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TNF-α Signals Apoptosis through a Bid-Dependent Conformational Change in Bax that Is Inhibited by E1B 19K

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Summary

The adenovirus E1B 19K gene product is an inhibitor of apoptosis induced by tumor necrosis factor-α (TNF-α) during viral infection. We report that E1B 19K inhibited neither caspase-8 activation nor caspase-8-dependent Bid cleavage by TNF-α. Rather, TNF-α induced a tBid-dependent conformational change in Bax that allowed an interaction between E1B 19K and conformationally altered Bax, which caused inhibition of cytochrome c release and caspase-9 activation. E1B 19K expression interrupted caspase-3 processing, permitting cleavage to remove the p12 subunit but not the prodomain consistent with caspase-8 and not caspase-9 enzymatic activity. Thus, E1B 19K blocks TNF-α-mediated death signaling by inhibiting a specific form of Bax that interrupts caspase activation downstream of caspase-8 and upstream of caspase-9.

Introduction

TNF-α is a proinflammatory cytokine that triggers receptor-mediated apoptosis in response to viral infection (Wold et al., 1999). TNF-α acts as a ligand for two ubiquitously expressed receptors, TNFR-1 and TNFR-2, which belong to a family of death receptors that include Fas and TRAIL receptors that function to induce apoptosis. Viruses have evolved mechanisms for evading this host immune surveillance to ensure efficient viral replication, propagation, and persistent infection.

TNFR-1 signals apoptosis through recruitment of the adaptor proteins TRADD and FADD that facilitate multimerization, autocatalytic cleavage, and activation of caspase-8 (Nagata, 1997). One known functional target of caspase-8 is the proapoptotic Bcl-2 family member Bid. Cytosolic Bid is cleaved by caspase-8 at the amino terminus to generate a truncated form of Bid (tBid) that translocates to and mediates cytochrome c release from mitochondria (Li et al., 1998; Luo et al., 1998). Cytochrome c acts as a cofactor for Apaf-1-dependent caspase-9 activation, which serves as an amplification signal by activating downstream effector caspases through cleavage, including caspase-3 (Li et al., 1997; Wolf and Green, 1999). Pro-caspase-3 can also be directly cleaved by caspase-8 in a mitochondria-independent manner (Stennicke et al., 1998). In turn, effector caspases are responsible for cleaving cellular proteins such as poly (ADP-ribose) polymerase (PARP) and lamin, which implement cell death.

The adenovirus encodes genes within E3 and E1B that function to block TNF-α- and Fas-mediated apoptosis as a mechanism to counteract this antiviral host response (Wold et al., 1999). The E3-10.4 K/14.5 K (RID) gene products internalize and promote the degradation of Fas and TRAIL receptors (Wold et al., 1999). E3 14.7 K may prevent apoptosis by binding to pro-caspase-8 and other cellular mediators of death receptor–induced apoptosis (Wold et al., 1999). E1B encodes a p53 inhibitor (E1B 55K) and a Bcl-2 homolog (E1B 19K) to block apoptosis by other mechanisms (White, 1998).

Utilizing an adenovirus with a deletion in E3 (Ad5*dl*309), it was revealed that E1B 19K also blocks TNF-α-mediated cytolysis and DNA fragmentation during viral infection of many human cell lines (Gooding et al., 1991; White et al., 1992). Unlike the E3 RID complex, E1B 19K did not cause internalization of TNF-α receptors on the cell surface (White et al., 1992) and was also capable of blocking death signaling by Fas and TRAIL (Wold et al., 1999). Furthermore, transient or stable expression of the *E1B 19K* gene was sufficient to confer resistance of human cells to apoptosis induced by TNF-α (or TNF-α plus cycloheximide to block the NF-κB-mediated survival pathway induced by TNF-α) (White et al., 1992; Chiou et al., 1994). Thus, the 19K protein functions downstream at a point in the signaling pathway common to many, if not all, death receptors.

In addition to inhibiting apoptosis by TNF-α, E1B 19K also functions to block apoptosis induced by other apoptotic stimuli. The adenovirus E1A gene deregulates the cell cycle by driving cells into S phase to facilitate viral DNA replication. In response, the host cell activates apoptosis that must be inhibited by E1B 19K to prevent premature death of the infected host cell, which can impair virus production (White, 1998). E1A induces apoptosis by several mechanisms, one of which is mediated by the stabilization and accumulation of p53 (White, 1998). Likewise, p53 has multiple mechanisms for inducing apoptosis, one of which is through transactivation of the gene encoding the proapoptotic protein Bax (Miyashita and Reed, 1995).

Bax is a member of a subset of the Bcl-2 family that includes Bak, Bid, and NBK/BIK and that possesses a conserved Bcl-2 homology region (BH3) (Gross et al., 1999). BH3 is essential for killing and for mediating an interaction with death antagonists (i.e., Bcl-2-like proteins). Bax interferes with mitochondrial function to activate downstream effector caspases and apoptosis (Gross et al., 1999).

E1B 19K is functionally homologous to other antiapoptotic Bcl-2 family members. 19K interacts with and inhibits Bax-induced apoptosis. It also binds to and inhibits other BH3-containing proteins, such as Bak and NBK/BIK (White, 1998). However, the relation of the binding activities to the inhibition of apoptosis by death receptors was unknown.

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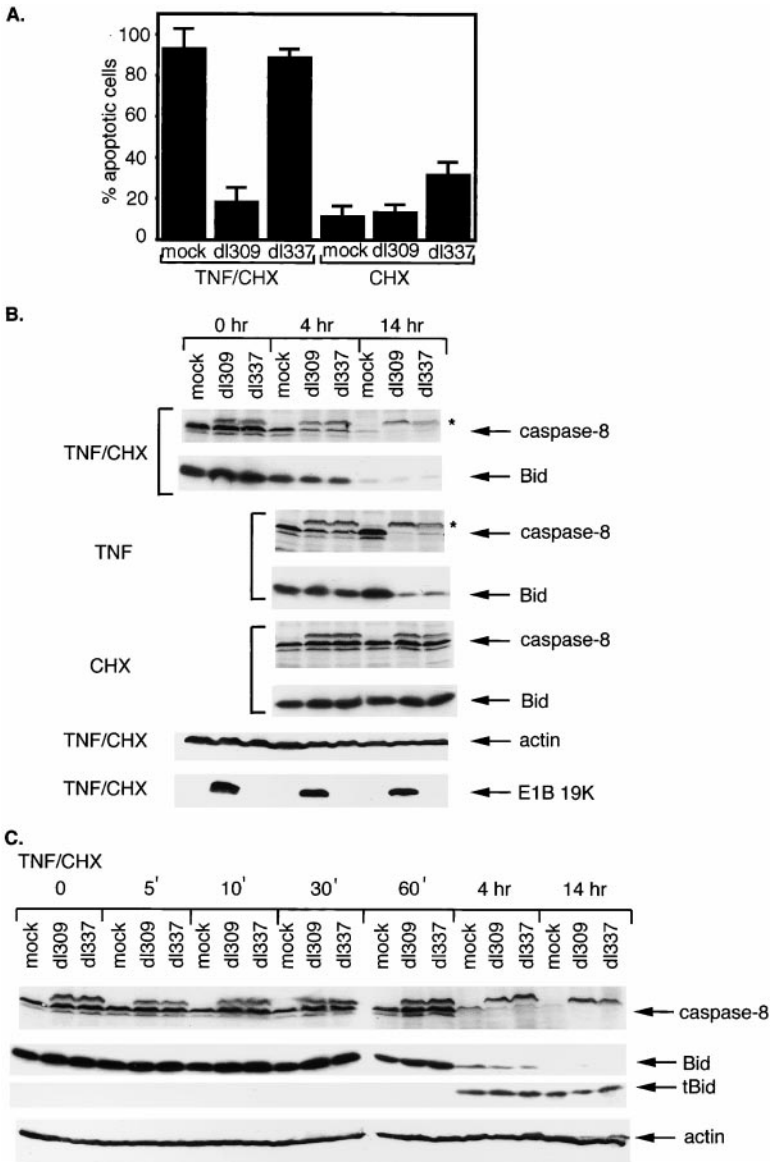

Figure 1. E1B 19K Inhibits Cell Death but Does Not Block Caspase-8 Activation or Bid Cleavage

(A) HeLa cells were mock-, Ad5*dl*309-, or Ad5*dl*337 infected for 24 hr and treated with TNF/CHX or CHX alone for 14 hr. Cells were harvested, and viability was assessed by trypan blue exclusion assay.

(B) Whole-cell extracts from mock-, Ad5*dl*309-, or Ad5*dl*337-infected HeLa cells (24 hr) that were treated with TNF/CHX, TNF-α alone, or CHX alone for 0, 4, and 14 hr. Extracts were analyzed by immunoblotting with an anti-caspase-8, anti-Bid (recognizes only Bid), anti-E1B 19K, or anti-actin antibodies to confirm equal protein levels. (Asterisk) The band above caspase-8 in the infected samples represents a nonspecific cross-reactivity with a viral protein.

(C) E1B 19K does not attentuate caspase-8 processing or Bid cleavage. Whole-cell extracts were prepared at 5, 10, 30, and 60 min, and 4 and 14 hr post-TNF/CHX treatment from mock-, Ad5*dl*309-, and Ad5*dl*337-infected cells as stated above. Extracts were analyzed by immunoblotting with an anti-caspase-8, anti-Bid (recognizes Bid and tBid), or anti-actin antibodies.

We report here that E1B 19K expression during adenovirus infection does not prevent TNF-α from stimulating caspase-8-dependent Bid cleavage. TNF-α also induced a tBid–Bax interaction, a conformational change in Bax, and binding between E1B 19K and Bax. Cytochrome c release and caspase-9 activation were inhibited, resulting in incomplete processing and activation of pro-caspase-3. Thus, through an in vivo analysis of TNF-α signaling, we have determined a mechanism by which E1B 19K abrogates TNF-α-mediated apoptosis during adenovirus infection.

Results

E1B 19K Inhibits TNF-α-Mediated Apoptosis during Adenovirus Infection Downstream of Caspase-8 Activation and Bid Cleavage

HeLa cells were either mock infected or infected with wild-type Ad5*dl*309 or an E1B 19K deletion mutant virus, Ad5*dl*337, and treated with TNF-α and a protein synthesis inhibitor cycloheximide (TNF/CHX), and cell viability was determined. Approximately 90% of the cells underwent apoptosis in mock- and Ad5*dl*337-infected cells, whereas only 18% of the wild-type adenovirus-infected cells were not viable (Figure 1A). Apoptosis was not due to inhibition of protein synthesis, since CHX alone did not induce cell death in mock- and Ad5*dl*309-infected cells. Approximately 30% of the Ad5*dl*337-infected cells were apoptotic, since adenovirus infection induces apoptosis in the absence of E1B 19K expression (White, 1998).

To evaluate where apoptosis was inhibited, HeLa cells were either mock infected or infected with Ad5*dl*309 or Ad5*dl*337, treated with TNF/CHX, TNF-α, or CHX, and caspase-8 activation was then examined. Reduced levels of pro-caspase-8 are indicative of autocatalytic activation due to proteolytic self-processing (Nagata, 1997). Western analysis with an anti-caspase-8 monoclonal

(Figure 1B) and polyclonal antibodies (data not shown) revealed that the proform of caspase-8 was partially processed at 4 hr, and by 14 hr of TNF/CHX treatment almost no pro-caspase-8 was detectable in all samples. The band above caspase-8 in the virus-infected cells was most likely the result of cross-reactivity of the antibody with a viral protein. The disappearance of caspase-8 was not due to inhibition of protein synthesis, since cells treated with CHX alone showed no effect on caspase-8 levels (Figure 1B). These results suggested that E1B 19K did not block caspase-8 activation and thus must inhibit further downstream in the signaling pathway.

Interestingly, both Ad5*dl*309- and Ad5*dl*337-infected cells treated with TNF-α alone showed caspase-8 processing (Figure 1B). This is likely due to E1A, which sensitizes cells to TNF-α, obviating the need for protein synthesis inhibitors for cell killing (Wold, 1999). 19K expression during infection also did not block this mechanism of E1A sensitization to TNF-α (Figure 1B).

To investigate the next signaling event downstream, Bid, a direct substrate of caspase-8, was analyzed. Bid is cleaved by caspase-8 near the amino terminus, which generates truncated tBid that translocates to the mitochondria (Li et al., 1998; Luo et al., 1998). Western analysis with a Bid monoclonal antibody that recognizes full-length Bid revealed that Bid levels were reduced at 4 hr and were negligible by 14 hr of TNF/CHX treatment, indicative of Bid cleavage in all samples (Figure 1B). The failure of the E1B 19K protein to inhibit caspase-8-dependent Bid cleavage was not due to a decrease in 19K expression during TNF/CHX treatment, nor was Bid disappearance due to inhibition of protein synthesis (Figure 1B). These results are consistent with caspase-8 activation and Bid processing in both 19K-expressing and null adenovirus-infected cells.

Adenovirus infection caused reduced Bid levels compared to mock-infected cells when treated with TNF-α alone (Figure 1B). As in the case of caspase-8, E1A may sensitize cells to TNF-α by permitting Bid cleavage that is independent of E1B 19K function.

Since E1B 19K expression during adenovirus infection did not alter caspase-8 or Bid processing by TNF-α at either 4 or 14 hr after treatment, we wanted to examine whether E1B 19K attenuated caspase-8 activation at earlier time points. There was no difference in the kinetics of caspase-8 processing in mock-, Ad5*dl*309-, or Ad5*dl*337-infected cells (Figure 1C). An anti-Bid polyclonal antibody that was specific for both Bid and tBid revealed the conversion of Bid to tBid by 4 hr post-TNF/CHX treatment, which was not inhibited by E1B 19K expression (Figure 1C). Since 19K did not bind tBid (see below), the TNF-α pathway must be inhibited further downstream.

TNF-α Signaling Activates an E1B 19K–Bax Protein Interaction

The E1B 19K protein binds to and inhibits Bax (Han et al., 1996; Han et al., 1998), and to investigate whether Bax may play a role in TNF-α-mediated apoptosis, endogenous Bax was immunoprecipitated in Ad5*dl*309-infected HeLa cells in the absence and presence of TNF/CHX. Bax immunoprecipitates were then examined for 19K–Bax association. In untreated cells, E1B 19K did not detectably coimmunoprecipitate with Bax, whereas TNF/CHX-treated cells displayed an induction of 19K–Bax binding (Figure 2). Bax immunoprecipitates were immunoblotted with an anti-Bax antibody to verify equivalent levels of Bax (Figure 2). As a control for nonspecific E1B 19K protein interaction, Jun (which does not bind E1B 19K) was immunoprecipitated in both conditions, and the 19K–Bax interaction induced by TNF/CHX was above nonspecific background levels of E1B 19K (Figure 2). In the converse experiment, an E1B 19K antibody coimmunoprecipitated endogenous Bax only in TNF/CHX-treated Ad5*dl*309-infected cells (data not shown). Thus, TNF-α induced a 19K–Bax interaction in adenovirus-infected cells that may be the point of inhibition of death signaling.

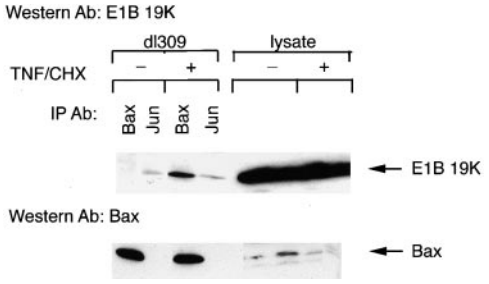

Figure 2. E1B 19K Coimmunoprecipitates with Bax Only upon TNF/CHX Treatment

HeLa cells were infected with Ad5*dl*309 for 24 hr and were either left untreated or treated with TNF/CHX for 4 hr. Lysates were immunoprecipitated with an anti-Bax antibody (N-20), and immune complexes were resolved by SDS–PAGE and probed with an anti-Bax (N-20) and an E1B 19K polyclonal antibody. Whole-cell lysates were generated to verify equal protein levels.

TNF-α Induces a Conformational Change in Bax

It was curious that Bax only interacted with E1B 19K when cells were treated with TNF-α. Treating cells with Staurosporine causes Bax to undergo a conformational change that results in the exposure of an amino-terminal epitope, and antibodies directed against the amino terminus of Bax can recognize this conformational change (Hsu et al., 1997; Desagher et al., 1999). We therefore tested whether TNF-α could mediate an alteration in Bax conformation that permitted E1B 19K to recognize and bind to Bax.

HeLa cells were either mock infected or infected with either Ad5*dl*309 or Ad5*dl*337, treated with TNF/CHX, and immunostained with a Bax polyclonal antibody (NT) that recognizes the amino terminus of Bax. No Bax immunoreactivity was detected in the mock-, Ad5*dl*309-, or Ad5*dl*337-infected cells, whereas TNF/CHX treatment caused mock- and both the Ad5*dl*309- and Ad5*dl*337-infected cells to exhibit bright Bax staining localized to mitochondria (Figure 3A). Furthermore, adenovirus infection and 19K expression did not inhibit this conformational change in Bax. Adenovirus infection in the absence of 19K (Ad5*dl*337), which also triggers apoptosis, did not induce a Bax conformational change (Figure 3A). This raised the possibility that Bax becomes activated

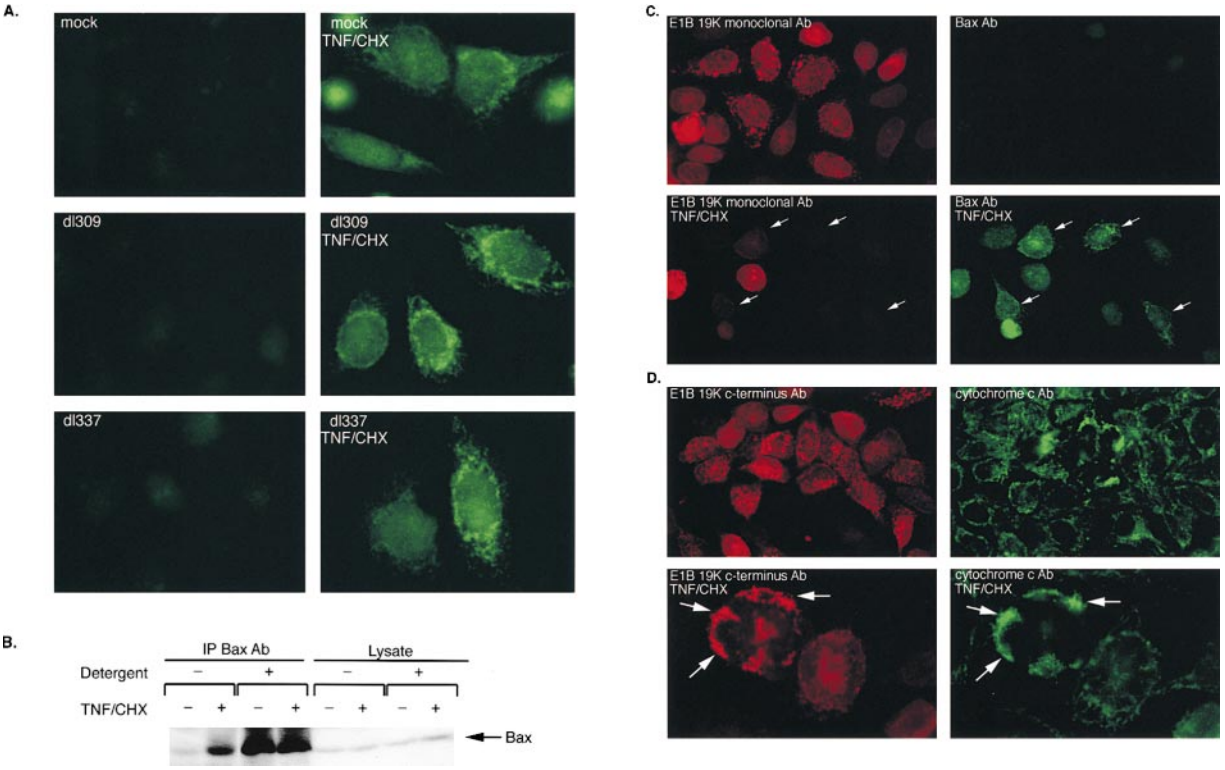

Figure 3. Bax Undergoes a Conformational Change in Response to TNF-α Activation

(A) HeLa cells were mock-, Ad5*dl*309-, or Ad5*dl*337 infected for 24 hr and were left untreated or treated with TNF/CHX for 4 hr. Cells were fixed and stained with a Bax antibody (NT) to recognize conformationally altered Bax.

(B) Bax immunoprecipitates with a Bax conformation-specific antibody upon TNF/CHX treatment. Lysates from HeLa cells in the absence or presence of TNF/CHX (4 hr) were prepared in buffer with or without Triton X-100 detergent. Bax was immunoprecipitated with a Bax antibody (NT). Lysate was removed to verify equal amounts of Bax and analyzed by Western blotting with an anti-Bax antibody (N-20).

(C) The E1B 19K/Bax association masks the epitope for the E1B 19K monoclonal antibody (2F3) during TNF-α activation. HeLa cells were infected with Ad5*dl*309 and treated with TNF/CHX as described above. Cells were double stained with E1B 19K (2F3) and Bax antibodies (NT).

(D) E1B 19K translocates to mitochondria upon TNF/CHX treatment. HeLa cells were infected with Ad5*dl*309 for 24 hr and were either left untreated or treated with TNF/CHX for 4 hr. Cells were double stained with an E1B 19K antibody raised against the carboxyl terminus of E1B 19K and an anti-cytochrome c antibody.

in the TNF-α pathway through alteration in its conformation.

In nondetergent conditions, the amino terminus of Bax is inaccessible for immunoprecipitation, whereas nonionic detergents expose this epitope and allow Bax to be immunoprecipitated (Hsu and Youle, 1997). To biochemically assess whether TNF/CHX treatment causes exposure of the Bax amino terminus, untreated and TNF/CHX-treated HeLa cells were immunoprecipitated in the absence or presence of detergent with an anti-Bax antibody (NT). Bax from untreated cells was not immunoprecipitated in nondetergent conditions but was pulled down effectively when cells were treated with TNF/CHX (Figure 3B). In the presence of detergent, Bax was immunoprecipitated equally in both untreated and TNF/CHX-treated cells, and lysates made in the absence or presence of detergent had equal levels of Bax (Figure 3B). Thus, TNF-α causes exposure of the Bax amino terminus.

The ability of E1B 19K to bind Bax only in a specific conformation may explain why 19K is not normally found localized to mitochondria yet can bind and inhibit the function of a mitochondrial protein (Bax). During adenovirus infection, E1B 19K localizes predominantly to the nuclear lamina, endoplasmic reticulum, and nuclear membranes (White et al., 1984). Although the 19K protein can be redirected partially to mitochondria by overexpressing Bax in cells, this may not be physiological (Han et al., 1998). Alternatively, a specific proapoptotic stimulus (TNF-α) may be required to render Bax capable of binding 19K, and under those circumstances E1B 19K may colocalize with Bax in mitochondria. We therefore examined the intracellular localization of E1B 19K in Ad5*dl*309-infected HeLa cells treated with TNF-α.

HeLa cells infected with Ad5*dl*309 were fixed and stained with both an anti-19K mouse monoclonal antibody (2F3) and an anti-Bax antibody (NT). In untreated cells, E1B 19K localized primarily to the nuclear envelope and cytoplasmic membranes as previously reported (Figure 3C) (White et al., 1984), and mock- and Ad5*dl*337-infected cells were not stained with this antibody (data not shown). Surprisingly, however, TNF/CHX

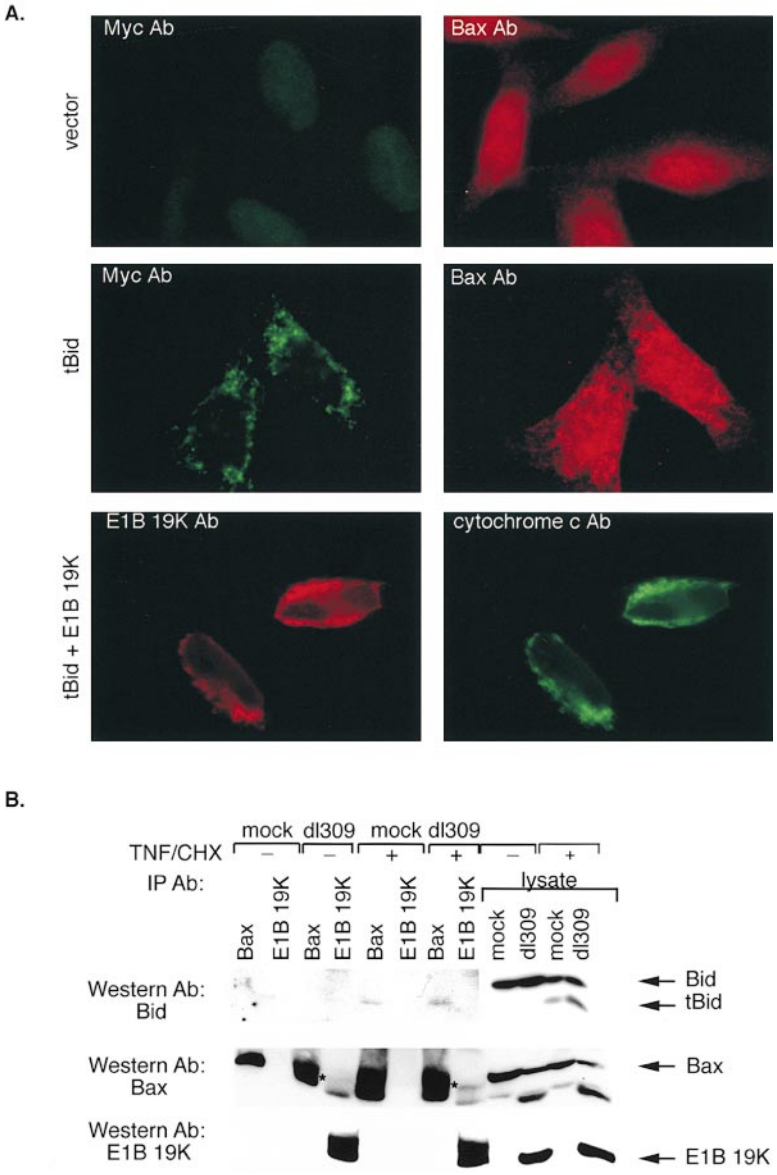

Figure 4. tBid Triggers a Bax Conformational Change In Vivo

(A) HeLa cells were transfected with either vector control, Myc-tagged tBid alone, or Myc-tagged tBid and E1B 19K as indicated. Twenty-four hours posttransfection, cells were double stained with an anti-Myc antibody and a Bax antibody (NT). Myc-tagged tBid plus E1B 19K-transfected HeLa cells were stained with a carboxyl terminus–specific E1B 19K antibody and an anti-cytochrome c antibody.

(B) Bax interacts with tBid in vivo in response to TNF/CHX treatment. HeLa cells were mock- or Ad5*d*l309 infected for 24 hr and lysates made in the absence or presence of TNF/CHX (4 hr). Lysates were immunoprecipitated with a Bax antibody (N-20) or an E1B 19K polyclonal antibody, analyzed by SDS–PAGE, and Western blotted with an anti-Bid antibody (recognizes Bid and tBid), an anti-Bax antibody (N-20), and an E1B 19K polyclonal antibody. The asterisk is placed to mark the Bax band of interest. Cell lysates are shown to verify equal protein levels.

treatment of Ad5*d*l309-infected cells caused a drastic decrease in the number of cells positive for 19K staining (Figure 3C). The few cells that were positive for 19K staining showed E1B 19K localized exclusively to the nuclear envelope, with only a few cells displaying a cytoplasmic membrane staining pattern. The reduction in E1B 19K staining upon TNF/CHX treatment of Ad5*d*l309-infected cells was not due to a reduction in the expression level of E1B 19K (Figure 1B). Frequently, the cells that displayed the brightest Bax immunoreactivity were also most negative for E1B 19K staining (Figure 3C). The converse immunofluorescence pattern, bright 19K staining and weak or no Bax staining, was also observed (Figure 3C, arrows). These results suggested that the epitope for the E1B 19K monoclonal antibody (2F3) may become masked during TNF/CHX treatment. The 2F3 epitope resides between amino acids 70–93 of 19K, which encompasses BH1 (Chiou et al., 1994). Since these residues on E1B 19K are required for Bax binding (Han et al., 1996), the E1B 19K–Bax protein interaction probably conceals this epitope during TNF-α signaling, accounting for the reduction in 19K staining.

Since the E1B 19K antibody 2F3 was largely incapable of detecting the 19K protein in TNF/CHX-treated infected cells by immunofluorescence, we utilized an antibody raised against the carboxyl terminus of E1B 19K that is dispensable for Bax binding and function (Han et al., 1996). Double labeling with the Bax antibody (NT) was not possible because both antibodies were raised in rabbits. Thus, we stained for the mitochondrial protein cytochrome c to examine whether E1B 19K translocates to the mitochondria during TNF-α signaling. HeLa cells were infected with Ad5*d*l309 and then left untreated or treated with TNF/CHX and stained simultaneously for cytochrome c and the 19K carboxyl terminus. E1B 19K local-

ized predominantly to the cytoplasmic membranes in untreated cells (Figure 3D), and uninfected cells were not stained (data not shown). Costaining with cytochrome c indicated no detectable colocalization between E1B 19K and mitochondria in cells that were not treated with TNF/CHX (Figure 3D). In contrast, TNF/CHX treatment caused E1B 19K to colocalize with cytochrome c in mitochondria in some cells (Figure 3D, arrows). Thus, E1B 19K translocated to the mitochondria and interacted with Bax in response to activation of the TNF-α pathway.

tBid Induces an Alteration in Bax Conformation

Since Staurosporine treatment can induce a tBid-dependent conformational change in Bax in vitro (Desagher et al., 1999), we investigated whether tBid transient expression could induce a conformational change in Bax in vivo. Control vector, Myc-tagged tBid, or Myc-tagged tBid and E1B 19K expression vectors were transfected into HeLa cells and immunostained for Bax with the amino-terminal Bax antibody (NT). No Myc or Bax immunostaining was detectable in vector alone–transfected cells (Figure 4A). Double labeling with an anti-Myc monoclonal antibody to detect tBid and an anti-Bax antibody (NT) to detect endogenous conformationally altered Bax demonstrated that cells overexpressing tBid strongly stained for Bax, and Bax colocalized with tBid in mitochondria (Figure 4A). The same subcellular localization was observed for Bax and tBid in cells transfected with plasmids expressing Myc-tagged tBid and E1B 19K (data not shown). Thus, tBid expression was sufficient to induce a conformational change in Bax.

To ascertain whether E1B 19K localized to the mitochondria upon tBid expression, tBid- and E1B 19K-cotransfected cells were examined. Double labeling with a carboxy-terminal 19K antibody and an anti-cytochrome c antibody revealed that E1B 19K colocalized with cytochrome c in mitochondria in tBid-expressing cells but not in the absence of tBid (Figure 4A). These results suggested that E1B 19K localized to the mitochondria upon tBid-dependent activation of Bax.

TNF-α Induced a tBid and Bax Interaction In Vivo

To test if tBid and Bax interacted directly, HeLa cells were either mock or Ad5*dl*309 infected, then left untreated or treated with TNF/CHX and subjected to immunoprecipitation. An anti-Bax antibody (N-20) was used to immunoprecipitate endogenous Bax and was probed with an antibody that recognizes both Bid and tBid. tBid immunoprecipitated with Bax only in the presence of TNF/CHX, and E1B 19K expression had no effect on tBid/Bax association (Figure 4B). Equal amounts of Bax were immunoprecipitated in all conditions (Figure 4B). Thus, TNF-α signaling induces a tBid–Bax interaction that is not affected by E1B 19K expression. This interaction may, however, serve to promote a conformational change in Bax, thereby permitting recognition and binding by E1B 19K.

To determine if E1B 19K is also in this tBid/Bax protein complex, lysates were subjected to immunoprecipitation studies with the anti-19K polyclonal antibody. Neither Bid nor tBid coimmunoprecipitated with E1B 19K even upon TNF/CHX treatment, although 19K was immunoprecipitated equally (Figure 4B). E1B 19K, however, was able to coimmunoprecipitate Bax (Figure 4B). Although it remains possible that E1B 19K, Bax, and tBid form a ternary complex that evades our detection, most likely a tBid-dependent Bax conformational change may result in the displacement of tBid, which would then allow E1B 19K to recognize and interact with a newly exposed epitope on Bax.

E1B 19K Blocks Cytochrome c Release during TNF-α Signaling

Bax can mediate the release of cytochrome c from mitochondria, although the mechanism by which this occurs is not fully understood (Gross et al., 1999). We investigated if E1B 19K could block cytochrome c release during adenovirus infection and activation of the TNF-α signaling pathway.

HeLa cells were mock, Ad5*dl*309, or Ad5*dl*337 infected and immunostained with an antibody directed against cytochrome c. Bright mitochondrial staining was observed in mock- and adenovirus-infected cells (either Ad5*dl*309 or Ad5*dl*337) (Figure 5A). In contrast, TNF/CHX treatment caused a dramatic relocalization of cytochrome c in mock- and Ad5*dl*337-infected cells (Figure 5A). Cytochrome c staining displayed a dim and diffuse staining pattern in the cytosol of most cells or displayed punctate nuclear staining. In Ad5*dl*309-infected cells, however, cytochrome c staining clearly remained mitochondrial (Figure 5A). Thus, E1B 19K expression blocked cytochrome c release from mitochondria by TNF-α.

Since dispersed cytochrome c was difficult to see in the TNF/CHX-treated cells, subcellular fractionation was performed. In healthy cells, cytochrome c cofractionates with mitochondria in the pelleted membrane fraction and does not appear in the soluble S-100 fraction (Figure 5B). In response to TNF/CHX treatment, cytochrome c was no longer observed in the membrane fraction but was detected in the soluble S-100 cytosolic fraction (Figure 5B). Infection with wild-type Ad5*dl*309 inhibited the release of cytochrome c to the cytosolic fraction by TNF/CHX (Figure 5B). Ad5*dl*337-infected cells displayed a modest increase in cytochrome c release, which was not apparent by immunofluorescence. Cell death occurs as result of E1A-mediated apoptosis, since this virus is defective for E1B 19K expression. TNF/CHX further elevated the level of soluble cytochrome c in Ad5*dl*337-infected cells (Figure 5B). These experiments demonstrate that E1B 19K expression effectively blocks cytochrome c release by TNF/CHX.

E1B 19K Inhibits Caspase-9 Activation

Cytochrome c is a cofactor for Apaf-1-dependent caspase-9 activation (Li et al., 1997). To test if E1B 19K expression blocked caspase-9 activation, HeLa cells were mock, Ad5*dl*309, or Ad5*dl*337 infected, treated with TNF/CHX, and cell lysates were immunoblotted with an anti-caspase-9 antibody that recognizes a carboxy-terminal epitope that becomes exposed upon caspase-9 activation. Four hours post-TNF/CHX treatment, the 30 kDa cleavage product of caspase-9 was evident in mock- and Ad5*dl*337-infected cells, and the levels were

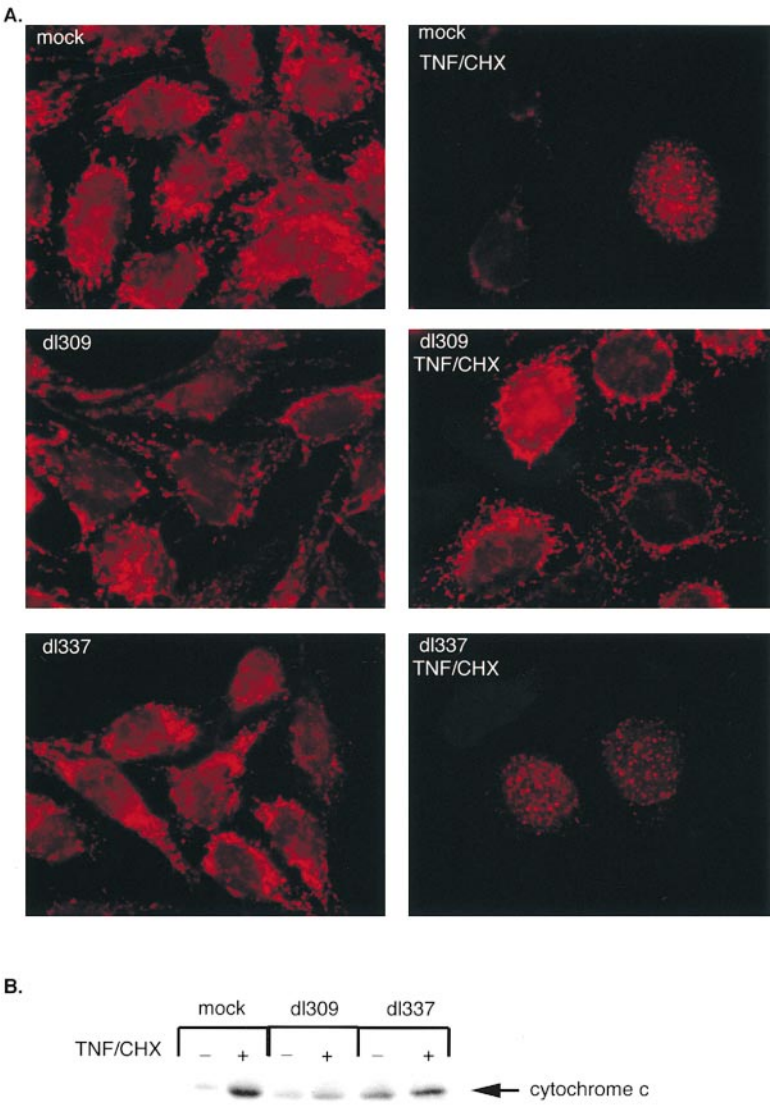

Figure 5. E1B 19K Inhibits Cytochrome c Release from Mitochondria by TNF-α

(A) HeLa cells were mock-, Ad5*dl*309-, or Ad5*dl*337 infected for 24 hr and were left untreated or treated with TNF/CHX for 4 hr. Cells were fixed and stained with a cytochrome c antibody.

(B) E1B 19K inhibits release of cytochrome c into the cytosolic fraction. HeLa cells treated as in (A) were subjected to subcellular fractionation. The soluble S-100 fraction was analyzed by Western blotting with an anti-cytochrome c antibody.

further increased at 14 hr (Figure 6A). In contrast, caspase-9 cleavage was strikingly absent in Ad5*dl*309-infected cells even at 14 hr post-TNF/CHX treatment (Figure 6A), although the samples contained equal protein levels (Figure 1B). Treatment of cells with TNF-α or CHX alone had little effect on caspase-9 activation, with the exception that Ad5*dl*337-infected plus TNF-α-treated cells showed a low level of activation at 14 hr (Figure 6A). This demonstrated that caspase-9 activation was inhibited by E1B 19K expression during TNF-α signaling and was consistent with the absence of cytochrome c release in these cells.

E1B 19K Prevents Prodomain but Not p12 Cleavage of Pro-Caspase-3

Caspase-3 is a downstream effector caspase that is a target for both caspase-8- and caspase-9-mediated processing (Li et al., 1997; Kuida et al., 1998; Stennicke et al., 1998). Since E1B 19K differentially blocks caspase-9 but not caspase-8 activation during TNF-α signaling, we determined if caspase-3 activation was also inhibited. To evaluate the status of caspase-3 processing and activation, extracts from mock-, Ad5*dl*309-, and Ad5*dl*337-infected cells were treated with TNF/CHX and immunoblotted with an anti-caspase-3 antibody (Figure 6B). Four hours post TNF/CHX treatment, mock- and Ad5*dl*337-infected cells displayed a decrease in pro-caspase-3 levels and the appearance of two cleavage products, p20 and p17 (Figure 6B). p20 represents the product of the cleavage event that removes the carboxy-terminal p12. The caspase-3 antibody was not able to recognize the smaller p12. p17 is generated by cleavage to remove the prodomain from p20 (Figure 6B). Although Ad5*dl*309-infected cells also displayed a decrease in pro-caspase-3 levels upon TNF/CHX treatment, only p20 and not p17 was observed after 4 hr of TNF/CHX treatment. By 14 hr, p20 disappeared; however, p17 was still not observed (Figure 6B). p20 may be highly unstable since it is an intermediate in caspase-3 processing. The difference in caspase-3 processing in Ad5*dl*309- and Ad5*dl*337-infected cells was similar to TNF-α treatment alone as with TNF/CHX (Figure 6B), which is likely due to the sensitization to TNF-α caused by E1A. In contrast, mock-infected cells displayed no

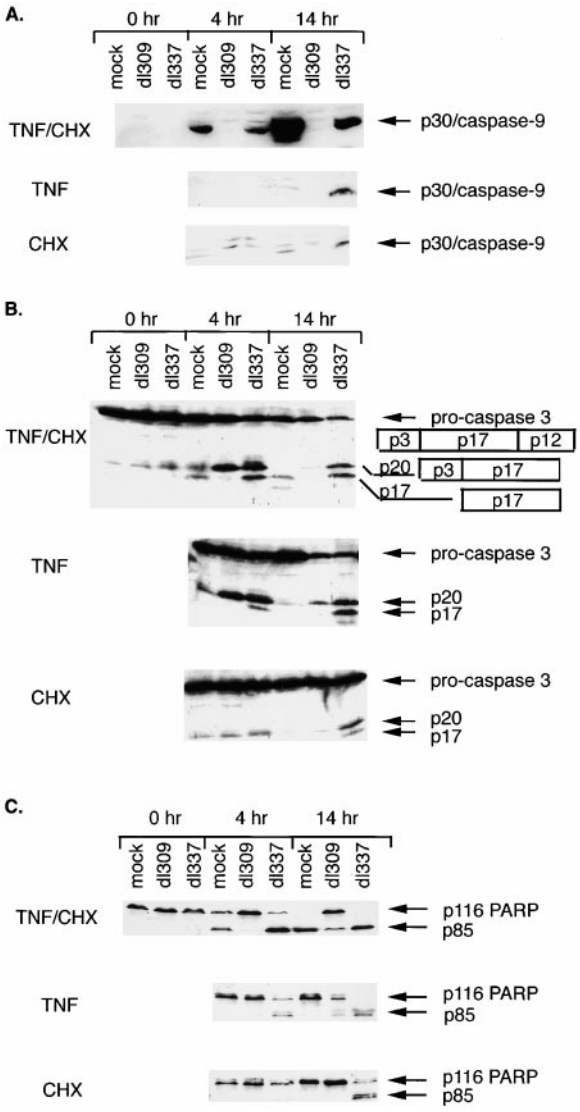

Figure 6. E1B 19K Inhibits Caspase-9 Processing

(A) Whole-cell extracts were generated from mock-, Ad5*dl*309-, or Ad5*dl*337 infected HeLa cells (24 hr) that were treated with TNF/CHX, TNF-α alone, or CHX alone for 0, 4, and 14 hr. Extracts were analyzed by immunoblotting with an anti-caspase-9 antibody that recognizes only the processed form of caspase-9.

(B) E1B 19K inhibits caspase-3 processing. Extracts were generated as stated above and analyzed by immunoblotting with an anti-caspase-3 antibody. The presence of pro-caspase-3, p20, and p17 are indicated.

(C) E1B 19K inhibits PARP cleavage. Extracts were analyzed by immunoblotting with an anti-PARP antibody. The presence of full-length p116 PARP and cleaved p85 PARP are indicated.

caspase-3 cleavage products (Figure 6B). In CHX alone–treated cells, only Ad5*dl*337-infected cells showed processing by 14 hr, likely due to a slight induction of E1A-mediated apoptosis, as had been shown in Figure 1A. These results suggested that the E1B 19K protein altered the normal processing of caspase-3.

E1B 19K Blocks Caspase-3 Substrate Cleavage

As E1B 19K expression permitted partial processing of caspase-3 into p20 but not p17, we investigated whether this partial processing was nonetheless sufficient for caspase-3 activity in vivo. Caspase-3 activity was assessed by examining one of its substrates, PARP, for cleavage by Western blot analysis. Four hours post-TNF/CHX treatment, an 85 kDa PARP cleavage product was detectable in mock- and Ad5*dl*337-infected cells (Figure 6C). By 14 hr post-TNF/CHX treatment, all full-length PARP was converted into the 85 kDa processed form. In Ad5*dl*309-infected cells, only a very small amount of the processed form was detected at 14 hr post-TNF/CHX treatment. This analysis provides evidence that the caspase-3 p20 is not significantly active in vivo and that a second cleavage event to remove the prodomain to generate p17 must occur to render caspase-3 capable for efficient substrate cleavage. Thus, E1B 19K inhibits PARP from being processed by blocking the removal of the prodomain of caspase-3.

Discussion

E1B 19K was shown to block TNF-α-mediated apoptosis downstream of caspase-8-dependent Bid cleavage most likely by directly interacting with a conformationally altered Bax in mitochondria (Figure 7). 19K–Bax association blocked death signaling at mitochondria as demonstrated by inhibition of cytochrome c release and inhibition of caspase-9 activation, which prevented complete processing and activation of pro-caspase-3. Since the mitochondrial pathway is required for induction of TNF-α-mediated apoptosis in HeLa cells, E1B 19K can block cell death in type II cells (Scaffidi et al., 1998). Whether E1B 19K can also inhibit receptor-mediated apoptosis from mitochondria-independent cell types (type I) is not yet known.

E1B 19K can sequester overexpressed FADD and inhibit caspase-8 activation in some situations (Perez and White, 1998), and another protein, FLASH, is proposed to mediate the 19K-FADD-caspase-8 interaction (Imai et al., 1999). E1B 19K, however, does not block FADD recruitment to the death-inducing signaling complex (Perez and White, 1998). Data presented here demonstrate that E1B 19K does not hinder autocatalytic processing of caspase-8 during TNF-α signaling, suggesting that FADD is not targeted by E1B 19K in the TNF-α signaling pathway. Whether FADD and caspase-8 are inhibited by E1B 19K in other death receptor–mediated signaling pathways remains to be determined.

Thus, Bax has been implicated as a component for the TNF-α apoptotic signaling pathway. Since HeLa cells remain viable despite high Bax levels, a mechanism for regulating Bax activity must exist. Two proposed mechanisms for Bax activation are Bax dimerization and translocation to the mitochondria, and/or Bax conversion to an active conformation (Wolter et al., 1997; Gross et al., 1998; Desagher et al., 1999). Based on the structural analysis of the antiapoptotic molecule Bcl-x_L, Bax is predicted to be in a closed structural configuration with its BH3 domain buried (Muchmore et al., 1996). An apoptotic stimulus may expose the amino terminus and the BH3 domain to facilitate heterodimerization with antiapoptotic molecules and oligomerization with itself (Hsu and Youle, 1997; Gross et al., 1998; Eskes et al., 2000). tBid may recruit and translocate Bax to mitochondria where induction of a conformational change allows

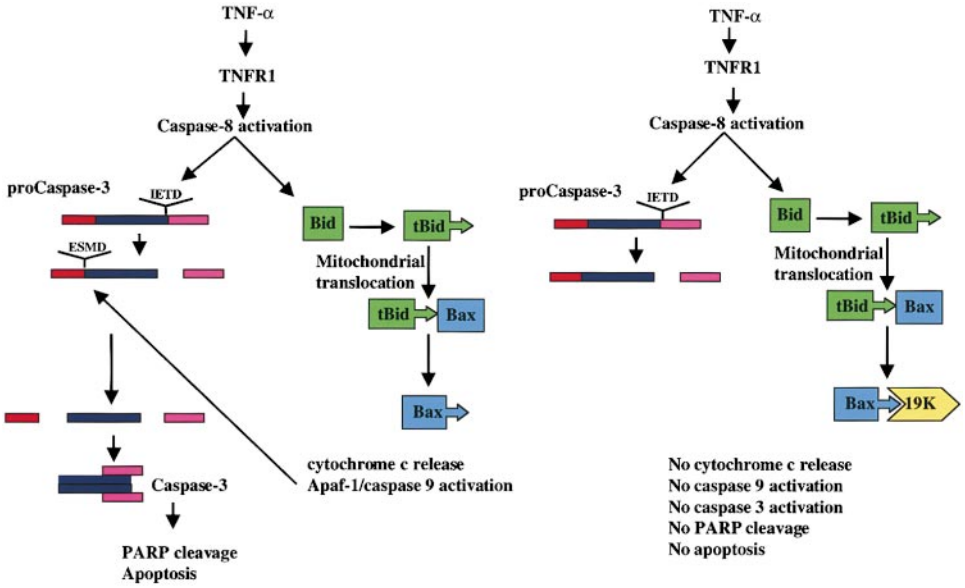

Figure 7. Pathway for Inhibition of the TNF-α Signaling Cascade by E1B 19K
See text for explanation.

Bax to insert into mitochondria. Alternatively, tBid may bind Bax in mitochondria and induce a conformational change that results in exposure of its BH3 and multimerization with itself. As we have failed to detect a ternary complex of tBid, Bax, and E1B 19K, it may be that E1B 19K displaces tBid from Bax and binds Bax BH3. Alternatively, exposure of BH3 may allow Bax to multimerize, which then permits E1B 19K to recognize and associate with Bax. Since oligomerization may be required for Bax activity, inhibition of multimerization by E1B 19K may be a mechanism for ablating Bax function.

Caspase-8-deficient cells are resistant to TNF-α- and Fas-mediated apoptosis, and Fas-mediated apoptosis was attenuated by Bid deficiency in vivo (Varfolomeev et al., 1998; Yin et al., 1999). Alternatively, induction of TNF-α-mediated apoptosis is tissue specific in caspase-3-deficient cells. One prediction from our results is that Bax-deficient cells may be resistant to TNF-α-mediated apoptosis. However, it would not be surprising if cell death were only attenuated or not inhibited at all by Bax deficiency, as redundant proapoptotic proteins such as Bak may also be activated by tBid. Staurosporine induces a conformational change in Bak as well as Bax (Desagher et al., 1999; Griffiths et al., 1999), and E1B 19K binds and inhibits Bak (Farrow et al., 1995). It is not yet possible to discern whether inhibition of TNF-α-mediated apoptosis is a sole function of the 19K–Bax interaction or whether it also relies on 19K–Bak binding.

Although E1B 19K expression during adenoviral infection in human cells blocks TNF-α- and Fas-mediated apoptosis, the ability of cellular antiapoptotic Bcl-2 family members to block receptor-mediated apoptosis has remained controversial. These discrepancies may be due to cell type specificity, strength of the apoptotic stimulus, or differential functions and/or expression levels of Bcl-2 family members. Indeed, low sequence homology among Bcl-2 family members may be indicative of different functional capacities. Bcl-2 binds Bid and has a weaker affinity for tBid (Luo et al., 1998). In contrast, Bcl-x_L binds tBid better than Bid (Li et al., 1998). E1B 19K functions differently from Bcl-2 or Bcl-x_L, since it does not bind to either Bid or tBid in vivo. Bcl-2, Bcl-x_L, and E1B 19K are all capable of interacting with Bax, but Bcl-2 and Bcl-x_L may interact prior to a Bax conformational change (Desagher et al., 1999). In contrast, E1B 19K is capable of interacting with Bax in mitochondria only after a conformational change has occurred in response to TNF-α signaling. As a viral protein, the mechanism of E1B 19K inhibition of apoptosis may represent a gain of function over cellular Bcl-2-related apoptotic inhibitors. Alternatively, other Bcl-2-like proteins may indeed function similarly to E1B 19K.

The interaction between E1B 19K and Bax results in inhibition of downstream mitochondrial apoptotic events: cytochrome c–dependent caspase-9 processing and caspase-3 activation. Interestingly, dissection of the TNF-α pathway revealed that cooperation between caspase-8 and caspase-9 is required for complete processing and activation of caspase-3. In vitro studies with immunodepleted caspase-9 extracts suggest that caspase-8 can directly process pro-caspase-3 into its active form (Stennicke et al., 1998). Also, in vitro analysis with caspase inhibitors provides evidence that caspase-3 is activated upon two distinct cleavage events, and that the second cleavage activity is autocatalytic (Slee et al., 1999). However, in vivo studies with caspase-9 null mice demonstrate that caspase-9 is essential for caspase-3 activation (Kuida et al., 1998**)**. We demonstrate that pro-caspase-3 can be activated via a branched caspase cascade that requires both a mitochondria-dependent and independent caspase-specific cleavage.

Cleavage of pro-caspase-3 between p17 and p12 results in an intermediate p20 (Stennicke et al., 1998).

Since caspase-8 activation was not abrogated by E1B 19K, processing of pro-caspase-3 at the IETD site could generate p20 (Thornberry et al., 1997). p20 was probably not an active form of caspase-3, since little PARP cleavage was detected in the presence of E1B 19K. Another caspase-3 cleavage event removes the prodomain from p20, generating p17, which in cooperation with p12 renders the caspase enzymatically active. This processing event appears to be caspase-9 dependent. Caspase-9 may be required for caspase-3 activation, since inhibition by E1B 19K renders caspase-3 in an intermediate state of processing that is not capable of cleaving either itself or its downstream substrates. Whether caspase-9 directly catalyzes caspase-3 activation or whether the effect of caspase-9 on caspase-3 is indirect remains to be determined. Although this cleavage site (ESMD) is not an ideal caspase-9 consensus recognition site, studies have shown that caspase-9 can directly activate caspase-3 in vitro (Slee et al., 1999). Nonetheless, caspase processing appears to be a highly specific and ordered event that can be interrupted at the level of the mitochondria by E1B 19K interacting with an altered form of Bax. This function contributes to the repertoire of activities that the adenovirus has evolved to conceal itself from immune surveillance by the host.

Experimental Procedures

Antibodies

The following antibodies were used: anti-E1B 19K 2F3 mouse monoclonal antibody (clone #3D11) that recognizes amino acids 70–93 (Han et al., 1996) (Calbiochem-Novabiochem Corp., San Diego, CA), rabbit polyclonal antibody generated against full-length E1B 19K (Han et al., 1996), rabbit polyclonal E1B 19K antibody raised against six carboxy-terminal amino acids of E1B 19K (Dr. Phillip Branton, McGill University, Montreal, Canada), rat anti-human caspase-8 monoclonal antibody (clone #1H10E4H10) raised against amino acids 217–375, rat anti-Bid monoclonal antibody (clone #5C4A11) that recognizes only the full-length Bid (Zymed Laboratories, San Francisco, CA), rat anti-Bid polyclonal antibody that recognizes Bid and tBid (Dr. Junying Yuan, Harvard Medical School, Boston, MA), mouse monoclonal antibodies directed against the native (65971A) and the denatured (65981A) cytochrome c (PharMingen, San Diego, CA), rabbit polyclonal Bax antibodies N-20 (directed against amino acids 11–30) and NT (directed against amino acids 1–21) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, and Upstate Biotechnology, Lake Placid, NY, respectively), rabbit anti-caspase-9 polyclonal antibody raised against a CPEPD peptide that recognizes this epitope at the carboxyl terminus of the large subunit (Mesner et al., 1999) (Dr. Scott H. Kauffman, Mayo Clinic, Rochester, MN), rabbit anti-human caspase-3 polyclonal antibody (cat. #65906E) (PharMingen, San Diego, CA), mouse C2-10 monoclonal antibody directed against PARP (Dr. Guy Poirier, Centre de Recherche du Chul, Quebec, Canada), anti-actin mouse monoclonal antibody (Amersham Pharmacia Biotechnology, Buckingshire, England), anti-Myc tag mouse monoclonal antibody (cat. #R950-25, Invitrogen, Carlsbad, CA), and anti-Jun mouse monoclonal antibody (Transduction Laboratories, San Diego, CA).

Plasmids and Transfection

tBid was generated by a PCR reaction from a HeLa library and was generously supplied by Dr. Arun Gaur. A 5′ BamHI and a 3′ EcoRI linker was added to the carboxy-terminal Myc-tagged tBid product for ligation into the pcDNA3.1 expression vector (Invitrogen, San Diego, CA). pcDNA3.1-V5/His-tagged E1B 19K was described previously (Kasof et al., 1998). pcDNA3 was purchased from Invitrogen (San Diego, CA) and used as a control vector. HeLa cells were electroporated with 18 μg of pcDNA3 control vector, 6 μg of pcDNA3.1-Myc-tagged tBid, and 12 μg of pcDNA3.1-V5-His-tagged E1B 19K expression plasmids. The total amount of DNA was kept constant at 18 μg by addition of the pcDNA3 vector.

Adenovirus Infection

Adenoviruses Ad5*dl*309 and Ad5*dl*337 were obtained from Dr. T. Shenk (Princeton University, Princeton, NJ). Ad5*dl*309 has a deletion in the *E3* gene and is used as the wild-type virus (Jones and Shenk, 1979). Ad5*dl*337 was derived from Ad5*dl*309 and has a deletion in the *E1B 19K* gene (Pilder et al., 1984). HeLa cells were infected as previously described (White et al., 1984).

TNF-α Apoptosis Induction Assay

Twenty-four hours postinfection, cells were treated with 2000 units/ml of TNF-α (Boehringer Mannheim, Indianapolis, IN) and/or 30 μg/ml of CHX (Sigma, St. Louis, MO) as indicated. Cell viability was determined by trypan blue exclusion.

Subcellular Fractionation

1×10^7 HeLa cells were harvested and resuspended at a cell density of 5×10^7 cell/ml in a hypotonic lysis buffer containing protease inhibitors (10 mM HEPES [pH 7.4], 38 mM NaCl, 2 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A) and fractionated as previously described (Hsu and Youle, 1997).

Indirect Immunofluorescence and Western Blotting

Cells were fixed (Desagher et al., 1999) and stained (Perez and White, 1998) as previously described. Whole-cell extracts were generated and analyzed by SDS–PAGE and semidry blotted onto polyvinylidene fluoride (PVDF) membrane (Schleicher & Schuell, Keene, NH) as previously described (Perez and White, 1998).

Immunoprecipitation

1×10^7 HeLa cells were mock- or Ad5*dl*309 infected for 24 hr and were either left untreated or treated with TNF/CHX for 4 hr. All cells were harvested and resuspended in lysis buffer (20 mM Tris [pH 7.4], 137 mM NaCl, 2 mM EDTA, 10% glycerol, and 1% Triton X-100) containing protease inhibitors. Lysates were precleared, incubated with antibody overnight, and protein A Sepharose (Pharmacia, Piscataway, NJ) was added to pull down immune complexes. The Sepharose was washed three times in a 0.2% Triton X-100 buffer. Immunoprecipitation with an antibody that recognizes the conformation-specific form of Bax was performed as follows (Hsu and Youle, 1997): HeLa cells (1×10^7) were harvested and resuspended in detergent-containing lysis buffer or in a nondetergent-containing buffer (as stated above) with protease inhibitors and sonicated (Fischer Sonic Dismembrator model 300) and immunoprecipitated as stated above.

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