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E1A Sensitizes Cells to Tumor Necrosis Factor Alpha by Downregulating c-FLIP$_S$

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Tumor necrosis factor alpha (TNF-α) activates both apoptosis and NF-κB-dependent survival pathways, the former of which requires inhibition of gene expression to be manifested. *c-FLIP* is a TNF-α-induced gene that inhibits caspase-8 activation during TNF-α signaling. Adenovirus infection and E1A expression sensitize cells to TNF-α by allowing apoptosis in the absence of inhibitors of gene expression, suggesting that it may be disabling a survival signaling pathway. E1A promoted TNF-α-mediated activation of caspase-8, suggesting that sensitivity was occurring at the level of the death-inducing signaling complex. Furthermore, E1A expression downregulated c-FLIP$_S$ expression and prevented its induction by TNF-α. c-FLIP$_S$ and viral FLIP expression rescued E1A-mediated sensitization to TNF-α by restoring the resistance of caspase-8 to activation, thereby preventing cell death. E1A inhibited TNF-α-dependent induction of c-FLIP$_S$ mRNA and stimulated ubiquitination- and proteasome-dependent degradation of c-FLIP$_S$ protein. Since elevated c-FLIP levels confer resistance to apoptosis and promote tumorigenicity, interference with its induction by NF-κB and stimulation of its destruction in the proteasome may provide novel therapeutic approaches for facilitating the elimination of apoptosis-refractory tumor cells.

Viral infection elicits a variety of host responses that enable the infected cell to induce programmed cell death or apoptosis, which can limit or alter the course of a productive infection (37). It is an intrinsic apoptotic cellular response coupled with activation of the immune surveillance mechanisms that facilitates elimination of virally infected cells. Expression of the adenovirus *E1A* gene triggers these proapoptotic host cell responses during adenovirus infection as a consequence of its cell cycle deregulation functions, which are required for replication of viral DNA (52).

E1A is alternatively spliced into several transcripts, but the 12S transcript alone is sufficient for both induction of proliferation and apoptosis in the host cell (52). The amino terminus of the 12S transcript contains two highly conserved regions that are binding sites for the transcriptional coactivator p300 and the retinoblastoma tumor suppressor protein (Rb), which activate both the proliferative and apoptotic functions of E1A through intrinsic pathways (52). Thus, the intrinsic apoptotic pathway may be a consequence of cell cycle deregulation by E1A.

In addition to the activation of an intrinsic apoptotic pathway, the host activates an extrinsic apoptotic pathway by mounting an immune response, which triggers the release of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) (55). TNF-α binds to its cognate receptors TNF receptors 1 and 2 (TNFR-1 and TNFR-2) and activates both apoptosis and an NF-κB-mediated survival pathway. TNFR-1 signals apoptosis through the recruitment of the adaptor molecules TRADD and FADD and procaspase-8 to the death-inducing signaling complex (DISC), which facilitates autocatalytic activation of procaspase-8 (48). Active caspase-8 facilitates Bid cleavage into tBID (25), which in turn promotes a conformational change in the mitochondrial proapoptotic proteins Bax and Bak (33, 44, 51). Activation of either Bax or Bak triggers cytochrome *c* release, which promotes the processing of downstream effector caspases that aid in the dismantling of the cell (7, 50).

TNF-α signals both an NF-κB-dependent survival and an apoptotic pathway. The outcome of survival over cell death is dependent upon NF-κB-mediated transcriptional induction of antiapoptosis genes (1, 31). Activation of the NF-κB-mediated survival pathway is initiated through ubiquitin-dependent degradation of phosphorylated IκB, which promotes the release and translocation of NF-κB into the nucleus. Consequently, NF-κB transcriptionally activates antiapoptotic genes that function at the DISC as well as at the mitochondria. IAP-1, IAP-2, c-FLIP, TRAF1, and TRAF2 coordinate their inhibitory effects to hinder receptor-mediated death at the DISC. IAP-1 and IAP-2 can interact with caspases to block their activation directly and may facilitate their degradation (8, 15, 36, 39, 56). Moreover, TRAF2, a putative substrate for IAP-1-dependent degradation, has been reported to augment NF-κB activation (19, 26).

In addition, cellular FLICE-inhibitory protein (c-FLIP, Casper, FLAME-1, I-FLICE, CASH, CLARP, MRIT, and Usurpin), the cellular homologue of viral FLIPs, has been shown to block TNF-α-mediated apoptosis by preventing autocatalytic activation of procaspase-8 at the DISC (23, 28, 40, 46). NF-κB also transactivates the antiapoptotic genes *Bfl-1/A1* and *Bcl-x$_L$* which act at the mitochondria to prevent cytochrome *c* release (4, 58). Thus, NF-κB induces multiple independent and likely redundant mechanisms for disabling apo-

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ptotic signaling. As a consequence of NF-κB activity, most cell types require inhibition of gene expression, such as the protein synthesis inhibitor cycloheximide, in conjunction with TNF-α to induce receptor-mediated apoptosis.

Induction of the apoptotic pathway by TNF-α alone, however, occurs in cells derived from tumors or cells infected by adenovirus. This sensitization of cells to TNF-α is a function of E1A in adenovirus-infected cells, and E1A expression alone in cell lines is sufficient for this activity (10, 38, 42). The molecular mechanism for this sensitization has not been elucidated, but E1A expression is sufficient to sensitize cells to apoptosis mediated not only by TNF-α but also by TNF-related apoptosis-inducing ligand (TRAIL) (5, 10, 38, 42). Moreover, p300 and Rb binding to E1A is required to confer this sensitization phenotype on adenovirus-infected cells (42).

Here we report that E1A expression confers susceptibility to TNF-α-mediated apoptosis by preventing c-FLIP$_S$ mRNA induction and promoting ubiquitination and proteasome-mediated degradation of c-FLIP$_S$ protein. This multilevel regulatory mechanism for inhibiting c-FLIP$_S$ expression facilitates E1A-mediated, TNF-α-dependent caspase-8 activation in the DISC. Thus, the ability of E1A to modulate c-FLIP$_S$ levels, and thereby function, suggests a novel target for regulating the sensitivity of tumor cells to apoptotic stimuli.

MATERIALS AND METHODS

Antibodies. The following antibodies were used: anti-caspase-8 polyclonal antibody (Pharmingen, San Diego, Calif.); anti-adenovirus 2 E1A (clone M73) and antiactin (Ab-1) monoclonal antibodies (Calbiochem-Novabiochem Corp., San Diego, Calif.); anti-c-FLIP$_L$ polyclonal antibody (CT) generated against amino acids 447 to 464 and anti-c-FLIP monoclonal antibody (clone Dave-2) generated against full-length c-FLIP (Alexis Corp., San Diego, Calif.); anti-c-FLIP mouse monoclonal antibody NF6 generated against amino acids 1 to 194 (Peter H. Krammer, German Cancer Research Center, Heidelberg, Germany); anti-IκBα and anti-TRAF2 polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.); anti-HA.11 polyclonal antibody (BabCo, Richmond, Calif.); anti-FADD and anti-RIP monoclonal antibodies (Transduction Laboratories, San Diego, Calif.); and anti-IAP-1 and anti-IAP-2 polyclonal antibodies (Trevigen Inc., Gaithersburg, Md.).

Plasmids. pcDNA3-HA-tagged MC159 was generated by digesting hemagglutinin (HA)-tagged MC159 from pCi-HA-tagged MC159, generously provided by Michael Lenardo (National Institutes of Health, Bethesda, Md.) at the *Eco*RI and *Xba*I restriction sites. The gene was subsequently ligated into the pcDNA3 expression vector (Invitrogen, San Diego, Calif.). The cytomegalovirus-driven pBlueScriptSK$^-$ HA-tagged ubiquitin expression plasmid was generously provided by Peter Howley (Harvard Medical School, Boston, Mass.). The cytomegalovirus-driven pc-DNA3-c-FLIP$_S$ expression plasmid was described previously (21) and generously provided by Peter Krammer (German Cancer Research Center, Heidelberg, Germany).

Cell lines and adenovirus infection. HeLa cells were electroporated with 1 μg of linearized pcDNA3 vector control or pcDNA3-HA-tagged MC159. At 48 h posttransfection, neomycin-resistant cells were selected and maintained in Dulbecco's modified Eagle's medium containing 1.25 mg of G418 (Gibco-BRL, Carlsbad, Calif.) per ml. Ad5*dl*309 has a deletion in the *E3* gene and was used as the wild-type virus (18). Ad5*dl*337 was derived from Ad5*dl*309 and has a deletion in the *E1B 19K* gene (35). Ad5*dl*309 and Ad5*dl*337 were obtained from T. Shenk (Princeton University, Princeton, N.J.). 12S E1B$^-$ viruses contain a missense mutation in the 12S *E1A* gene and a deletion in the *E3* and *E1B* genes and were previously described (6). Ad5PAC3 was derived from Ad5*dl*309 and has the *E1A* and *E1B* genes deleted (6). HeLa cells were infected as previously described (6).

TNF-α apoptosis assay and Western blotting. Subsequent to infection, HeLa cells were treated with 1,000 U of TNF-α (Boehringer Mannheim, Indianapolis, Ind.) and/or 30 μg of cycloheximide (Sigma, St. Louis, Mo.) per ml, as indicated. Cell viability was assessed by trypan blue dye exclusion (53). In conjunction, whole-cell extracts were generated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and semidry blotted onto polyvinylidene fluoride or nitrocellulose (c-FLIP$_S$ analysis) membranes (Schleicher & Schuell, Keene, N.H.) as previously described (32). Extracts were immunoblotted with antiactin antibody to confirm equal protein loading. In Fig. 3C and D, HeLa cells were transiently transfected with 10 μg of pcDNA3 or pcDNA3-c-FLIP$_S$ for 24 h and subsequently infected and treated with TNF-α as indicated in the legend.

Taqman real-time quantitative RT-PCR. Taqman reverse transcription (RT)-PCR was performed on the ABI Prism 7700 sequence detector (PE Applied Biosystems, Foster City, Calif.). Primers and probes were designed for c-FLIP$_L$, c-FLIP$_S$, IAP-2, and Iκ-Bα through the use of Primer Express version 1.5 (Applied Biosystems). c-FLIP$_L$, c-FLIP$_S$, IAP-2, and IκBα probes were labeled with the 5′ fluorescent reporter dye 6-carboxy-fluorescein (FAM) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with JOE (2,7-dimethoxy-4,5-dichloro-6-carboxy-fluorescein). All reporter dyes were quenched with the 3′ quencher 6-carboxy-tetramethylrhodamine (TAMRA).

The following primer and probe sequences were used. For c-FLIP$_L$, the primers were 5′-TTGGCCAATTTGCCTGTATG-3′ and 5′-TCGGCTCACCAGGACACA-3′ and the probe was 6FAM-CGAGCACCGAGACTACGACAGCTTTGT-TAMRA. For c-FLIP$_S$, the primers were 5′-GCAGGGACAAGTTACAGGAATGT-3′ and 5′-GGACAATGGGCATAGGGTGT-3′ or 5′-CAGTCTGTTCAAGGAGCAGGG-3′ and 5′-TTTCAGATCAGGACAATGGGC-3′ and the probe was 6FAM-CTCCAAGCAGCAATCCAAAAGAGTCTCAAG-TAMRA. For IAP-2, the primers were 5′-GGACTCAGGTGTTGGGAATCTG-3′ and 5′-CAAGTACTCACACCTTGGAAACCA-3′ and the probe was 6FAM-AGATGATCCATGGGTTCAACATGCCAA-TAMRA. For IκBα, the primers were 5′-AATGGCCGGACTGCCC-3′ and 5′-CAGGAGTGACACCAGGTCAGG-3′ and the probe was 6FAM-TCACCTCGCAGTGGACCTGCAAAA-TAMRA.

GAPDH control primers and probe were obtained from Applied Biosystems. RNA was obtained from mock- or adenovirus-infected HeLa cells treated with 2,000 U of TNF-α per ml for 4 h. Total RNA was isolated with an RNeasy mini kit (Qiagen, Valencia, Calif.), and contaminating DNA was digested with the RNase-free DNase kit (Qiagen, Valencia, Calif.).

A total of 100 ng of RNA sample was reverse-transcribed, and real-time PCR was performed according to the directions provided by the Taqman EZ-RT PCR kit (PE Applied Biosystems, Foster City, Calif.). RT-PCR of c-FLIP$_S$ was performed with 5 mM manganese acetate. All reactions were done in triplicate. The threshold cycle represents the cycle at which an increase in fluorescence was statistically significant. The difference in one cycle value represents a twofold difference in message levels. The induction or repression of mRNA levels in each sample was determined from the change in fluorescence relative to that of the untreated mock-infected samples. Subsequently, the untreated mock-infected samples were normalized to zero, and the change in mRNA levels was graphed.

Proteasome inhibition and ubiquitination assay. In Fig. 5A, HeLa cells were infected as indicated, and at 8 h postinfection, the cells were incubated in 200 nM or 500 nM epoxomicin (Alexis Corp., San Diego, Calif.) solubilized in dimethyl sulfoxide or dimethyl sulfoxide alone for 12 h and subsequently treated with TNF-α for 10 h. Whole-cell extracts were prepared, analyzed on SDS-PAGE, and subjected to Western blot analysis. In Fig. 5B, HeLa cells were infected as indicated, and at 8 h postinfection, cells were incubated in 200 nM epoxomicin for 12 h, and whole-cell extracts prepared. In Fig. 5D, HeLa cells were infected as indicated for 42 h, and whole-cell extracts were prepared, analyzed on SDS-PAGE, and subjected to Western blot analysis with anti-c-FLIP and antiactin antibodies.

In Fig. 5E, HeLa cells were electroporated with 20 μg of pcDNA3, 10 μg of pBlueScriptSK$^-$ HA-tagged ubiquitin and/or 10 μg of pcDNA3-c-FLIP$_S$ expression plasmid, as indicated in the legend. The total amount of DNA was kept constant at 20 μg by the addition of pcDNA3 vector. At 24 h posttransfection, cells were infected as indicated for 16 h and treated with 200 nM epoxomicin for 8 h. Whole-cell extracts were prepared for Western blot analysis. In Fig. 6A and B, HeLa cells were incubated with 200 nM epoxomicin for 12 h and subsequently treated with TNF/cycloheximide or cycloheximide alone for 10 h. Whole-cell extracts were prepared for Western blot analysis, and cell viability was assessed by trypan blue exclusion.

RESULTS

E1A expression during adenovirus infection promotes caspase-8 activation by TNF-α. It has been well established that E1A expression induces cells to become susceptible to apoptosis induced by TNF-α (5, 10, 42). Although many studies with wild-type adenovirus and various adenovirus mutants

have demonstrated that E1A sensitizes cells to TNF-α-mediated apoptosis (33, 42, 54), the precise mechanism has not been elucidated. Since many of the components of death receptor signaling pathways have been identified, we investigated whether alterations in the levels of DISC components occurred in response to adenovirus infection and E1A expression.

The wild-type adenovirus Ad5*dl*309, which has a deletion within the *E3* gene but expresses the antiapoptotic adenovirus Bcl-2 homologue E1B 19K, blocks TNF-α death signaling downstream of the DISC at Bax and Bak in mitochondria (33, 43, 44). The Ad5*dl*337 mutant virus was derived from Ad5*dl*309 and has the *E1B 19K* gene deleted (35). Infection with Ad5*dl*337 induces cells to undergo apoptosis upon E1A expression and allows all TNF-α-mediated events upstream and downstream of mitochondria to proceed normally.

To identify the TNF-α-mediated signaling events that were altered by E1A expression, HeLa cells were infected with Ad5*dl*309 or Ad5*dl*337 or mock infected and either left untreated or treated with TNF-α for 4 and 12 h. No procaspase-8 processing was observed in mock- or virus-infected cells in the absence of TNF-α (Fig. 1A). Whereas mock-infected cells treated with TNF-α were unaffected, procaspase-8 levels were reduced at 4 h and disappeared at 12 h post-TNF-α treatment in cells infected with adenovirus (Fig. 1A). Concurrent with the reduction in procaspase-8, a cleaved intermediate p43 formed, and the p24 prodomain of caspase-8 appeared 4 and 12 h post-TNF-α treatment in adenovirus-infected cells (Fig. 1A). These results are TNF-α dependent, since adenovirus infection alone does not induce caspase-8 activation.

In conjunction with procaspase-8 processing, we observed cleavage of a direct substrate of caspase-8, Bid, during TNF-α treatment and adenovirus infection (33). These results demonstrate that adenovirus infection sensitizes cells to TNF-α by promoting the processing and activation of procaspase-8 by TNF-α, despite the absence of a protein synthesis inhibitor. Furthermore, the facilitation of caspase-8 activation by E1A and TNF-α indicates that E1A is acting at the DISC to sensitize cells to TNF-α, and other possible mechanisms of sensitization that would function downstream of the DISC, at the mitochondria, for example, can be excluded.

TNF-α-mediated caspase-8 activation cosegregates with Rb and p300 binding to E1A. E1A binding to both Rb and p300 has been linked to sensitization to TNF-α-mediated apoptosis (42). Therefore, we wanted to examine whether point mutations within the Rb and p300 binding sites of E1A would disrupt the ability of E1A to promote TNF-α-mediated activation of caspase-8. HeLa cells were infected with viruses that expressed either wild-type E1A or specific E1A missense mutations that disrupt Rb and/or p300 binding in the background of an *E3* and *E1B* gene deletion (6) and were either left untreated or treated with TNF-α for 10 h.

In contrast to mock-infected cells, Ad5*dl*309 or Ad5*dl*337 infection enabled caspase-8 activation by TNF-α (Fig. 1B). However, cells infected with Ad5*dl*337 alone were slightly apoptotic because this virus lacks the viral Bcl-2 homologue, the *E1B 19K* survival gene. This facilitation of TNF-α-dependent caspase-8 activation was a function of E1A, in that a mutant virus with E1A and E1B deleted, Ad5PAC3, showed little procaspase-8 processing by TNF-α (Fig. 1B). As expected, infection with the 12S E1B$^-$ virus, which expresses the 12S

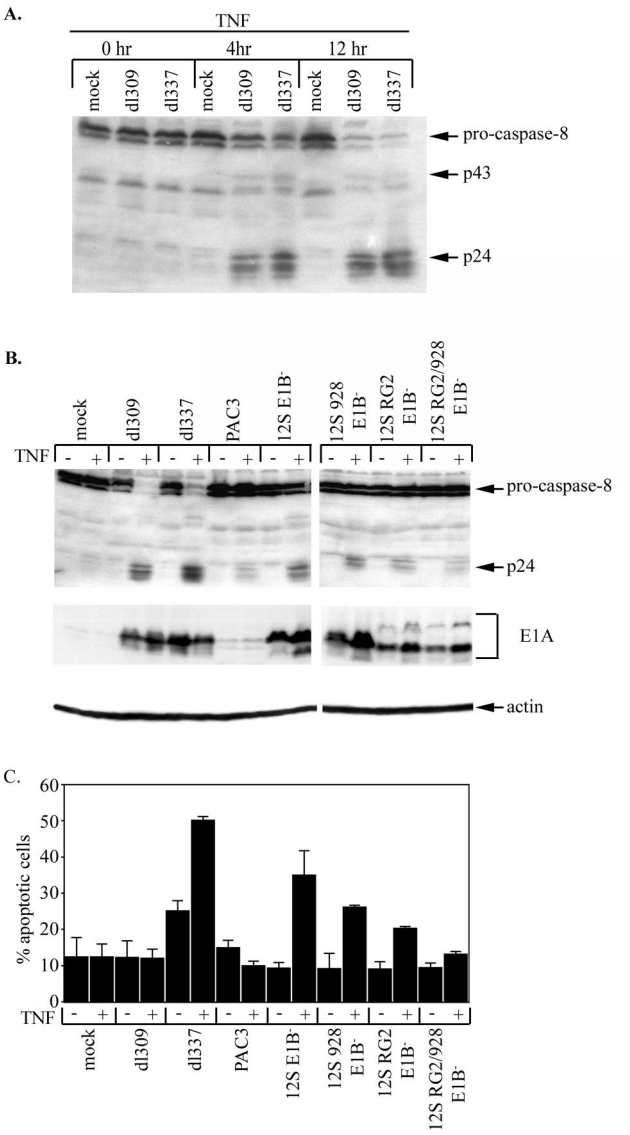

FIG. 1. E1A promotes caspase-8 activation by TNF-α, and its function is dependent upon Rb and p300 binding. (A) HeLa cells were mock, Ad5*dl*309, or Ad5*dl*337 infected for 24 h and treated with TNF-α for 0, 4, or 12 h. Whole-cell extracts were analyzed by immunoblotting with an anti-caspase-8 antibody. The positions of procaspase-8 and the processed p43 and p24 forms are indicated, (B) HeLa cells infected with adenoviruses containing Rb (928) and/or p300 (RG2) binding point mutations within E1A and treated with TNF-α for 10 h were analyzed by immunoblotting with anti-caspase-8, anti-E1A, and antiactin antibodies. (C) HeLa cells were infected and treated with TNF-α as in the experiments shown in panel B. Viability was assessed by the trypan blue dye exclusion assay.

E1A gene product and has E1B deleted (6), promoted caspase-8 activation in response to TNF-α (Fig. 1B). Cells infected with 12S E1B$^-$-expressing viruses that contain missense mutations in either the Rb (12S 928 E1B$^-$) or the p300 (12S RG2 E1B$^-$) binding site showed a decrease in caspase-8 activation compared to 12S E1B$^-$-infected cells (Fig. 1B). A substantial decrease in procaspase-8 processing, similar to that seen in mock-infected cells, was observed in cells infected with an Rb and p300 binding site double mutant virus (12S 928/

RG2 E1B$^-$) (Fig. 1B). E1A levels were very high in cells infected with all the viruses, as expected for a typical productive infection (Fig. 1B). E1A is observed as multiple bands because it is alternatively spliced and posttranslationally modified. These observations indicate that disruption of either the Rb or p300 binding site on E1A caused a significant reduction in caspase-8 processing and that both interactions are required for abolition of TNF-α-mediated caspase-8 activation.

Since disruption of Rb and p300 binding to E1A altered caspase-8 activation, we determined if abolition of binding was sufficient to block the susceptibility of cells to TNF-α-mediated apoptosis. HeLa cells were infected and treated as stated above, and viability was assessed by trypan blue exclusion. During TNF-α signaling, approximately 50% of Ad5*dl*337-infected cells underwent apoptosis, compared to only approximately 10% of mock- and Ad5*dl*309-infected cells (Fig. 1C). No loss in viability was detected in the virus with E1A and E1B deleted (Ad5PAC3) treated with TNF-α, whereas 12S E1B$^-$-infected cells underwent TNF-α-mediated apoptosis (Fig. 1C). The Rb (12S 928 E1B$^-$) and p300 (12S RG2 E1B$^-$) E1A binding mutants displayed a decrease in apoptosis, and complete abolition of apoptosis was detected in the Rb and p300 binding double E1A mutant (12S 928/RG2 E1B$^-$) during TNF-α signaling (Fig. 1C). Thus, binding of both p300 and Rb to E1A contributes to the enablement of caspase-8 activation by TNF-α and to the sensitization of cells to TNF-α-mediated apoptosis. However, we cannot rule out the possibility that other E1A binding proteins may contribute to the sensitization of cells to TNF-α.

Adenovirus infection downregulates c-FLIP$_S$ protein expression. Since adenovirus infection promotes caspase-8 activation during TNF-α signaling, we investigated whether inhibitory proteins that block caspase-8 activation at the DISC are differentially regulated in response to E1A expression. FLIPs were discovered in viruses, and viral FLIPS (v-FLIPs) contain two death effector domains. Death effector domains normally mediate an interaction with Fas-associated death domain and procaspase-8 that is required for multimerization and activation of caspase-8. By virtue of containing death effector domains, v-FLIPs interfere with normal procaspase-8 recruitment to the DISC, thereby preventing autocatalytic processing of procaspase-8, which inhibits death receptor-mediated cell death (2, 14, 45).

A cellular homologue of v-FLIPs, *c-FLIP* is an NF-κB-regulated gene with multiple mRNA splice variants, from which two distinct isoforms, long and short (c-FLIP$_L$ and c-FLIP$_S$), are expressed in cells (23, 28, 46). c-FLIP$_L$ resembles procaspase-8, with two death effector domains at its amino terminus and an inactive carboxy-terminal catalytic caspase domain. c-FLIP$_S$ is composed of only the death effector domains, thereby structurally resembling v-FLIP (46). Both c-FLIP$_L$ and c-FLIP$_S$ are recruited to the DISC upon death receptor activation and inhibit caspase-8 activation (24, 40) and prevent cell death in cultured cells (23, 28, 40) and in vivo (9, 27). However, for the regulation of lymphocyte homeostasis, it is the specific upregulation of c-FLIP$_S$ that confers resistance to CD95-mediated apoptosis in activated T cells (21).

Since c-FLIP is an inhibitor of caspase-8 activation and c-FLIP-deficient cells are highly sensitive to TNF-α alone (57), we tested whether c-FLIP protein levels were affected by E1A

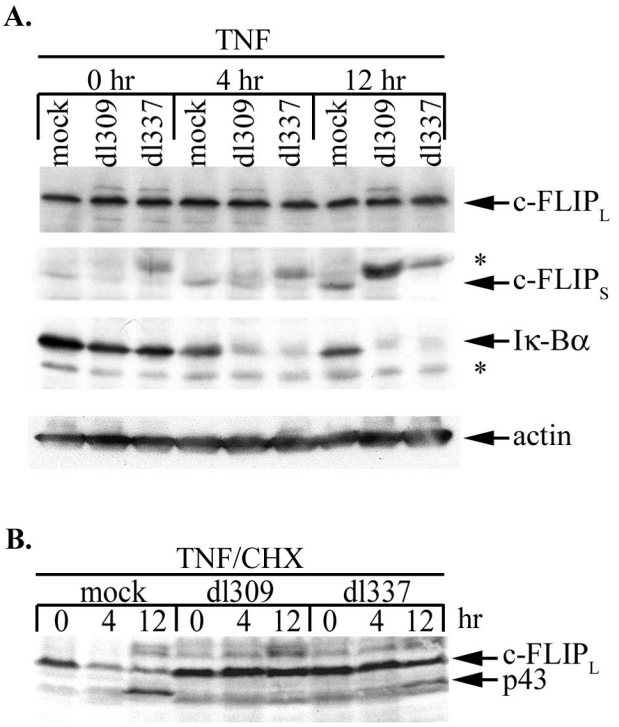

FIG. 2. Adenovirus infection prevents c-FLIP$_S$ accumulation during TNF-α signaling. (A) HeLa cells were mock, Ad5*dl*309, or Ad5*dl*337 infected for 24 h and treated with TNF-α for 0, 4, or 12 h. Whole-cell extracts were immunoblotted with anti-c-FLIP$_L$ polyclonal (CT) (c-FLIP$_L$), anti-c-FLIP monoclonal (Dave-2) (c-FLIP$_S$), anti-IκBα, and antiactin antibodies. The band below IκBα represents a nonspecific cross-reactive band (asterisk). (B) HeLa cells were mock, Ad5*dl*309, or Ad5*dl*337 infected for 24 h and treated with TNF/cycloheximide for 0, 4, or 12 h. Whole-cell extracts were immunoblotted with an anti-c-FLIP (Dave-2) monoclonal antibody.

expression during adenovirus infection. HeLa cells were either mock infected or infected with Ad5*dl*309 or Ad5*dl*337 and treated with TNF-α. No changes in c-FLIP$_L$ protein levels were detected in TNF-α-treated and/or infected cells immunoblotted with both an anti-c-FLIP$_L$ polyclonal (Fig. 2A) and the anti-c-FLIP monoclonal (Dave-2) antibodies (data not shown). However, c-FLIP$_S$ was induced by TNF-α. Also, adenovirus infection alone promoted the downregulation of c-FLIP$_S$ protein levels and prevented the induction of c-FLIP$_S$ by TNF-α (Fig. 2A). The band above c-FLIP$_S$ in adenovirus-infected cells (asterisk) may represent a cross-reaction with a viral protein or a modified form of c-FLIP$_S$. Thus, TNF-α induced the specific upregulation of c-FLIP$_S$ without affecting the levels of c-FLIP$_L$. Furthermore, adenovirus infection alone both specifically promoted the downregulation of constitutive c-FLIP$_S$ expression and also blocked TNF-α-dependent accumulation of c-FLIP$_S$.

Since overexpression of c-FLIP$_L$ allows partial cleavage of procaspase-8 into the p43 subunit while high levels of c-FLIP$_S$ completely abolish this cleavage event (24), our results are consistent with the preferential downregulation of c-FLIP$_S$ by E1A's promoting caspase-8 activation (Fig. 1A). Thus, c-FLIP$_S$ and not c-FLIP$_L$ may be the significant regulator of procaspase-8 processing for TNF-α-mediated sensitization.

Stable E1A expression in cell lines has been reported to inhibit IκBα phosphorylation, which normally targets degradation of IκBα, thereby inducing NF-κB activation (41). This possible mechanism by which E1A could potentially inhibit survival signaling by NF-κB does not occur in virus-infected cells, as adenovirus infection alone activates NF-κB DNA-binding activity (P. Sabbatini and E. White, unpublished observations). Consistent with this observation, adenovirus infection alone also promoted the downregulation of IκBα protein levels, which was further accentuated by TNF-α (Fig. 2A). Thus, adenovirus infection, like many cellular stresses, promotes the degradation of IκBα, which leads to NF-κB translocation to the nucleus and NF-κB DNA-binding activity. Hence, if E1A is interfering with survival signaling by NF-κB, it must be exerting its function downstream of NF-κB activation.

Others have reported that c-FLIP$_L$ is cleaved into a p43 product during TNF/cycloheximide treatment or anti-Fas signaling. Since we did not observe c-FLIP$_L$ cleavage in adenovirus-infected cells treated with TNF-α alone, we examined TNF/cycloheximide-treated cells for c-FLIP$_L$ cleavage. HeLa cells were mock, Ad5*dl*309, or Ad5*dl*337 infected and treated with TNF/cycloheximide. Full-length c-FLIP$_L$ levels were dramatically reduced, and the appearance of the p43 cleaved product was observed at 4 and 12 h post-TNF/cycloheximide treatment in mock-infected cells (Fig. 2B). No cleavage was detected in Ad5*dl*309-infected cells, whereas cleavage was observed at 12 h post-TNF/cycloheximide treatment in Ad5*dl*337-infected cells (Fig. 2B). These results suggest that addition of cycloheximide and apoptotic events downstream of the mitochondria may be required for c-FLIP$_L$ cleavage in adenovirus-infected cells.

Expression of v-FLIP or c-FLIP$_S$ rescues E1A-mediated sensitization to TNF-α. The downregulation of constitutive and NF-κB-inducible c-FLIP$_S$ protein expression by E1A may provide a mechanism for the potentiation of caspase-8 activation during TNF-α-mediated signaling. Since v-FLIP is structurally homologous to c-FLIP$_S$ and is a known inhibitor of TNF-α-dependent caspase-8 activation, we investigated if v-FLIP would substitute for the absence of c-FLIP$_S$ and reverse the ability of E1A to promote caspase-8 activation and abolish sensitization by TNF-α. We generated stable HeLa cell lines that express HA-tagged MC159 v-FLIP in parallel with pcDNA3 vector controls. Vector control and MC159-expressing cell lines were mock, Ad5*dl*309, or Ad5*dl*337 infected and either left untreated or treated with TNF-α for 10 h.

Procaspase-8 processing was dramatically induced in vector control cells infected with adenovirus and treated with TNF-α compared to mock-infected cells (Fig. 3A). In contrast, expression of MC159 reversed the ability of E1A to promote caspase-8 activation during TNF-α treatment, as indicated by the absence of the cleaved caspase-8 p24 product (Fig. 3A). Interestingly, v-FLIP protein levels were downregulated upon adenovirus infection, although not as dramatically as endogenous c-FLIP$_S$. Nonetheless, the levels were still apparently sufficient to block procaspase-8 processing. Thus, MC159 v-FLIP expression rescued caspase-8 activation by E1A and TNF-α.

To test if the abolition of procaspase-8 processing by MC159 blocked the ability of adenovirus to sensitize cells and induce apoptosis via the TNF-α signaling pathway, pcDNA3 or HA-MC159 expressing HeLa cells were mock or Ad5*dl*337 infected and either left untreated or treated with TNF-α for 10 h. Trypan blue exclusion demonstrated that approximately 38% of the infected vector control cells underwent apoptosis in the presence of TNF-α, compared to 7% of mock-infected cells (Fig. 3B). However, only 18% of the infected MC159 cells underwent apoptosis during TNF-α treatment, which was similar to what was observed in untreated infected cells (Fig. 3B). Cells infected with Ad5*dl*337 alone were slightly apoptotic, since infection induces death in the absence of E1B 19K expression. Thus, expression of the v-FLIP MC159 conferred resistance to apoptosis in cells infected with adenovirus and treated with TNF-α. These data demonstrate that expression of v-FLIP reverses the ability of E1A to sensitize cells to TNF-α by blocking TNF-α-mediated caspase-8 activation and apoptosis.

To demonstrate if expression of c-FLIP$_S$ in addition to v-FLIP rescued E1A sensitization to TNF-α, HeLa cells were transiently transfected with pcDNA3 control or c-FLIP$_S$. Vector control- and c-FLIP$_S$-expressing cells were mock, Ad5*dl*309, or Ad5*dl*337 infected and either left untreated or treated with TNF-α for 10 h. c-FLIP$_S$ reversed the ability of E1A to promote caspase-8 processing during TNF-α signaling compared to vector control-expressing cells (Fig. 3C). Furthermore, the trypan blue exclusion assay demonstrated one-third fewer dead cells among the c-FLIP$_S$-transfected and Ad5*dl*337-infected cells compared to vector control cells (Fig. 3D), which corresponds to a transfection efficiency of 30%. These data demonstrate that c-FLIP$_S$ reversed the ability of E1A to promote caspase-8 activation and to induce apoptosis via the TNF-α signaling pathway.

Adenovirus infection prevents NF-κB-mediated induction of c-FLIP$_S$ mRNA levels. In HeLa cells, TNF-α-mediated activation of NF-κB induces transcriptional activation and a threefold induction of c-FLIP mRNA levels (23). Adenovirus infection alone reduces IκBα levels (Fig. 2) and induces NF-κB DNA binding (data not shown), suggesting that if E1A is interfering with survival signaling by NF-κB, it is doing so downstream of NF-κB activation. Indeed, E1A may interfere with NF-κB-mediated transcriptional activation, since E1A interacts with p300, a putative cofactor for NF-κB-dependent transactivation (12). As inhibition of NF-κB activation is known to sensitize cells to TNF-α (47, 49) inhibition of target gene transcription by E1A expression would be expected to do the same. Therefore, we determined if adenovirus infection prevented the induction of c-FLIP mRNA levels, as well as other NF-κB target genes, in response to TNF-α treatment.

We employed the Taqman real-time quantitative RT-PCR assay to assess mRNA expression levels of c-FLIP$_L$, c-FLIP$_S$, IAP-2, and IκBα during adenovirus infection and/or TNF-α treatment. HeLa cells were mock or Ad5*dl*309 infected and subsequently left untreated or treated with TNF-α. Four hours post-TNF-α treatment, cells were harvested for RNA isolation, and real-time quantitative RT-PCR was performed. Real-time RT-PCR was not performed on Ad5*dl*337-infected cells because the cells begin to undergo apoptosis by 24 h. The change in mRNA expression for the genes examined was expressed relative to that of the untreated mock-infected sample (see Materials and Methods). As a control, GAPDH mRNA levels

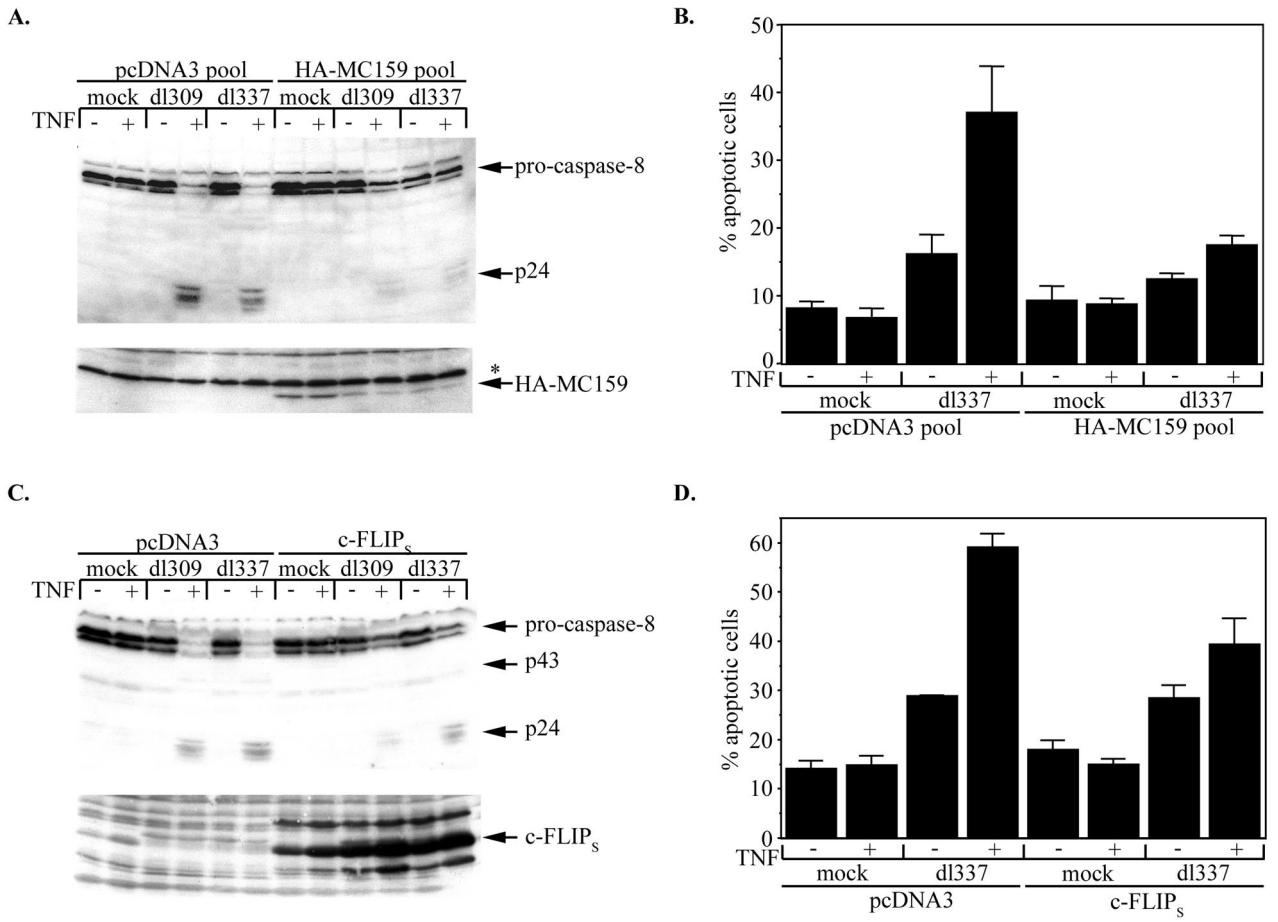

FIG. 3. v-FLIP and c-FLIP$_S$ rescue TNF-α-mediated E1A-dependent caspase-8 activation and apoptosis. (A) Stable HeLa cell lines expressing either vector control or HA-tagged MC159 were mock, Ad5*dl*309, or Ad5*dl*337 infected for 24 h and treated with TNF-α for 10 h. Extracts were analyzed by immunoblotting with anti-caspase-8 and anti-HA antibodies. (B) Viability was assessed by the trypan blue exclusion assay in stable cell lines that were mock or Ad5*dl*337 infected for 24 h and treated with TNF-α for 10 h. The band above HA-MC159 represents a nonspecific cross-reactive band (asterisk). (C) Vector control- and c-FLIP$_S$-overexpressing HeLa cells were mock, Ad5*dl*309, or Ad5*dl*337 infected for 24 h and treated with TNF-α for 10 h. Extracts were analyzed by immunoblotting with anti-caspase-8 and anti-c-FLIP monoclonal (Dave-2) antibodies. (D) Viability was assessed by the trypan blue exclusion assay in HeLa cells that were mock or Ad5*dl*337 infected and treated with TNF-α for 10 h.

were assessed, and the basal expression level of GAPDH was not affected by adenovirus infection and/or TNF-α treatment (Fig. 4A).

As expected, c-FLIP$_L$ mRNA levels were induced twofold upon TNF-α treatment compared to mock-infected cells. In contrast, Ad5*dl*309 infection repressed c-FLIP$_L$ mRNA levels by fourfold, and the levels could not be restored by TNF-α (Fig. 4A). Despite this dramatic repression of c-FLIP$_L$ mRNA expression, c-FLIP$_L$ protein levels remained unchanged (Fig. 2A), suggesting that the protein may have a long half-life. c-FLIP$_S$ mRNA levels were induced sixfold by TNF-α, and consistent with that of c-FLIP$_L$, c-FLIP$_S$ was dramatically repressed by Ad5*dl*309 infection in the absence and presence of TNF-α (Fig. 4A). As a control, other known NF-κB target genes, IAP-2 and IκBα, were assessed, and as expected, nine- and fivefold increases in mRNA levels, respectively, were observed in response to TNF-α (Fig. 4A). IAP-2 and IκBα mRNA levels were constitutively repressed and TNF-α-mediated induction was inhibited by adenovirus infection (Fig. 4A). Thus, wild-type Ad5*dl*309 infection alone repressed constitutive expression of c-FLIP$_L$ and c-FLIP$_S$ mRNAs, as well as that of other NF-κB-regulated genes. In addition, adenovirus infection prevented the induction of c-FLIP$_L$ and c-FLIP$_S$ mRNAs and that of IAP-2 and IκBα by TNF-α.

Since infection with 12S E1B$^-$ virus, which expresses only the 12S E1A product, also sensitized cells to TNF-α by promoting caspase-8 activation, we determined if 12S E1B$^-$ exerted similar effects on the message levels of NF-κB-regulated genes as did Ad5*dl*309. Real-time RT-PCR was performed on HeLa cells infected with 12S E1B$^-$ virus that were then treated with TNF-α for 4 h. No significant changes in mRNA levels were observed in the GAPDH control (Fig. 4B). Induction of c-FLIP$_L$, c-FLIP$_S$, IAP-2, and Iκ-Bα mRNAs was observed in mock-infected cells in response to TNF-α signaling (Fig. 4B). In contrast to the constitutive repression of mRNA levels for all of these genes by Ad5*dl*309, no difference in the basal expression level of c-FLIP$_L$, c-FLIP$_S$, IAP-2, and IκBα was detected with 12S E1B$^-$ infection alone (Fig. 4B). However, 12S E1B$^-$ infection abolished the induction of c-FLIP$_L$, c-FLIP$_S$, IAP-2, and IκBα mRNA levels upon TNF-α signaling

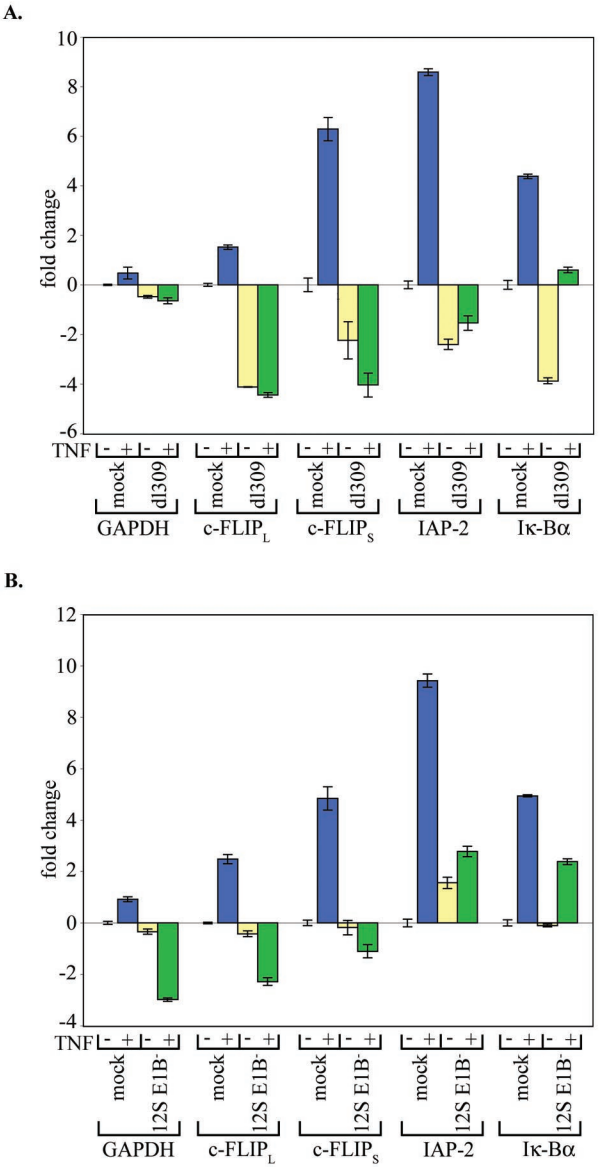

FIG. 4. Adenovirus infection prevents NF-κB-mediated induction of c-FLIP and IAP-2 mRNA levels. Real-time RT-PCR was performed on HeLa cells that were mock or Ad5*dl*309 infected for 24 h and treated with TNF-α for 4 h. (A) The change in mRNA levels of GAPDH, c-FLIP$_L$, c-FLIP$_S$, IAP-2, and IκBα was determined as a change in fluorescence relative to that of the untreated mock-infected sample (see Materials and Methods). Fold change is defined as the change in mRNA levels for each sample relative to the untreated mock-infected sample. (B) HeLa cells were mock or 12S E1B$^-$ infected for 34 h and treated with TNF-α for 4 h. The change in mRNA levels was assessed for GAPDH, c-FLIP$_L$, c-FLIP$_S$, IAP-2, and IκBα as stated above.

(Fig. 4B). In conclusion, adenovirus infection blocked TNF-α-dependent mRNA induction of multiple prosurvival genes.

Proteasome inhibitors restore c-FLIP$_S$ levels and inhibit E1A-dependent TNF-α-mediated caspase-8 activation. E1A expression downregulated c-FLIP$_S$ protein levels and abolished its induction but had no effect on c-FLIP$_L$ protein levels despite abolition of NF-κB-mediated induction of both mRNAs. This raised the possibility that in addition to affecting NF-κB-mediated induction of c-FLIP$_S$ and c-FLIP$_L$ mRNAs, adenovirus infection also differentially regulated c-FLIPs at the posttranscriptional level. Although others have reported that p53 overexpression enhanced ubiquitin-mediated proteasome-dependent degradation of c-FLIP$_L$ in colorectal cells (11), we did not observe changes in c-FLIP$_L$ protein turnover in HeLa cells infected with adenovirus. Therefore, we tested whether c-FLIP$_S$ was also regulated by proteasome-dependent turnover and whether adenovirus infection enhanced this activity.

Mock- or Ad5*dl*309-infected HeLa cells were treated with the proteasome inhibitor epoxomicin or lactacystin and subsequently left untreated or treated with TNF-α for 10 h. TNF-α-dependent procaspase-8 processing occurred only in Ad5*dl*309-infected cells, whereas procaspase-8 was unaffected in mock-infected cells (Fig. 5A). In contrast, epoxomicin (Fig. 5A) or lactacystin (data not shown) treatment resulted in complete abolition of procaspase-8 processing by TNF-α. Western analysis with both anti-c-FLIP$_L$ (CT) polyclonal (Fig. 5A) and anti-c-FLIP monoclonal (Dave-2) (data not shown) antibodies revealed that c-FLIP$_L$ protein levels remained unchanged with epoxomicin. However, c-FLIP$_S$ levels were significantly elevated in both mock- and Ad5*dl*309-infected cells treated with epoxomicin (Fig. 5A) or lactacystin (data not shown). These data illustrate that in HeLa cells, c-FLIP$_S$ but not c-FLIP$_L$ levels are constitutively regulated by proteasome-dependent degradation and that this turnover is relieved by TNF-α treatment and enhanced by E1A expression. The restoration of c-FLIP$_S$ expression by proteasome inhibitors correlated with the abolition of caspase-8 activation, which would terminate the death signal in the TNF-α pathway.

c-FLIP$_S$ is only one of a number of proteins recruited to the DISC upon TNF-α signaling. Therefore, we determined if other DISC components were constitutively susceptible to degradation in proteasomes and/or in response to adenovirus infection. We did not observe a decrease in RIP or IAP-1 levels during Ad5*dl*309 infection compared to mock-infected cells, although in infected cells, RIP and IAP-1 levels decreased upon TNF-α treatment, and this decrease was prevented by epoxomicin (Fig. 5A). Since epoxomicin blocks caspase-8 activation by TNF-α in infected cells, the reduction in IAP-1 and RIP levels may merely be a consequence of caspase-8 activity. No changes in IAP-2 protein levels were observed with epoxomicin treatment in mock-infected cells (Fig. 5A). However, we detected reduced IAP-2 levels in response to adenovirus infection alone, and this reduction was prevented by epoxomicin (Fig. 5A). These results illustrate that, in contrast to c-FLIP$_S$, IAP-2 was not constitutively degraded in the proteasome and that adenovirus infection promoted its degradation at the proteasome. In contrast, TRAF2 and FADD protein levels remained unaltered in the presence or absence of epoxomicin and with or without TNF-α treatment or adenovirus infection (Fig. 5A).

In addition, v-FLIP MC159 protein levels were downregulated with Ad5*dl*309 infection (Fig. 3A) and levels were restored with epoxomicin (data not shown). These results suggest that E1A expression may not only repress mRNA expression but may also enhance proteasome-dependent degradation of c-FLIP$_S$, IAP-2, and v-FLIP. IAP-2 expression, however, does

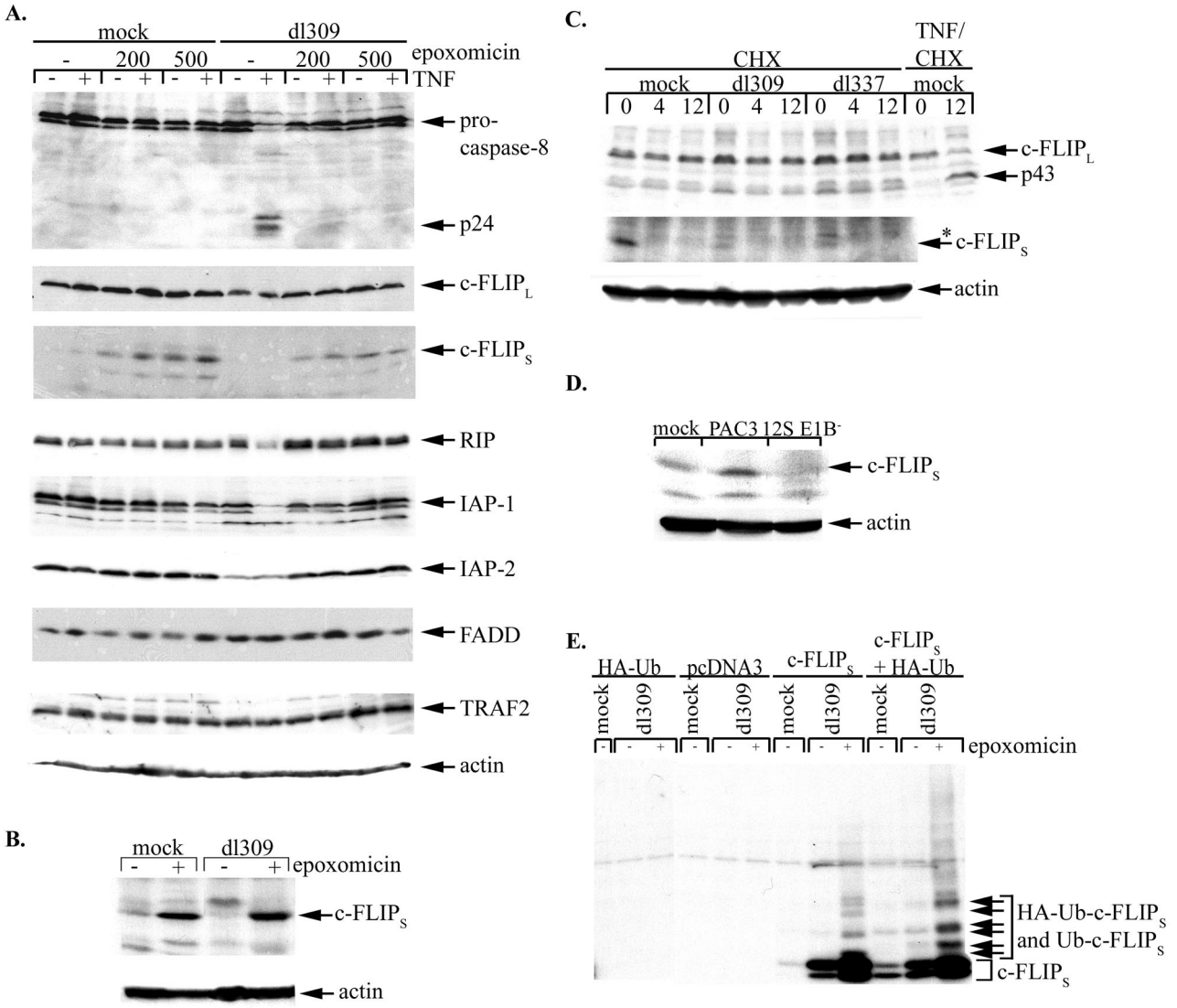

FIG. 5. Adenovirus infection enhances c-FLIP$_S$ degradation through a ubiquitin-dependent proteolysis pathway. (A) HeLa cells were mock or Ad5*dl*309 infected and treated with epoxomicin and subsequently TNF-α for 10 h. Whole-cell extracts were analyzed by immunoblotting with anti-caspase-8, anti-c-FLIP$_L$ polyclonal (CT) (c-FLIP$_L$), anti-FLIP monoclonal (Dave-2) (c-FLIP$_S$), anti-RIP, anti-IAP-1, anti-IAP-2, anti-TRAF2, anti-FADD, and antiactin antibodies. (B) HeLa cells were mock or Ad5*dl*309 infected and treated with epoxomicin. Whole-cell extracts were analyzed by immunoblotting with anti-FLIP (Dave-2) and antiactin monoclonal antibodies. (C) HeLa cells were mock, Ad5*dl*309, or Ad5*dl*337 infected for 24 h and treated with cycloheximide for 0, 4, or 12 h. As a control, mock-infected HeLa cells were treated with TNF/cycloheximide for 0 or 12 h. Whole-cell extracts were immunoblotted with anti-c-FLIP (Dave-2) and antiactin monoclonal antibodies. (D) Whole-cell extracts from mock-, Ad5PAC3-, and 12SE1B$^-$-infected HeLa cells were analyzed by immunoblotting with anti-c-FLIP monoclonal (Dave-2) and antiactin antibodies. (E) HeLa cells were transfected with vector control, HA-tagged ubiquitin, c-FLIP$_S$, or c-FLIP$_S$ and HA-tagged ubiquitin. Transfectants were mock or Ad5*dl*309 infected for 16 h and treated with epoxomicin for 8 h as indicated. Whole-cell extracts were prepared and immunoblotted with an anti-c-FLIP monoclonal (NF6) antibody. High-molecular-mass forms of c-FLIP$_S$ that correspond to ubiquitinated products are indicated.

not confer resistance to E1A sensitization (data not shown). Since IAP-2 is an E3 ubiquitin ligase (15), it may facilitate the degradation of c-FLIP$_S$, which is the functional target of E1A, in addition to itself.

To address whether proteasome inhibitors could rescue the loss of basal c-FLIP$_S$ expression in E1A-expressing cells, mock- and Ad5*dl*309-infected cells were either left untreated or were treated with epoxomicin. Lysates probed with an anti-FLIP monoclonal antibody demonstrated that adenovirus infection significantly downregulated c-FLIP$_S$ levels compared to mock-infected cells and that epoxomicin efficiently rescued c-FLIP$_S$ levels to that observed in mock-infected cells treated with epoxomicin (Fig. 5B). These results suggest that the loss of c-FLIP$_S$ induced by E1A is also due to proteasome-mediated degradation and not solely to transcriptional repression. Although our data demonstrate that c-FLIP$_S$ but not c-FLIP$_L$ is constitutively regulated by proteasome-dependent degradation, others have reported both c-FLIP$_L$ and c-FLIP$_S$ have equivalent short half-lives.

To address this discrepancy, we assessed the half-life of

c-FLIP$_L$ and c-FLIP$_S$ in HeLa cells by addition of the metabolic inhibitor cycloheximide. Mock-, Ad5*dl*309-, or Ad5*dl*337-infected HeLa cells were left untreated or treated with cycloheximide. c-FLIP$_S$ decreased to undetectable levels by 4 h post-cycloheximide treatment, but c-FLIP$_L$ remained stable even after 12 h in both mock- and adenovirus-infected cells (Fig. 5C). Once again, c-FLIP$_S$ but not c-FLIP$_L$ levels were downregulated with adenovirus infection alone (Fig. 5C). The band above c-FLIP$_S$ in adenovirus-infected cells (asterisk) may represent a cross-reaction with a viral protein or a modified form of c-FLIP$_S$. Thus, c-FLIP$_S$ has a strikingly shorter half-life than c-FLIP$_L$.

Since infection with the wild-type virus Ad5*dl*309 promoted the transcriptional repression of c-FLIP$_S$ mRNA, we wanted to assess whether infection with the 12S E1B$^-$-expressing virus, which does not affect c-FLIP$_S$ mRNA basal expression, enhanced c-FLIP$_S$ protein degradation. To assess the contribution of E1A-mediated repression of c-FLIP$_S$ expression versus c-FLIP$_S$ turnover mediated by proteasomes, Western analysis was performed on whole-cell lysates made from mock- and 12S E1B$^-$-infected HeLa cells. 12S E1B$^-$ infection alone promoted c-FLIP$_S$ downregulation compared to mock- or Ad5PAC3-infected samples (Fig. 5D). c-FLIP$_S$ levels appear to be slightly increased with Ad5PAC3 infection, but this induction has not been consistently observed and was not significant (Fig. 5D). Thus, c-FLIP$_S$ protein expression was downregulated in an E1A-dependent, transcription-independent manner upon 12S E1B$^-$ infection alone. Furthermore, 12S E1B$^-$ infection downregulated HA-MC159 protein levels in stably transfected HeLa cells, and its levels were rescued by epoxomicin (data not shown). Since HA-MC159 expression is under the control of a cytomegalovirus promoter, regulation of its expression is independent of NF-κB. These results illustrate that, independent of transcriptional regulation, adenovirus infection alone facilitates the degradation of c-FLIP$_S$ and v-FLIP MC159 and thus initiates a second level of regulation by promoting protein turnover.

Since proteasome inhibitors were able to rescue c-FLIP$_S$ degradation in response to adenovirus infection, we determined whether this was caused by c-FLIP$_S$ ubiquitination. Negative control and c-FLIP$_S$ expression vectors were transfected into HeLa cells, and transfectants were mock or Ad5*dl*309 infected and incubated with epoxomicin. Lysates were probed with anti-FLIP NF6 (Fig. 5E) or anti-FLIP Dave-2 (data not shown) monoclonal antibodies, which recognized c-FLIP$_S$ and multiple high-molecular-mass forms of c-FLIP$_S$ in epoxomicin-treated Ad5*dl*309-infected cells transfected with c-FLIP$_S$. Both antibodies also recognized a band below c-FLIP$_S$, which may be merely a degradation product of overexpressed c-FLIP$_S$. Furthermore, the upregulation of transiently expressed c-FLIP$_S$ observed in adenovirus-infected cells is very likely due to transactivation of the cytomegalovirus promoter by E1A, which is commonly observed in transfection-infection experiments.

The sizes of the multiple high-molecular-mass forms of c-FLIP$_S$ are consistent with ubiquitinated forms of c-FLIP$_S$ (Fig. 5E). To confirm that these bands represented ubiquitinated c-FLIP$_S$, an HA-tagged ubiquitin expression plasmid was cotransfected with the c-FLIP$_S$ expression vector. Again, slower-migrating protein forms of c-FLIP$_S$ were observed in both mock- and Ad5*dl*309-infected cells, which were greatly increased by epoxomicin treatment (Fig. 5E). Furthermore, the increase in the molecular mass of c-FLIP$_S$ was consistent with the conjugation of c-FLIP$_S$ to HA epitope-tagged ubiquitin compared to endogenous ubiquitin. These results demonstrate that adenovirus infection enhanced ubiquitin-mediated targeting of c-FLIP$_S$, which subsequently acts as a signal for proteasome-dependent degradation.

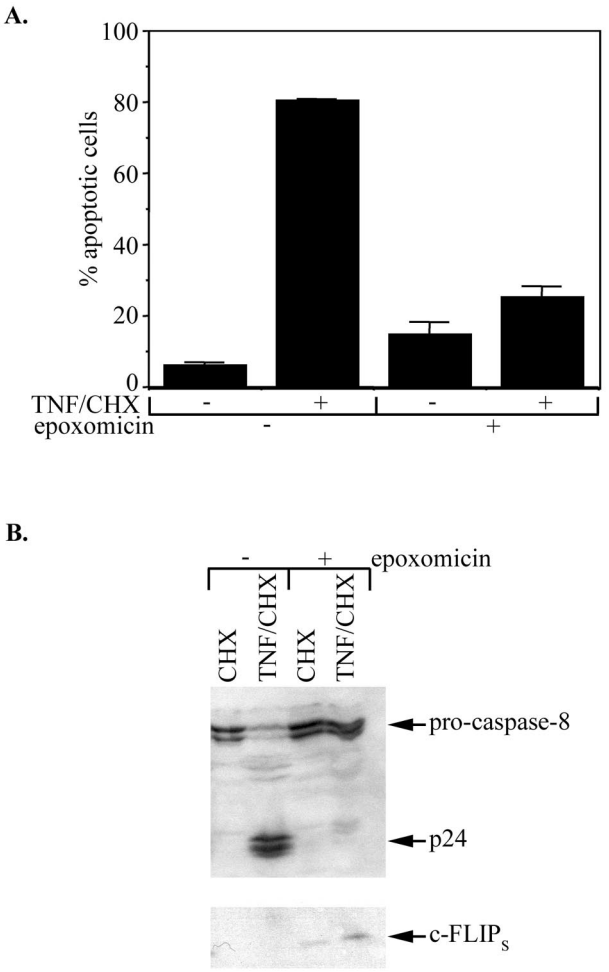

FIG. 6. Epoxomicin blocks caspase-8 processing and TNF/cycloheximide-mediated apoptosis by preventing c-FLIP$_S$ degradation. (A) HeLa cells were incubated with epoxomicin for 12 h and then treated with TNF/cycloheximide or cycloheximide alone for 10 h. Viability was assessed by trypan blue exclusion. (B) Whole-cell extracts were prepared from HeLa cells treated as in panel A and analyzed by immunoblotting with anti-caspase-8 and anti-c-FLIP monoclonal (Dave-2) antibodies.

Proteasome inhibitors increase c-FLIP$_S$ levels and confer resistance to TNF/cycloheximide-mediated apoptosis. Since proteasome inhibitors blocked E1A-mediated sensitization of cells to TNF-α, we wanted to determine whether proteasome inhibitors could also rescue TNF-α-treated cells whose survival pathway had been abolished by the protein synthesis inhibitor cycloheximide. HeLa cells were incubated with epoxomicin prior to TNF/cycloheximide or cycloheximide treatment, and viability was assessed by trypan blue. A total of 80% of the

TNF/cycloheximide-treated cells underwent apoptosis, compared to only 10% of cycloheximide-treated cells (Fig. 6A). Epoxomicin treatment dramatically rescued TNF/cycloheximide-treated cells from apoptosis (Fig. 6A). Therefore, proteasome inhibitors can effectively abolish the TNF-α death pathway despite the inhibition of newly synthesized antiapoptotic proteins.

Since epoxomicin treatment blocked apoptosis in the presence of a protein synthesis inhibitor, we wanted to determine if the mechanism of inhibition was homologous to inhibition of E1A-mediated sensitization to TNF-α. Therefore, we performed Western analysis on TNF/cycloheximide- or cycloheximide-treated HeLa cells preincubated with epoxomicin. Procaspase-8 processing was clearly detected in TNF/cycloheximide-treated cells compared to the cycloheximide-alone control (Fig. 6B). However, a dramatic absence of caspase-8 activation was observed when cells were incubated with epoxomicin (Fig. 6B). This lack of procaspase-8 processing correlated with the upregulation of c-FLIP$_S$ in response to epoxomicin (Fig. 6B). Thus, inhibition of proteasomes elevates c-FLIP$_S$ levels, which result in the abolition of TNF-α-mediated caspase-8 activation and apoptosis.

DISCUSSION

We have established that adenovirus infection and E1A expression sensitize cells to death receptor signaling by facilitating the activation of caspase-8. This activity was a function of both the Rb and p300 binding activities of E1A, suggesting that interference with Rb and p300 function may be responsible for the altered sensitivity of tumor cells to TNF-α and other death receptor ligands. The mechanism by which E1A facilitated TNF-α-dependent caspase-8 activation was attributed to the abolition of NF-κB-mediated transcriptional induction of c-FLIP$_S$ in conjunction with ubiquitination and proteasome-dependent degradation of c-FLIP$_S$. The rescue of both E1A-dependent caspase-8 activation and sensitization to TNF-α-mediated apoptosis by c-FLIP$_S$ and v-FLIP expression demonstrates that c-FLIP$_S$ is the functional target for modulation of death receptor signaling by E1A. Thus, infected cells are sensitized to the host's immune surveillance response via a multitiered regulatory mechanism, whereby E1A facilitates c-FLIP$_S$ mRNA downregulation and enhances c-FLIP$_S$ protein turnover in the proteasome.

The sensitization of cells to death receptor-mediated cell death is conferred by E1A's ability to sequester p300 and Rb, and the latter activity facilitates activation of E2F-1 (42). Consistent with our observations, E2F-1 overexpression has been shown to sensitize cells to TNF-α, and Rb deficiency in mouse embryo fibroblasts results in increased susceptibility to TNF-α-mediated apoptosis (34). Although tetracycline-inducible E2F-1 expression promotes the degradation of TRAF2, which may lead to inhibition of the NF-κB survival response (34), TRAF2 levels were not decreased by E1A expression during adenovirus infection (Fig. 5A). The possibility, however, that another TRAF family member is also degraded in response to E1A expression cannot be ruled out. Furthermore, although E2F-1 overexpression did not alter mRNA expression of NF-κB-regulated genes (34), E2F-1 may transcriptionally activate genes that facilitate proteasome-dependent protein degradation of c-FLIP$_S$ and IAP-2. E1A may also interfere with c-FLIP and IAP-2 mRNA induction by sequestering p300, a putative cofactor for NF-κB-mediated transactivation (12). Thus, E1A may alter the expression of NF-κB-regulated genes through sequestration of coactivators.

c-FLIP is an NF-κB-inducible gene, but differential expression of c-FLIP$_L$ and c-FLIP$_S$ in response to TNF-α and T-cell activation and in various cell types has suggested the existence of additional posttranscriptional mechanisms for the regulation of protein levels (21, 23, 28). Others have reported that p53 overexpression enhances proteasome-dependent degradation of c-FLIP$_L$ (11). We have demonstrated that c-FLIP$_S$ is subjected to ubiquitination and proteasome-dependent degradation which is enhanced by E1A. E1A also facilitated proteasome-dependent degradation of IκBα and IAP-2 (Fig. 2A and 5A) and has previously been reported to induce the ubiquitin-mediated degradation of topoisomerase IIα (29, 30). Thus, E1A may facilitate ubiquitination and degradation of a number of cellular proteins as a means to alter cellular metabolism during viral infection.

The ability of E1A to differentially regulate c-FLIP$_L$ and c-FLIP$_S$ expression levels serves as a highly efficient mechanism for sensitizing HeLa cells to TNF-α. c-FLIP$_L$ but not c-FLIP$_S$ has been shown to be required for caspase-8 activation (3). Thus, preferential downregulation of c-FLIP$_S$ by E1A promotes the removal of a caspase-8 inhibitor while allowing a positive regulator of caspase-8 activation, c-FLIP$_L$, to remain intact. Thus, modulation of c-FLIP$_S$ expression levels is sufficient to determine the fate of a cell during TNF-α signaling in HeLa cells.

IAP-2, a component of the TNF-α receptor complex, contains a ring finger domain associated with E3 protein ligase activities for the ubiquitination of substrates as well as itself (8, 15, 36). Moreover, ubiquitination and degradation of IAP family members has been linked to apoptotic progression (26, 56). Since IAP-2 as well as c-FLIPs has been reported to bind to the other ring domain-containing proteins, TRAF1 and TRAF2 (20, 36), the regulation of c-FLIP$_S$ protein may occur at this complex. Whether IAP-2 functions in response to adenovirus infection by serving as an E3 ligase for the ubiquitin-dependent degradation of c-FLIP$_S$, thereby sensitizing cells to TNF-α, remains to be determined.

Elevated c-FLIP levels have been implicated in tumorigenesis and resistance of various tumor cell lines to Fas ligand-, TRAIL-, and TNF-α-mediated cell death (13, 16, 17). For example, c-FLIP expression was detected in metastatic melanoma lesions but not in the surrounding melanocytes (17). Furthermore, elevated c-FLIP levels in numerous melanoma cell lines have been correlated with resistance to Fas ligand and TRAIL-induced death (13, 17). Most importantly, elevated c-FLIP levels have been shown to promote tumor establishment and progression (9, 27). One proposed mechanism for the enhancement of tumor formation by c-FLIP is through its ability to protect tumor cells from T-cell-mediated CD95-dependent apoptosis in vivo (9, 27). Thus, escape from immune surveillance by elevated c-FLIP levels appears to be a critical factor in tumor progression.

Elucidating the mechanism by which E1A sensitizes cells to proinflammatory cytokines has provided new insights into possible therapeutic means to alter the apoptotic propensity of

tumor cells. In addition to treating tumors with chemotherapeutic drugs, selectively infecting tumor cells with replication-selective adenoviruses may provide a means for sensitizing tumor cells to the patient's own inflammatory response mechanism and thus providing synergistic efficacy for tumor destruction (22). Thus, in melanomas, where elevated c-FLIP levels may provide a mechanism for immune evasion, adenovirus could reverse these protective effects and facilitate receptor-mediated cell death. Furthermore, resistance to TRAIL-induced death in a melanoma cell line was reversed when infected with adenovirus or when E1A was stably expressed (38). Thus, tumors resistant to TRAIL-induced death may become sensitized when used in combination with adenovirus-specific virotherapy. Similarly, drugs that act as p300 or Rb antagonists may mimic the ability of E1A to downregulate c-FLIP expression via transcriptional repression and ubiquitin-mediated proteolysis and thus may provide a novel therapeutic treatment for highly resistant tumorigenic cells.

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REFERENCES

1. **Barkett, M., and T. D. Gilmore.** 1999. Control of apoptosis by Rel/NF-κB transcription factors. Oncogene **18:**6910–6924.
2. **Bertin, J., R. C. Armstrong, S. Ottilie, M. D., Y. Wang, S. Banks, G. H. Wang, T. G. Senkevich, E. S. Alnemri, B. Moss, M. J. Lenardo, K. J. Tomaselli, and J. I. Cohen.** 1997. Death effector domain-containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR-1-induced apoptosis. Proc. Natl. Acad. Sci. USA **94:**1172–1176.
3. **Chang, D. W., Z. Xing, Y. Pan, A. Algeciras-Schimnich, B. C. Barnhart, S. Yaish-Ohad, M. E. Peter, and X. Yang.** 2002. cFLIP$_L$ is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. EMBO J. **21:**3704–3714.
4. **Chen, C., L. Edelstein, and C. Gelinas.** 2000. The Rel/NF-κB family directly activates expression of the apoptosis inhibitor Bcl-x(L). Mol. Cell. Biol. **20:**2687–2695.
5. **Chen, M. J., B. Holskin, J. Strickler, J. Gorniak, M. A. Clark, P. J. Johnson, M. Mitcho, and D. Shalloway.** 1987. Induction by E1A oncogene expression of cellular susceptibility to lysis by TNF. Nature (London) **330:**581–583.
6. **Chiou, S.-K., and E. White.** 1997. p300 binding by E1A cosegregates with p53 induction but is dispensable for apoptosis. J. Virol. **71:**3515–3525.
7. **Degenhardt, K., R. Sundararajan, T. Lindsten, C. B. Thompson, and E. White.** 2002. Bax and Bak independently promote cytochrome-c release from mitochondria. J. Biol. Chem. **277:**14127–14134.
8. **Deveraux, Q. L., and J. C. Reed.** 1999. IAP family proteins—suppressors of apoptosis. Genes Dev. **13:**239–252.
9. **Djerbi, M., V. Screpanti, C. A., B. Bogen, P. Biberfeld, and A. Grandien.** 1999. The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. J. Exp. Med. **190:**1025–1031.
10. **Duerksen-Hughes, P., W. S. M. Wold, and L. R. Gooding.** 1989. Adenovirus E1A renders infected cells sensitive to cytolysis by tumor necrosis factor. J. Immunol. **143:**4193–4200.
11. **Fukazawa, T., T. Fujiwara, F. Uno, F. Teraishi, Y. Kadowaki, T. Itoshima, Y. Takata, S. Kagawa, J. A. Roth, J. Tschopp, and N. Tanaka.** 2001. Accelerated degradation of cellular FLIP protein through the ubiquitin-proteasome pathway in p53-mediated apoptosis of human cancer cells. Oncogene **20:**5225–5731.
12. **Gerritsen, M. E., A. J. Williams, A. S. Neish, S. Moore, Y. Shi, and T. Collins.** 1997. CREB-binding protein/p300 are transcriptional coactivators of p65. Proc. Natl. Acad. Sci. USA **94:**2927–2932.
13. **Griffith, T. S., W. A. Chin, G. C. Jackson, D. H. Lynch, and M. Z. Kubin.** 1998. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. Immunology **161:**2833–2840.
14. **Hu, S., C. Vincenz, M. Buller, and V. M. Dixit.** 1997. A novel family of viral death effector domain-containing molecules that inhibit both CD-95- and tumor necrosis factor receptor-1-induced apoptosis. J. Biol. Chem. **272:**9621–9624.
15. **Huang, H.-k., C. Joazeiro, E. Bonfoco, S. Kamada, J. Leverson, and T. Hunter.** 2000. The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes *in vitro* monoubiquitination of caspases 3 and 7. J. Biol. Chem. **275:**26661–26664.
16. **Irisarri, M., J. Plumas, T. BonneFoix, M. C. Jacob, C. Roucard, M. A. Pasquier, J. J. Sotto, and A. Lajmanovich.** 2000. Resistance to CD95-mediated apoptosis through constitutive c-FLIP expression in a non-Hodgkin's lymphoma B cell line. Leukemia **14:**2149–2158.
17. **Irmler, M., M. Thome, M. Hahne, P. Schneider, K. Hofmann, V. Steiner, J.-L. Bodmer, M. Schröter, K. Burns, C. Mattmann, D. Rimoldi, L. E. French, and J. Tschopp.** 1997. Inhibition of death receptor signals by cellular FLIP. Nature (London) **388:**190–195.
18. **Jones, N., and T. Shenk.** 1979. An adenovirus type 5 early gene function regulates expression of other early viral genes. Proc. Natl. Acad. Sci. USA **76:**3665–3669.
19. **Karin, M., and A. Lin.** 2002. NF-κB at the crossroads of life and death. Nat. Immunol. **3:**221–227.
20. **Kataoka, T., R. C. Budd, N. Holler, M. Thome, F. Martinon, M. Irmler, K. Burns, M. Hahne, N. Kennedy, M. Kovacsovics, and J. Tschopp.** 2000. The caspase-8 inhibitor FLIP promotes activation of NF-κB and Erk signaling pathways. Curr. Biol. **10:**640–648.
21. **Kirchhoff, S., W. Müller, A. Krueger, I. Schmitz, and P. Krammer.** 2000. TCR-mediated upregulation of c-FLIP$_{short}$ correlates with resistance toward CD95-mediated apoptosis by blocking death-inducing signaling complex activity. J. Immunol. **165:**6293–6300.
22. **Kirn, D.** 2000. Replication-selective oncolytic adenoviruses: virotherapy aimed at genetic targets in cancer. Oncogene **19:**6660–6669.
23. **Kreuz, S., D. Siegmund, P. Scheurich, and H. Wajant.** 2001. NF-κB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. Mol. Cell. Biol. **21:**3964–3973.
24. **Krueger, A., I. Schmitz, S. Baumann, P. H. Krammer, and S. Kirchhoff.** 2001. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. J. Biol. Chem. **276:**20633–20640.
25. **Li, H., H. Zhu, C.-J. Xu, and J. Yuan.** 1998. Cleavage of BID by caspase8. mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell **94:**491–501.
26. **Li, X., Y. Yang, and J. D. Ashwell.** 2002. TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. Nature **416:**345–347.
27. **Medema, J., J. de Jong, T. van Hall, C. Melief, and R. Offrings.** 1999. Immune escape of tumors in vivo by expression of cellular FLICE-inhibitory protein. J. Exp. Med. **7:**1033–1038.
28. **Micheau, O., S. Lens, O. Gaide, K. Alevizopoulos, and J. Tschopp.** 2001. NF-κB signals induce the expression of c-FLIP. Mol. Cell. Biol. **21:**5299–5305.
29. **Nakajima, T., M. Kimura, K. Kuroda, M. Tanaka, A. Kikuchi, H. Seino, F. Yamao, and K. Oda.** 1997. Induction of ubiquitin conjugating enzyme activity for degradation of topoisomerase IIα during adenovirus E1A-induced apoptosis. Biochem. Biophys. Res. Commun. **239:**823–829.
30. **Nakajima, T., K. Morita, N. Oh, T. Arai, N. Nozaki, A. Kikuchi, F. Osaka, F. Yamao, and K. Oda.** 1996. Degradation of topoisomerase IIα during adenovirus E1A-induced apoptosis is mediated by the activation of the ubiquitin proteolysis system. J. Biol. Chem. **271:**23842–24849.
31. **Pahl, H. L.** 1999. Activators and target genes of Rel/NF-κB transcription factors. Oncogene **18:**6853–6866.
32. **Perez, D., and E. White.** 1998. E1B 19K inhibits Fas-mediated apoptosis through FADD-dependent sequestration of FLICE. J. Cell Biol. **141:**1255–1266.
33. **Perez, D., and E. White.** 2000. TNF-α signals apoptosis through a Bid-dependent conformational change in Bax that is inhibited by E1B 19K. Mol. Cell **6:**53–63.
34. **Phillips, A. C., M. K. Ernst, S. Bates, N. R. Rice, and K. H. Vousden.** 1999. E2F-1 potentiates cell death by blocking antiapoptotic signaling pathways. Mol. Cell **4:**771–781.
35. **Pilder, S., J. Logan, and T. Shenk.** 1984. Deletion of the gene encoding the adenovirus 5 early region 1B 21,000-molecular-weight polypeptide leads to degradation of viral and cellular DNA. J. Virol. **52:**664–671.
36. **Rothe, M., M. G. Pan, W. J. Henzel, T. M. Ayres, and D. V. Goeddel.** 1995. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell **83:**1243–1252.
37. **Roulston, A., R. C. Marcellus, and P. E. Branton.** 1999. Viruses and apoptosis. Annu. Rev. Microbiol. **53:**577–628.
38. **Routes, J., S. Ryan, A. Clase, T. Miura, A. Kuhl, T. Potter, and J. Cook.** 2000. Adenovirus E1A oncogene expression in tumor cells enhances killing by TNF-related apoptosis-inducing ligand (TRAIL). J. Immunol. **165:**4522–4527.
39. **Roy, N., Q. Deveraux, R. Takahashi, and J. Reed.** 1997. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J. **16:**6914–6925.

40. **Scaffidi, C., I. Schmitz, P. Krammer, and M. Peter.** 1999. The role of c-FLIP in modulation of CD95-induced apoptosis. J. Biol. Chem. **274:**1541–1548.
41. **Shao, R., M. C. T. Hu, B. P. Zhou, S.-Y. Lin, P. J. Chiao, R. H. von Lindern, B. Spohn, and M.-C. Hung.** 1999. E1A sensitizes cells to tumor necrosis factor-induced apoptosis through inhibition of IκB kinases and nuclear factor κB activities. J. Biol. Chem. **274:**21495–21498.
42. **Shisler, J., P. Duerksen-Hughes, T. M. Hermiston, W. S. Wold, and L. R. Gooding.** 1996. Induction of susceptibility to tumor necrosis factor by E1A is dependent on binding to either p300 or p105-Rb and induction of DNA synthesis. J. Virol. **70:**68–77.
43. **Sundararajan, R., A. Cuconati, D. Nelson, and E. White.** 2001. Tumor necrosis factor-α induces Bax-Bak interaction and apoptosis, which is inhibited by adenovirus E1B 19K. J. Biol. Chem. **276:**45120–45127.
44. **Sundararajan, R., and E. White.** 2001. E1B 19K blocks Bax oligomerization and tumor necrosis factor alpha-mediated apoptosis. J. Virol. **75:**7506–7516.
45. **Thome, M., P. Schneider, K. Hofmann, H. Fickenscher, E. Meinl, F. Neipel, C. Mattmann, K. Burns, J.-L. Bodmer, M. Schröter, C. Scaffidl, P. H. Krammer, M. E. Peter, and J. Tschopp.** 1997. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature (London) **386:**517–521.
46. **Tschopp, J., M. Irmler, and M. Thome.** 1998. Inhibition of Fas death signals by FLIPs. Curr. Opin. Immunol. **10:**552–558.
47. **Van Antwerp, D. J., S. J. Martin, T. Kafri, D. R. Green, and I. M. Verma.** 1996. Suppression of TNF-α-induced apoptosis by NF-κB. Science **274:**787–789.
48. **Wallach, D., E. E. Varfolomeev, N. L. Malinin, Y. V. Goltsev, A. V. Kovalenko, and M. P. Boldin.** 1999. Tumor necrosis factor receptor and Fas signaling mechanisms. Annu. Rev. Immunol. **17:**331–367.
49. **Wang, C.-Y., M. W. Mayo, and A. S. Baldwin, Jr.** 1996. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. Science **274:**784–787.
50. **Wei, M., W.-X. Zong, E. Cheng, T. Lindsten, V. Panoutsakopoulou, A. Ross, K. Roth, G. MacGregor, C. Thompson, and S. Korsmeyer.** 2001. Proapoptotic Bax and Bak: a requisite gateway to mitochondrial dysfunction and death. Science **292:**727–730.
51. **Wei, M. C., T. Lindsten, V. K. Mootha, S. Weiler, A. Gross, M. Ashiya, C. B. Thompson, and S. J. Korsmeyer.** 2000. tBid, a membrane-targeted death ligand, oligomerizes Bak to release cytochrome *c*. Genes Dev. **14:**2060–2071.
52. **White, E.** 1998. Regulation of apoptosis by adenovirus E1A and E1B oncogenes. Semin. Virol. **8:**505–513.
53. **White, E., and R. Cipriani.** 1990. Role of adenovirus E1B proteins in transformation: altered organization of intermediate filaments in transformed cells that express the 19-kilodalton protein. Mol. Cell. Biol. **10:**120–130.
54. **White, E., P. Sabbatini, M. Debbas, W. S. M. Wold, D. I. Kusher, and L. Gooding.** 1992. The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor alpha. Mol. Cell. Biol. **12:**2570–2580.
55. **Wold, W. S. M., K. Doronin, K. Toth, M. Kuppuswamy, D. L. Lichtenstein, and A. E. Tollefson.** 1999. Immune responses to adenoviruses: viral evasion mechanisms and their implications for the clinic. Curr. Opin. Immunol. **11:**380–386.
56. **Yang, Y., S. Fang, J. P. Jensen, A. M. Weissman, and J. D. Ashwell.** 2000. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science **288:**874–877.
57. **Yeh, W.-C., A. Itie, A. Elia, M. Ng, H.-B. Shu, A. Wakeham, C. Mirtsos, N. Suzuki, M. Bonnard, D. Goeddel, and T. Mak.** 2000. Requirement for casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. Immunity **12:**633–642.
58. **Zong, W. X., L. C. Edelstein, C. Chen, J. Bash, and C. Gelinas.** 1999. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-κB that blocks TNF alpha-induced apoptosis. Genes Dev. **13:**382–387.