E1A-induced Processing of Procaspase-8 Can Occur Independently of FADD and Is Inhibited by Bcl-2*

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Expression of the 243-residue form of the adenovirus E1A protein in the absence of other viral proteins triggers apoptosis by a pathway that requires p53. This pathway includes processing and activation of initiator procaspase-8, redistribution of cytochrome c, and activation of procaspase-3. Bcl-2 functions at or upstream of procaspase-8 processing to inhibit all of these events and prevent cell death. This contrasts with the anti-apoptotic influence of Bcl-2 family proteins in the cell death pathway induced by Fas ligand or tumor necrosis factor (TNF), in which Bcl-2 typically acts downstream of Fas/TNFFR1-mediated activation of caspase-8. Moreover, E1A induces procaspase-8 processing and cell death in cells deleted of FADD, an adaptor protein critical for Fas/TNFFR1 activation of caspase-8. The results indicate that E1A is capable of activating caspase-8 by a Bcl-2-inhibitable pathway that does not involve autocrine stimulation of FADD-dependent death receptor pathways.

Although there are notable examples of apoptotic cell deaths that occur in the apparent absence of induction of caspases (1–3), most death pathways in metazoans involve this family of cysteine proteases in the execution process (4–6). Execution begins with the recruitment of initiator procaspases such as procaspases-8, -9, and -10 into caspase activation complexes in response to a death signal. This in turn initiates downstream activation of effector caspases, resulting in apoptotic cell death. Among the best understood of the caspase activation complexes is the TNF1 family of death receptors located at the cell surface, prototypically represented by CD95/Fas/Apo-1 and TNFRI,

which recruit procaspases-8 and -10 (7). Here, the mechanism of receptor coupling to death signaling is clear. Binding of the cognate ligand to the receptor induces receptor oligomerization (8), recruitment of procaspases-8 and -10 via the adaptor molecule FADD/MORT1 (7, 9, 10), and activation of the associated procaspase probably by oligomerization-induced autocatalytic processing (11, 12). While the ligands are typically presented to the receptor from a heterotypic extracellular source (7), cell surface death receptors can also respond to autocrine stimulation by ligand produced and externalized from the same cell (13). This in turn raises the possibility that these death receptors can be coupled to a diverse repertoire of death signals. Recent evidence, for example, has implicated autocrine stimulation of Fas as an important contribution to cell death induced by Myc (14) and radiation (15).

In all cases examined to date, the Bcl-2 family of apoptosis suppressors function upstream of effector caspase-3 to inhibit cell death (16–18), by a process that includes inhibition of cytochrome c release from mitochondria (19, 20). This aborts downstream activation of the Apaf-1-procaspase-9 caspase activation complex, which requires cytochrome c as a constituent cofactor, and subsequent processing of procaspase-3 by caspase-9 (21–23). In contrast, the death inhibitory activity of Bcl-2 family suppressors in response to Fas ligand or TNF occurs downstream of receptor-activated caspase-8 in most instances (24–26), due in part to protection of mitochondria against the death stimulating influences of this initiator caspase (24, 27). Here, we have examined the contribution of the FADD-dependent death receptor and cytochrome c-Apaf-1-procaspase-9 caspase activation complexes to apoptosis induced by a model oncogene product in rodents, the 12 S E1A product of adenovirus type 5, and at what point Bcl-2 intercedes and blocks E1A-induced apoptosis.

Depending on the cellular and viral contexts, the products of early region 1A mRNAs (12 S E1A and 13 S E1A) of adenovirus stimulate DNA synthesis, cell proliferation, and cellular transformation or apoptosis (28, 29). In contrast to the longer product of 13 S E1A mRNA, the 243-residue product of 12 S E1A, which lacks conserved region 3 (CR3), is incapable of activating most other early viral units. Thus, virus that produces 12 S E1A and that lacks the E1B region, whose 19- and 55-kDa protein products are inhibitors of apoptosis, represents a viral vector that efficiently delivers 12 S E1A as the main vector expression product. This product is a potent inducer of apoptosis in a wide variety of human and rodent cells, for which it exhibits an absolute dependence on functional p53 tumor suppressor protein (30, 31). Bcl-2 or E1B 19K protects cells against E1A (32) by inhibiting caspase activation (16, 33). Virus that expresses both 12 S and 13 S spliceoforms of E1A, and lacks E1B, induces additional apoptotic pathways as a result of expression of other viral proteins, e.g. the adenoviral death factor, E4orf4, which triggers p53- and caspase-independent apoptosis (3, 41). In this report, we demonstrate that 12 S E1A induces apoptosis by a pathway that does not require autocrine stimulation of FADD-dependent cell surface death receptors. In contrast to the caspase cascade activated by these cell surface receptors in response to ligand, Bcl-2 acts upstream rather than downstream of procaspase-8 in the E1A pathway.

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1 The abbreviations used are: TNF, tumor necrosis factor; DN, dominant-negative.

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EXPERIMENTAL PROCEDURES

General—KB human epithelial cells stably expressing Bcl-2 have been described previously (34). Those stably expressing dominant-negative C287A procaspase-9 (35) were created by co-expression with a neomycin resistance gene, selection in G418, and screening with anti-T7 to detect the epitope-tagged protein. Infection with adenovirus type 5 $dI_52OE1B^2$ (expressing only 12 S E1A and no E1B products) (36) was performed as described (16, 37) and cell viability determined by trypan blue exclusion. Immunoblotting and microscopic immunofluorescence was conducted as described previously (3, 37). Additional details are provided in the figure legends.

FADD-null Mouse Embryo Fibroblasts—Primary embryo fibroblasts were prepared from 9.5-day-old embryos of mice carrying a homozygous deletion of the entire coding region of $FADD$ (38). They were cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum.

RESULTS

Ad5 $dI_52OE1B^-$ is an adenovirus type 5 vector that produces the 243-amino acid product of 12 S E1A mRNA as the major vector expression product (36). As documented previously (37), 243R E1A triggers apoptosis in KB human epithelial cells by a pathway that is inhibited by Bcl-2. This pathway includes activation of initiator procaspase-8 and cleavage of the caspase-8 target, Bap31 (Fig. 1). Bap31 is a 28-kDa polytopic integral protein of the endoplasmic reticulum that functions as a predicted regulator of apoptosis. Following induction of apoptosis, it is cleaved within its cytosolic tail by caspase-8, generating a 20-kDa membrane-integrated fragment (p20 Bap31) (37). As shown in Fig. 1, Bcl-2 inhibits processing of procaspase-8 to the p18 catalytic subunit induced by 12 S E1A and blocks the appearance of the p20 Bap31 product. 12 S E1A expression results in a dramatic but transient increase in p53 levels (30, 31, 39); however, this is not influenced by Bcl-2 (not shown), indicating that Bcl-2 acts downstream of this event on the E1A pathway.

Immunostaining of KB cells with antibody against cytochrome c revealed a redistribution of this mitochondrial protein throughout the cell in response to E1A expression. This began within 24 h (Fig. 2), at a time when most cells retained an intact plasma membrane, as judged by exclusion of trypan blue (Fig. 1). In contrast, Bcl-2 inhibited these E1A-induced events. Even after 48 h of E1A expression in the presence of Bcl-2, cells remained normal and retained cytochrome c in a punctate mitochondrial pattern, whereas control cells were obviously apoptotic (Fig. 2).

To examine if cytochrome c redistribution in response to E1A expression plays a direct role in the E1A apoptotic pathway, or results indirectly as a consequence of apoptosis induced by a parallel pathway, we examined the requirement for cytochrome c’s immediate downstream target, procaspase-9.

Processing of procaspase-9 occurs by oligomerization-induced autocatalytic activation (35) following its recruitment into the Apaf-1-cytochrome c caspase activation complex in the cytosol (23, 35). As a consequence, co-expression of catalytically inactive proenzyme exerts a dominant-negative (DN) influence on pro-
caspase-9 processing. To that end, KB cells were created that stably express procaspase-9 DN-C287A (35) at levels approximately three times that of the endogenous wild type proenzyme. As shown in Fig. 3, the procaspase-9 DN significantly retarded the death of these cells in response to E1A.

Finally, we examined the contribution of FADD-dependent cell surface death receptors to the E1A apoptotic pathway. Mice harboring a homozygous deletion of the FADD gene have been created recently, from which embryonic fibroblasts can be obtained (38). In contrast to fibroblasts taken from FADD+/+ mice, FADD-null fibroblasts were resistant to cell death induced by TNFα, whose receptor depends on FADD for activation of caspase-8. However, both FADD+/+ and FADD−/− cells exhibited equivalent sensitivity to death signaling by adenovirus lacking the E1B region (38). Fig. 4 extends these findings and shows that 12 S E1A expression in the FADD-null cells resulted in processing of procaspase-8 and cell killing. As demonstrated previously (38), these cells were resistant to killing by TNFα in the presence of the protein synthesis inhibitor, cycloheximide, which inhibits TNFα-induced survival pathways (Fig. 4).

DISCUSSION

E1A proteins are transcriptional regulators whose influence on cell proliferation depends on discrete regions within the molecule, which include conserved regions 1 and 2 (CR1 and CR2), together with a less well conserved amino terminus. These regions are common to the two forms of the protein (derived from 12 S and 13 S E1A mRNA) and function by binding to a family of proteins related to retinoblastoma protein. This releases the otherwise Rb-sequestered E2F group of transcription factors that turn on genes that stimulate cell growth. Additionally, CR1 and the amino terminus bind the p300/CREB family of transcription factor co-activators, resulting in repression of various genes contributing to terminal cellular differentiation (28, 29). How these responses of the cell to E1A expression are tied to the apoptosis-inducing properties of E1A is not presently known, but one possibility may involve regulation of gene products in concert with p53 that influence one or more of the caspase activation complexes of the cell (33). In the case of apoptosis induced by coupling Myc expression with survival factor withdrawal, for example, it has been suggested that cell death involves Myc-induced autocrine stimulation of the cell surface death receptor, CD95/Fas/Apo-1 (14). The Fas-dependent caspase activation complex, or DISC (death-inducing signaling complex), is coupled to activation of caspase-8 via the adaptor molecule, FADD (7). However, Myc is cytotoxic in mouse cells devoid of FADD (38), suggesting that other death pathways are influenced by Myc, including a pathway that does not involve caspases (2). Here, we show that E1A not only is capable of inducing cytotoxicity in the absence of FADD but that this is accompanied by activation of procaspase-8, indicating that this initiator procaspase is capable of being processed by a FADD-independent mechanism.

One possibility is that processing of procaspase-8 is an end or by-product of Fas signaling via the adaptor protein, Daxx, and the JNK kinase cascade (43), although such a link has not yet been described. Another is that processing of procaspase-8 is a by-product of activation of effector caspase-3 via the Apaf-1-cytochrome c-procaspase-9 caspase activation complex (23, 35). E1A induces release of cytochrome c from mitochondria (Fig. 2) and activation of caspase-3 (16, 33). Moreover, our finding that a dominant-negative mutant of procaspase-9 retards cell death in response to E1A (Fig. 3) indicates that this caspase activation complex contributes directly to the E1A-induced apoptotic pathway. A third possibility is that procaspase-8 is activated by a separate FADD-independent caspase activation complex and that caspase-3 is activated either as a consequence of cytochrome c release from mitochondria induced by caspase-8 (27) or by a parallel pathway. One candidate is the Bap31 complex in the endoplasmic reticulum, a Bcl-2/Bcl-XL-associated protein that can recruit procaspase-8 and a Ced-4-like adaptor (37, 40). Bap31 is also a target of caspase-8 or related caspase during apoptosis induced by E1A, generating a proapoptotic fragment of the protein (37). Importantly, however, we demonstrate here that Bcl-2 acts upstream to prevent E1A-induced activation of caspase-8 and cell death, which differs from the Fas/TNFRI pathway in which Bcl-2 suppressors typically function downstream of the active enzyme (24–26), if enzyme activation occurs at the level of the receptor (44). This ability of Bcl-2 to block activation of both initiator and effector caspases in response to signaling by an oncogene like E1A likely contributes to the sustained suppression of apoptosis that is required to support manifestation of the cellular transforming properties of these otherwise death-inducing oncogenes.

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FIG. 3. Expression of procaspase-9 dominant-negative mutant retards cell death induced by E1A. Human KB cells were created that stably express the neomycin resistance gene with or without co-expression of mutant procaspase-9 carrying an alanine substitution for the catalytic cysteine residue (proc-9 DN). Following infection with adenovirus type 5 d152OE1B− (expressing only 12 S E1A and no E1B product) for the indicated times, the cells were assessed for viability by trypan blue exclusion (% Viable Cells).

FIG. 4. E1A, but not TNFα, induces procaspase-8 processing and cell death in FADD-null cells. Mouse embryonic fibroblasts that do not express FADD due to targeted disruption of the FADD gene (38) were infected with adenovirus type 5 d152OE1B− (200 plaque-forming units/cell) for the indicated periods of time, or they were treated with mouse TNFα (10 ng/ml) plus the indicated concentrations of cycloheximide for a period of 12 h. Cells were analyzed for viability by trypan blue exclusion (% Viable Cells) and for the presence of p18 caspase-8 catalytic subunit, as described in the legend to Fig. 1. Immunoblot signals for p18 caspase-8 were quantified using a Power Macintosh 7200/120 and NIH Image v1.61 image analysis software and the maximum assigned a value of 100.
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