The adenovirus E1A protein has been implicated in increasing cellular susceptibility to apoptosis induced by tumor necrosis factor (TNF); however, its mechanism of action is still unknown. Since activation of nuclear factor κB (NF-κB) has been shown to play an anti-apoptotic role in TNF-induced apoptosis, we examined apoptotic susceptibility and NF-κB activation induced by TNF in the E1A transfectants and their parental cells. Here, we reported that E1A inhibited activation of NF-κB and rendered cells more sensitive to TNF-induced apoptosis. We further showed that this inhibition was through suppression of IκB kinase (IKK) activity and IκB phosphorylation. Moreover, deletion of the p300 and Rb binding domains of E1A abolished its function in blocking IKK activity and IκB phosphorylation, suggesting that these domains are essential for the E1A function in down-regulating IKK activity and NF-κB signaling. However, the role of E1A in inhibiting IKK activity might be indirect. Nevertheless, our results suggest that inhibition of IKK activity by E1A is an important mechanism for the E1A-mediated sensitization of TNF-induced apoptosis.

E1A sensitizes cells to tumor necrosis factor-induced apoptosis through inhibition of IκB kinases and nuclear factor κB activities.*

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activation. We found that the cells transfected with E1A, but not E1A mutants, became very sensitive to TNF-induced apoptosis. Furthermore, we found that this E1A-mediated sensti-

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cultures**—The establishment and culture conditions of ip1-E1A2, ip1-Efs, and SKOV3.ip1 cell lines have been described previously (4). To establish the Edl.0108/ip1 cell line, the SKOV3.ip1 cells were transfected with the pE1A mutant DNA whose p300 and Rb binding domains were deleted. All of these stable cell lines were cell clones which were isolated by G418 selection. The culture conditions for the Edl.0108/ip1 cells, a human prostate cancer cell line (PC3), and its transfectants (PC3-E1A1 and PC3-neo) were the same as those for ip1-E1A2, ip1-Efs, and SKOV3.ip1 cells.

**Apoptosis Assays**—The luciferase-based *in vitro* cell viability assay was performed as described previously (23). Specifically, ip1-E1A2, ip1-Efs, and Edl.0108/ip1 cells were transfected with the cytomegalovirus (CMV) promoter-luciferase expressing vector (pCMV-luc), using liposome as a gene delivery vehicle. About 36 h after transfection, the cells were treated with or without TNF (20 ng/ml). After incubation for an additional 12 h, the cells were lysed, and the luciferase activity was determined. The percentage of luciferase activity of the TNF-treated cells was normalized by using the percentage of luciferase activity of the untreated cells (100%) as base line. Standard deviations were calculated from three independent experiments. The apoptotic cells were also analyzed by the deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay as described previously (24). Briefly, the cells were seeded in an eight-chamber slide (1.6 × 10^4 cells/chamber) for 4 h before they were treated with or without TNF. The cells were cultured for 3 days followed by a TUNEL assay. Quantitation of apoptotic cells was indicated as a percentage over the total cell number per field (100%), and error bars depict S.D. of triplicate samples.

**Electrophoretic Mobility Shift Assay (EMSA) and Western Blot Analysis**—The cells were treated with TNF (20 ng/ml) for 30 min or untreated (as controls). Cell extracts were prepared, and EMSA for NF-κB was performed as described previously (9). For Western blot analysis, the cells were treated with TNF (20 ng/ml) for 4 min or left untreated (as controls), cell extracts were prepared, and Western blot analysis was performed as described previously (4). Antibodies against RelA (SC-109, Santa Cruz) and IκB-α (SC-371, Santa Cruz) were used.

**Dephosphorylation Assay**—IκB-α was immunoprecipitated from the cell lysates using an anti-IκB-α antibody (SC-371, Santa Cruz). The precipitates were then incubated with calf intestinal phosphatase (Promega) at 37 °C for 30 min. Subsequently, the samples were dissolved in the loading buffer and subjected to 12% SDS-polyacrylamide gel electrophore-
The precipitates were incubated with or without calf intestine phosphatase (CIP) for 30 min at 37 °C, before they were subjected to SDS-PAGE (12% gel). The phosphorylation of IκBα (p-IκBα) and IκBα were detected by Western blot with an anti-IκBα antibody (top two panels). As a control, the same blot was probed with an anti-actin antibody (bottom panel). B, phosphorylation of IκBα induced by TNF could be dephosphorylated by a phosphatase. Equal amount of total cell lysates from the TNF-induced ip1-Efs and SKOV3.ip1 cells were immunoprecipitated with an anti-IκBα antibody. The precipitates were incubated with or without calf intestine phosphatase (CIP) for 30 min at 37 °C, before they were subjected to SDS-PAGE (12% gel). IκBα was detected by Western blot with an anti-IκBα antibody. C, phosphorylation of IκBα induced by TNF in PC3 cell was detected by Western blot analysis. The PC3-E1A1, PC3-neo, and PC3 cells were treated with or without TNF, and p-IκBα and IκBα were detected by Western blot analysis as described above.

sis (PAGE) and immunoblot analysis using the same anti-IκB-α antibody. 

Transient Transfections and Immunocomplex Kinase Assays—The various ip1 cell lines were plated the day before transfection at a density of 2 × 10^6 cells per 100-mm dish. Cells were transfected with either an IKK-α or IKK-β expression vector or an empty vector (control), using liposome as described above. Cell extracts were prepared 48 h after transfection, and immunocomplex kinase assays were performed as described previously (25).

RESULTS AND DISCUSSION

To test whether E1A could affect cellular susceptibility to cell death induced by TNF, an in vitro cell viability assay was performed using a luciferase assay (23). We used a human ovarian cancer cell line derivative, SKOV3.ip1, which was stably transfected with the wild-type Ad5 E1A (ip1-E1A2) or an E1A frameshift Efs mutant (ip1-Efs) that has lost the E1A function, or an E1A deletion mutant (Edl.0108/ip1) whose p300 and Rb binding domains were deleted (10). When the cells were transiently transfected with the luciferase reporter gene (pCMV-luc) and treated with TNF, the luciferase activity was strongly reduced in the ip1-E1A2 cells compared with the parental SKOV3.ip1 and the mutant ip1-Efs cells (Fig. 1A). This suggests that E1A sensitizes cells to TNF-induced cell death. However, this E1A-mediated sensitization of TNF-induced cell death was abolished in the Edl.0108/ip1 cells, suggesting that the p300 and Rb binding domains of E1A are required for this sensitization. This is consistent with the previous finding that the E1A function in increasing cellular susceptibility to cell death induced by TNF depends on its binding to either p300 or p105Rb (10). As a control, we examined the expression of E1A in these cell lines (ip1-E1A2, ip1-Efs, SKOV3.ip1, and Edl.0108/ip1) by Western blot analysis using an anti-E1A monoclonal antibody (mAb) (M58). These results indicated that the ip1-E1A2 and Edl.0108/ip1 cells expressed a significant amount of E1A, but the parental (SKOV3.ip1) and the mutant (ip1-Efs) cells did not (insert, Fig. 1A). As another control, we also examined the expression of TNF receptor (TNFR) in these cell lines (ip1-E1A2, ip1-Efs, SKOV3.ip1, and Edl.0108/ip1) by Western blot analysis using an anti-TNFR1 antibody (R&D System) and showed that the levels of TNFR were similar in these cell lines (data not shown). To confirm the E1A-mediated sensitization of TNF-induced apoptosis, we examined these TNF-treated ip1-E1A2 cells by a TUNEL assay. As shown in Fig. 1B, TNF induced apoptotic DNA breakage in the ip1-E1A2 cells. As negative controls, the ip1-Efs and SKOV3.ip1 cells did not show apoptotic phenotypes with the TNF treatment. Moreover, many of the ip1-E1A2 cells treated with TNF showed the morphologic changes associated with apoptosis, including cell shrinking and apoptotic body formation (data not shown). Taken together, these results suggest that E1A sensitizes cells to TNF-induced apoptosis.

Recently, NF-κB has been shown to have an important role in the antiapoptotic pathway (9, 18–22). Although NF-κB plays a role in blocking apoptosis induced by TNF (19–22), the involvement of NF-κB in the E1A-mediated cellular susceptibility to TNF has not been examined. Therefore, the ip1-E1A2, ip1-Efs, and Edl.0108/ip1 cells for their NF-κB DNA binding activities before and after TNF treatment were analyzed by EMSA. Our results showed that TNF induced NF-κB DNA binding activity in the ip1-Efs and Edl.0108/ip1 cells, but not in the ip1-E1A2 cells (Fig. 2A). The activated NF-κB complex...
induced by TNF in the ip1-Efs cells was eliminated in the presence of excess cold wild-type, but not mutant, NF-\(\kappa\)B oligonucleotides, suggesting that the activated NF-\(\kappa\)B DNA binding activity is NF-\(\kappa\)B DNA specific (Fig. 2A). To confirm the presence of the RelA subunit of NF-\(\kappa\)B, we performed the same EMSA in the presence of an anti-RelA polyclonal antibody. A RelA-specific super-shifted complex was detected, indicating that the binding complex is indeed an activated NF-\(\kappa\)B (Fig. 2A), presumably the RelA/p50 heterodimer as reported previously (9). Thus, these results indicate that E1A is capable of inhibiting NF-\(\kappa\)B activation induced by TNF. To test whether inhibition of NF-\(\kappa\)B activity correlates with inhibition of NF-\(\kappa\)B protein expression, whole-cell extracts from the ip1-E1A2, SKOV3.ip1, ip1-Efs, and Edl.0108/ip1 cells with or without TNF treatment were examined by Western blot analysis using an anti-RelA antibody. While NF-\(\kappa\)B (RelA) protein expression was not inhibited by E1A, its expression was slightly enhanced by TNF stimulation (Fig. 2B). Although this increase of NF-\(\kappa\)B protein by TNF may partially contribute to the TNF-induced NF-\(\kappa\)B activity, it does not account for the inhibition of TNF-induced NF-\(\kappa\)B activity by E1A. Our data suggest that E1A inhibits NF-\(\kappa\)B activation but not its protein expression.

To further study how E1A might inhibit the TNF-induced NF-\(\kappa\)B activity, we investigated whether E1A could down-regulate NF-\(\kappa\)B activity through inhibition of I\(\kappa\)B phosphorylation. The changes of phosphorylation and expression of I\(\kappa\)B-\(\alpha\) in the ip1-E1A2, SKOV3.ip1, ip1-Efs, and Edl.0108/ip1 cells treated with TNF for 4 min were examined by Western blot analysis. As shown in Fig. 3A, there was only one I\(\kappa\)B-\(\alpha\) band observed in the ip1-E1A2 cells with or without treatment with TNF. The same band was also detected in the non-TNF-treated SKOV3.ip1, ip1-Efs, and Edl.0108/ip1 cells. However, two I\(\kappa\)B-\(\alpha\) bands were detected in the TNF-treated SKOV3.ip1, ip1-Efs, and Edl.0108/ip1 control cells (Fig. 3A); the upper band should be the phosphorylated form of I\(\kappa\)B-\(\alpha\). To confirm this, we immunoprecipitated I\(\kappa\)B-\(\alpha\) from the cell extracts with an anti-I\(\kappa\)B-\(\alpha\) antibody. Then the precipitated pellets were treated with or without calf intestinal phosphatase and subjected to immunoblotting with the same anti-I\(\kappa\)B-\(\alpha\) antibody. The results showed that the upper band disappeared after the calf intestinal phosphatase treatment (Fig. 3B), indicating that the upper band is indeed the phosphorylated form of I\(\kappa\)B-\(\alpha\), and E1A inhibits TNF-induced I\(\kappa\)B-\(\alpha\) phosphorylation. The finding that the level of I\(\kappa\)B-\(\alpha\) protein was elevated in the ip1-E1A2 cells might be due to slower degradation of the nonphosphorylated I\(\kappa\)B-\(\alpha\) protein (Fig. 3A). Furthermore, the E1A-mediated suppression of I\(\kappa\)B phosphorylation induced by TNF was confirmed by using a human prostate cancer cell line PC3 and its E1A transfectants (Fig. 3C) and a human ovarian cancer cell line 2774 (data not shown).

It has been well documented that TNF induces the activation of IKK, which in turn phosphorylate I\(\kappa\)B-\(\alpha\) with the subsequent activation of NF-\(\kappa\)B (11, 14). To examine whether E1A could regulate IKK activity, the ip1-E1A2, SKOV3.ip1, ip1-Efs, and Edl.0108/ip1 cells were treated with TNF, and the endogenous IKK-\(\alpha\) and IKK-\(\beta\) activities were determined by immunocomplex kinase assays. The endogenous IKK-\(\alpha\) (Fig. 4A) and IKK-\(\beta\) (Fig. 4B) activities were readily detected in the SKOV3.ip1, ip1-Efs, and Edl.0108/ip1 cells, whereas the activities of both IKKs in the ip1-E1A2 cells were inhibited. To confirm that E1A could inhibit the activities of IKK-\(\alpha\) and IKK-\(\beta\) expressed ectopically, we transfected an IKK-\(\alpha\)-FLAG or a HA-IKK-\(\beta\) into 293T cells. Whole cell lysates were immunoprecipitated with an anti-E1A mAb (M58), and the immunoprecipitates were subjected to Western blot analysis with either an anti-FLAG or anti-HA mAb. While M58 mAb immunoprecipitated E1A protein (∼43 kDa) from the cell lysates, neither anti-FLAG nor anti-HA mAb detected IKK-\(\alpha\)-FLAG or HA-IKK-\(\beta\) immunoprecipitated with E1A in Western blot analysis (data not shown). Similarly, cell lysates were immunoprecipitated with either an anti-FLAG or anti-HA mAb, and the immunoprecipitates were then subjected to Western blot analysis with M58 mAb. Again, no detectable E1A protein was immunoprecipitated with either IKK-\(\alpha\)-FLAG or HA-IKK-\(\beta\) (data not shown). These results indicated no direct binding between E1A and IKK, suggesting that E1A might act on IKK indirectly. Further investigation of the direct cellular target(s) of E1A in this signaling pathway is necessary to elucidate the mechanism underlying the E1A-regulated IKK activity and apoptosis.

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