Tumor necrosis factor-α (TNF-α) mediates death signaling in cells, which induces the oligomerization of pro-apoptotic Bcl-2 family member Bax into a high molecular mass protein complex in mitochondrial membranes. Bax complex formation is associated with the release of cytochrome c, which propagates death signaling by acting as a cofactor for caspase-9 activation. The adenovirus Bcl-2 homologue E1B 19K blocks TNF-α-mediated apoptosis by preventing cytochrome c release, caspase-9 activation, and apoptosis of virus-infected cells. TNF-α induces E1B 19K-Bax interaction and inhibits Bax oligomerization. Oligomerized Bax may form a pore to release mitochondrial proteins, analogous to the homologous pore-forming domains of bacterial toxins. E1B 19K can also bind to pro-apoptotic Bax, but the functional significance is not known. TNF-α signaling induced Bak-Bax interaction and both Bak and Bax oligomerization. E1B 19K was constitutively in a complex with Bak, and blocked the Bak-Bax interaction and oligomerization of both. The TNF-α-mediated cytochrome c and Smac/DIABLO release from mitochondria was inhibited by E1B 19K expression in adenovirus-infected cells. Since either Bax or Bak is essential for death signaling by TNF-α, the interaction between E1B 19K and both Bak and Bax may be required to inhibit their cooperative or independent oligomerization to release proteins from mitochondria which promote caspase activation and cell death.

TNF-α is a cytokine produced by the immune system in response to viral infection. Signaling by TNF-α through the TNF R1 induces a variety of cellular responses including the recruitment and activation of caspase-8 in the receptor complex, which initiates a pathway to implement cell death by apoptosis. Caspase-8, which is a cysteine protease (2, 3), then initiates a pathway to implement cell death by recruiting and activating caspase-8 in the receptor complex. TNF R1 induces a variety of cellular responses including the recruitment and activation of caspase-8 in the receptor complex. Oligomerized Bax may form a pore to release mitochondrial proteins, analogous to the homologous pore-forming domains of bacterial toxins. E1B 19K can also bind to pro-apoptotic Bax, but the functional significance is not known. TNF-α signaling induced Bak-Bax interaction and both Bak and Bax oligomerization. E1B 19K was constitutively in a complex with Bak, and blocked the Bak-Bax interaction and oligomerization of both. The TNF-α-mediated cytochrome c and Smac/DIABLO release from mitochondria was inhibited by E1B 19K expression in adenovirus-infected cells. Since either Bax or Bak is essential for death signaling by TNF-α, the interaction between E1B 19K and both Bak and Bax may be required to inhibit their cooperative or independent oligomerization to release proteins from mitochondria which promote caspase activation and cell death.

Changes in Bax and Bak, and their homodimerization and oligomerization into large protein complexes, which may be analogous to membrane pores (7–10). These putative Bax and Bak pores reside in the outer mitochondrial membrane, but whether Bax or Bak oligomeric complexes that may make up these pores contain other proteins, is not known. TNF-α signaling, however, requires either Bax or Bak, which implement the release of cytochrome c from mitochondria. Released cytochrome c functions, perhaps in concert with other released mitochondrial proteins, as a cofactor for caspase-9 activation. Active caspase-9 then facilitates caspase-3 activation, substrate cleavage, and cell death by apoptosis.

How Bax or Bak oligomerize to form pores to release cytochrome c, and possibly other proteins such as Smac/DIABLO, which is an inhibitor of a caspase-9 inhibitor (12, 13), from the mitochondrial intermembrane space, is not known. It is also unclear whether Bax or Bak generate separate pores, or how their function is regulated. The adenovirus E1B 19K protein, however, is a potent inhibitor of TNF-α-mediated apoptosis and has been a useful tool in elucidating death signaling events at the level of the mitochondria (7, 10, 14, 15).

DNA viruses, including adenovirus, have evolved multiple functions to disable apoptotic signaling by TNF-α, likely as part of their repertoire of functions to sustain the viability of infected host cells until the virus life cycle is complete (16). The adenovirus E1B 19K protein functions as an apoptosis inhibitor during productive virus infection of human cells, and by the p53 tumor suppressor protein in oncogenic transformation of primary rodent epithelial cells (17). In infected cells, E1B 19K blocks apoptosis induced by deregulation of the cell cycle by adenovirus E1A expression, as well as that induced by TNF-α. Infection with viruses that lack E1B 19K causes infected cells to die by E1A-mediated apoptosis, and infected cells become exquisitely sensitive to TNF-α-mediated death signaling (7, 14, 15). E1B 19K expression alone is sufficient to confer resistance to TNF-α, demonstrating that this function is independent of the presence of any other adenovirus proteins (15).

E1B 19K is homologous in sequence and in function to the anti-apoptotic Bcl-2 protein (17). Examination of the point at which E1B 19K blocked death signaling by TNF-α during virus infection revealed that inhibition of the pathway took place at the level of the mitochondria (7). Those events upstream of the mitochondria, specifically caspase-8 activation and BID cleavage to tBid, take place normally whether viruses expressed E1B 19K or not. In contrast, mitochondrial events, and those downstream, namely cytochrome c release, caspase-9 and -3 activation, poly(ADP-ribose) polymerase and lamin cleavage, and apoptosis, are inhibited in wild-type but not E1B 19K.
mutant virus-infected cells (7). These observations led to scrutiny of mitochondrial death signaling events as likely targets for EIB 19K inhibition.

TNF-α-induced generation of tBid causes tBid to bind to Bax, and probably also Bak, which results in a conformational change in the Bax amino terminus by a hit-and-run mechanism (7). EIB 19K neither binds Bid or tBid, nor does its expression prevent Bid-Bax interaction or the conformational change in the Bax amino terminus (7). Rather, this amino-terminal altered Bax binds EIB 19K, and Bax oligomerization into a 500-kDa high molecular mass complex is inhibited in infected cells treated with TNF-α (10). EIB 19K-Bax complex formation also blocks the occurrence or detection of the conformational change in the Bax carboxyl terminus (10). These profound effects of EIB 19K on Bax protein complex formation in mitochondria indicated a role for Bax in death receptor signaling pathways.

How Bcl-2 family members may regulate mitochondrial death signaling was suggested from studies of homologous bacterial proteins. Bax and other Bcl-2 family members share structural homology to the pore-forming domains of bacterial toxins, which also undergo changes in conformation followed by membrane insertion and oligomerization to form a membrane pore (18–24). These structure-function parallels are also mirrored by EIB 19K and bacterial immunity proteins (10), which can block membrane pore formation by interacting with and inhibiting oligomerization of the toxin pore-forming domain (25–29). Whether the EIB 19K-Bax interaction is sufficient to explain inhibition of TNF-α-mediated apoptosis remained to be determined.

We report here that TNF-α induced an interaction between Bak and Bax and both proteins oligomerize. In wild-type virus-infected cells, EIB 19K was constitutively bound to Bak and prevented Bak-Bax interaction and oligomerization stimulated by TNF-α. The TNF-α-stimulated release of not only cytochrome c but also Smac/DIABLO was specifically inhibited by EIB 19K expression in virus-infected cells. Although tBid can independently bind Bak and alter its conformation to permit EIB 19K binding, the redundant functional activities of Bak and Bak may require inhibition of both by EIB 19K to effect inhibition of cytochrome c and Smac/DIABLO release from mitochondria in TNF-α-mediated apoptosis. Finally, cooperative binding between Bax and Bak may facilitate Bax oligomerization and efficient formation of mitochondrial membrane pores.

EXPERIMENTAL PROCEDURES

Antibodies—The following antibodies were used: rabbit polyclonal EIB 19K antibody generated against baculovirus expressed, full-length recombinant EIB 19K protein (10); rabbit polyclonal Bax antibody Bax(11–30) directed against amino acids 11–30 of human Bax (Bax N-29, Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rabbit polyclonal Bax antibody Bax(43–61) directed against amino acids 43–61 of human Bax (Pharmmingen, San Diego, CA); rabbit polyclonal Bax antibody Bax(11–30) directed against amino acids 23–37 of human Bax (Upstate Biotechnology, Lake Placid, NY); rabbit polyclonal Bax antibody Bax(14–36) directed against 14–36 of human Bax (Pharmmingen, San Diego, CA); rabbit polyclonal Bax antibody Bax NT directed against amino acids 53–37 of human Bax (Pharmmingen, San Diego, CA); mouse monoclonal anti-cytochrome c antibody which recognized the denatured protein (Pharmmingen, San Diego, CA); mouse monoclonal anti-cytochrome oxidase subunit II (COXII) (Molecular Probes). The anti-Smac/DIABLO rabbit polyclonal antibody was generated as follows: sequences encoding amino acids 95–239 of human Smac/DIABLO were polymerase chain reaction-subcloned into the pCRT7/NT-TOPO vector (InVitrogen, Carlsbad, CA) using the primers 5′-GCCTGTTAGT-GAGGTATTACTGAAATATCGACGGC-3′ and 5′-AGACAGGGCGATT-GTGTCAAGCAGCTACTA-3′. Recombinant histidine-tagged fusion protein was produced in Escherichia coli BL21, and purified using the Xpress system for protein purification (InVitrogen), according to the manufacturer’s instructions using a denaturing purification scheme.

AdenoVirus Infection and TNF-α Treatment—Adenoviruses Ad5dl309 and Ad5dl337 were obtained from Dr. T. Shenk (Princeton University, Princeton, NJ). Ad5dl309 has a deletion in the E3 gene and a protein synthesis inhibitor cycloheximide (TNF/CHX) (2000 units/ml TNF-α (Roche Molecular Biochemicals, Indianapolis, IN) and 30 μg/ml CHX (Sigma)) to block the NFκB-activated survival pathway as previously described (7, 10, 15). Treatment of cells with CHX alone has no effect on caspase activation, Bak conformation, or cell viability (7, 10, 15).

Gel Filtration Chromatography—For gel filtration chromatography, 2.5 × 107 HeLa cells were mock, Ad5dl309-, or Ad5dl337-infected for 24 h and were then untreated or treated with TNF/CHX for 4 h. Lysates prepared in CHAPS (Calbiochem, La Jolla, CA) lysate buffer (20 mM triis, pH 7.4, 137 mM NaCl, 2 mM EDTA, 10% glycerol, and 2% CHAPS) at a density of 1 × 105 cells/ml were centrifuged at 14,000 rpm for 20 min and the supernatant was loaded onto the column. The Sepharyl S-300 gel filtration chromatography was carried out as previously described (10). Fractions were analyzed by SDS-PAGE and Western blotting as previously described (33) and probed with the Bax(11–30) or Bak NT antibodies.

In Vitro Cross-linking—1 × 107 HeLa cells were mock or Ad5dl309 infected for 24 h and were then untreated or treated with TNF/CHX for the indicated time periods. Cell lysates prepared in HEPES-CHAPS lysis buffer (10 mM HEPES, pH 7.4, 137 mM NaCl, 2 mM EDTA, 10% glycerol, and 2% CHAPS) at a density of 1 × 106 cells/ml were incubated with 1 or 5 mM 1,6-bismaleimidohexane (BMH) ( Pierce, Rockford, IL) or Me2SO alone for 2 h at 4 °C, and quenching carried out as previously described (8). Microprecipitates formed were spun down at 14,000 rpm for 20 min. 23 μl of the 1 ml of cross-linked lysate was resolved by SDS-PAGE and then analyzed by Western blotting with the Bax(11–30) antibody, Bak NT antibody, or EIB 19K polyclonal antibody.

Immunoprecipitation—HeLa cell immunoprecipitations were carried out as previously described (7), except that all cells were lysed by resuspended in CHAPS lysis buffer with protease inhibitors, as previously described (7, 10). The protein A-Sepharose was washed four times in a 0.5% CHAPS buffer. Whole cell extracts and immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blotting with the Bax(11–30), Bak NT, and EIB 19K antibodies.

Subcellular Fractionation—7 × 107 HeLa cells were either mock infected or infected with Ad5dl309 or Ad5dl337 for 24 h, followed by treatment with either TNF/CHX or CHX alone for 4 h. Attached and floating cells were harvested by scraping into the growth medium and centrifugation, washed with PBS, and resuspended at a density of 1 × 105 cells/ml in Buffer I (10 mM HEPES, pH 7.4, 0.32 M sucrose, 42 mM KCl, 5 mM MgCl2, 2 mM EDTA, 1 mM dithiothreitol, plus protease inhibitors as above). The suspension was incubated on ice 20 min. Using a 1-ml syringe, cells were lysed by passage through a 26-gauge needle 10 times, followed by passage through a 30-gauge needle 30 times. A 100-μl sample of whole cell (total) extract was retained, and the extract was centrifuged at 400 × g for 10 min, 4 °C. The supernatant was centrifuged at 15,000 × g for 10 min, 4 °C, and the resulting pellet was resuspended in 100 μl of Buffer II (identical to Buffer I, minus sucrose and including 1.0% Triton X-100 for solubilization of the pellet) and retained as the heavy membrane/mitochondrial fraction. The supernatant was centrifuged at 100,000 × g, and the S-100 was retained as the cytosolic fraction. 10 μl of total extract, and 20 μl each of the mitochondrial/heavy membrane and cytosolic fractions, respectively, were subjected to 17% SDS-PAGE, and transferred to 0.2-μm pore size polyvinylidene difluoride membrane (Schleicher and Schuell, Keene, NH) for 1 h in TBE (25 mM Tris, pH 8.3, 200 mM boric acid, and 25 mM EDTA) blotting, and probed with Smac/DIABLO antibody or COXII antibody.
infected for 24 h. Cells were treated with TNF/CHX, or with CHX alone, for 4 h. Cells were then fixed in 4% paraformaldehyde, and indirect immunofluorescence was performed as described previously (33), with the following modifications. Coverslips for Smac/DIABLO staining were subjected to epitope retrieval by heating them to 91 °C for 15 min in PBS, followed by immediate transfer to PBS at 25 °C. Coverslips were blocked with 4% bovine serum albumin/PBS at 37 °C for 1 h and stained with Smac/DIABLO antisera diluted 1:200 in PBS with 1 mg/ml bovine serum albumin. Unheated coverslips were stained with anti-native cytochrome c antibody (Pharmingen, San Diego, CA), diluted 1:60 as above. Staining was visualized by epifluorescence microscopy as described previously (33), and percentages of cells with cytosolic or mitochondrial Smac/DIABLO or cytochrome c staining were determined by scoring ~250 cells on duplicate coverslips.

**RESULTS**

TNF-α Induces a Bax-Bak Co-immunoprecipitation—Antibodies directed against specific epitopes have indicated that Bax undergoes defined and discrete conformational changes revealed by differential exposure of epitopes in the absence and presence of death signaling (4, 7, 10, 34, 35). Death signaling by TNF-α induces a tBid-dependent conformational change in the Bax amino terminus indicated by exposure of an amino-terminal Bax epitope (7). Bak also undergoes an amino-terminal conformational change in TNF/CHX cells (data not shown), which is likely also due to tBid-Bak interaction (6). A second conformational change in the carboxy-terminal BH2 region of Bax is also revealed by exposure of that epitope upon treatment of cells with TNF/CHX (10). An epitope in an unstructured loop region of Bax between amino acids 43 and 61, however, remains exposed independent of a death stimulus and Bax is efficiently immunoprecipitated from cells in the absence or presence of TNF/CHX (Fig. 1 (10)). Infection of HeLa cells with wild-type adenovirus Ad5dl309 and expression of the E1B 19K protein, or infection with the E1B 19K deletion mutant virus Ad5dl337 also did not affect Bax immunoprecipitation by the Bax-(43-61) loop antibody (Fig. 1 (10)). When the Bax immunoprecipitates were subjected to Western blotting with an anti-Bak antibody, Bak was specifically co-immunoprecipitated with Bax only from cells treated with TNF/CHX (Fig. 1). Infec- tion with Ad5dl309 and E1B 19K expression greatly diminished co-immunoprecipitation of Bax with Bak induced by TNF/CHX (Fig. 1), while inducing an E1B 19K-Bak association (Fig. 1) as reported previously (10). Infection with the E1B 19K viral mutant Ad5dl337 induced Bax-Bak co-immunoprecipitation even in the absence of TNF/CHX, that was further stimulated by TNF/CHX (Fig. 1). As this virus induces E1A-mediated apoptosis because it lacks E1B 19K, it may also do so by promoting Bax-Bak complex formation in a similar manner to TNF/CHX.

**E1B 19K Binds Bak and Conformationally Altered Bax and Inhibits Bak-Bax Interaction—Immunoprecipitation of Bak similarly revealed TNF/CHX-dependent or Ad5dl337-induced co-immunoprecipitation of Bak with Bax, which was greatly diminished by E1B 19K expression during viral infection (Fig. 1). As Ad5dl337 lacks E1B 19K, apoptosis is induced by E1A in infected cells. E1B 19K, however, co-immunoprecipitated with Bak equally in the presence and absence of TNF/CHX (Fig. 1). Thus, death signaling by either TNF/CHX or E1A induced Bax-Bak complex formation that was inhibited by E1B 19K expression during virus infection. Since E1B 19K is constitutively bound to Bak in infected cells, 19K-Bak complex formation may prevent Bak as well as Bax oligomerization, Bak-Bax complex formation, and co-oligomerization and apoptosis.

**E1B 19K Inhibits Bax and Bak Oligomerization—Gel filtration chromatography has been useful in the characterization of changes in Bax and Bak protein complexes stimulated by death signaling through mitochondria (9, 10). To evaluate the potential for modulation of Bak protein complexes by TNF/CHX and E1B 19K, HeLa cells were infected with the wild-type virus Ad5dl309 or the E1B 19K deletion mutant virus Ad5dl337, and were then untreated or treated with TNF/CHX for 4 h. Cell lysates were prepared in CHAPS lysis buffer and were immunoprecipitated with the Bax-(43-61) antibody, rabbit polyclonal Bak antibody, and a negative control antibody (BRCA-2). Immune complexes were resolved by SDS-PAGE and subjected to Western blotting by probing with the Bax-(11-30) antibody. Bak and E1B 19K antibodies as indicated. Equivalent levels of Bak, Bak, and E1B 19K were present in all lysates (bottom panels).

![Fig. 1. TNF-α induces Bax-Bak co-immunoprecipitation that is inhibited by E1B 19K during adenovirus infection](http://www.jbc.org/)

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**Fig. 1. TNF-α induces Bax-Bak co-immunoprecipitation that is inhibited by E1B 19K during adenovirus infection.** HeLa cells were mock, Ad5dl309, or Ad5dl337 infected for 24 h and then untreated or treated with TNF/CHX for 4 h. Cell lysates were prepared in CHAPS lysis buffer and were immunoprecipitated with the Bax-(43-61) antibody, rabbit polyclonal Bak antibody, and a negative control antibody (BRCA-2). Immune complexes were resolved by SDS-PAGE and subjected to Western blotting by probing with the Bax-(11-30), Bak, and E1B 19K antibodies as indicated. Equivalent levels of Bak, Bak, and E1B 19K were present in all lysates (bottom panels).

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HeLa cells were mock, Ad5dl309, or Ad5dl337 infected for 24 h and then untreated or treated with TNF/CHX for 4 h. Cell lysates were prepared in CHAPS lysis buffer and were immunoprecipitated with the Bax-(43-61) antibody, rabbit polyclonal Bak antibody, and a negative control antibody (BRCA-2). Immune complexes were resolved by SDS-PAGE and subjected to Western blotting by probing with the Bax-(11-30), Bak, and E1B 19K antibodies as indicated. Equivalent levels of Bak, Bak, and E1B 19K were present in all lysates (bottom panels).
the higher molecular mass range of the column, with a peak at 70 kDa that trailed off into the 500-kDa molecular mass range that overlapped with the 500-kDa Bak peak (Fig. 2). Although E1B 19K expression during virus infection blocked the formation of the 500-kDa Bax complex (Fig. 2) (10), the shift in the Bak elution profile was not affected by E1B 19K expression in this assay (Fig. 2). E1B 19K elutes with a molecular mass of 43 kDa in healthy cells, which is only slightly increased upon TNF/CHX treatment (10). This is consistent with constitutive E1B 19K-Bak heterodimer formation and E1B 19K trapping both Bax and Bak in low molecular mass protein complexes.

E1B 19K expression selectively inhibited the TNF/CHX-stimulated recruitment of Bax but not Bak into a higher molecular mass protein complex. However, protein cross-linking studies were undertaken to better characterize the composition of the Bax, Bak, and 19K complexes.

Induction of the formation of Bax and Bak homodimers and homo-oligomers by various death stimuli has been demonstrated by protein cross-linking studies in vivo and in vitro (8, 10). Mock-infected HeLa cells untreated or treated with TNF/CHX for 4 h, were lysed in a CHAPS lysis buffer and then incubated with the chemical cross-linking reagent BMH or vehicle alone. Bax, Bak, and E1B 19K cross-linking was then interrogated by SDS-PAGE and Western blotting. To determine what effect E1B 19K would have on Bax and Bak cross-linking, Ad5dl309-infected cells untreated or treated with TNF/CHX were examined in parallel (10).

TNF/CHX treatment induces disuccinimidyl suberate cross-linked Bax dimers and oligomers, the formation of which is inhibited by E1B 19K expression in adenovirus infection (10). Disuccinimidyl suberate, however, does not work well for cross-linking Bak protein complexes, which are better visualized by the cross-linking agent BMH (8). BMH similarly induces the same Bax dimer cross-linked complex (Bax-Bax) and higher molecular mass protein complexes.
molecular weight complexes Bax-A and Bax-B (Fig. 3A). Bax-A migrated at about 40 kDa, just above the Bax dimer, whereas Bax-B migrated at ~55 kDa (Fig. 3A). E1B 19K expression in infected cells inhibited TNF/CHX-dependent Bax protein cross-linking by BMH (Fig. 3A) as well as disuccinimidyl suberate (10). Probing the same cross-linked extracts for Bak revealed multiple Bak cross-linked protein complexes induced by TNF/CHX (Fig. 3A) as the asterisks, migrated similarly to Bax-A and Bax-B, E1B 19K expression during virus infection greatly diminished the TNF/CHX-stimulated formation of Bak cross-linked complexes (Fig. 3A). Although E1B 19K expression did not block the TNF/CHX-mediated recruitment of Bak in a higher molecular mass protein complex by gel filtration chromatography, as it did to Bax, (Fig. 2), the nature of the Bak complex generated in E1B 19K expressing cells appears distinct from that associated with productive propagation of the death signal through mitochondria. It is possible that the change in Bak elution position in extracts from TNF/CHX-treated cells detected by gel filtration is indicative of a post-translational modification of the protein rather than oligomerization which occurs independently of E1B 19K expression.

To determine whether Bax-A and Bax-B complexes contained Bak, the Bax Western blot in the top panel of Fig. 3A was reprobed directly for Bak. Bax-A and Bax-B complexes did correspond directly to Bak-containing complexes induced by TNF/CHX (Fig. 3A). Bax-A has a molecular mass consistent with a Bax-Bak heterodimer, whereas Bax-B is much larger and may represent Bax and Bak oligomers or Bax-Bak heterodimers in a complex with other proteins. Taken together with the co-immunoprecipitation of Bak and Bak shown in Fig. 1, this suggests that death signaling by TNF/CHX induces the formation of a high molecular weight oligomeric complex of Bax and Bak which is prevented by E1B 19K expression.

To further study the Bax-Bak heterodimers, Bak was immu-
norpncipitated from cross-linked lysates prepared from mock infected and wild-type adenovirus Ad5dl309-infected cells treated or untreated with TNF/CHX. When the Bak immunoprecipitates were subjected to Western blotting with an anti-Bax antibody, monomeric Bax was specifically co-immunoprecipitated with Bak only from cells treated with TNF/CHX as in Fig. 1. Interestingly, the Bax-Bax cross-linked dimer induced by TNF-α treatment, shown in Fig. 3A, also specifically co-immunoprecipitated with Bak (Fig. 3B). Thus Bak was found complexed with both Bax monomers and dimers, the formation of which was induced by TNF-α death signaling. Finally, E1B 19K expression in wild-type adenovirus-infected cells prevented Bak-Bak and Bak-Bax dimer interactions (Fig. 3B). These results support a model whereby TNF-α death signaling induces Bax-Bak complex formation and oligomerization which is inhibited by E1B 19K-Bax and E1B 19K-Bak binding.

Since E1B 19K interacted with Bak constitutively and with Bak upon TNF/CHX treatment, and E1B 19K blocked the Bax-Bak interaction, we investigated the E1B 19K-Bax and E1B 19K-Bak interaction by chemical cross-linking. While we have been unable to cross-link E1B 19K and Bax with disuccinimidyl suberate or BMH under any conditions (10) (data not shown), a novel Bak cross-linked complex Bak-A was detected in BMH-treated infected cells (Fig. 3C). Bak-A was present in infected cells in the absence or presence of TNF/CHX, and Western blotting for E1B 19K revealed that Bak-A corresponded to a prominent E1B 19K immunoreactive band (Fig. 3C). Thus, Bak can be cross-linked to Bax only in TNF/CHX-treated cells, whereas E1B 19K can be cross-linked to Bak, but not Bax, in the absence or presence of TNF/CHX. By binding to Bak, E1B 19K may prevent both Bak binding to Bax, and Bax and Bak co-oligomerization to form a pore to release mitochondrial components that signal caspase-9 activation.

**E1B 19K Blocks TNF-α-mediated Cytochrome c and Smac/DIABLO Release from Mitochondria**—HeLa cells infected with the wild-type Ad5dl309, the E1B 19K deletion mutant Ad5dl337, or mock infected, were treated with TNF/CHX or CHX alone and analyzed by subcellular fractionation. As reported previously (7), TNF/CHX treatment of mock or Ad5dl337-infected cells resulted in a marked increase in the amount of cytochrome c in the cytosolic fraction, as compared with mock or Ad5dl337-infected, CHX-treated cells (Fig. 4A). A corresponding increase in the cytosolic levels of Smac/DIABLO also occurred under the same conditions (Fig. 4A). However, when cells infected with Ad5dl309 were treated with TNF/CHX, there was a significant reduction in the amounts of both cytochrome c and Smac/DIABLO released into the cytosol (Fig. 4A). These results demonstrated that E1B 19K does indeed block the release of both cytochrome c and Smac/DIABLO from mitochondria.

The amount of Smac/DIABLO in the total extracts was reduced under conditions where it is released from mitochondria (Fig. 4A). This reduction was abrogated by Ad5dl309 infection, suggesting that Smac/DIABLO may be degraded upon release, and that by inhibiting Smac/DIABLO release the E1B 19K protein may prevent its degradation (Fig. 4A). Thus, the degradation of Smac/DIABLO may be an indicator of its release from mitochondria.

To corroborate that TNF/CHX promoted cytochrome c and Smac/DIABLO release that was inhibited by E1B 19K expression, we performed indirect immunofluorescence with antibodies to either cytochrome c or Smac/DIABLO on mock, Ad5dl337-, or Ad5dl309-infected HeLa cells treated with TNF/CHX or CHX alone. We observed only mitochondrial staining for cytochrome c in infected or mock infected cells that were treated with CHX alone (Fig. 4B). Likewise, cells infected with Ad5dl309 and treated with TNF/CHX exhibited mitochondrial localization of cytochrome c (Fig. 4B). In contrast, treatment of mock or Ad5dl337-infected cells with TNF/CHX resulted in the presence of flat cells with weak and diffuse cytochrome c staining throughout the cell, a pattern consistent with its release from mitochondria (Fig. 4B).

The localization of Smac/DIABLO was examined under the same conditions described above. By indirect immunofluorescence, Smac/DIABLO appeared to be released from mitochondria in both mock-infected, TNF/CHX-treated cells, and in Ad5dl337-infected, TNF/CHX-treated cells, as evidenced by diffuse staining throughout the cell (Fig. 4C). In contrast, infection with Ad5dl309 caused cells to retain a mitochondrial staining pattern for Smac/DIABLO when treated with TNF/CHX (Fig. 4C).

To quantify these observations, we counted cells in each condition, distinguishing the cells exhibiting release from those that appear intact. We were unable to score the Ad5dl337, TNF/CHX-treated cells, due to the extremely low number of viable cells remaining on the coverslips under that condition. Of the mock-infected, TNF/CHX-treated cells, ~7% were scored as having released cytochrome c 4 h post-treatment. In the Ad5dl309-infected TNF/CHX-treated cells, only 2.5% showed cytochrome c release (Fig. 4D). These observations agree with our earlier report, in which E1B-19K expression blocks the cytosolic redistribution of cytochrome c (7).

When intact and Smac/DIABLO-released cells were counted, ~9% of mock infected TNF/CHX-treated cells were scored as having cytosolic staining 4 h post-treatment. In contrast, less than 4% of Ad5dl309-infected, TNF/CHX-treated cells exhibited release of Smac/DIABLO (Fig. 4D). Thus, E1B 19K blocked the mitochondrial release of Smac/DIABLO as well as that of cytochrome c, suggesting that the two events may have a common mechanism of action.

**DISCUSSION**

**TNF-α Induces Oligomerization of Both Bak and Bax—TNF-α death signaling activates caspase-8 to cleave Bid to tBid, and tBid interacts with Bax and Bak. Evidence suggests that tBid acts by a hit-and-run mechanism to alter the conformation of the Bak and Bak amino termini as revealed by exposure of otherwise buried epitopes (7, 8). Bax undergoes a second and distinct conformational change near the carboxyl terminus (10), but whether a similar change in the conformation of the Bak carboxyl terminus also occurs has not been established. While tBid is sufficient to induce the amino-termini conformational changes in Bax and Bak, it is not clear if the Bax carboxyl-terminal change results directly from tBid-Bak binding or if it is mediated by another protein interaction. Conformational changes in Bax and probably Bak occur in amphipathic helices that surround two central helices, in a fashion analogous to the mechanism for membrane insertion and oligomerization proposed for bacterial toxin pore forming domains (21, 24). Bax oligomerizes into a 500-kDa complex (10) that lacks tBid (7), but contains Bak, by gel filtration chromatography, co-immunoprecipitation, and chemical cross-linking. Bak also oligomerizes into a complex that contains Bax. Indeed, both Bak monomers and Bax-Bak cross-linked dimers co-immunoprecipitate with Bak. Bak and Bak, however, appear to be minor components of each others oligomers, which raises the possibility that three types of complexes may form: Bax, Bax plus Bak, and Bak oligomers.

**Bak and Bax May Independently Signal Cell Death or Function Cooperatively**—TNF-α death signaling may be unique in that the signal propagator, tBid, interacts with both Bax and Bak (6). This may result in simultaneous activation of both, and perhaps functional cooperation to facilitate pore formation.
FIG. 4. E1B 19K expression during adenovirus infection inhibits TNF-α-dependent release of cytochrome c and Smac/DIABLO from mitochondria. HeLa cells were mock, Ad5dl309, or Ad5dl337 infected for 24 h and then untreated or treated with TNF/CHX for 4 h. A, subcellular fractionation of cells as indicated into total (T), mitochondrial/heavy membrane (M), and cytosolic (C) fractions, prepared as described under “Experimental Procedures.” Fractions were analyzed by Western blotting for cytochrome c, Smac/DIABLO, and the mitochondrial marker COXII. B, localization of cytochrome c; and C, Smac/DIABLO by indirect immunofluorescence in HeLa cells infected and treated as in A. D, percentage of HeLa cells scored for cytosolic staining of cytochrome c and Smac/DIABLO.
Studies with Bax, Bak, and Bax plus Bak-deficient animals, and cells derived from them, have been illuminating in that regard. Deficiency of either Bax or Bak does not prevent apoptosis induction by TNF-α, but deficiency of both renders cells resistant to TNF-α-mediated apoptosis.2 Similar results have been observed for apoptosis induction by Fas stimulation which similarly activates caspase-8 to cleave Bid to tBid (36). Bax or Bak-deficient cells are capable of releasing cytochrome c, but those deficient for both do not (36).2 This suggests that Bax and Bak can function independently to release cytochrome c from mitochondria. Indeed, mice deficient for both Bax and Bak die neonatally and suffer more profound developmental defects than those animals deficient for only Bax or Bak (37). Bak-deficient cells, however, are retarded in their ability to implement TNF-α-mediated apoptosis relative to Bax deficient or wild-type cells.2 Thus Bax function is not equivalent to Bak function in vivo. One explanation is that although Bax and Bak may be independently capable of pore formation, Bax may require Bak for optimal pore formation. Alternatively Bax and Bak may form different kinds of pores separately and together. The nature of the pore formed may depend on whether Bax or Bak or both are activated by upstream signaling events. As TNF-α activates both Bax and Bak, cooperative pore formation may yield a more stable, rapidly forming, or larger pore to facilitate cytochrome c release, than either Bax or Bak alone could produce.

**E1B 19K Interacts with Both Bak and Bax**—The dependence of TNF-α death signaling on either Bax or Bak explains why the E1B 19K protein interacts with and inhibits both (Fig. 5). Based on the studies of the Bax and Bak-deficient cells, if E1B 19K interacted only with Bax, then Bak would still be available to promote cytochrome c release and apoptosis by TNF-α. The constitutive interaction between Bak and E1B 19K and the TNF-α-inducible interaction between Bax and E1B 19K raises some interesting issues. Bak in healthy HeLa cells is apparently not in a conformation that will permit E1B 19K binding (7, 10). Since Bax BH3 is necessary and sufficient for E1B 19K binding to Bax (11, 38), Bax BH3 may not be in a configuration in the absence of a death stimulus to permit E1B 19K binding. Once tBid is bound to Bax and has altered the conformation of the Bax amino terminus adjacent to BH3, this may expose the E1B 19K-binding domain to permit Bax-E1B 19K complex formation. Bak may already be in a conformation competent to bind E1B 19K prior to the presence of tBid. Alternatively, Bax may bind E1B 19K through Bak through the formation of a E1B 19K-Bak-Bax ternary complex (Fig. 5). Indeed, E1B 19K was cross-linked to Bak but not to Bax. Since TNF-α induces E1B 19K-Bax and also Bak-Bax interaction by co-immunoprecipitation, and Bax, Bak, and E1B 19K are all capable of binding to each other, this has been difficult to resolve. E1B 19K interaction with Bak and Bax has a profound inhibitory effect on Bax-Bak complex formation and on the generation of Bak and Bak oligomers. This conclusion is supported by gel filtration chromatography, co-immunoprecipitation, and chemical cross-linking of protein complexes. Since E1B 19K is a potent inhibitor of Bax and Bak proapoptotic function, this suggests that Bax-Bak interaction and the oligomerized forms of Bak and Bax are the effectors of cytochrome c and Smac/DIABLO release from mitochondria that propagate the apoptotic signal. It will be interesting to determine whether E1B 19K-Bax and/or -Bak interaction is also responsible for inhibition of p53-dependent apoptosis during transformation, or E1A-mediated apoptosis during productive viral infection.

**Fig. 5. Model for inhibition of Bax and Bak complex formation and apoptosis by E1B 19K. Asterisks indicate changes in protein conformation. See text for explanation.**
Tumor Necrosis Factor-α Induces Bax-Bak Interaction and Apoptosis, Which Is Inhibited by Adenovirus E1B 19K
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