that cell-type-specific factors may affect active nuclear import of CrmA.

CrmA does not contain a classical NLS, such as the short stretch of basic amino-acids (Lys^{74}-Lys-Arg-Lys) that mediates nuclear import of the cellular serpin PI-10 [30]. However, other cellular members of the serpin family, such as PI-9, have been shown to enter the nucleus using a non-conventional mechanism of active import that does not rely on the presence of such sequences [22]. We show here that, like PI-9, CrmA is able to undergo CRM1-mediated nuclear shuttling and it is possible that both serpins also share similar import mechanisms. Blocking CRM1-dependent nuclear export with LMB induced only a partial relocation of CrmA to the nucleus, comparable with the LMB effect we have previously observed on another shuttling protein, BRCA1 [25], but smaller than the effect on HIV Rev [25] or survivin [33]. Thus in comparison with that of PI-9, nuclear import of CrmA seems to be a slow process. Interestingly, a CrmA deletion mutant lacking the N-terminal 100 residues rapidly accumulates into the nucleus upon LMB treatment, suggesting that the CrmA N-terminal region may negatively regulate its nuclear import.

We have identified a motif in CrmA (G^{242}SYN**L**VDA**L**VK-**L**G**L**TEVF; one-letter amino acid symbols, with bold letters highlighting residues of interest) that constitutes an autonomous, transferable leucine-rich NES. The activity of this novel NES is comparable with that of HIV Rev export signal when tested using an *in vivo* nuclear export assay [24]. Furthermore, we show that the PI-9 sequence (E^{276}DYD**M**ESV**L**RH**L**G**I**VDAF), homologous with CrmA NES, is also a functional, although weaker, NES. No other export signals have been identified in this cellular serpin, and the role of the NES reported here in the context of full-length PI-9 remains to be established. Nevertheless, by comparison with CrmA NES, we would predict that mutation of hydrophobic residues conforming to the NES consensus sequence, such as Leu^{287} and Ile^{289}, would decrease PI-9 nuclear export.

Despite its strong activity in the nuclear export assay, the NES appears to play only a partial role in CrmA nuclear export in HeLa cells, a process that seems to be largely mediated by additional sequences in the amino acid segment 200–303, flanking the NES. This region does not contain other active leucine-rich export signals but might represent a binding site for another NES-containing protein, which may bridge the interaction with CRM1. It is possible that the contribution of the NES to CrmA export changes under different conditions. One must consider, in this regard, that a prominent characteristic of serpins is their conformational instability [34]. This property allows serpins to adopt different conformations and may conceivably lead to exposure or masking of different motifs, including the NES, under different conditions. In this regard, we noted that mutational inactivation of the NES had a dramatic effect in the context of CrmA-(100–341). This observation suggests that deletion of the N-terminal end leads not only to increased nuclear import of CrmA, but also to exposure of the NES. More puzzling is the observation that NES-inactivating mutations appear to reduce nuclear import of CrmA in MCF-7 cells. The mechanism underlying this effect remains to be established, but the different consequences of the NES mutation in HeLa and MCF-7 cells constitute a clear example that the cellular context may influence the dynamics of CrmA nucleocytoplasmic transport.

The nuclear shuttling ability of CrmA raises the possibility that it may carry out its anti-apoptotic function in the cytoplasm and the nucleus. Testing this possibility was hampered by the fact that the NES is not the only motif mediating CrmA export. It was therefore not possible to completely abrogate this process without deleting a large amino acid segment. This gross deletion would presumably alter CrmA structure and function, preventing a meaningful comparison with wild-type CrmA. As an alternative approach to gain some insight into the functionality of CrmA in the nucleus, we generated a chimaeric protein, fusing the NLS of SV40 large T antigen to the C-terminal end of CrmA. Although CrmA + NLS still has the potential to undergo nuclear export, the strong NLS results in its rapid re-import into the nucleus. Thus whereas YFP-CrmA is predominantly cytoplasmic, YFP-CrmA + NLS accumulates strongly into the nucleus. Consistent with the view that CrmA may have an anti-apoptotic activity in the nucleus, we found that the ability of YFP-CrmA + NLS to prevent Fas-induced apoptosis is fully preserved.

In summary, our results reveal that the subcellular localization of CrmA is regulated in a more complex way than previously suspected. Its ability to shuttle between nucleus and cytoplasm may enable CrmA to efficiently target the pro-apoptotic proteins caspase 1, caspase 8 and granzyme B for inactivation in either cellular compartment. The two novel leucine-rich NESs reported here are, to our knowledge, the first NESs to be identified in the serpin family.

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REFERENCES

1 Nagata, S. (1999) Fas ligand-induced apoptosis. Annu. Rev. Genet. **33**, 29–55
2 Robertson, J. D., Orrenius, S. and Zhivotovsky, B. (2000) Review: nuclear events in apoptosis. J. Struct. Biol. **129**, 346–358
3 Rout, M. P. and Aitchison, J. D. (2001) The nuclear pore complex as a transport machine. J. Biol. Chem. **276**, 16593–16596
4 Mattaj, I. W. and Englmeier, L. (1998) Nucleocytoplasmic transport: the soluble phase. Annu. Rev. Biochem. **67**, 265–306
5 Görlich, D. and Kutay, U. (1999) Transport between the cell nucleus and the cytoplasm. Annu. Rev. Cell Dev. Biol. **15**, 607–660
6 Macara, I. G. (2001) Transport into and out of the nucleus. Microbiol. Mol. Biol. Rev. **65**, 570–594
7 Ray, C. A., Black, R. A., Kronheim, S. R., Greenstreet, T. A., Sleath, P. R., Salvesen, G. S. and Pickup, D. J. (1992) Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1B converting enzyme. Cell **69**, 597–604
8 Komiyama, T., Ray, C. A., Pickup, D. J., Howard, A. D., Thornberry, N. A., Peterson, E. P. and Salvesen, G. S. (1994) Inhibition of interleukin-1B converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. J. Biol. Chem. **269**, 19331–19337
9 Quan, L. T., Caputo, A., Bleackley, R. C., Pickup, D. J. and Salvesen, G. S. (1995) Granzyme B is inhibited by the cowpox virus serpin cytokine response modifier. J. Biol. Chem. **270**, 10377–10379

10 Tewari, M., Telford, W. G., Miller, R. A. and Dixit, V. M. (1995) CrmA, a poxvirus-encoded serpin, inhibits cytotoxic T-lymphocyte-mediated apoptosis. J. Biol. Chem. **270**, 22705–22708
11 Tewari, M. and Dixit, V. M. (1995) Fas- and Tumor Necrosis Factor-induced apoptosis is inhibited by the poxvirus *crmA* gene product. J. Biol. Chem. **270**, 3255–3260
12 Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V. M. and Salvesen, G. S. (1997) Target protease specificity of the viral serpin CrmA. Analysis of five caspases. J. Biol. Chem. **272**, 7797–7800
13 Garcia-Calvo, M., Peterson, E. P., Leiting, B., Ruel, R., Nicholson, D. W. and Thornberry, N. A. (1998) Inhibition of human caspases by peptide-based and macromolecular inhibitors. J. Biol. Chem. **273**, 32608–32613
14 Ekert, P. G., Silke, J. and Vaux, D. L. (1999) Inhibition of apoptosis and clonogenic survival of cells expressing crmA variants: optimal caspase substrates are not necessarily optimal inhibitors. EMBO J. **18**, 330–338
15 Trapani, J. A., Browne, K. A., Smyth, M. J. and Jans, D. A. (1996) Localization of granzyme B in the nucleus. A putative role in the mechanism of cytotoxic lymphocyte-mediated apoptosis. J. Biol. Chem. **271**, 4127–4133
16 Pinkoski, M. J., Heibein, J. A., Barry, M. and Bleackley, R. C. (2000) Nuclear translocation of granzyme B in target cell apoptosis. Cell Death Differ. **7**, 17–24
17 Nakagawara, A., Nakamura, Y., Ikeda, H., Hiwasa, T., Kuida, K., Su, M. S., Zhao, H., Cnaan, A. and Sakiyama, S. (1997) High levels of expression and nuclear localization of interleukin-1β converting enzyme (ICE) and CPP32 in favorable human neuroblastomas. Cancer Res. **57**, 4578–4584
18 Mao, P.-L., Jiang, Y., Wee, B. Y. and Porter, A. G. (1998) Activation of caspase-1 in the nucleus requires nuclear translocation of pro-caspase-1 mediated by its prodomain. J. Biol. Chem. **273**, 23621–23624
19 Stegh, A. H., Herrmann, H., Lampel, S., Weisenberger, D., Andra, K., Seper, M., Wiche, G., Krammer, P. H. and Peter, M. E. (2000) Identification of the cytolinker plectin as a major early *in vivo* substrate for caspase 8 during CD95- and tumor necrosis factor-receptor-mediated apoptosis. Mol. Cell. Biol. **20**, 5665–5679
20 Qin, Z.-H., Wang, Y., Kikly, K. K., Sapp, E., Kegel, K. B., Aronin, N. and DiFiglia, M. (2001) Pro-caspase-8 is predominantly localized in mitochondria and released into cytoplasm upon apoptotic stimulation. J. Biol. Chem. **276**, 8079–8086
21 Benchoua, A., Couriaud, C., Guegan, C., Tartier, L., Couvert, P., Friocourt, G., Chelly, J., Menissier-de Murcia, J. and Onteniente, B. (2002) Active caspase-8 translocates into the nucleus of apoptotic cells to inactivate poly (ADP-ribose) polymerase-2. J. Biol. Chem. **277**, 34217–34222

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22 Bird, C. A., Blink, E. J., Hirst, C. E., Buzza, M. S., Steele, P. M., Sun, J., Jans, D. A. and Bird, P. I. (2001) Nucleocytoplasmic distribution of the ovalbumin serpin PI-9 requires a nonconventional nuclear import pathway and the export factor Crm1. Mol. Cell. Biol. **21**, 5396–5407
23 Kalderon, D., Roberts, B. L., Richardson, W. D. and Smith, A. E. (1984) A short amino acid sequence able to specify nuclear location. Cell **39**, 499–509
24 Henderson, B. R. and Eleftheriou, A. (2000) A comparison of the activity, sequence specificity and CRM1-dependence of different nuclear export signals. Exp. Cell Res. **256**, 213–224
25 Rodriguez, J. A. and Henderson, B. R. (2000) Identification of a functional nuclear export sequence in BRCA1. J. Biol. Chem. **275**, 38589–38596
26 Huisman, C., Ferreira, C. G., Broker, L. E., Rodriguez, J. A., Smit, E. F., Postmus, P. E., Kruyt, F. A. E. and Giaccone, G. (2002) Paclitaxel triggers cell death primarily via caspase-independent routes in the non-small cell lung cancer cell line NCI-H460. Clin. Cancer Res. **8**, 596–606
27 Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E. P., Yoneda, Y., Yanagida, M., Horinouchi, S. and Yoshida, M. (1998) Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. Exp. Cell Res. **242**, 540–547
28 Wen, W., Meinkoth, J. L., Tsien, R. Y. and Taylor, S. S. (1995) Identification of a signal for rapid export of proteins from the nucleus. Cell **82**, 463–473
29 Richards, S. A., Lounsbury, K. M., Carey, K. L. and Macara, I. G. (1996) A nuclear export signal is essential for the cytosolic localization of the Ran binding protein, RanBP1. J. Cell Biol. **134**, 1157–1168
30 Chuang, T. L. and Schleef, R. R. (1999) Identification of a nuclear targeting domain in the insertion between helices C and D in protease inhibitor-10. J. Biol. Chem. **274**, 11194–11198
31 Grigoryev, S. A., Bednar, J. and Woodcock, C. L. (1999) MENT, a heterochromatin protein that mediates higher order chromatin folding, is a new serpin family member. J. Biol. Chem. **274**, 5626–5636
32 Haines, B. P., Voyle, R. B. and Rathjen, P. D. (2000) Intracellular and extracellular leukemia inhibitory factor proteins have different cellular activities that are mediated by distinct protein motifs. Mol. Biol. Cell **11**, 1369–1383
33 Rodriguez, J. A., Span, S. W., Ferreira, C. G. M., Kruyt, F. A. E. and Giaccone, G. (2002) CRM1-mediated nuclear export determines the cytoplasmic localization of the antiapoptotic protein Survivin. Exp. Cell Res. **275**, 44–53
34 Janciauskiene, S. (2001) Conformational properties of serine proteinase inhibitors (serpins) confer multiple pathophysiological roles. Biochim. Biophys. Acta **1535**, 221–235