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Distinct cell death pathways triggered by the adenovirus early region 4 ORF 4 protein

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In transformed cells, induction of apoptosis by adenovirus type 2 (Ad2) early region 4 ORF 4 (E4orf4) correlates with accumulation of E4orf4 in the cell membrane–cytoskeleton fraction. However, E4orf4 is largely expressed in nuclear regions before the onset of apoptosis. To determine the relative contribution of nuclear E4orf4 versus membrane-associated E4orf4 to cell death signaling, we engineered green fluorescent fusion proteins to target E4orf4 to specific cell compartments. The targeting of Ad2 E4orf4 to cell membranes through a CAAX-box or a myristylation consensus signal sufficed to mimic the fast Src-dependent apoptotic program induced by wild-type E4orf4. In marked contrast, the nuclear targeting of E4orf4 abolished the early induction of extranuclear apoptosis. However, nuclear E4orf4 still induced a delayed cell death response independent of Src-like activity and of E4orf4 tyrosine phosphorylation. The zVAD.fmk-inhibitable caspases were dispensable for execution of both cell death programs. Nevertheless, both pathways led to caspase activation in some cell types through the mitochondrial pathway. Finally, our data support a critical role for calpains upstream in the death effector pathway triggered by the Src-mediated cytoplasmic death signal. We conclude that Ad2 E4orf4 induces two distinct cell death responses, whose relative contributions to cell killing may be determined by the genetic background.

Introduction

Adenovirus type 2 (Ad2)* early region 4 ORF 4 (E4orf4) is a small 14-kD Ad protein that may play multiple roles during Ad infection (Muller et al., 1992; Kleinberger and Shenk, 1993; Bondesson et al., 1996; Estmer Nilsson et al., 2001). In mammalian cell lines, expression of Ad2 E4orf4 triggers a p53-independent cell death program which is selective to transformed cells (Shtrichman et al., 1999; unpublished data). Thus, uncovering the molecular mechanisms involved in E4orf4-mediated death may help in deciphering the cellular components linking cell division to cell death programs and unravel novel strategies for tumor therapy.

Early work in mammalian cells revealed that cell death induced by Ad2 E4orf4 is associated with classic apoptotic hallmarks, including nuclear condensation, cell shrinkage, and externalization of phosphatidylserines (Lavoie et al., 1998). Nevertheless, activation of the zVAD.fmk-inhibitable caspases is not required for execution of the death program in most cell types (Lavoie et al., 2000; Livne et al., 2001; unpublished data). E4orf4 binding to the Bα subunit of protein phosphatase (PP)2A was linked to cell death induction in mammalian cells (Shtrichman et al., 1999; Marcellus et al., 2000), and to induction of irreversible growth arrest in yeast (Kornitzer et al., 2001; Roopchand et al., 2001). However, the exact mechanisms involved remain ill defined. In addition, these studies indicated that extra functions are required for cell killing, and furthermore, that a PP2A/Cdc55-independent function also exists. Recently, Ad2 E4orf4 was found to induce a Src-mediated cytoplasmic apoptotic signal requiring the tyrosine phosphorylation of E4orf4, which rapidly leads to caspase-independent membrane blebbing and cell death (Lavoie et al., 2000; Gingras et al., 2002). Analysis of E4orf4 distribution in various cell lines revealed that the protein is first expressed preferentially in nuclear regions; however, induction of early membrane blebbing correlates with rapid accumulation of E4orf4 in cytoplasmic membrane regions. In the present study, we provide strong evidence that both the cytoplasmic membrane-associated and the nuclear E4orf4 induce specific cell death programs in human transformed cells. Our data indicate that the cell death programs

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*Abbreviations used in this paper: Ad2, adenovirus type 2; AIF, apoptosis-inducing factor; E4orf4, early region 4 ORF 4; Myr, myristylation; NES, nuclear exclusion signal; PP, protein phosphatase; WT, wild-type.

Key words: Ad2 E4orf4; apoptosis; Src kinases; caspase; calpastatin

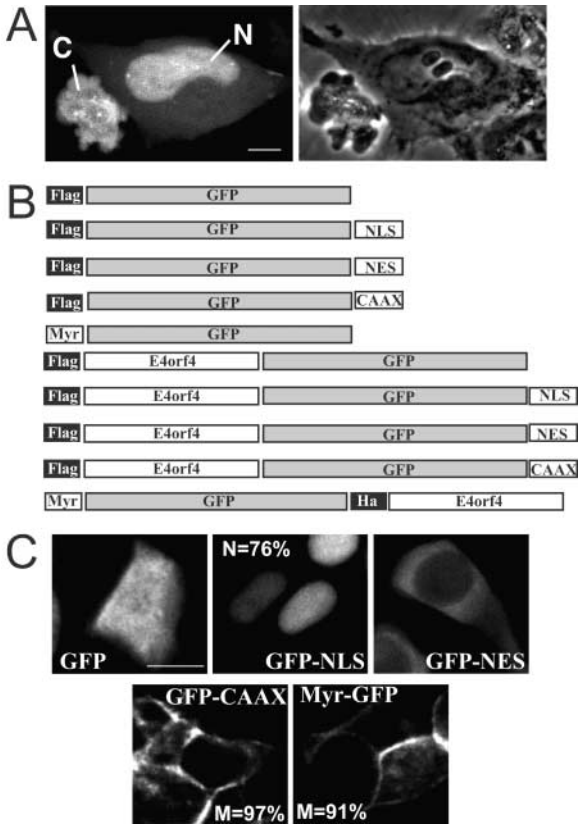

Figure 1. **GFP fusions and protein targeting.** (A) Immunostaining of transfected E4orf4 protein in H1299 cells using rabbit anti-2418 E4orf4 antibody (Lavoie et al., 2000); C, cell cytoplasm of a blebbing cell; N, nucleus. (B) GFP and E4orf4-GFP constructs engineered to target proteins to the cytoplasm (NES), to cell membranes (CAAX and Myr), and to the nucleus (NLS). (C) In vivo localization of GFP control proteins expressed in 293T cells (confocal micrographs). Protein targeting efficiencies were evaluated 24 h after transfection by counting positive cells expressing the GFP proteins in the targeted cell compartment, by fluorescence microscopy in live cells, $n = 600$. Bars, 10 μm.

induced by the differentially localized E4orf4 proteins are associated with distinct morphological features and kinetics and involve different death effector pathways.

Results

Expression of Ad2 E4orf4 in cell membranes is sufficient to trigger a cytoplasmic death signal

When expressed in mammalian cells, Ad2 E4orf4 shows a typical nucleocytoplasmic distribution and the onset of extranuclear apoptosis correlates with its accumulation in the cytoplasm and membranes (Fig. 1 A) (Lavoie et al., 2000; Gingras et al., 2002). To address the significance of the nuclear versus the cytoplasm membranes distribution of E4orf4 for its killing function, E4orf4-GFP proteins were engineered to target or enrich the protein in the different cell compartments, using specific targeting sequences (nuclear exclusion signal [NES], CAAX, myristylation [Myr], NLS) as described in Materials and methods (Fig. 1 B). The blebbing-inducing activity in live cells and the appearance of nuclear condensation were measured in cells expressing comparable levels of the E4orf4 proteins in the proper cell compartments, in the three transformed cell lines 293T, 293, and C-33A. As expected, the GFP control proteins did not induce membrane blebbing or nuclear condensation over a period of 3 d (Figs. 1 C and 2; see Table II). Remarkably, overexpression of the E4orf4-GFP-NES or the membrane-anchored E4orf4-GFP proteins (E4orf4-GFP-CAAX and Myr-GFP-E4orf4) induced membrane blebbing to equivalent or even higher efficiency relative to the wild-type (WT) E4orf4-GFP protein (Fig. 2 A; Table I). Furthermore, nuclear condensation typical of that induced by WT E4orf4 was triggered by the cytoplasmic and membrane-anchored E4orf4 proteins (Fig. 2 A; Table II). In contrast, the nuclear E4orf4 (E4orf4-GFP-NLS) did not lead to early induction of membrane blebbing. Nevertheless, nuclear condensation was induced by E4orf4-GFP-NLS, but cell death was markedly delayed relative to that induced by WT and membrane-associated E4orf4-GFP (Fig. 2 A; Table II). Thus, the results strongly suggest that membrane-associated E4orf4 directly signals membrane blebbing followed by rapid cell death, and that nuclear E4orf4 in¬duces a distinct death process.

The cytoplasm membrane–associated and the nuclear Ad2 E4orf4 death activities are distinct

Recent work indicated that Src-regulated Ad2 E4orf4 phosphorylation is a prerequisite for induction of the cytoplasmic death signal (Gingras et al., 2002). To delineate the mechanisms involved in induction of cell death mediated by the different E4orf4-GFP proteins, we looked at the effect of tyrosine phosphorylation of E4orf4. We used a previously characterized nonphosphorylatable E4orf4 (3Y-F)-GFP, in which tyrosine 26, 42, and 59 are replaced by phenylalanines; this mutant was found to accumulate to higher levels in the cell nucleus (Gingras et al., 2002). As shown previously, apoptosis induced by mutant E4orf4 (3Y-F)-GFP was severely impaired in 293T cells (Table II). In 293 and

Table I. **Targeting efficiencies and blebbing-inducing activities of the E4orf4-GFP proteins**

Constructs	293T Targeting	C-33A Targeting	C-33A Blebbing	293 Targeting	293 Blebbing
			% cells		
E4orf4-GFP	N = 35	–	61	N = 42	39
E4orf4-GFP-NLS	N = 94	N = 65	18	N = 91	3
E4orf4-GFP-NES	C = 92	–	–	–	–
E4orf4-GFP-CAAX	M = 96	M = 93	55	M = 92	76
Myr-GFP-E4orf4	M = 33	–	–	–	–
	$n > 850$	$n > 600$		$n = 500$	$n > 150$

Transfection of the indicated GFP constructs was performed in 293T, 293, and C-33A, and the protein targeting efficiencies and blebbing-inducing activities (percentage blebbing) were determined by fluorescence microscopy in live cells 24 h after transfection; C, cytoplasmic; M, cell membranes; N, nuclear.

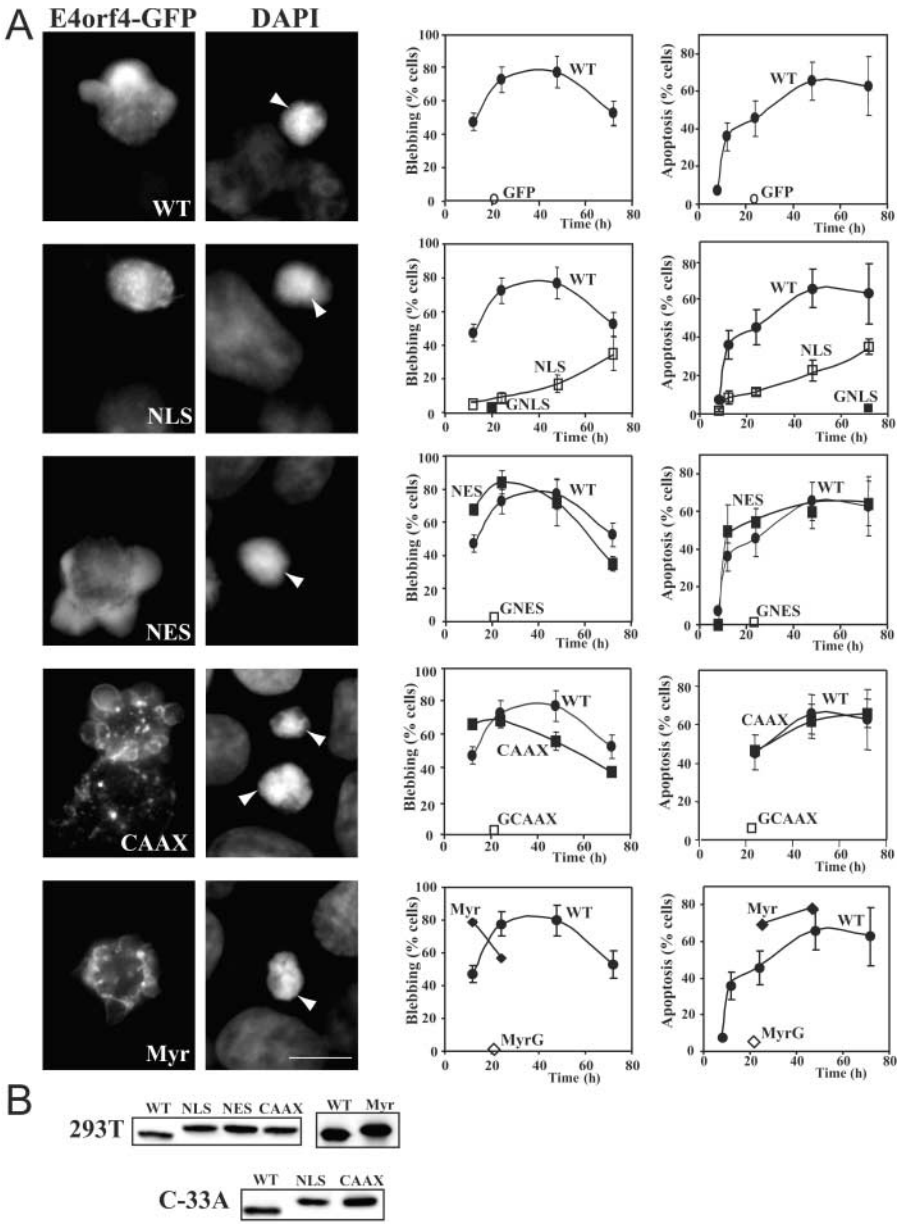

Figure 2. **Functional activities of the E4orf4-GFP proteins.** (A) 293T cells were transfected with the indicated Flag-E4orf4-GFP constructs (WT, NLS, NES, CAAX, and Myr), or with the relevant Flag-GFP controls (GFP, GNLS, GNES, GCAAX, and MyrG). At various times after transfection, the amounts of GFP-positive cells undergoing membrane blebbing were determined by fluorescence microscopy in live cells. Data are expressed as percent blebbing cells relative to the total number of GFP-positive cells. Other cultures of transfected cells were fixed and counterstained with DAPI and the nuclear morphology of GFP-positive cells was analyzed by fluorescence microscopy (representative phenotypes are shown). Apoptosis was determined by counting the number of cells expressing the relevant GFP proteins strictly in the targeted cell compartments that presented nuclear shrinkage and condensation (arrows). Data are expressed as the number of apoptotic cells relative to the total number of GFP-positive (means ± SE of at least four independent experiments, $n > 2{,}000$). Bars, 10 μm. (B) Aliquots of transfected cells were kept for Western blot analyses of expression levels using anti-Flag M2 antibody, or anti-GFP, 16–24 h after transfection.

Table II. **The tyrosine phosphorylation of Ad2 E4orf4 regulates its cytoplasmic membrane activity but not its nuclear activity**

Constructs	C-33A			293T			293	
	NL	Apoptosis		NL	Apoptosis		NL	Apoptosis
				% cells				
		24 h	48 h		48 h	72 h		24 h
GFP	–	10	8	–	4	–	–	9
GFP-NLS	69	5	4	76	3	4	–	11
GFP-CAAX	<1	11	13	1	7	–	–	7
orf4-GFP	29	37	56	35	56	–	42	37
3YF-GFP	64	21	41	77	13	26	–	20
orf4-GFP-NLS	65	14	34	94	–	33	92	21
3YF-GFP-NLS	92	17	34	91	–	30	–	–
orf4-GFPCAAX	2	39	51	2	42	–	–	48
3YF-GFPCAAX	9	13	16	19	14	–	–	10
	$n > 600$	$n > 900$		$n > 600$	$n > 900$		$n = 500$	$n = 1{,}000$

293T, 293, and C-33A cells were transfected with the indicated GFP constructs; (3Y-F), nonphosphorylated mutant E4orf4. At the indicated times after transfection, cells were fixed and DNA was labeled with DAPI. Protein targeting efficiencies and apoptotic activities (nuclear condensation and fragmentation) were evaluated by fluorescence microscopy and are expressed as the percentage of nuclear localization (NL), or the percentage of apoptosis, relative to the total number of GFP-positive cells. Data are the means ± SE of four independent experiments.

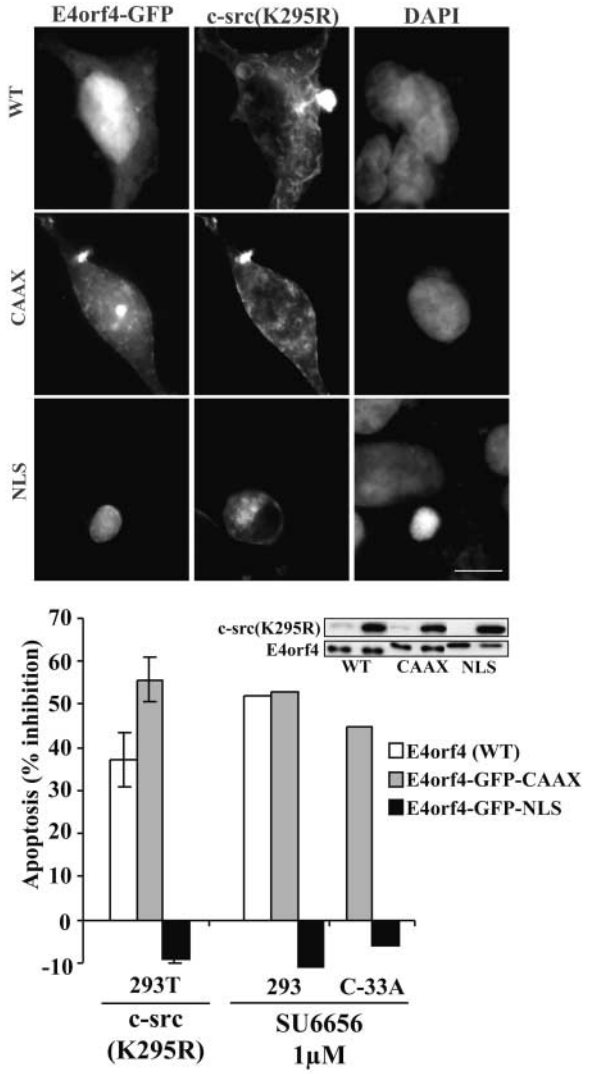

Figure 3. **Role of Src kinases in regulation of both cell death pathways.** 293T, 293, and C-33A cells were transfected with the indicated E4orf4-GFP constructs with or without c-src (K295R). The Src inhibitor SU6656 was added at a concentration of 1 μM 6 h after the transfection. Apoptosis was evaluated after cell fixation and DAPI staining, couple to immunostaining of c-src (K295R). Data are expressed as the percent inhibition of apoptosis (of apoptotic nuclei) relative to cells expressing equivalent levels of E4orf4-GFP proteins only (means ± SE of two independent experiments). Aliquots of transfected cells were kept for Western blot analyses of E4orf4-GFP (anti-Flag M2) and c-src (K295R) (anti–v-src Ab1). The effect of SU6656 was determined only in those cells expressing equivalent levels (intensity) of GFP proteins in presence and absence of the inhibitor and data are representative of two independent experiments, $n > 800$. Bar, 10 μm.

C-33A cells, apoptosis induced by the nonphosphorylatable E4orf4 was also delayed, but reached comparable levels later on. Notably, E4orf4 phosphorylation was not required to drive its nuclear activity, as the nuclear targeting of the nonphosphorylatable E4orf4 (E4orf4 [3Y-F]-GFP-NLS) did not affect its apoptotic activity relative to the WT E4orf4-GFP-NLS, or to the parental nonphosphorylatable E4orf4. Furthermore, the nonphosphorylatable membrane-anchored E4orf4 protein (E4orf4 [3Y-F]-GFP-CAAX) was completely deficient, indicating that expression of Ad2 E4orf4 in the cell nucleus is required to trigger the Src-independent cell death signal. As another approach, we measured the effects of a kinase-deficient c-src and of a selective Src family kinase inhibitor, SU6656 (Blake et al., 2000), on apoptosis induced by both pathways. In agreement with our previous findings, overexpression of kinase-deficient c-src markedly inhibited the appearance of nuclear condensation in cells expressing the WT E4orf4-GFP (Lavoie et al., 2000) and a similar effect was measured in presence of SU6656 (Fig. 3). Importantly, these treatments also inhibited apoptosis induced by the E4orf4-GFP-CAAX, but not apoptosis induced by the nuclear E4orf4 (E4orf4-GFP-NLS). This confirmed that the nuclear death pathway induced by E4orf4 was distinct from the Src-regulated cytoplasmic pathway.

Caspase activation by Ad2 E4orf4 is dispensable for execution of both cell death programs

Our previous work indicates that apoptosis mediated by Ad2 E4orf4 is resistant to caspase inhibition (Lavoie et al, 1998, 2000; unpublished data); however, inhibition of E4orf4-induced nuclear condensation by caspase inhibitors was reported in 293T cells (Livne et al., 2001). Thus, we readdressed this question by looking at apoptosis induced by both E4orf4-dependent cell death pathways in presence and absence of two different broad-spectrum cell-permeable irreversible caspase inhibitors, zVAD.fmk and BocD.fmk. In 293T cells, no significant inhibition of nuclear condensation was detected in cells expressing either of the E4orf4-GFP proteins, even when zVAD.fmk or BocD.fmk were added back every 24 h (Fig. 4 A). Moreover, no activation of the effector caspase-3 was detected in the apoptotic condensed cells, when using a specific antibody against the activated/cleaved form of caspase-3 (Fig. 4 C; Table III). In marked contrast, high levels of active caspase-3 were detected in 293T cells overexpressing Bax-GFP. Because DNA fragmentation was observed along with nuclear condensation in C-33A, but not in 293 and 293T cells (Fig. 4 C, compare c with f), these experiments were repeated in C-33A cells. Not surprisingly, BocD.fmk and zVAD.fmk potently inhibited DNA fragmentation in cells expressing the various E4orf4-GFP proteins (Fig. 4 B). However, caspase inhibitors had a very different effect in these cells when compared with Fas- or Bax-expressing cells. Indeed, caspase inhibition decreased the overall number of apoptotic cells expressing Fas or Bax by 70% to 50%, respectively, whereas no protection was observed in E4orf4-expressing cells. In fact, the overall number of apoptotic cells was not diminished and inhibition of DNA fragmentation was associated with increased nuclear condensation (Fig. 4 B). Additionally, high levels of active caspase-3 were detected, but only in those cells presenting the fragmented phenotype; active caspase-3 was completely absent in condensed cells (in presence of caspase inhibitors), thus reproducing the apoptotic process observed in 293T cells (Fig. 4 C; Table III). A similar switch of apoptotic phenotypes was observed in C-33A cells coexpressing E4orf4 and dominant negative caspase-9, or Bcl-2, but not by treatment with caspase-8 inhibitor (zIETD-fmk) (Figs. 4 B and 5). Thus, caspase-dependent DNA fragmentation and activation of the effector caspase-3 were likely triggered through activation of the mitochondria–apoptosome pathway in C-33A cells, but not in 293T cells.

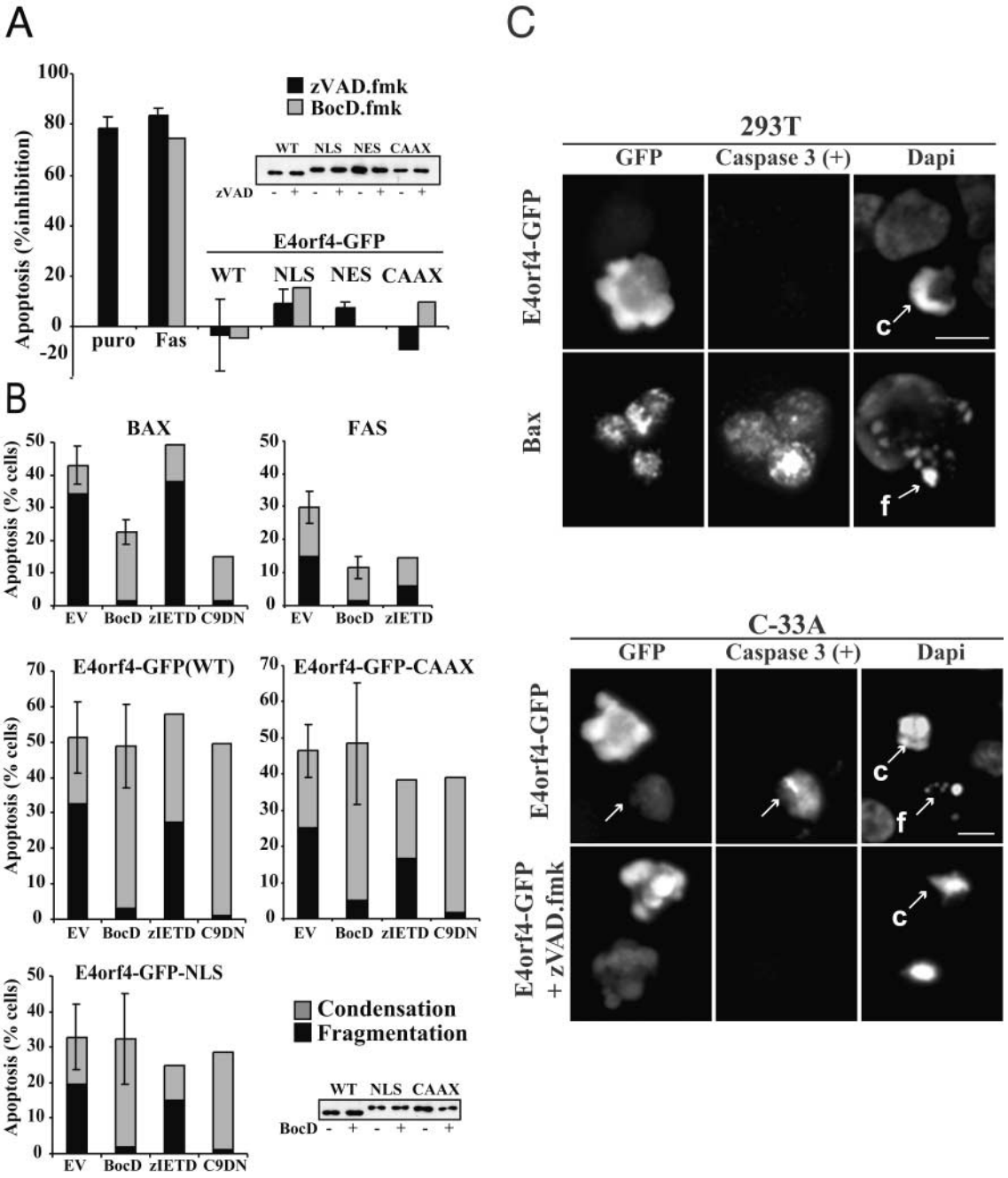

Figure 4. **Effect of caspase inhibitors and caspase activation in cells expressing E4orf4-GFP proteins.** (A and B) 293T and C-33A cells were transfected with the indicated E4orf4-GFP constructs in presence or absence of different caspase inhibitors (as indicated), or cotransfected with dominant-negative caspase-9 (C9DN) at a plasmid DNA ratio of 1:2. As controls, cells were transfected with Fas or Bax-GFP or incubated in presence of puromycin (10 μg/ml). Cells were fixed at various times after transfection; 24 (puro, Fas, Bax-GFP), 48 h (WT, NES, CAAX in 293T and C-33A; NLS in C-33A), or 72 h (NLS in 293T). The number of apoptotic nuclei (% apoptosis) was determined after DAPI staining. (A) Data are expressed as percent inhibition of apoptosis relative to untreated cells (DMSO only) and are the means ± SE of three independent experiments, $n = 500$. Aliquots of transfected cells were kept for Western blot analysis of E4orf4-GFP proteins using anti-Flag M2 (insert). (B) Apoptosis is expressed as the number of cells presenting DNA fragmentation (black bars), or nuclear condensation (gray bars) relative to the total number of transfected cells expressed relative to untreated cells empty vector (EV) (cells expressing E4orf4-GFP proteins alone). Data are representative of three independent experiments (means ± SE, BocD.fmk, $n = 1,000$); zIETD.fmk, $n = 200$; C9DN, $n = 350$. (C) 293T or C-33A cells transfected with E4orf4-GFP were fixed at 24 (C-33A) or 48 h (293T) after transfection, and endogenous active caspase-3 was detected in single cells by immunolabeling using anti-cleaved caspase-3 (Asp175) and DNA was labeled with DAPI. C, nuclear condensation; f, DNA fragmentation. Bars, 10 μm.

Calpains are involved in execution of the Src-regulated death pathway induced by Ad2 E4orf4

Because overexpression of the antiapoptotic protein Bcl-2 did not inhibit the caspase-independent nuclear condensation induced by the various E4orf4-GFP proteins in either 293T or C-33A (Fig. 5), we envisioned that execution of E4orf4-

Table III. **Analysis of activated caspase-3 in cells expressing E4orf4-GFP proteins**

	C-33A			293T		
	Caspase 3	Apoptosis		Caspase 3	Apoptosis	
	+	% cells		+	% cells	
	% cells	−zVAD	+zVAD	% cells	−zBocD	+BocD
GFP	7[b]	7	6	0.2[c]	2	3
E4orf4-GFP	24[b]	33	38	0.8[c]	52	48
GFP-CAAX	7[b]	10	8	0.4[c]	5	6
E4orf4-GFP-CAAX	24[b]	34	32	2.4[c]	40	41
GFP-NLS	12[b]	10	9	1.0[d]	2	3
E4orf4-GFP-NLS	23[b]	23	20	–	–	–
3YF-GFP-NLS	–	–	–	2.1[d]	27	34
Bax-GFP	93[a]	98	51	80.0[a]	100	21
		$n > 350$			$n > 500$	

[a]Cells fixed at 24 h.
[b]Cells fixed at 36 h.
[c]Cells fixed at 48 h.
[d]Cells fixed at 72 h.
Apoptosis was determined from the total number of E4orf4-expressing cells with apoptotic nuclei (including condensed "c" or fragmented "f", Fig. 4 C) and the percentages of apoptotic cells showing positive staining for active caspase-3 (Caspase-3 [+]) were determined in absence and presence (data not shown) of caspase inhibitors. Data are representative of at least two independent experiments.

mediated death involves some nonmitochondrial death effectors. Indeed, analysis of the subcellular distribution of mitochondrial proapoptotic factors confirmed that expression of E4orf4-GFP led to the release of both cytochrome c and apoptosis-inducing factor (AIF) in the cytosol of C-33A cells. However, overexpression of Bcl-2, a critical regulator of the permeabilization of the outer mitochondrial membrane (for review see Martinou and Green, 2001), inhibited their release, although no effect was observed on the overall level of apoptosis (unpublished data). Thus, AIF, a mediator of caspase-independent cell death (for review see Daugas et al., 2000), is likely not required for execution of either caspase-independent death pathways induced by Ad2 E4orf4. In marked contrast, we found that overexpression of calpastatin, the endogenous inhibitor of calpains that binds to the activated calpain as a suicide substrate (for review see Donkor, 2000), dramatically inhibited the caspase-independent apoptosis induced by the WT and membrane-anchored E4orf4-GFP proteins (60–80% inhibition; Fig. 6). Relatively, overexpression of the calpain inhibitor had little effect on apoptosis induced by the nuclear and the nonphosphorylatable E4orf4-GFP (~30% inhibition), or on that induced by Bax-GFP that was completely inhibitable by coexpression of Bcl-2 (Fig. 5). Thus, the results strongly suggest that calpains are playing a critical role upstream in execution of the Src-regulated cytoplasmic death pathway, but not in the Src-independent nuclear death pathway induced by Ad2 E4orf4, which likely involve other nonmitochondrial death effectors.

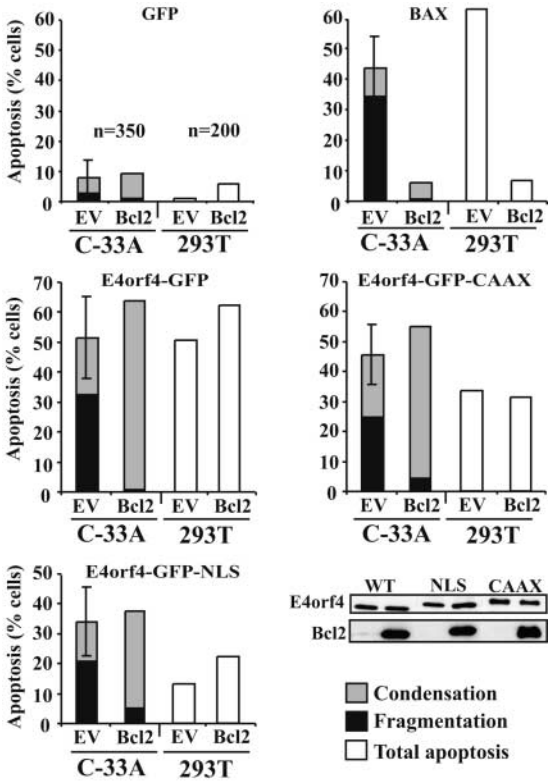

Figure 5. **Caspase-independent apoptosis induced by E4orf4-GFP proteins is not inhibited by Bcl-2.** 293T and C-33A were transfected with the indicated E4orf4-GFP constructs alone, or with an HA-Bcl-2 construct using a plasmid DNA ratio of 1:1. The percentages of apoptotic cells were determined after cell fixation and immunodetection of Bcl-2 using mouse anti-HA antibody followed by DAPI staining in cells expressing E4orf4-GFP proteins (EV), as compared with cells coexpressing E4orf4-GFP and HA-Bcl-2 (Bcl-2). Data are representative of at least three independent experiments.

Discussion

The cytoplamic membrane and the nuclear localization of Ad2 E4orf4 are both relevant to its killing function

When expressed in mammalian cells, Ad2 E4orf4 distributes in both the cell nucleus and the cytoplasm and membranes. The nuclear accumulation of Ad2 E4orf4 is regulated at the molecular level through a stretch of basic residues spanning amino acids 68–75 (KRRDRRRR) that serves as an NLS (unpublished data), whereas its cytoplasmic accumulation likely results from a passive diffusion out of the nucleus. Indeed, the

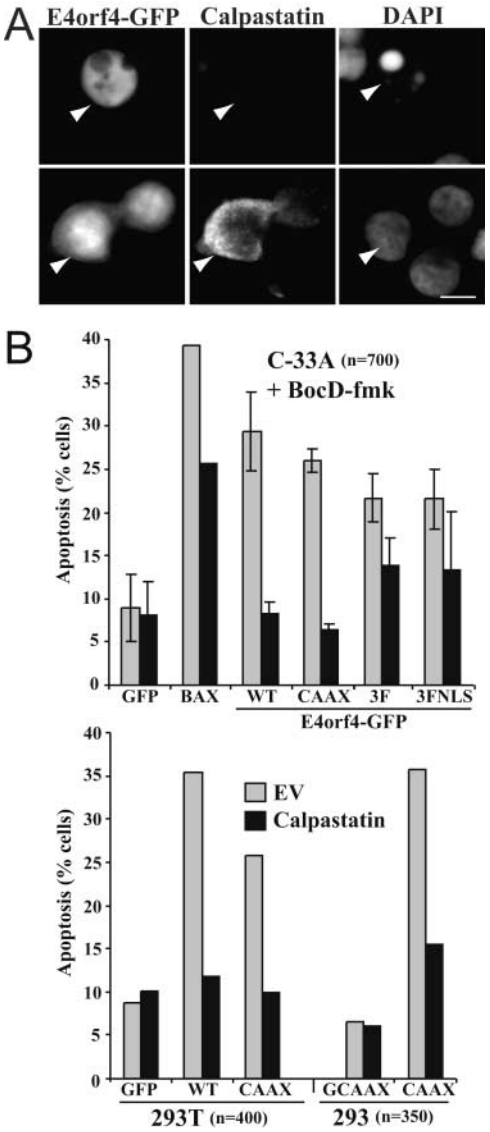

Figure 6. **Inhibition of E4orf4-mediated caspase-independent apoptosis by calpastatin.** Cells were transfected with the Bax-GFP or E4orf4-GFP constructs alone, or together with a HA-calpastatin construct at a plasmid DNA ratio of 1:2, in the presence of caspase inhibitor (C-33A). The percentages of apoptotic cells were determined in cells expressing E4orf4 or Bax proteins alone (EV), as compared with cells coexpressing the GFP proteins and HA-calpastatin, after cell fixation and immunodetection of the exogenous calpastatin using mouse anti-HA followed by DAPI staining. Data are representative of two independent experiments (means ± SE, C-33A, $n = 700$).

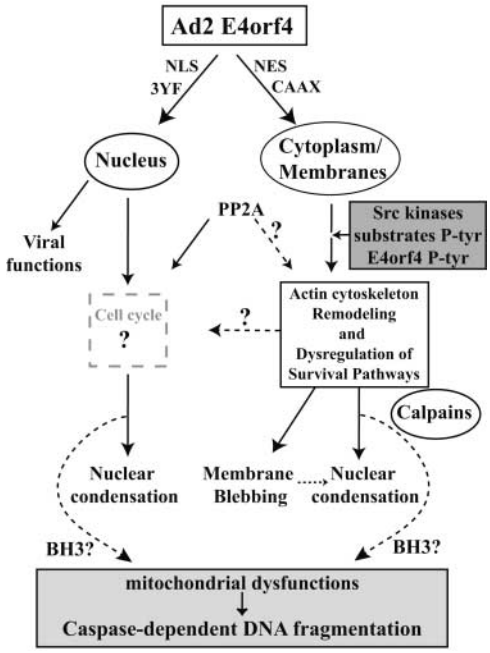

Figure 7. **Working model for the mechanisms of Ad2 E4orf4–induced cell death.** Based on the results obtained in the present study and those reported before (Shtrichman et al., 1999; Lavoie et al., 2000; Marcellus et al., 2000; Estmer Nilsson et al., 2001; Gingras et al., 2002), we propose that Ad2 E4orf4 triggers two distinct cell death pathways that do not require caspase activation for execution, but can connect to the intrinsic caspase-activating pathway in competent cells (e.g., C-33A cells). One is initiated from the cytoplasm membranes and involves some calpain activity; another is signaled from the cell nucleus and relies on unknown mitochondrial-independent death effector (see Discussion for details).

cytoplasmic distribution of the protein is not affected by leptomycin B, an inhibitor of the CRM1-mediated nuclear export pathway (Ossareh-Nazari et al., 1997; unpublished data). It has been suggested that E4orf4 binding to Src kinases followed by tyrosine phosphorylation inhibits the nuclear import of E4orf4 and promotes its cytoplasmic retention (Gingras et al., 2002). Because the onset of the apoptotic response mediated by E4orf4 correlates better with its accumulation in the cell cytoplasm membranes, the relevance of E4orf4 nuclear localization for cell killing is somewhat unclear. Surprisingly, E4orf4 targeting to different cell compartments has uncovered a delayed novel killing function associated with its nuclear localization. This approach also confirmed that the presence of E4orf4 in the cell cytoplasm membranes is necessary and sufficient for triggering the Src-regulated cytoplasmic death signal that rapidly leads to cell death. Several observations indicated that the cell death pathway induced by nuclear E4orf4 is functionally distinct. When expressed in 293T, C-33A, or 293 cells, the E4orf4-GFP-NLS was mainly nuclear in the majority of cells over a period of 3 d. Presumably, the strong NLS from large T antigen took over the natural molecular determinants of Ad2 E4orf4 localization and allowed for continual import of the fusion protein into the cell nucleus, at least in the cell types used. In these cells, the onset of apoptosis was not associated with a dramatic extranuclear apoptotic phase (early membrane blebbing), did not require tyrosine phosphorylation of E4orf4 nor Src kinase activity, and was markedly delayed relative to the cytoplasmic Src-regulated cell death pathway. Furthermore, inhibition of calpains had little effect on apoptosis induced by the nuclear and nonphosphorylatable E4orf4 proteins, whereas it dramatically inhibited the caspase-independent apoptosis mediated by the membrane-anchored E4orf4. Although fluorescence microscopy is not sensitive enough to exclude the possibility that small amounts of E4orf4-GFP-NLS may accumulate in the cytoplasm to some extent, altogether, these mechanistic data strongly support the existence of two distinct E4orf4-mediated cell death pathways and make it very unlikely that delayed apoptosis resulted from protein leakage out of the nucleus.

Toward a better understanding of Ad2 E4orf4–mediated cell death

As summarized in a working model (Fig. 7), we propose that Ad2 E4orf4 triggers two caspase-independent cell death programs that rely on distinct death effectors for execution, which can connect to the mitochondrial caspase-activating pathway in competent cells. An Src-regulated death pathway requires E4orf4 accumulation in the cell cytoplasm membranes and involves calpains. A distinct Src-independent death signal results from E4orf4 accumulation in the cell nucleus and is mediated by unknown nonmitochondrial death effectors. We believe that the contribution of either pathway to cell death will be determined by the availability of specific targets of Ad2 E4orf4 and the activity of signaling pathways involved in a given transformed cell types (e.g., Src kinases). Accordingly, the low killing efficacy of the nuclear E4orf4 in 293T relative to 293 cells may result from a decreased accessibility of some critical nuclear target of E4orf4, which may be sequestered by simian virus large T antigen. Whatever the case, the contribution of Src kinases and the role of E4orf4 tyrosine phosphorylation have been firmly assigned exclusively to regulation of the cytoplasmic pathway. It remains to be determined whether PP2A is involved in the Src-regulated cytoplasmic, and/or the nuclear cell death pathways mediated by E4orf4, and whether the tumor-selective killing of Ad2 E4orf4 can be assigned to one specific pathway or both. Nevertheless, the data obtained using E4orf4 as a model system suggest that specific modulation of Src family kinases may induce efficient tumor-selective calpain-regulated cell death.

The results obtained clearly establish that the general caspase pathways (the extrinsic and intrinsic pathways) are dispensable for execution of both death processes induced by Ad2 E4orf4. Even caspase-2, a more atypical caspase does not appear to play a role in either pathway (no inhibition using specific caspase-2 inhibitor; unpublished data). Our results are in contradiction with another study showing a critical role for caspase-8 in 293T cells (Livne et al., 2001). The reasons for this discrepancy are unknown, but could relate to clonal cell variability and culture conditions. Based on our data, we rather believe that caspase activation through the intrinsic-mitochondrial pathway more generally serves to accelerate and amplify the apoptotic responses induced by E4orf4 in cell types more competent to apoptosis induction (e.g., C-33A). In fact, in C-33A cells expressing E4orf4, caspase inhibition (using caspase inhibitors, Bcl-2, or dominant-negative caspase-9) did not change the overall rate of apoptosis but only switched the apoptotic phenotype toward pure caspase-independent nuclear condensation, like the apoptotic response observed in 293 or 293T cells. In these cells transformed with viral oncogenes, the overexpressed Bcl-2 functional homologue E1B-19K could be responsible for the lack of caspase activation in response to E4orf4 (White et al., 1992). Thus, both the cytoplasmic and the nuclear death pathways induced by E4orf4 rather rely on other caspase- and mitochondria-independent execution processes.

Finally, using the highly specific endogenous calpain inhibitor, calpastatin, we obtained strong evidence that some calpain activity is critical for execution of the Src-dependent cytoplasmic death pathway, but not as such for the nuclear pathway. As suggested in other systems, calpains may connect E4orf4 signaling (both the cytoplasmic and nuclear) to the mitochondrial pathway via regulation of the proapoptotic activity of some Bcl-2 family, thus amplifying the release of mitochondrial factors leading to caspase-dependent and -independent cell death (Wood et al., 1998; Gao and Dou, 2000; Chen et al., 2001; Gil-Parrado et al., 2002; Mandic et al., 2002). Whatever the case, calpains appear to serve a critical function for execution of the cytoplasmic death pathway mediated by E4orf4. E4orf4-Src–mediated calpain activation (either via protein phosphorylation and/or calcium transient) may be critical for triggering actin remodeling and polymerization required for dynamic apoptotic membrane blebbing. Calpain activation by v-src is believed to promote focal adhesion disruption, loss of substrate adhesion and migration of v-src–transformed cells (Carragher et al., 2002). Indeed, calpains are Ca^{2+}-dependent intracellular proteases that cleave substrate proteins localized near the membranes and cytoskeleton in a limited manner, and may thus act to regulate protein functions like caspases (for review see Glading et al., 2002). Additionally, some proteins involved in motility and adhesion are substrates for both calpains and caspases (e.g., FAK, actin, and cortactin), and some of these proteins are also substrates of Src (for review see Tatosyan and Mizenina, 2000), whose phosphorylation is modulated by E4orf4 expression (positively and negatively for cortactin and FAK, respectively) (Lavoie et al., 2000). Interestingly, changes in tyrosine phosphorylation can modify susceptibility of some substrates to calpain, suggesting that a specific modulation of Src couple to calpain activation could function coordinately in initiation of the cytoplasmic death pathway mediated by E4orf4 (Huang et al., 1997). Alterations of the actin polymerization status could then directly induce apoptosis (Rao et al., 1999). For instances, disinhibition of DNase I (which binds to actin) is associated with actin hyperpolymerization in posttraumatic apoptotic cell death, and human DNase I family endonucleases can play a role in apoptotic cell death programs (Kayalar et al., 1996; Villa et al., 1998; Bareyre et al., 2001; Oliveri et al., 2001; Shiokawa and Tanuma, 2001). In future work, we hope to clarify the mechanisms that may mediate calpain activation by E4orf4-Src signaling and the downstream events that trigger caspase-independent and -dependent apoptotic alterations.

Materials and methods

Expression vectors and mutagenesis

The Flag-E4orf4-GFP and Flag-E4orf4 (3Y-F)-GFP were described previously (Gingras et al., 2002). The expression vectors Flag-GFP-NLS, which contain the NLS from SV40 large T antigen (PKKKRKKV) (Kalderon et al., 1984) and the Flag-GFP-NES, containing the leucine-rich nuclear export signal from REV-1 (LPPLERLTL) (Fischer et al., 1995), were described previously (Lee et al., 1999) and were obtained from Dr. Stephen Lee (McGill University, Montréal, Québec, Canada). The Flag-GFP-CAAX contains a COOH terminus CAAX-box from ras (CMSCKCVLS) (Ruta et al., 1986) and was obtained by inserting a peptide sequence coding for the CAAX-box with cohesive extensions into EcoRI-EcoRV sites of the Flag-GFP vector. To generate the Flag-E4orf4-GFP constructs, Ad2 E4orf4 sequence was amplified by the PCR method using an HA-E4orf4/PUHD10-3 as the template as described (Gingras et al., 2002) and inserted into ApaI-XhoI sites of the Flag-GFP vectors. The Flag-E4orf4 (3Y-F)-GFP-NLS and Flag-E4orf4 (3Y-F)-CAAX were obtained using the same method except that Flag-E4orf4 (3Y-F) was used as the template. The Myr-GFP construct containing the Myr con-

sensus signal from chicken c-src (MGSSKSK) (Resh, 1999) was generated by standard PCR method and subcloned into the EGFP-C1 expression vector (CLONTECH Laboratories). The Myr-GFP-HA-E4orf4 construct was generated by digesting pHAO4 hygromycin (unpublished data) to obtain HA-tagged E4orf4, which was subcloned into the ecl136II site of Myr-GFP. The cDNA coding for dominant-negative caspase-9 was obtained from Dr. Y.A. Lazebnik (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (Fearnhead et al., 1998) and was subcloned into the pCI-neo-myc expression vector. The human HA-tagged calpastatin cDNA (Hitomi et al., 1998) was subcloned into the pCI-neo expression vector (Invitrogen). The human HA-tagged Bcl-2 was a gift from Dr. G.C. Shore (McGill University, Montréal, Québec, Canada) and was described previously (Nguyen et al., 1994). The chicken c-src(K295R) in pLNCX vector was provided by Dr. J.S. Brugge (Harvard Medical School, Boston, MA) and was described previously (Thomas et al., 1991). Human Fas in pCI-neo and human Bax in pEGFP-C3 vector were described previously (Wolter et al., 1997; Charette et al., 2000).

Cell culture and transfection

293 cells were derived from human embryonic kidney cells and express Ad5 E1A and E1B proteins and so are 293T cells that also express large T antigen (Graham et al., 1977). Human C-33A (American Type Culture Collection HTB-31) are from cervical carcinoma and are deficient for p53 expression. 293 and 293T were maintained in DME and C-33A were culture in α-modified Eagle medium, both supplemented with 10% fetal bovine serum and streptomycin sulfate-penicillin (100 U/ml). Transfections were performed by the calcium phosphate method as described (Gingras et al., 2002). Transfections were processed during 5 to 6 h in presence of chloroquine (25 μM for 293T and 50 μM for 293 and C-33A; Sigma-Aldrich). Cotransfection of E4orf4 constructs with HA-Bcl2, dominant-negative caspase-9, or HA-calpastatin were performed using a plasmid DNA ratio of 1:1, or 1:2, in 5–7 μg total plasmid DNA. For all experiments using 293T and C-33A, the cells were seeded on polylysine-coated culture dishes as described previously (Lavoie et al., 2000).

In vivo localization, morphological assays, and apoptosis assays

Live cells expressing the various GFP fusion proteins were observed using a Nikon TE-300 inverted microscope equipped with a ×60 0.85 MA objective. Images were captured as 16-bit TIFF files with a Micromax 1300YHS (B/W) cooled CCD camera (−30°C; Princeton Instruments) driven by Metamorph software version 4.5 (Universal Imaging Corp.). Confocal microscopy was performed using a Bio-Rad MRC-1024 imaging system mounted on a Nikon Diaphot-TMD equipped with a ×20 objective lens. The blebbing-inducing activity was measured in live cells, and apoptosis was determined after cell fixation by analyzing the nuclear morphology as described (Gingras et al., 2002). Aliquots of transfected cells were kept for Western blot analysis of protein expression. The broad spectrum caspase inhibitors, z-Val-Ala-Asp-(Ome)-CH_2F (zVAD.fmk), Boc-Asp-(OMe)-CH_2F (BocD.fmk), and the caspase-8 inhibitor II, z-IETD-fmk (z-Ile-Glu[Ome]-Thr-Asp[Ome]-CH_2F; Calbiochem) were dissolved at 50 mM in DMSO, and were added to the cell cultures during and after transfection at a concentration of 75 μM; fresh inhibitors were added back every 24 h. Immunodetection was performed as described (Lavoie et al., 2000). At the indicated times, cells were washed in PBS containing 1 mM $MgCl_2$, fixed in 3.7% formaldehyde/PBS for 20 min and postfixation was performed in ice-cold 70% ethanol for 12 h at 4°C. Immunofluorescence analyses were performed using the following antibodies: chicken c-src (K295R) was revealed using rabbit anti-SRC2 antibody (Santa Cruz Biotechnology); rabbit anti-GFP was used occasionally to label the GFP fusion proteins (ClONETECH Laboratories, Inc.); active caspase-3 was revealed with rabbit anticleaved caspase-3 (Asp175) antibody (Cell signaling; NEB); cytochrome c was revealed using mouse anti-cytochrome c (clone 6H2.B4; Pharmingen); AIF was monitored using a rabbit anti-AIF antibody raised against a COOH-terminal peptide (LNEVAKLFNIHED) and was a gift from Dr. J Landry (Laval University, Québec, Canada), followed by either ALEXA-594- or ALEXA-488-labeled goat anti–rabbit, or anti–mouse immunoglobulin G (Molecular Probes). DNA was labeled using DAPI (Molecular Probes).

Western blotting

Cells were washed in PBS and lysed in SDS sample buffer (Lavoie et al., 2000). Equal amounts of total cell proteins (determined using the Bio-Rad D_c Protein Assay) were loaded in SDS-PAGE gels (12% acrylamide) and transferred onto nitrocellulose membrane. Blots were developed with mouse monoclonal anti-Flag M2 (Sigma-Aldrich), mouse Ab-1 anti–v-src (Calbiochem; Novabiochem), or mouse HA.11 anti-HA antibody (BabCO). Horseradish peroxidase–linked goat anti–mouse immunoglobulin G (Jackson ImmunoResearch Laboratories), revealed by the enhanced chemiluminescence detection system (Renaissance; NEN Life Science Products) was used for detection.

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