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Caspase Activation by Adenovirus E4orf4 Protein Is Cell Line Specific and Is Mediated by the Death Receptor Pathway

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Adenovirus E4orf4 protein has been shown to induce transformed cell-specific, protein phosphatase 2A-dependent, and p53-independent apoptosis. It has been further reported that the E4orf4 apoptotic pathway is caspase-independent in CHO cells. Here, we show that E4orf4 induces caspase activation in the human cell lines H1299 and 293T. Caspase activation is required for apoptosis in 293T cells, but not in H1299 cells. Dominant negative mutants of caspase-8 and the death receptor adapter protein FADD/MORT1 inhibit E4orf4-induced apoptosis in 293T cells, suggesting that E4orf4 activates the death receptor pathway. Cytochrome *c* is released into the cytosol in E4orf4-expressing cells, but caspase-9 is not required for induction of apoptosis. Furthermore, E4orf4 induces accumulation of reactive oxygen species (ROS) in a caspase-8- and FADD/MORT1-dependent manner, and inhibition of ROS generation by 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron) inhibits E4orf4-induced apoptosis. Thus, our results demonstrate that E4orf4 engages the death receptor pathway to generate at least part of the molecular events required for E4orf4-induced apoptosis.

Apoptosis is a genetically regulated cellular process which plays an important role in conservation of homeostasis and protection from tumorigenesis. Apoptosis can be induced by various signals that trigger activity of the evolutionarily conserved core cell death apparatus that commits the cells to die. The commitment to cell death leads to a cascade of events that result in the acquisition of morphological and biochemical features unique to apoptotic cells.

Caspases constitute one class of components belonging to the core death machinery. They are cysteine proteases that cleave proteins after aspartic acid residues. Caspases are constitutively expressed as catalytically inactive proenzymes and are activated by proteolytic processing (reviewed in reference 5). Several apoptosis-inducing signals have been shown to activate caspases. However, various recent reports indicated the existence of caspase-independent apoptosis (11, 27, 30). Thus, whereas in some cases inhibition of caspases prevented cell death (39), in others, death was delayed but not abolished (42).

Currently, two caspase-activating pathways that regulate apoptosis are well characterized. One is initiated from cell surface death receptors, and the other is triggered by various stress signals and involves changes in mitochondrial integrity (reviewed in reference 4). The death receptors are a family of transmembrane proteins, including Fas/APO-1/CD95 and the tumor necrosis factor (TNF) receptor, which share a region of homology at the cytoplasmic face, termed the death domain. Upon binding of ligands to the receptors and subsequent receptor trimerization, the death domains recruit adapter proteins containing a death domain and a death effector domain. The death domain of Fas, for example, recruits an adapter protein called FADD/MORT1, and the FADD/MORT1 death effector domain is critical for recruiting an upstream procaspase, such as procaspase-8 or -10, to form the death-inducing signaling complex (DISC) (29, 41). Immediately after recruitment, the procaspase is proteolytically processed to the active caspase form. Activation of caspase-8 can lead to a direct cleavage and activation of downstream caspases, such as caspase-3, -6, and -7, or can activate the mitochondrial apoptotic pathway (41). Caspase-8 can be efficiently inhibited by CrmA, a viral protein of the serpin family (20, 31).

The second caspase activation cascade involves mitochondrial changes induced by several stress signals, such as serum starvation, oncogene activation, DNA-damaging agents, kinase inhibitors, etc., as well as activation of cell surface death receptors (32, 43). Cytochrome *c* is released from the mitochondria and associates with two cytosolic proteins, Apaf-1 and procaspase-9. Caspase-9 is activated in this complex, and is released to further activate downstream caspases, such as caspase-3, -6, and -7 (reviewed in reference 4). Bcl-2 family members regulate cytochrome *c* release from the mitochondria. Overexpression of Bcl-2 or Bcl-xL blocks the release, whereas the proapoptotic Bcl-2 proteins, including Bax and Bid, promote it.

Reactive oxygen species (ROS), which are formed in organisms exposed to molecular oxygen, have been reported to be important players in apoptosis (8). ROS or H_2O_2 can induce apoptosis, and the antiapoptotic effect of Bcl-2 appears to be at least partially the result of its antioxidant properties (14, 16). Two reports have recently suggested that generation of ROS is a key event in evolutionary early apoptotic mechanisms. In the first, ROS was shown to accumulate in yeast cells induced to undergo apoptosis by Bax or by a CDC48 mutation, whereas radical depletion prevented apoptosis (24). In the second report, the mammalian FADD death effector domain was shown to induce bacterial cell death. FADD toxicity was suppressed by various oxidoreductases of *Escherichia coli* (23). The death

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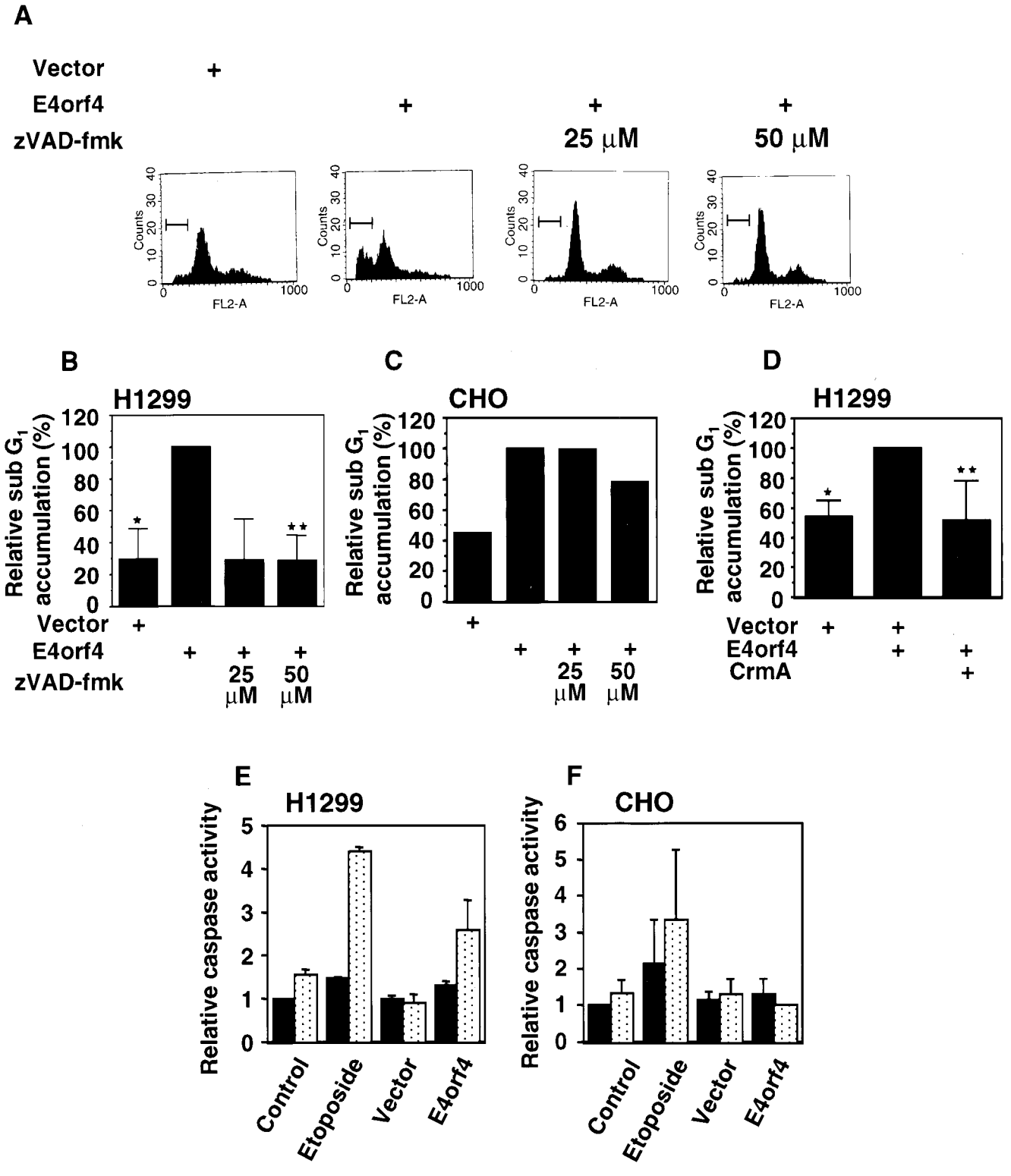

FIG. 1. E4orf4 induces caspase activation in H1299 cells but not in CHO cells. E4orf4 or the empty vector was cotransfected into H1299 (A and B) or CHO (C) cells with a membrane-anchored GFP, and zVAD-fmk was added where indicated, at concentrations of 25 or 50 μM. Cells were collected 48 h posttransfection and subjected to FACS analysis. (A) The bars mark the position of the sub-G_1 cell population. (B and C) Relative accumulation of cells in sub-G_1 is shown, and the sub-G_1 fraction of E4orf4-expressing cells is defined as 100%. The average of two to three experiments is shown. One-tailed *P* value: asterisk (the difference between the empty vector and E4orf4), $P = 0.006$; two asterisks (the difference between E4orf4 and E4orf4 plus 50 μM zVAD-fmk), $P = 0.0005$. (D) H1299 cells were cotransfected with GFP and the empty vector or E4orf4, and one group of E4orf4-transfected cells was cotransfected with a plasmid expressing CrmA as well. The cells were analyzed as in panels A to C. The average of three experiments is shown. One-tailed *P* value: asterisk (the difference between empty vector and E4orf4), *P* =

receptor pathway in mammalian cells was shown to involve ROS generation as well (12, 38). In mammalian cells, ROS can be generated in the mitochondria but also in the ER and the plasma membrane.

Adenovirus E4orf4 protein has been shown to induce p53-independent apoptosis in transformed cells (18, 22, 25, 34). Its proapoptotic activity is higher in transformed cells than in normal cells (35), suggesting that it may be potentially useful in cancer gene therapy. E4orf4 associates with several populations of protein phosphatase 2A (PP2A), and its association with the PP2A molecules that contain a B/PR55 subunit is required for induction of apoptosis (35, 36). On the basis of studies utilizing CHO cells, it has been suggested that E4orf4-induced apoptosis is caspase independent (22). Here, we show that caspase activation by E4orf4 is cell line specific and is essential for cell death in some cells, while being dispensable in others. Moreover, E4orf4 induces caspase activation by the death receptor pathway, leading to ROS accumulation and cell death.

MATERIALS AND METHODS

Plasmids and cells. The following plasmids have been previously described: pCMV-E4orf4 (34), the pCMV/neo vector (13), pEGFP-C1 (Clontech), pEGFP-Spectrin (i.e., membranal green fluorescent protein [GFP]) (15), pBabe-puro (28), the plasmids expressing CrmA, caspase-8 (Mach a1), caspase-8 dominant negative (Mach a1 c360s), p55-TNF-R1, p55-FAS, MORT1-DD (3), caspase-9, and its dominant-negative mutant (7), HA-Bax (from A. Gross).

Human 293T cells were derived by introducing the simian virus 40 T antigen into 293 cells. 293 cells are human embryonic kidney cells that express Ad5 E1A and E1B proteins (9). 293T cells, the human non-small-cell lung carcinoma H1299 cells (26), and CHO cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Transfections, Western blot analysis, and antibodies. Cells were plated in 60-mm-diameter culture dishes, and transfections were carried out by the standard method of calcium phosphate precipitation of DNA for 293T cells (10) and with Lipofectamine plus (Gibco BRL) for CHO and H1299 cells. The amounts of DNA used per dish for each of the following were as indicated: GFP or membrane-anchored GFP, 2 μg; E4orf4, 1.5 μg; and effector plasmid, 4.5 μg. Twenty four hours posttransfection, cells were harvested, washed with phosphate-buffered saline (PBS), and lysed with lysis buffer containing 50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.5% Nonidet P-40, 2 μg of leupeptin per ml, 2 μg aprotinin per ml, and 0.5 mM phenylmethylsulfonyl fluoride. The levels of the proteins were analyzed by Western blot analysis. Expression of E4orf4 was detected by a rabbit polyclonal antibody previously described (35). Other antibodies used were against caspase-3 and cytochrome *c* (Santa Cruz Biotechnologies), caspase-9 (Y. Lazebnik), and PP2A-C (34).

Flow cytometry. Cells were cotransfected with E4orf4, effector plasmids, and a plasmid expressing a membrane-anchored GFP (15), into H1299 cells. Where indicated, zVAD-fmk (Biomol, Plymouth Meeting, Pa.) was added. At the indicated times, cells were harvested and fixed with methanol for at least 30 min at −20°C. The cells were subsequently washed in PBS, resuspended in PBS containing 50 μg of RNase A per ml, and incubated 30 min at 37°C. To stain the DNA, propidium iodide (Sigma) was added to a final concentration of 25 μg per ml. Transfected cells manifesting high green fluorescence levels were gated separately, and their DNA content was analyzed using the CellQuest software on a FACSCalibur (Becton Dickinson).

DAPI assay. Cells were transfected as described above. Twenty-four hours posttransfection, the cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, washed again, and stained with 0.1 μg of 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma). A cover slide was mounted on the plates, using Fluoromount-G (Southern Biotechnology Associates, Birmingham, Ala.). The fluorescent cells were visualized, using a ×600 magnification on a Zeiss axioskop. In each experiment, 100 transfected nuclei were counted.

Trypan blue exclusion assay. Nonadherent and adherent cells were collected, and aliquots were mixed with an equal volume of 0.4% trypan blue. The percentage of cells that took up the dye was calculated by counting at least 200 cells. Percent E4orf4-induced apoptosis was determined by normalizing these numbers to transfection efficiency, determined by expression of cotransfected GFP.

Caspase assays. Cells were transfected with the appropriate plasmids together with a plasmid expressing LacZ from an immediate-early cytomegalovirus promoter, or treated with 50 μM etoposide (Sigma). At the indicated times, cells were collected and washed with PBS, followed by centrifugation at 500 × *g* for 5 min. Half the cells were used to measure β-galactosidase (β-Gal) activity, and the other half was used to measure caspase activity. For caspase activity assays, the cell pellet was suspended in 0.1 ml of buffer containing 10 mM HEPES (pH 7.5), 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma), 5 mM dithiothreitol, leupeptin (20 μg/ml), aprotinin (10 μg/ml), and 0.5 mM phenylmethylsulfonyl fluoride. Following a 20-min incubation on ice, cells were disrupted by sonication. Cell extracts were subjected to centrifugation at 20,000 × *g* for 20 min at 4°C, and the supernatants were collected. Caspase assays included 200 μg of cell lysate, 5 μl of 5 mM Ac-DEVD-pNA (Biomol), and reaction buffer (100 mM HEPES [pH 7.5], 20% glycerol, 5 mM dithiothreitol, 0.5 mM EDTA) in a total volume of 0.2 ml for each reaction. Samples were incubated at 37°C for 5 h and enzyme-catalyzed release of *p*-nitroanilide was measured at 400 nm. For the samples from transfected cells, the results of the caspase assay were normalized to transfection efficiencies, assessed by the β-Gal assay.

Viability assays. Cells were cotransfected with 2 μg of pBabe-puro, 1.5 μg of pCMV or pCMV-E4orf4, and 4.5 μg of effector plasmid or the empty vector. Stable colonies were selected in puromycin (2 μg per ml for H1299 and 3 μg per ml for 293T) for 2 weeks. Colonies were detected by Giemsa staining (H1299) or by staining with neutral red (293T).

Detection of ROS. Cells were cotransfected with 2 μg of pEGFP-C1 and 2.5 μg of pCMV/neo vector or vector expressing wild-type E4orf4. At 12 h posttransfection, the cells were incubated at room temperature with dihydroethidium (hydroethidine; Sigma) at 5 μg per ml, from a 5-mg/ml stock solution. Cells were washed with PBS after 10 min of incubation and viewed through a rhodamine optical filter. Where indicated, the antioxidant 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron; Sigma), was added to the cells 6 h posttransfection, at a concentration of 1 mM.

Preparation of subcellular fractions. Cells were washed with PBS, resuspended in sucrose buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.25 M sucrose, 1 μg each of aprotinin and leupeptin per ml, 0.5 mM phenylmethylsulfonyl fluoride), and homogenized by passing seven times through a 25-gauge needle. The homogenates were centrifuged at 750 × *g* for 5 min at 4°C to collect nuclei. The supernatants were centrifuged at 10,000 × *g* for 5 min at 4°C to collect microsomal fractions. The final supernatants are referred to as cytosolic fractions.

RESULTS

Caspase activation by E4orf4 is cell line specific. It has been previously reported that caspase-3 was not activated during the E4orf4 apoptotic process in CHO cells (22). It was further shown in these cells that the broad-range caspase inhibitor, zVAD-fmk, did not inhibit the appearance of apoptotic morphologies, such as DNA degradation, chromatin condensation, and loss of mitochondrial membrane potential (22). We first tested whether zVAD-fmk inhibited DNA degradation in the human lung carcinoma H1299 cell line, as measured by appearance of a sub-G_1 cell population. (Cells with degraded DNA lose some of their DNA during the preparation for fluorescence-activated cell sorter [FACS] analysis, leading to

0.0045; two asterisks (the difference between E4orf4 and E4orf4 plus CrmA), $P = 0.038$. (E and F) H1299 (E) and CHO (F) cells were treated with 50 μM etoposide or cotransfected with a plasmid expressing LacZ and vector or E4orf4. Cell extracts were prepared 24 (black bars) or 48 (dotted bars) h later, and β-Gal and caspase assays were performed. Caspase activity was normalized to transfection levels as assayed by β-Gal expression. Caspase activity in untreated cells at the 24 h time point was defined as 1. An average of two experiments is shown. (B and D to F) Error bars, standard deviation.

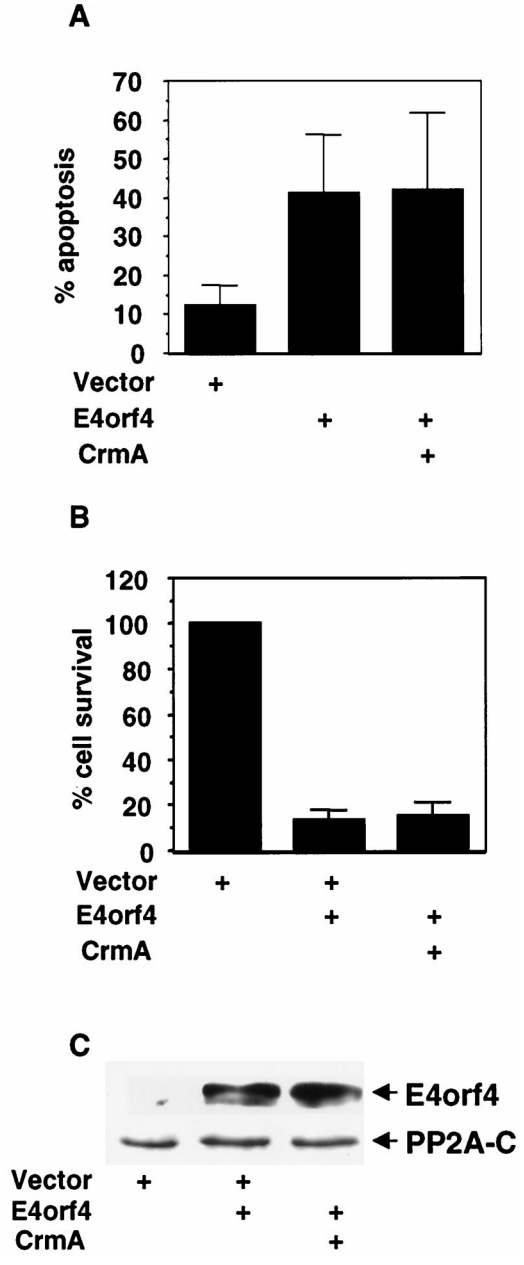

FIG. 2. CrmA does not inhibit E4orf4-induced nuclear condensation and cell death in H1299 cells. (A) H1299 cells were cotransfected with GFP and the empty vector or E4orf4, and one group of E4orf4-transfected cells was cotransfected with a plasmid expressing CrmA as well. Twenty-four hours posttransfection, nuclei were stained with DAPI and percent apoptosis was determined by counting nuclei with apoptotic morphology in the transfected cell population, identified by the green fluorescence. One hundred nuclei were counted for each point, and the graph shows the average of four experiments (error bars, standard deviation). (B) Cells were cotransfected with pBabe-puro and combinations of vector, E4orf4 and vector, or E4orf4 and CrmA. Cells were collected 48 h posttransfection and plated in medium containing 2 μg of puromycin per ml. Resistant colonies were visualized 2 weeks later by Giemsa staining and counted. The number of colonies obtained from the transfection with the vector was defined as 100%. The average of five experiments is shown (error bars, standard deviation). (C) Protein extracts were prepared from an aliquot of the cells used for the clonogenic assay described for panel B, and E4orf4 was detected by a Western blot analysis. Detection of PP2A-C indicated that equal protein quantities were loaded in each lane.

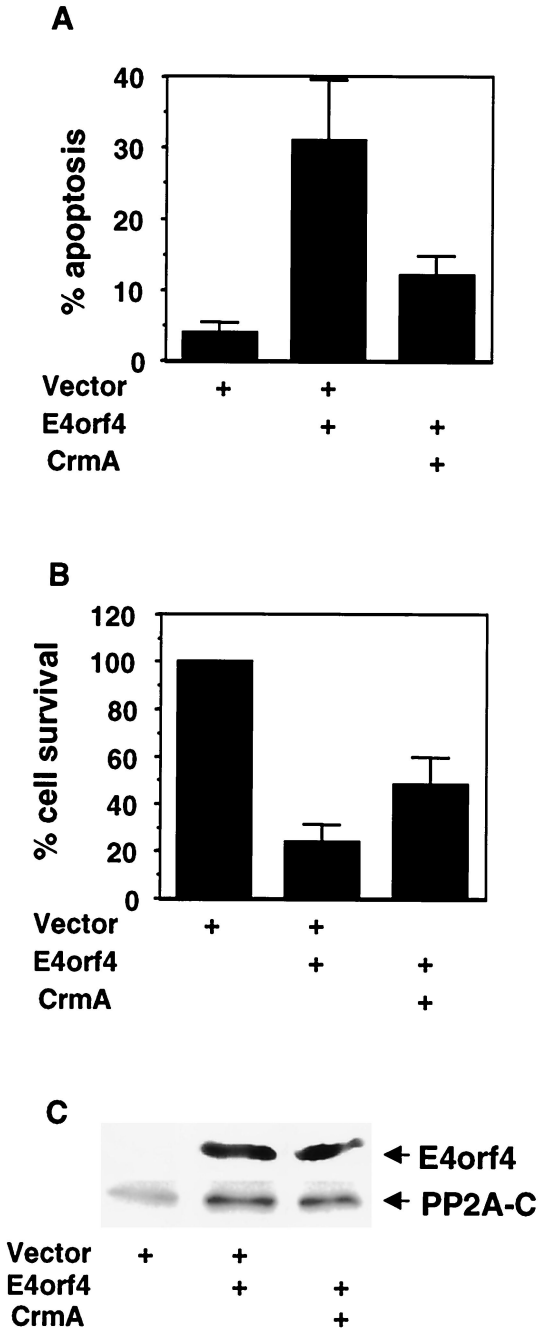

FIG. 3. CrmA inhibits E4orf4-induced apoptosis in 293T cells. The experiments were carried out as described in the legend to Fig. 2, except that selection of resistant colonies was carried out in medium containing 3 μg of puromycin per ml. The average of two (A) or three (B) experiments is shown (error bars, standard deviation). (C) Protein extracts were prepared from an aliquot of the cells used for the clonogenic assay described for panel B, and E4orf4 was detected by a Western blot analysis. Detection of PP2A-C indicated that equal protein quantities were loaded in the presence or absence of CrmA.

the appearance of cells with less than G_1 DNA content.) Results shown in Fig. 1A and B demonstrate that zVAD-fmk abolished E4orf4-induced DNA degradation. However, as previously reported (22), the same concentrations of the drug failed to inhibit DNA degradation in CHO cells (Fig. 1C). The

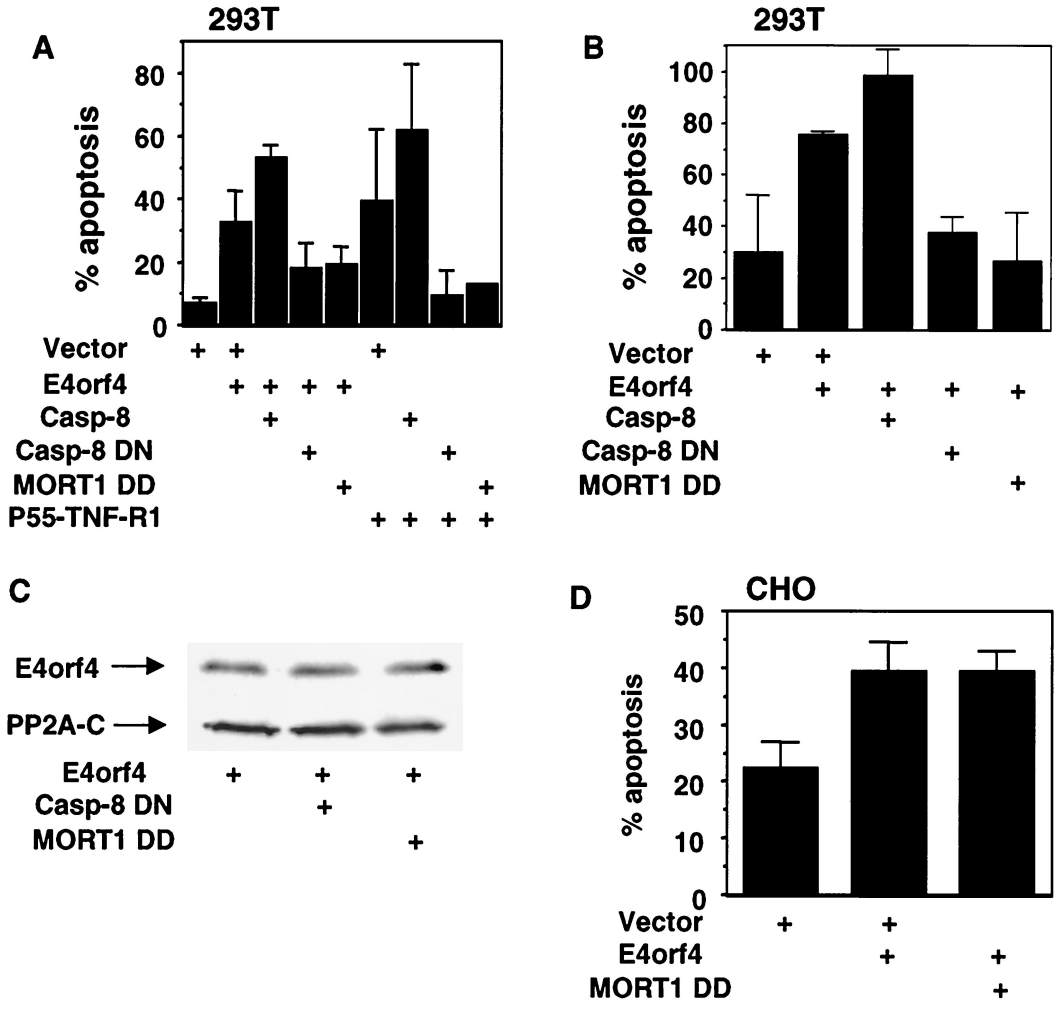

FIG. 4. The death receptor pathway mediates E4orf4-induced apoptosis in 293T cells. (A) The various constructs mentioned in the figure were cotransfected into 293T cells with GFP, and all transfections contained identical amounts of DNA. Nuclei were stained with DAPI 24 h posttransfection, and the graph shows the percent of transfected nuclei with apoptotic morphology. The average of three experiments is shown. (B) Cells were transfected as described for panel A and were harvested 24 h posttransfection. Percent apoptosis was determined by trypan blue exclusion, and the percentage of cells that took up the dye was normalized to the percentage of transfection, determined by GFP. At least 200 cells were counted in each experiment, and the average of two experiments is shown. (C) Proteins were extracted from cells transfected in parallel to the experiment shown in panel A, and separated on a sodium dodecyl sulfate–15% polyacrylamide gel. E4orf4 was detected with specific antibodies, and detection of PP2A-C in the same blot indicated that equal protein levels were loaded in each lane. (D) CHO cells were cotransfected with GFP and combinations of vector, E4orf4 and vector, or E4orf4 and MORT1-DD, and were analyzed as described for A. The average of two sets of triplicates is shown. (A, B, and D) Error bars, standard deviation.

caspase inhibitor CrmA also inhibited DNA degradation in H1299 cells when cotransfected with E4orf4 (Fig. 1D). Next, we measured caspase activity in cell extracts. H1299 and CHO cells were cotransfected with a transfection marker (*lacZ*) and with an E4orf4-expressing plasmid or with the empty vector, and caspase activity assays were carried out using cell extracts and the colorimetric caspase-3 substrate Ac-DEVD-pNA. Caspase activity was normalized to levels of transfection measured by the β-Gal assay in the same cells. Figure 1E demonstrates that caspase activity was enhanced in E4orf4-transfected H1299 cells at 24 h and more dramatically at 48 h posttransfection, compared to cells transfected with the empty vector. By contrast, caspase activity was undetectable in E4orf4-transfected CHO cells at both time points tested (Fig. 1F). Etoposide treatment resulted in caspase activation in both cell lines, indicating that lack of caspase activation in CHO cells did not result from caspase deficiency in these cells.

We next determined whether caspase activation by E4orf4 in H1299 cells led to additional apoptotic manifestations. Cells transfected with an E4orf4-expressing plasmid or the empty vector were stained with DAPI 24 h posttransfection to visualize their nuclear morphology, as previously described (34). Nuclear condensation was efficiently induced by E4orf4. However, cotransfection with CrmA or treatment with zVAD-fmk did not affect the number of cells manifesting condensed nuclei (Fig. 2A and results not shown), similar to the results reported for CHO cells (22). Thus, E4orf4-induced caspase activation in H1299 cells appeared to be required for DNA degradation but not for chromatin condensation. Similar results were obtained 48 h posttransfection (results not shown).

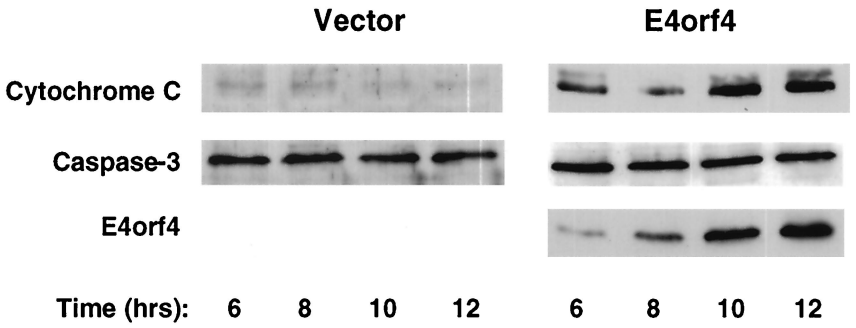

FIG. 5. E4orf4 induces cytochrome *c* release into the cytosol in H1299 cells. Empty vector or E4orf4 was transfected into H1299 cells, and cells were harvested at the indicated times posttransfection. Cell extracts were fractionated into nuclear, cytoplasmic, and microsomal fractions, and cytosolic proteins were separated on a sodium dodecyl sulfate–15% polyacrylamide gel. The Western blot from this gel was stained three consecutive times with antibodies specific to cytochrome *c*, caspase-3, or E4orf4.

To examine whether caspase activation was required for loss of viability and cell death, a clonogenic assay was carried out. The puromycin-resistance gene (pBabe-puro) and E4orf4 were cotransfected into H1299 cells either with the empty vector or with a plasmid expressing the caspase inhibitor CrmA, and the colonies appearing within 2 weeks of growth in the presence of puromycin were counted. As demonstrated in Fig. 2B, CrmA did not affect loss of cell viability of the E4orf4-transfected H1299 cells, although it inhibited toxicity caused by overexpression of the TNF receptor in these cells (results not shown), as well as E4orf4-induced DNA degradation (Fig. 1D). CrmA did not affect E4orf4 protein levels in these cells (Fig. 2C). Thus, E4orf4-induced caspase activation in H1299 cells is required for some of the morphological features that accompany apoptosis but not for cell death.

A second human cell line, 293T, was also used to test whether caspase activation was required for E4orf4-induced apoptosis. In these cells, unlike in H1299 cells, CrmA inhibited E4orf4-induced nuclear condensation (Fig. 3A) and reduced cell death frequency as measured by the number of puromycin-resistant colonies derived from cells cotransfected with the puromycin resistance gene (Fig. 3B). The reduced number of colonies obtained in the presence of CrmA did not result from decreased E4orf4 levels in cells cotransfected with E4orf4 and CrmA, as determined by the results of a Western blot analysis shown in Fig. 3C. Thus, activation of caspases appeared to be important for E4orf4-induced apoptosis in 293T cells.

E4orf4 induces caspase activation through the death receptor pathway. Data shown in Fig. 1D and 3 demonstrated that CrmA, a potent inhibitor of caspase-8 (20, 31), inhibited E4orf4-induced apoptosis in 293T cells and E4orf4-induced DNA degradation in H1299 cells. To further investigate whether caspase-8 was involved in E4orf4-mediated events, we tested whether a dominant-negative caspase-8 mutant affected E4orf4-induced apoptosis. 293T cells were cotransfected with E4orf4 together with an empty vector, wild-type caspase-8, or a dominant-negative form of this caspase (3). Induction of apoptosis was measured by counting DAPI-stained apoptotic nuclei. As seen in Fig. 4A, the dominant-negative caspase-8 mutant inhibited apoptosis induced by the TNF alpha receptor (p55-TNF-R1) and by E4orf4, whereas the wild-type enzyme increased cell death. Caspase-8 is known to be activated by the death receptor pathway. The receptor death domain recruits an adapter protein, which further recruits caspase-8 through an interaction of the caspase with the death effector domain of the adapter (reviewed in reference 41). To test whether upstream activators of caspase-8 were involved in E4orf4-induced apoptosis, E4orf4 was cotransfected with a dominant-negative mutant of the FADD/MORT1 protein (MORT1-DD), which contains the MORT1 death domain but not its death effector domain (3). The mutant suppressed cell death induced by p55-TNF-R1 and by E4orf4 (Fig. 4A), suggesting that E4orf4 acts upstream of the DISC. These results were verified by using an additional assay to measure apoptosis, the trypan blue exclusion assay (Fig. 4B). Suppression of E4orf4-induced cell death by the various dominant-negative mutants was not due to decreased cellular levels of E4orf4, as demonstrated by results of the Western blot shown in Fig. 4C. MORT1-DD did not affect induction of apoptosis in CHO cells (Fig. 4D), confirming that E4orf4 did not operate via the death receptor pathway in these cells.

E4orf4 induces release of cytochrome *c* from the mitochondria. Activation of caspase-8 can lead to direct cleavage and activation of downstream caspases and can also activate the mitochondrial apoptotic pathway (41). To determine whether the mitochondrial pathway is activated and cytochrome *c* is released into the cytosol upon E4orf4 expression, H1299 cells were transfected with E4orf4 and cell extracts were fractionated by centrifugation into nuclear, cytosolic, and microsomal fractions. Western blots of the cytosolic fractions extracted at various times posttransfection revealed almost no cytochrome *c* in cytosol-derived fractions from vector-transfected cells, whereas it was detected in cytosols of E4orf4-transfected cells as early as 6 h posttransfection, further increasing at later times (Fig. 5). The levels of caspase-3 remained unaltered in the cytosol during the same period of time. Similar results were obtained with 293T cells (not shown). These results suggest that the mitochondrial pathway is activated in E4orf4-transfected cells.

Activation of caspase-9 is not part of the E4orf4 apoptotic pathway. Release of cytochrome *c* from the mitochondria is required for activation of caspase-9. To test whether caspase-9 is activated upon E4orf4 expression, H1299 cells were cotransfected with E4orf4 and a dominant-negative mutant of caspase-9 (7), and the proportion of a sub-G_1 cell population was measured by FACS analysis. Data shown in Fig. 6A dem-

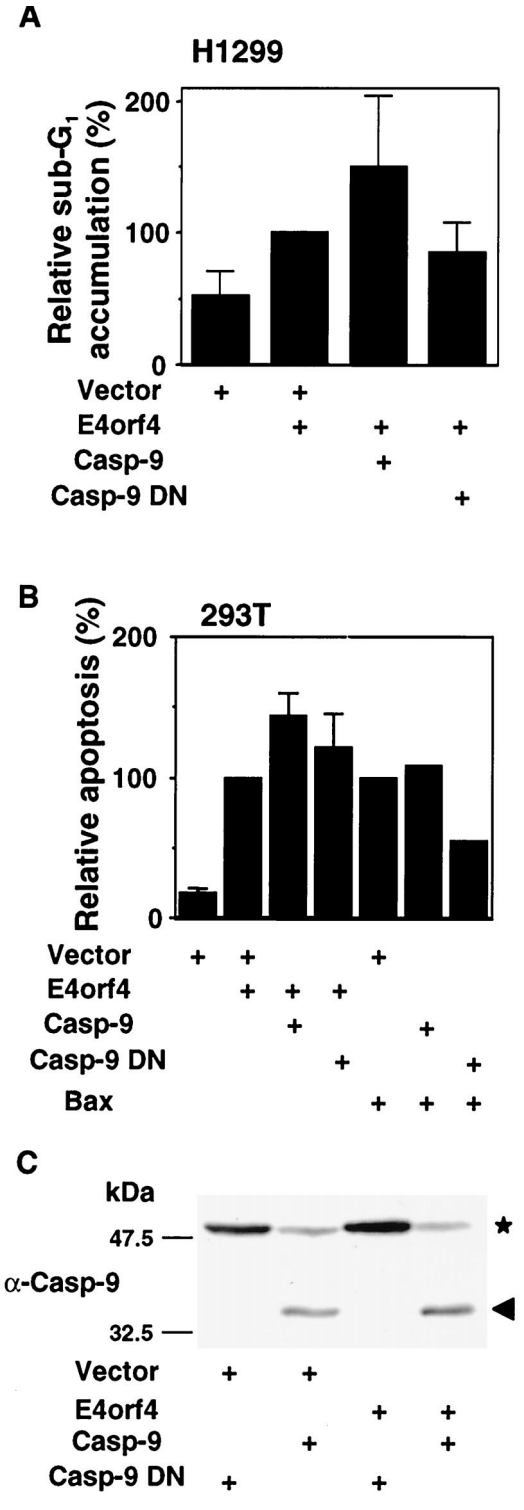

FIG. 6. Activation of caspase-9 is not required for E4orf4-induced apoptosis. (A) The constructs mentioned in the figure (casp-9, caspase-9; casp-9 DN; caspase-9 dominant negative) were cotransfected into H1299 cells with membrane-anchored GFP, and analysis was carried out by FACS as described in the legend to Fig. 1A. The sub-G_1 fraction of E4orf4-expressing cells is defined as 100%, and the average of three experiments is shown. (B) 293T cells were transfected with the constructs mentioned in the figure and with GFP, and analysis was carried out by the DAPI assay, as described in the legend to Fig. 2A. Apoptosis induced by E4orf4 cotransfected with the empty vector was defined as 100%. The average of three experiments is shown. (A and B) Error bars, standard deviation. (C) Vectors expressing caspase-9 or dominant-negative caspase-9 were cotransfected with an empty vector or with a vector expressing E4orf4. Processing of caspase-9 was assessed by immunoblotting. The caspase precursor is indicated by a star, and the processed caspase is indicated by an arrowhead.

onstrated that the mutant caspase-9 did not significantly diminish E4orf4-induced DNA degradation in H1299 cells, although this apoptotic morphology was inhibited by zVAD-fmk (Fig. 1). In 293T cells, dominant-negative caspase-9 did not reduce E4orf4-induced apoptosis, although this mutant inhibited Bax-induced apoptosis in the same cells, as measured by the DAPI assay (Fig. 6B). Figure 6A and B further demonstrated that overexpression of wild-type caspase-9 increased apoptosis in the presence of E4orf4. However, when ectopically expressed caspase-9 was visualized by a Western blot (Fig. 6C), no significant differences in its processing were observed in the presence or absence of E4orf4. These results indicate that caspase-9 and E4orf4 do not work in synergy. Thus, E4orf4 did not appear to enhance caspase-9 activation in these cells, and this caspase was not required for E4orf4-induced apoptosis.

E4orf4 induces high ROS levels, which are required for E4orf4-induced apoptosis. Since ROS accumulation in cells appears to be a common event occurring in cell death pathways throughout evolution (24), we inquired whether E4orf4 expression led to ROS accumulation in the cells. 293T cells were transfected with either E4orf4 or an empty vector and with the transfection marker GFP. Intracellular ROS accumulation was assessed by staining of the transfected cells with the ROS-sensitive fluorescent dye dihydroethidium. As seen in Fig. 7A and B, transfection of 293T cells with E4orf4 resulted in a sharp increase in the intracellular levels of ROS at 18 h posttransfection, relative to ROS levels in vector-transfected cells. Cotransfection of E4orf4 with CrmA or MORT1-DD resulted in decreased ROS levels, as did treatment with zVAD-fmk (Fig. 7B). To inquire whether increased ROS levels contributed to E4orf4-induced apoptosis, the antioxidant Tiron was added to the cells at 5 h posttransfection, and its effect on E4orf4-induced apoptosis was assessed by the DAPI assay. As demonstrated in Fig. 7C, Tiron had a substantial protective effect against E4orf4-induced apoptosis in 293T cells. Similar results were obtained with the antioxidant *N*-acetylcysteine (NAC) (results not shown). These data suggest that the generation of ROS is essential for E4orf4-induced apoptosis in 293T cells, and occurs downstream of caspase activation.

DISCUSSION

Two novel characteristics of the E4orf4 apoptotic pathway are described in this report. First, E4orf4 induces caspase activation in a cell line-specific manner, and this activation can be essential or dispensable for the apoptotic process, depending on the cell line investigated. Thus, caspase activation was detected in H1299 cells (Fig. 1E) but not in CHO cells (21) (Fig. 1F). Furthermore, caspase activation was required for induction of cell death in 293T cells (Fig. 3) but not in H1299 cells (Fig. 2). The second major finding presented here implicates

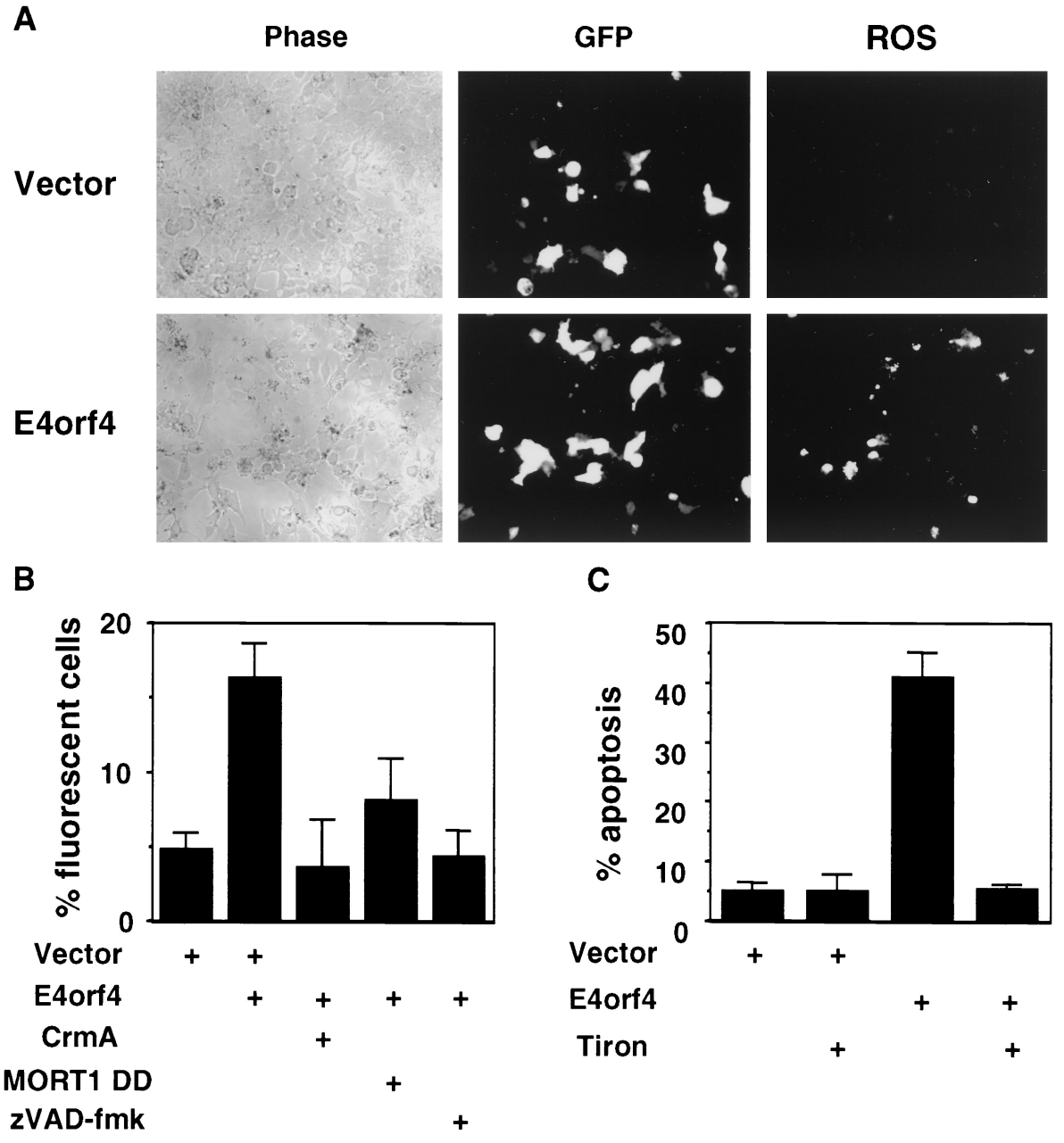

FIG. 7. Generation of ROS lies downstream of caspase activation and is required for E4orf4-induced apoptosis. (A) 293T cells were transfected with vector or E4orf4 and were stained with dihydroethidium for 10 min, 18 h posttransfection. Fluorescence was visualized by a Zeiss Axioscope (magnification, of ×170). (B) 293T cells were transfected with the constructs mentioned in the figure, and ROS detection was carried out as described for panel A. The number of fluorescent cells per 100 transfected cells was determined. The average of three experiments is shown. (C) 293T cells were transfected as described for panel A, and Tiron was added at a 1 mM final concentration for 16 h. The cells were fixed and stained with DAPI, and the number of nuclei with apoptotic morphology was determined. The average of two experiments is shown. (B and C) Error bars, standard deviation.

the death receptor pathway in mediating E4orf4-induced apoptosis in 293T cells (Fig. 4), and details of this pathway are described (Fig. 4 to 7).

The molecular basis for the high degree of variability in the response of cells to E4orf4 is currently unclear. Possibly, components of the mechanism involved in engaging the death receptor pathway, leading to caspase activation by E4orf4, are absent in CHO cells. Indeed, in these cells the dominant-negative mutant of the adapter molecule FADD/MORT1 did not affect E4orf4-induced apoptosis (Fig. 4D). The results obtained in H1299 cells indicated that at least two cell death pathways were affected by E4orf4: caspase activation by E4orf4 was required for DNA degradation, whereas nuclear condensation occurred by a caspase-independent mechanism. In these cells, a factor that lies on the pathway from caspase activation to nuclear condensation may be inactive. In 293T cells, both a

caspase-independent mechanism and a caspase-dependent pathway may operate as well, since the use of CrmA, caspase-8 dominant-negative mutant, or MORT1-DD did not completely abolish E4orf4-induced apoptosis in these cells (Fig. 3 and 4). However, the caspase-independent pathway in 293T cells might not be fully efficient and did not attain full induction of apoptosis in the absence of caspase activation (Fig. 3). The existence of multiple combinations of caspase-dependent and -independent pathways that can be activated by a single activator presents an added level of complexity in the cellular control of apoptotic processes.

Several lines of evidence indicate that E4orf4 operates through the death receptor pathway in 293T cells. First, CrmA, an efficient inhibitor of caspase-8 (20, 31), inhibited nuclear condensation and increased 293T cell survival measured by a clonogenic assay (Fig. 3). A dominant-negative mutant of caspase-8, which inhibited apoptosis induced by the death receptors Fas/APO-1 and p55-TNF-R1 (3) (Fig. 4A), inhibited E4orf4-induced apoptosis as well (Fig. 4A and B). Furthermore, a dominant-negative mutant of the adapter protein FADD/MORT1, which blocks signal transduction from the death receptors to caspase-8, also inhibited apoptosis induced by E4orf4 (Fig. 4A and B). These results suggested that E4orf4 acted as an activator of the DISC. The mechanisms by which E4orf4 affects the DISC are currently unknown. Observations from our laboratory (A. Livne and T. Kleinberger, unpublished data) and from others (21) indicated that E4orf4 translocated to the cell membrane at an early stage of the apoptotic process, thus potentially facilitating a direct interaction with components of the DISC or with DISC modulators. Alternatively, E4orf4 may enhance expression of death receptors or their ligands, or it may enhance membrane trafficking of the receptors, as was shown for p53 in human vascular smooth muscle cells (2). We have previously reported that E4orf4-induced apoptosis required an interaction between E4orf4 and PP2A (35). Whether or not the E4orf4-PP2A complex affects the death receptor signaling pathway by one of the mechanisms suggested above, the presence of E4orf4 in multiple cellular compartments during the apoptotic process suggests that it might target more than one pathway, combining with different protein partners, to attain full induction of apoptosis.

Caspase-8 is an upstream caspase that activates downstream caspases either directly or by activating the mitochondrial death pathway, leading to cytochrome *c* release and activation of caspase-9 (reviewed in reference 4). Our results demonstrated that cytochrome *c* was released into the cytoplasm in E4orf4-transfected H1299 (Fig. 5) and 293T (not shown) cells. However, a dominant-negative caspase-9 mutant, which inhibited Bax-induced apoptosis, did not affect apoptosis induction by E4orf4 in 293T cells (Fig. 6B) or DNA degradation in H1299 cells (Fig. 6A). Furthermore, processing of overexpressed caspase-9 was not significantly enhanced by E4orf4 in these cells above background levels (Fig. 6C). It is not currently understood why caspase-9 was not activated in the E4orf4-expressing cells upon cytochrome *c* release from the mitochondria. However, it is possible that an activity of E4orf4 or its downstream effectors uncouples cytochrome *c* release from activation of caspase-9. For example, it has been reported that Akt (also known as protein kinase B) phosphorylates caspase-9 and inactivates it downstream of cytochrome *c* release (reviewed in reference 6). It is possible that E4orf4 expression results in enhanced caspase-9 phosphorylation. However, since E4orf4 associates with PP2A and activates it (19), this possibility is less likely. Alternatively, E4orf4 may inhibit caspase-9 activation as a result of its ability to influence alternative splicing. It was shown that Apaf-1 binds to cytochrome *c*, and upon dATP addition this complex oligomerizes and activates procaspase-9. Alternative splicing gives rise to various Apaf-1 isoforms, and only those forms that contain a C-terminal WD-40 repeat activate procaspase-9 (1). It has been shown that E4orf4 regulates alternative splicing of late adenoviral mRNAs (17). Thus, it is possible that E4orf4 influences Apaf-1 alternative splicing and reduces the prevalence of the WD-40 repeat-containing Apaf-1 molecules, thus inhibiting procaspase-9 activation.

How does caspase-8 activate apoptosis in E4orf4-expressing cells? We found that E4orf4 enhanced ROS levels in 293T cells (Fig. 7A and B) and inhibition of ROS accumulation by the antioxidants suppressed E4orf4-induced apoptosis (Fig. 7C and results not shown). These results indicate that generation of ROS is essential for E4orf4-induced apoptosis in these cells. Furthermore, dominant-negative caspase-8 and MORT1-DD, as well as the broad-range caspase inhibitor, zVAD-fmk, reduced the accumulation of ROS in E4orf4-expressing cells (Fig. 7B), indicating that ROS generation lies downstream of caspase-8 activation. The generation of ROS plays a role in the apoptotic process both in metazoans and in *Saccharomyces cerevisiae* (14, 16, 24, 37). Several reports suggested that oxidative stress and ROS generation were involved in death receptor-mediated apoptosis. ROS levels were enhanced during death receptor-mediated apoptosis, and antioxidants, such as NAC, prevented Fas-mediated apoptosis in various cell lines (12, 40), although not in others (33). It was further demonstrated that an inhibitor of flavonoid-containing oxidases, such as NADPH oxidase and nitric oxide synthase, prevented Fas-mediated apoptosis in B lymphoma cell lines (38). Thus, our results further support a model by which E4orf4 activates the death receptor pathway, resulting in enhanced ROS generation and subsequent cell death.

In summary, our results demonstrate that, in certain cellular contexts, E4orf4 engages the death receptor pathway to generate at least part of the molecular events required for E4orf4-induced apoptosis. However, additional caspase-independent processes are also initiated by E4orf4 and contribute to the death process.

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