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 shuffling 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 hallmarksa 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.
1 To whom correspondence should be addressed (e-mail ja.rodriguez@vumc.nl).
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-FlagpGKuro-CrmAwt and pEF-FlagpKuro-CrmAmt, encoding Flag-tagged wild-type CrmA or an inactive point mutant (Thr291→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 JAR36 (Table 1). The PCR product, digested with NdeI and XbaI restriction enzymes (Roche Molecular Biochemicals), was used to replace an NdeI/XbaI-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) and JAR51/JAR83 (CrmA + NLS) and cloned as HindIII/KpnI fragments into the pEYFP-C1 expression plasmid (Clontech, Palo Alto, CA, U.S.A.). PCR was also used to generate the deletion segments 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 α-1-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 BamHI/Agel fragments into Rev(1,4)-GFP. The primer sets used in this case were JAR75/JAR76 (α-1-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 (Leu255→Arg) and 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.

Site-directed mutagenesis of CrmA NES

A two-step PCR-based site-directed mutagenesis approach was used to generate the NES-defective CrmANES by introducing two point mutations (Leu255→Ala and Leu255→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 NdeI and XbaI and used to replace the NdeI/XbaI-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\textsubscript{NESm} (primer set JAR51/JAR56), and the different YFP-Fusion fragments of CrmA containing the NES mutation: 100–341\textsubscript{NESm} (primer set JAR53/JAR56), 200–341\textsubscript{NESm} (primer set JAR55/JAR56), 200–303\textsubscript{NESm} (primer set JAR55/JAR72), and 200–270\textsubscript{NESm} (primer set

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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

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<tr>
<th>Cell line</th>
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<th>NC (%)</th>
<th>C (%)</th>
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</table>

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).

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.

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.
NES was classified as 9, i.e. experiments with less than 10% variation. More than 200 cells per sample were counted. NC, nuclear and cytoplasmic; C, cytoplasmic. Results are the mean of two independent assays. The subcellular distribution of each protein is indicated in the insets: N, nuclear; C, cytoplasmic. (negative control). In contrast, CrmA fragment 223–244 showed no export activity in this assay. 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. 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 (Leu253 and Leu255) (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 (Leu253 → Ala and Leu255 → 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 response to LMB cells was decreased, suggesting that the Leu253 → Ala and Leu255 → Ala mutations may interfere with the nuclear import of CrmA in MCF-7 cells.
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. 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 (S195MVVI LPDNIDGESIEQNL and 316HPFIYVIRHVDKGHKLFVGRYC; 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 (Leu253 → Ala and Leu255 → Ala) introduced by site-directed mutagenesis to generate NES-defective CrmANESm. (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 CrmANESm 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 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 nucleo-cytoplasmic 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 (Lys274-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 (G242SYNLVDALKYLGLTELVF: 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 transferable leucine-rich NES. The activity of this novel NES is highlighting residues of interest) that constitutes an autonomous, L

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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