Adenovirus E1B 19-kDa Death Suppressor Protein Interacts with Bax but Not with Bad*

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Adenovirus E1B 19-kDa protein (19K) is a member of the Bcl-2 family of suppressors of apoptosis. The suppressors function through heterodimerization with the death promoters, Bax and related proteins, thus establishing a set point within the cell that determines whether or not apoptosis is executed in response to a death signal. Sequence similarities between 19K and Bcl-2 are largely restricted to short Bcl-2 homology (BH) domains that mediate interaction with Bax. The BH1 sequence in 19K is degenerate but nevertheless contains a conserved glycine residue found in all family members that when mutated to alanine in Bcl-2 results in loss of Bcl-2 function and ability to dimerize with Bax (Yin, X.-M., Oltvai, Z. N., and Korsmeyer, S. J. (1994) Nature 369, 321–323). Here, we show that the analogous mutation in BH1 of 19K also abrogates the anti-apoptotic properties of 19K and its ability to interact with Bax, thus establishing the critical importance of this residue within BH1 and the likely similarity of Bcl-2 and 19K function. In distinct contrast to Bcl-2, however, 19K interaction was not detected with Bad, a Bcl-2/Bcl-XL dimerizing protein that can potentially regulate a Bax/Bcl-2/Bcl-XL survival set point and reinitiate susceptibility to a death signal. Furthermore, the anti-apoptotic function of 19K was not overcome by enforced expression of Bad in transfected cells. This feature of 19K may provide adenovirus with a selective advantage in evading premature induction of apoptosis by the host cell.

Apoptotic cell death is essential for tissue modeling and homeostasis in metazoans and provides the organism with the ability to selectively remove cells that are targets of various types of growth deregulating events (1, 2). Examples of such events include activation of oncogene expression and viral infection (3). Although the demise of the cell is initially triggered upon receipt of a specific death signal, execution of the apoptotic pathway occurs only upon activation of critical Ced-3/ICE cysteine proteases, which cleave and inactivate/activate specific target molecules (4–6). Whether or not the cell contains BH domains, is a direct regulator of the set point (15). It heterodimerizes with Bcl-2/Bcl-XL, but does not interact with itself or with Bax. Thus, it has the potential to readjust a Bax/Bcl-2/Bcl-XL set point that would otherwise specify cell survival following a death signal to one that specifies apoptosis (15). Although it is not presently known how the set point controls whether or not the cell will engage the apoptotic program, recent studies have shown that Bcl-2/Bcl-XL promotes either a subcellular cleavage process of proc-CPP32 apoptin/Yama (16, 17), a Ced-3/ICE protease that may be responsible for initiating the cascade of events that ultimately leads to the final execution of apoptosis (18–20). In this regard, the position of the set point within the apoptotic pathway differs from that of the viral apoptotic inhibitors, cowpox CrmA (4, 5) and Baculovirus p35 (21), which function as direct active site inhibitors of various Ced-3/ICE proteases. However, it is similar to that of E1B 19-kDa protein (16), an adenovirus suppressor of apoptosis that is functionally interchangeable with Bcl-2 in several different contexts (22–25).

Adenovirus has provided an important model system for studies of the relationship between cellular transformation and apoptosis, because the viral gene that induces host cell DNA synthesis and immortalization, E1A, is also a potent inducer of apoptosis (22). To permit the transforming properties of E1A to be manifested, adenovirus encodes two negative regulators of apoptosis: E1B 55-kDa protein, which binds p53 and impairs its ability to regulate gene expression (26, 36) and mediate E1A-induced apoptosis (27, 28), and E1B 19-kDa protein, a general suppressor of both p53-dependent and -independent cell death (27–30). Despite the functional similarities between Bcl-2 and E1B 19K, the two proteins are quite different over most of their respective polypeptide sequences, with the exception of regions that are related to BH1 and BH3 (31, 32). These regions in 19K, however, mediate interaction with Bax (31, 32), suggesting that Bax19K establishes an apoptotic control set point that is analogous to that of Bax/Bcl-2/Bcl-XL. Consistent

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1 The abbreviations used are: BH, Bcl-2 homology; 19K, 19-kDa protein encoded by adenovirus serotype 5 early region 1B; HA, influenza virus hemagglutinin epitope; PAGE, polyacrylamide gel electrophoresis.
with this thesis, enforced expression of either Bax or Bak overcomes the death protective effect provided by 19K (12, 31, 32). In the case of Bcl-2, a relatively conservative mutation within the BH1 domain, which changes glycine at position 145 to alanine, abrogates the death suppressor function and ability of Bcl-2 to interact with Bax (14). Although the corresponding 19K BH1 domain is relatively degenerate compared with other family members, it contains the analogous glycine. We show here that mutation of this glycine to alanine has the same detrimental effect on 19K, which implicates this highly conserved amino acid position within all known BH1 domains as critically important for function. Of note, however, we also find that in distinct contrast to Bcl-2 and Bcl-XL, interaction of 19K with Bad was not detected. This has particular significance because it suggests that the polypeptide sequence of 19K has evolved to avoid negative regulation by Bad, a feature that may provide adenovirus with a selective advantage in evading premature killing of the host cell.

MATERIALS AND METHODS

**Cells and Expression Vectors**—Standard recombinant polymerase chain reaction methodology was employed to insert oligonucleotides encoding the HA epitope, (M)AYPDYVPDYAV, at the 3′-end of the E1B 19K or BCL-2 cDNA (25, 30). cDNAs encoding human Bcl-2 (7) or HA Bcl-2 (25), adenovirus serotype 5 HA 19K (30), mouse Bax (10), or mouse Bad (15) were cloned into the Rc/RSV vector (Pharmacia Biotech Inc.) carrying a neomycin resistance gene. The authenticity of all constructs was verified by DNA sequencing. The vectors were transfected into CHO LR73 cells, as described previously (30), and stable transfectants obtained by selection in G418 or hygromycin B and identified by Western blot analysis. Cell lines expressing various levels of the individual proteins were isolated. To establish co-expressing lines, cellular clones that had been selected in one drug were subsequently transfected and selected in the other drug and characterized by Western blotting.

**Cell Viability Assays**—Cells were grown to ~80% confluency in α-minimum Eagle’s medium supplemented with 10% fetal calf serum and 100 units/ml penicillin and streptomycin. Puromycin (50 μg/ml) or staurosporin (6.0 μM) were added, total cells subsequently were collected at various times, and the percentage of the cells that excluded trypan blue was determined in two independent experiments, and the results were averaged as described (25, 30). All experiments were conducted 3–5 times and yielded results very similar to those presented.

**Western Blotting**—Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with P-9 rabbit polyclonal antibody against Bax, 12CA5 mouse monoclonal anti-HA, 6C8 hamster monoclonal anti-Bcl-2, rabbit polyclonal anti-19K, or rabbit polyclonal sc-943 anti-Bad. Following incubation with secondary antibody conjugated to alkaline phosphatase, color was developed with NBT/BCIP (Boehringer Mannheim) according to the manufacturer’s instructions.

**Co-immunoprecipitation**—Cell lines containing equivalent amounts of HA-Bcl-2, HA-19K, or neither protein (neo controls) were grown to ~80% confluency, collected following trypsinization, and washed, and the cells were homogenized at 4°C in medium containing 2% (w/v) Nonidet P-40, 140 mM KCl, 5 mM MgCl2, 1 mM ethylene glycol-bis-(β-aminoethylether)N,N,N′,N′-tetraacetate, 0.2 mM phenylmethylsulfonyl fluoride, 1.0 μg/ml leupeptin, and 10 μM Heps, pH 7.2. Following centrifugation at 12,000 × g for 15 min, supernatants were mixed with an equal volume of reticulocyte lysate containing [35S]Bax or [35S]Bad transcription-translation product (34), gradually diluted with 4.5 volumes of homogenization medium lacking detergent, and incubated for 1 h at 4°C prior to addition of 12CA5 anti-HA. Immune complexes were recovered with protein A-Sepharose and analyzed by SDS-PAGE and fluorography.

**RESULTS AND DISCUSSION**

Because Bcl-2 functions as a heterodimerizing partner with Bax (10), the ratio of the two proteins, rather than absolute levels, is an important determinant of the final outcome for a cell that receives a death signal (1, 10). This is also true for other Bax-interacting members of the Bcl-2 family (15, 31, 32). To study the ability of enforced expression of Bax to overcome the activity of a death suppressor, therefore, it is important to compare these co-expressing cells with ones that express the same level of the death suppressor in the absence of Bax. To that end, stable CHO LR73 cell lines were established that express equivalent levels of 19K plus or minus Bax (Fig. 1, upper panel, lanes 1 and 2) or equivalent levels of Bcl-2 plus or minus Bax (Fig. 1, upper panel, lanes 4 and 5), as judged by Western blot analysis. Cell lines were also generated that contain Bax alone, at levels that are equivalent to the level of Bax in the co-expressing cell lines (Fig. 1, lower panel, compare lane 2 with lane 3 and lane 5 with lane 6). A number of such cell lines were created and each gave relative responses to death signals that were comparable with those described below.

**Elevation of Bax Overcomes the Death Suppressor Effects of Both Bcl-2 and E1B 19K**—The stable cell lines that were analyzed in Fig. 1 by Western blotting were also examined for their susceptibility to a potent inducer of apoptotic cell death, puromycin (35). In common with other systems (10), enforced expression of BAX alone had only a slight enhancing effect on the rate of cell death in response to the drug relative to neo control cells but overcame the otherwise protective effect against the drug that was conferred by Bcl-2 (Fig. 2, upper graph). Whereas cells expressing neo, either alone or together with BAX, were
Cells were treated with 50 μg/ml puromycin, and at the indicated times following the addition of drug, cells were collected, and the percentage of viability determined by exclusion of Trypan Blue.

Almost all killed by 48 h after receiving 50 μg/ml of the drug, approximately 85% of the cells that express BCL-2 remained viable during this period. Co-expression of BAX with BCL-2, however, reduced the cell survival level to 30–35% at 48 h after receiving puromycin, and this was reduced to the level of neo controls by 72 h. A very similar pattern and extent of protection against puromycin and abrogation of this protection by Bax were also observed for E1B 19K (Fig. 2, lower panel).

A Degenerate BH1 Domain Contributes to the Anti-apoptotic Function of E1B 19K and Ability to Interact with Bax—Fig. 3 shows the sequences of BH1 domains in various Bcl-2 family members (from Ref. 1) and aligns these regions to a sequence in adenovirus serotype 5 E1B 19K (37) from amino acid 85 to 96 that shows similarity to the BH1 domains in these other proteins. The similarity, however, is relatively weak and in fact is less than that observed for the evolutionarily distant Bcl-2 homolog in Caenorhabditis elegans, Ced-9 (14, 33) (Fig. 3). Nevertheless, this predicted BH1 domain of 19K contains the highly conserved GR, which is present in all family members and whose glycine moiety is important for function. A relatively conservative change of this glycine to alanine at residue 145 of Bcl-2 results in loss of both Bcl-2 function and its ability to heterodimerize with Bax (14). Therefore, to determine if the predicted BH1 domain of 19K is of functional significance, the same mutation was introduced at residue 87 of 19K (designated 19K G87A) (Fig. 3). Several cell lines were created in CHO LR73 that express various levels of the mutant 19K protein, and in all cases, the G87A mutation was found to result in loss of 19K protection against puromycin treatment compared with an equivalent level of wild-type 19K. An example of such an analysis is shown in Fig. 4A, in which the level of HA 19K G87A was similar to or even greater than wild-type HA 19K as judged by immunoblotting with anti-HA antibody (Fig. 4A, inset). The 19K wild-type and G87A cell lines described in Fig. 4 were also examined for their susceptibility to staurosporin, a protein kinase inhibitor that induces apoptosis in many cell types (38, 39). Again, 19K G87A exhibited a significant loss in the ability to prevent cell death compared with wild-type 19K (Fig. 5). Consistent with the loss of death suppressing activity resulting from the G87A mutation of 19K, the mutant 19K in these cells exhibited a corresponding reduction in its ability to interact with Bax, relative to equivalent levels of wild-type 19K, in a co-immunoprecipitation assay in vitro (Fig. 4B, compare lanes 3 and 4).

Bcl-2, but Not E1B 19K, Interacts with Bad—Bad competes with Bax for heterodimerization with Bcl-2 or Bcl-XL and therefore has the potential to adjust a survival control set point to one that permits apoptosis to occur (15). In co-expressing cells, Bad overcomes the protective effects of Bcl-XL (15). However, despite repeated attempts, we have found that enforced expression of Bad does not overcome the death suppressor activity of E1B 19K (not shown). In distinct contrast to Bcl-XL and Bcl-2, interaction between 19K and Bad was not observed in a co-immunoprecipitation assay that readily detected interaction between Bcl-2 and Bad (Fig. 6, right panel, compare lanes 3 and 4) and interaction between 19K and Bax (Fig. 4B). In Fig. 6, cell extracts were employed that contained equivalent levels of HA Bcl-2 and HA 19K, as judged by Western blot analysis with 12CA5 anti-HA (Fig. 6, left panel). Similar results were obtained using different cell lines and cell types (CHO LR73 and human KB). The findings strongly suggest, therefore, that E1B 19K is not susceptible to regulation by Bad.

Conclusions—The early events of adenovirus infection require that the cytotoxic consequences of E1A expression are countered by negative regulators of apoptosis. To ensure this outcome, the virus encodes its own general suppressor of apoptosis, E1B 19K. The major role of 19K during viral infec-
tion is to block p53-dependent apoptosis induced by E1A (22, 27–29) and p53-independent cell death that would otherwise be induced by an E4 viral gene product that is regulated by E1A (29). Based on evidence presented here and elsewhere (31, 32), 19K is both a structural and functional homolog of Bel-2. Moreover, like Bel-2, 19K appears to function within a signaling pathway that prevents processing and activation of pro-CPP32 in response to a death stimulus (16). Because similarities between the two proteins are restricted to limited regions within the molecules, including BH1 and BH3 (31, 32), comparisons between the two proteins provide an opportunity to correlate function with domain structure. Regions of sequence conservation and divergence within BH1 of Bel-2 and 19K, for example, coupled with the deleterious mutation of the common glycine within this motif, help to define the essential features of this domain. Of particular relevance, however, we did not detect interaction between 19K and Bad, under conditions where 19K-Bax and Bel-2-Bad interactions are readily observed. This has obvious evolutionary implications for the ability of the virus to evade potential host defense mechanisms, because it permits 19K to target Bax while at the same time avoiding disruption of this interaction and re-instatement of the apoptotic program by Bad.

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FIG. 4. Mutation of glycine 87 inhibits E1B 19K function and ability to interact with Bax. A, standard polymerase chain reaction methodology was employed to convert codon 87 in the protein coding sequence of Bc/RSV HA 19K from glycine (GGG) to alanine (GCG). Stable CHO LR73 cell lines were then obtained that express HA 19K or HA 19K G87A (lane 2), or with wild type (lane 3), or with truncated HA 19K (lane 4). Following dilution of extracts to a final concentration of 0.1% detergent, 12CA5 anti-HA was added. Immunoblotting was conducted as described in Fig. 4B, except that [35S]Bax (lane 1, 2.5% of input) was added to cell extracts containing equivalent amounts of HA Bcl-2 (lane 3), or HA 19K (lane 4), or containing neither protein (control lane 2). The position of [35S]Bax is indicated (arrow). Left, Western blot of total cell extracts from HA 19K (lane 1) and HA Bcl-2 (lane 2) cells containing equivalent amounts of protein and visualized with anti-HA antibody (see “Materials and Methods”). B, [35S]Bax was produced by transcription-translation (lane 1) in a rabbit reticulocyte lysate, and equal volumes were added to extracts from the cell lines assayed in A. Extracts were prepared in 2% Nonidet P-40 and contained equivalent amounts of HA 19K (lane 3) or HA 19K G87A (lane 4), or they contained neither protein (control, lane 2). Following dilution of extracts to a final concentration of 0.1% detergent, 12CA5 anti-HA was added. Immunocomplexes were collected, resolved by SDS-PAGE, and analyzed by autoradiography. The position of [35S]Bax is indicated (arrow).

FIG. 5. Mutation of glycine 87 inhibits the ability of E1B 19K to counter staurosorin-induced cell death. The cell lines analyzed in Fig. 4A were also examined following treatment with 6.0 μM staurosorin, as indicated. Methodology and symbols are as described in the legend to Fig. 4A.
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