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13. ABSTRACT ***(Maximum 200 Words)***

MEK kinase 1 (MEKK1) is a serine threonine kinase that induces apoptosis. Activation of specific apoptotic pathways leads to cleavage of MEKK1 by caspase 3-like proteases into a 91kDa fragment containing the kinase domain. Upon cleavage, MEKK1 activates the caspase 3-like proteases in a feedback loop leading to apoptosis. MEKK1 is also cleaved into the 91kDa kinase domain in response to genotoxic agents such as etoposide or ultraviolet irradiation (UV). Thus, overexpression of kinase inactive MEKK1 inhibits both etoposide and UV-induced apoptosis. Akt is an anti-apoptotic serine threonine kinase that inhibits both etoposide and UV-induced apoptosis. We show that Akt blocks MEKK1-induced apoptosis in HEK 293 cells. MEKK1-induced caspase 3-like protease activation is inhibited with expression of Akt. This inhibition by Akt prevents cleavage of endogenous MEKK1 following exposure to etoposide and UV. Also, in an early signaling event, we show that MEKK1 leads to activation of death receptor 4 (DR4) and death receptor 5 (DR5). Expression of either the decoy receptor 1 (DcR1) or FADD dominant negative protein (FADD DN) inhibits MEKK1-induced apoptosis. Akt, however, failed to block etoposide induced upregulation of DR4 and DR5 expression and activation of caspase 8. In addition, Akt does not block MEKK1-induced JNK activation. Thus, we delineate that Akt inhibits MEKK1-induced apoptosis specifically through blocking caspase 3-like protease activation and amplification, resulting in inhibition of cleavage of MEKK1 to its 91 kDa fragment. .

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INTRODUCTION:

The uncontrolled growth of cancer cells can arise from either a loss of apoptotic signaling or proliferative signaling. With cancer chemotherapeutic and anti-angiogenic drugs, the shrinkage of tumors was found to be the result of increased apoptotic index. Thus, pathways influencing proliferation and apoptosis will complement the action of cancer therapies. Combined modality therapy that promotes pro-apoptotic signaling or inhibits proliferation in addition to cancer chemotherapeutic and anti-angiogenic drugs would form an efficacious strategy for breast cancer therapy. We were interested in understanding the interplay between MEKK1 and Akt kinases in order to determine the balance between specific pro- and anti- apoptotic pathways important in cancer cell survival and death. To accomplish this, we investigated the specific mechanisms by which MEKK1 induces apoptosis, and the ability of the oncogenic protein Akt to inhibit MEKK1's apoptotic induction.

Akt is a proto-oncogene upregulated in certain breast cancer cells that promotes cell survival. Lack of estrogen receptor expression and increased expression levels of the HER2/Neu receptor are frequent aberrations seen in breast cancer. In breast cancer cells lacking estrogen receptor, Akt activity is greatly increased when compared to estrogen receptor positive breast cancer cells. Also, heregulin is a potent activator of Akt; therefore, overexpression of HER2/Neu in breast cancer could lead to increased Akt activation and subsequent cell survival. Further, Akt is a key mediator of aberrant survival from cell detachment induced apoptosis (anoikis). This mechanism allows cells to survive in the absence of adhesion to the extracellular matrix, such as occurs with metastasis. Akt functions to inhibit apoptosis by inhibition of pro-apoptotic proteins and by activation of anti-apoptotic proteins. Pro-apoptotic proteins inhibited by Akt include caspase 9, BAD, and the Forkhead transcription factor. Akt phosphorylates pro-caspase 9, leading to inhibition of cytochrome c induced proteolytic caspase 9 activation. In specific cell types, Akt has also been shown to phosphorylate BAD. Phosphorylated BAD then binds to 14-3-3, preventing its ability to bind to and sequester the anti-apoptotic Bcl-2 protein. In addition, Akt phosphorylates the transcription factor Forkhead and prevents translocation of Forkhead from the cytoplasm to the nucleus where it can induce transcriptional upregulation of pro-apoptotic proteins. Akt phosphorylation also leads to the activation of specific anti-apoptotic factors. For example, Akt mediated phosphorylation of CREB causes increased transcription and expression Mcl-2, a Bcl-2 family member. In summary, Akt's mediation of cell survival following pro-apoptotic stimuli is through both inhibition of pro-apoptotic proteins and through activation of anti-apoptotic proteins (1,2). Alternatively, anoikis activates MEK kinase 1 (MEKK1), a serine threonine kinase that induces apoptosis through both transcriptional regulation and caspase amplification (5). Expression of MEKK1 leads to its cleavage at Asp874 by caspase 3-like proteases into a 91kDa fragment containing the kinase domain (6). Upon cleavage, MEKK1 activates the caspase 3-like proteases in a feedback loop leading to apoptosis. MEKK1 is also cleaved into its 91kDa kinase domain in response to genotoxic agents such as etoposide or ultraviolet irradiation (UV)(7). Mutation of the consensus caspase 3 cleavage site of MEKK1 inhibits its ability to induce apoptosis. Further, overexpression of a kinase inactive MEKK1 inhibits both etoposide and UV-induced apoptosis, suggesting that the kinase activity of MEKK1 is essential in etoposide and UV induced apoptosis(7). Therefore, MEKK1 exerts it pro-apoptotic effects both through through regulation of caspase activation.

BODY:

MEK kinase 1 (MEKK1) is a serine threonine kinase that induces apoptosis. Activation of specific apoptotic pathways leads to cleavage of MEKK1 by caspase 3-like proteases into a 91kDa fragment containing the kinase domain. Upon cleavage, MEKK1 activates the caspase 3-like proteases in a feedback loop leading to apoptosis (6). MEKK1 is also cleaved into the 91kDa kinase domain in response to genotoxic agents such as etoposide or ultraviolet irradiation (UV) (7). Thus, overexpression of kinase inactive MEKK1 inhibits both etoposide and UV-induced apoptosis. Akt is an anti-apoptotic serine threonine kinase that inhibits both etoposide and UV-induced apoptosis. We show that Akt blocks MEKK1-induced apoptosis in HEK 293 cells. MEKK1-induced caspase 3-like protease activation is inhibited with expression of Akt. This inhibition by Akt prevents cleavage of endogenous MEKK1 following exposure to etoposide and UV. Also, in an early signaling event, we show that MEKK1 leads to activation of death receptor 4 (DR4) and death receptor 5 (DR5). Expression of either the decoy receptor 1 (DcR1) or FADD dominant negative protein (FADD DN) inhibits MEKK1-induced apoptosis. Akt, however, failed to block etoposide induced upregulation of DR4 and DR5 expression and activation of caspase 8. In addition, Akt does not block MEKK1-induced JNK activation. Thus, we delineate that Akt inhibits MEKK1-induced apoptosis specifically through blocking caspase 3-like protease activation and amplification, resulting in inhibition of cleavage of MEKK1 to its 91 kDa fragment. .

Akt blocks MEKK1 induced apoptosis and caspase 3-like protease activation.

Human embryonic kidney (HEK) 293 cells were transiently transfected with full length MEKK1 in the presence or absence of constitutively active myristoylated Akt (myr-Akt), wild type Akt (wt-Akt), p35, or vector alone and stained for protein expression using anti-MEKK1 and anti-Akt antibodies. p35 is a protein previously found by our lab to inhibit MEKK1 cleavage and apoptosis, and is used as a control. Expression of myr-Akt and wt-Akt resulted in increased kinase activity as determined by an Akt kinase assay (data not shown). Percent apoptosis was quantified using a TdT-based TUNEL assay. The number of cells expressing MEKK1 and positive for TdT staining were then counted by fluorescence microscopy. At least 400 cells were counted for each condition in three separate experiments. 42.5% of cells expressing MEKK1 (with pCMV5 to equalize total DNA concentration) were apoptotic. In cells coexpressing MEKK1 with either myr-Akt or wt-Akt, the percentage of apoptotic cells was 8.5% and 7.6%, respectively. These results reflect an 80% inhibition of MEKK1-induced apoptosis by Akt. As a control, cells coexpressing p35 had a 14.7% apoptosis level (Figure 1A in appendix 1). Thus, Akt inhibits MEKK1-induced apoptosis.

To determine if Akt blocks MEKK1-induced caspase activation, HEK 293 cells were transiently transfected with full length MEKK1 with or without the expression of myr-Akt and wt-Akt. Caspase activity was determined by measuring cleavage levels of a caspase 3 consensus substrate (DEVE-AFC) as described in the Materials and Methods section. Expression of MEKK1 alone causes a 2.1 fold increase in caspase 3-like protease activity above baseline. This fold increase was reduced to below basal levels by coexpression with myr-Akt (0.87 fold) and to basal level by coexpression with wt-Akt (1.07 fold) (Figure 1B in appendix 1). These results are consistent with the hypothesis that Akt blocks MEKK1-induced apoptosis by inhibiting caspase 3-like protease activation.

Endogenous MEKK1 is cleaved by caspase 3-like proteases following treatment with genotoxic agents. Since Akt blocks caspase 3-like protease activation, Akt could potentially inhibit cleavage of endogenous MEKK1 following genotoxin treatment. HEK 293 cells expressing myr-Akt or vector alone were treated with etoposide (100μM) or UV-C (40 J/m^2). After treatment, cells were lysed and assayed for full-length MEKK1 expression by western blotting. After 48 hours of etoposide treatment, cleavage of endogenous MEKK1 was inhibited in the presence of myr-Akt when compared to cells expressing vector alone (Figure 2A). Following UV irradiation, cells expressing myr-Akt and wt-Akt also blocked cleavage of full length MEKK1 compared to cells expressing pCMV5 or β-gal alone (Figure 2B). This indicates that Akt blocks cleavage of endogenous MEKK1.

MEKK1 mediated apoptosis requires activation of death receptor 4 and death receptor 5.

MEKK1 kinase activity is activated following etoposide treatment and kinase inactive MEKK1 blocks etoposide induced upregulation of DR4 and DR5 expression and apoptosis. This suggest that MEKK1-induced apoptosis involves the activation of DR4 and DR5. To determine if DR4 and DR5 are involved in MEKK1-induced apoptosis, HEK 293 cells were stably transfected with cDNA for DcR1. DcR1 binds to the ligand for DR4 and DR5, TRAIL but fails to induce an apoptotic response. Full length MEKK1 was transiently transfected into these cells and the extent of apoptosis determined by TdT staining in a Tunel assay as described in Materials and Methods section. HEK 293 cells containing only empty vector showed 25% apoptosis when MEKK1 was expressed but HEK 293 cells expressing DcR1 showed only 5% apoptosis in the presence of MEKK1 (Figure 3A). In addition, HEK 293 cells were also transiently transfected with MEKK1 in the absence or presence of FADD DN. FADD DN inhibits the activation of death receptors including DR4 and DR5 by blocking caspase 8 activation. Expression of MEKK1 alone caused 43% apoptosis while in the presence of FADD DN MEKK1 induced only 20% apoptosis that is similar to apoptosis in untransfected cells (Figure 3B). FADD DN expressed alone also showed similar apoptosis as control. These results suggest that MEKK1-induced apoptosis involves activation of DR4 and DR5.

Akt fails to block etoposide mediated up-regulation of DR4 and DR5 and caspase 8 activation.

Kinase inactive MEKK1 inhibits etoposide-induced apoptosis and up-regulation of DR4 and DR5 expression. DR4 and DR5 activation plays a role in etoposide-induced apoptosis since blockage of the TRAIL binding to DR4 and DR5 inhibits etoposide-induced apoptosis. Since Akt blocks MEKK1-induced apoptosis, we determined if Akt blocks etoposide mediated up-regulation of DR4 and DR5. Etoposide treated cells expressing myr-Akt had increased expression of DR4 and DR5 compared to vector alone cells as determined by RNase protection assay (Figure 4A). Ligation of DR4 and DR5 leads to the activation of caspase 8 by cleavage to its active form. Caspase 8 activation in cells transfected with myr-Akt or vector alone was examined. The results show that caspase 8 is activated in both cells expressing myr-Akt and vector alone following etoposide treatment (Figure 4B). Over expression of Akt, however, did block etoposide-induced apoptosis (Figure 4C). This indicates that Akt inhibition of etoposide-induced apoptosis is not through prevention of DR4 and DR5 activation and probably is not involved in inhibiting MEKK1 mediated DR4 and DR5 activation.

Prevention of MEKK1-induced apoptosis by Akt is not mediated by inhibition of cytochrome c release from the mitochondria or JNK activation.

Akt blocks MEKK1 cleavage through inhibition of caspase 3-like proteases. A potential mechanism for this prevention could be inhibition of cytochrome c release from the mitochondria. Cytochrome c release from the mitochondria is essential for the activation of caspase 9. HEK 293 cells were transiently transfected with a HA-tagged 91 kDa MEKK1 cleavage fragment and stained for HA and cytochrome c. After 48 hours, 91kDa MEKK1 transfected cells underwent apoptosis (data not shown), but cytochrome c remained in the mitochondria as denoted as punctate structures in stained cells (Figure 5A). Cells transiently transfected with truncated BID (tBID) show that cytochrome c is released from the mitochondria and dispersed in the cell preventing bright staining of cytoplasmic cytochrome c (Figure 5A). Similar results were seen when full length MEKK1 was expressed in HEK 293 cells (data not shown). In addition, HEK 293 cells were transiently transfected with MEKK1 and lysed so that cytoplasmic and membrane fractions were separated. After western blotting for cytochrome c, there was no detectable cytochrome c in the cytoplasmic fraction following MEKK1 expression after 48 hours but cytochrome c was present in the cytosol fraction in cells transfected with tBID (Figure 5A). Thus, MEKK1-induced caspase activation is not mediated by release of cytochrome c from the mitochondria into the cytoplasm and subsequent activation of caspase 9. Akt's inhibition of MEKK1-induced caspase 3-like activation is thus not through blocking cytochrome c release from the mitochondria.

MEKK1 is known to activate c-Jun N-terminal kinase (JNK), a MAP kinase family member. JNK activation has been implicated in the induction of apoptosis. In HEK 293 cells transiently expressing MEKK1 and wt-Akt, the level of JNK activation was determined as described in the Material and Methods section.

Transfection of MEKK1 alone or with myr-Akt leads to equivalent levels of JNK activation (Figure 5B). Thus, Akt does not block MEKK1-induced JNK activation.

KEY RESEARCH ACCOMPLISHMENTS:

*Akt inhibits MEKK1 induced apoptosis

*MEKK1 induced caspase 3-like protease activation is suppressed by Akt

*Caspase inhibition by Akt prevents cleavage of endogenous MEKK1 following etoposide and UV irradiation treatment

*Earlier signaling events including etoposide induced MEKK1 kinase activity, MEKK1 mediated upregulation of DR4/5 expression, and activation of caspase 8 fail to be blocked by Akt

*MEKK1 does not induce cytochrome c release from the mitochondria, suggesting Akt is not inhibiting MEKK1 induced apoptosis by blocking BAD or caspase 9 (as these both exert their apoptotic functions by cytochrome c release)

*Akt does not inhibit MEKK1 induced JNK activity, suggesting JNK activation is not responsible for MEKK1's pro-apoptotic Effects

*MEKK1 functions to activate death receptor 4 and death receptor 5

*Expression of decoy receptor 1 or a FADD dominant negative protein inhibits MEKK1 induced apoptosis

*Akt inhibits MEKK1 induced apoptosis specifically through blocking caspase 3-like protease activation and amplification, resulting in inhibition of cleavage of MEKK1 to its 91 kDa fragment

REPORTABLE OUTCOMES:

Manuscript:

Andrea H. Bild, Erika M. Gibson, Jacylyn Onio, Timothy P. Garrington, Gary L. Johnson, and Spencer B. Gibson. Anti-apoptotic Akt/PKB blocks MEK kinase 1 (MEKK1) induced apoptosis by inhibiting MEKK1 cleavage. Evidence for a dual role for MEKK1 in cell death. *In preperation.*

Abstract:

MEKK1 and Akt: Opponents in Cell Death. Andrea H. Bild, Gary L. Johnson, and Spencer B. Gibson. Department of Defense, Era of Hope Meeting, Atlanta, GA, June 8-12, 2000.

CONCLUSIONS:

Overexpression of MEKK1 induces apoptosis in a variety of cell types. Expression of kinase inactive MEKK1 inhibits both genotoxin and anoikis-induced apoptosis. It is thought that MEKK1 apoptotic signaling involves its cleavage to a 91kDa kinase domain form that leads to activation of caspases. However there is evidence that MEKK1 may participate in apoptotic signaling prior to its cleavage. Following etoposide treatment, MEKK1 kinase activity is increased before it's cleaved by caspases. Expression of a kinase inactive form of MEKK1 blocks etoposide mediated increase in DR4 and DR5 expression, which has been shown to contribute to etoposide-induced apoptosis. This increase in DR4 and DR5 expression also occurs before cleavage of MEKK1. Since expression of kinase inactive MEKK1 also blocks etoposide-induced apoptosis, it is possible that MEKK1 mediated upregulation of DR4 and DR5 and their subsequent activation contributes to MEKK1-induced apoptosis. This theory is supported by the observation that blocking DR4 and DR5 activation through over expression of DcR1 or in the presence of FADD DN effectively blocks MEKK1-induced apoptosis. Thus, DR4 and DR5 activation is an early requirement for MEKK1-induced apoptosis. MEKK1 activation also increases FAS ligand expression in Jurkat T cells suggesting that MEKK1 regulation of death receptor activation is an important mechanism in its induction of apoptosis in different cell types. Only after 24 hours following etoposide treatment in HEK293 cells, MEKK1 cleavage into a 91-kDa kinase fragment by caspase 3-like proteases is detectable. This cleavage occurs parallel to etoposide-induced caspase 3-like activation (data not shown). Upon cleavage of MEKK1, further activation of caspases leads to the irreversible commitment of cells to undergo apoptosis. By inhibiting cleavage of MEKK1, amplification of caspase activation is prevented. Thus, MEKK1 could be involved in both early and late events in etoposide-induced apoptosis mediated by DR4 and DR5 activation and cleavage of MEKK1 into a pro-apoptotic 91-kDa kinase fragment (Figure 6).

Activation of Akt has been shown to block caspase activation and apoptosis following treatment with many different apoptotic stimuli, including anoikis and genotoxic agents. Therefore, Akt potentially contributes to survival of breast and other cancers after chemotherapy. Indeed, expression of myr-Akt prevents etoposide-induced apoptosis, a death pathway in which MEKK1 is involved. Since Akt inhibits MEKK1-induced cleavage and apoptosis, Akt protection against apoptosis could be acting through the prevention of protein cleavage such as MEKK1. MEKK1-induced apoptosis also involves activation of DR4 and DR5. Akt, however, fails to block MEKK1 activation (data not shown), up-regulation of DR4 and DR5 and caspase 8 activation following etoposide treatment. These results indicate that Akt blocks MEKK1-induced apoptosis at the level of caspase activation and amplification but after death receptor activation.

Etoposide increases the expression of DR4 and DR5, and is a proposed mechanism for genotoxin-induced apoptosis. The ligand for DR4 and DR5, TRAIL, is a potential new molecular based treatment for cancer due to its ability to reduce the size of human tumors in mice. In combination with genotoxic agents, TRAIL eradicates human tumors in mice and leads to a synergistic apoptotic response in breast cancer cell lines. These responses could at least partially be explained by the up-regulation of DR4 and DR5 expression. We have shown that Akt is effective at blocking MEKK1-induced apoptosis but not DR4 and DR5 activation. It follows that Akt will most likely not inhibit the early synergistic apoptotic response of TRAIL and genotoxin treatment, but instead will affect later apoptotic responses, when MEKK1 cleavage and caspase activation is occurring.

Akt inhibits caspase activation through a number of mechanisms. Akt phosphorylation of the Bcl-2 family member BAD prevents release of cytochrome c from the mitochondria and subsequent caspase activation. Akt can also prevent caspase activation through phosphorylation and inactivation of caspase 9. However, expression of the 91kDa kinase fragment or of full-length MEKK1 fails to release cytochrome c from the mitochondria. Given that both BAD and caspases 9 require release of cytochrome c from the mitochondria to exert their apoptotic functions, it is unlikely that Akt inhibits MEKK1-induced apoptosis by inactivating BAD or caspase 9. Akt also inactivates Forkhead transcription factor. This transcription factor has been implicated in the upregulation of Fas ligand expression. Increased Fas ligand expression has been shown to be involved in genotoxin-induced apoptosis similar to up-regulation of DR4 and DR5 expression. Following Fas ligation,

caspase 8 is specifically activated and leads to downstream apoptotic signaling and additional caspase activation. In view of the fact that Akt fails to block caspase 8 activation, it is unlikely that Akt's prevention of Forkhead mediated up-regulation of Fas ligand expression is the mechanism by which Akt inhibits MEKK1-induced apoptosis. Thus, instead of inhibiting the activity of pro-apoptotic proteins, Akt could be influencing the activity of anti-apoptotic proteins to prevent MEKK1-induced apoptosis. Akt activates the transcription factors CREB and NFκB. Both of these transcription factors have been shown to increase amounts of anti-apoptotic proteins such as Bcl-2 family members. Recently, it has been shown that inhibitor of apoptosis (IAP) proteins contain Akt phosphorylation sites that might activate their anti-apoptotic function. IAP family members (X-IAP, c-IAP1 and 2) can directly inhibit two members of the caspase 3-like family of proteases, caspase 3 and caspase 7, leading to inhibition of apoptosis. Activation of these anti-apoptotic proteins by Akt could be the mechanism for Akt inhibition of MEKK1-induced apoptosis; however, the exact mechanism remains to be determined.

Activation of MEKK1 leads to JNK activation. JNK has been implicated in the induction of apoptosis. In the presence of Akt, MEKK1 mediated JNK activation was not affected. This result suggests that JNK activation is not responsible for MEKK1's pro-apoptotic effects and that Akt's prevention of MEKK1-induced apoptosis is unrelated to MEKK1's regulation of JNK. It is still possible, however, that Akt could block downstream events following JNK activation leading to blockage of apoptosis. Since expression of kinase inactive MEKK1 fails to block JNK activation by etoposide (data not presented) but effectively blocks etoposide-induced apoptosis, it is unlikely that JNK is playing a major role in MEKK1-induced apoptosis. Indeed, MEKK1 knock out mice have decreased JNK response to cell stresses that alter the cytoskeleton and an increase apoptotic response to these stresses, suggesting that MEKK1-induced JNK activation is actually protective.

Our results show that Akt protects cells against MEKK1-induced apoptosis. This protection is through the inhibition of caspase 3-like protease activation. This inhibition prevents the cleavage of MEKK1 following genotoxin treatment, subsequently blocking further amplification of caspase activation. DR4 and DR5 activation is not blocked by over expression of Akt following etoposide treatment and is likely not the target of Akt prevention of MEKK1-induced apoptosis. These results provide evidence for a dual role for MEKK1in the induction of apoptosis, death receptor activation and caspase amplification in which Akt inhibits MEKK1-mediated caspase amplification. This identifies potential molecular targets that may be effective at regulating MEKK1 mediated apoptosis and overcoming survival signals, leading to more targeted breast cancer therapies.

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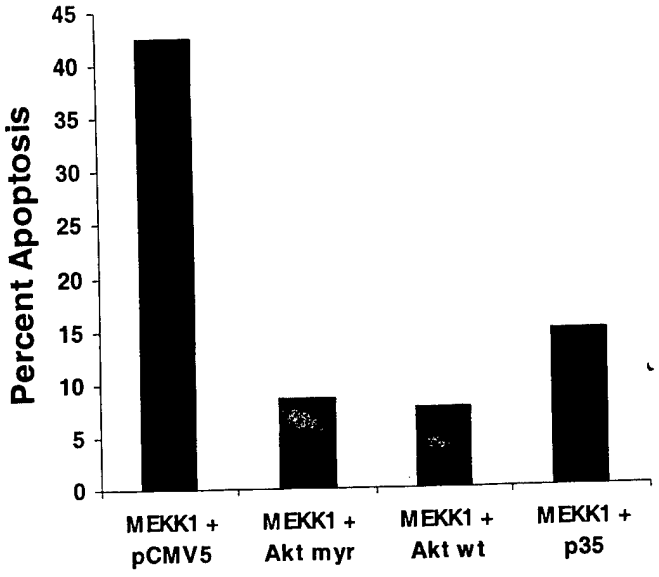
Figure 1 A)



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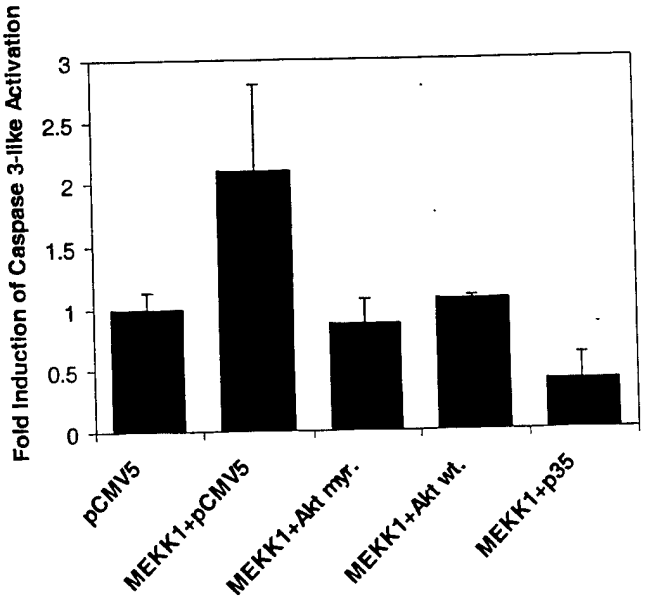

FIG. 1. Expression of myr-Akt and wt-Akt inhibit MEKK1-induced apoptosis and caspase activation. A) Apoptotic cells expressing MEKK1 and positive for the TUNEL based TdT staining were analyzed by fluorescence microscopy. The percentage of cells with apoptotic nuclei as determined by TdT staining for each condition was quantitated in a blind manner. At least 400 cells were counted for each condition in three different experiments. B) HEK 293 cells expressing full length MEKK1 and myr-Akt, wt-Akt, or p35 were lysed and measured for caspase activity by determining cleavage levels of a caspase 3 consensus substrate (DEVE-AFC) as described in the Material and Methods section. Levels of caspase activation were determined in respect to cells expressing pCMV5 vector.

Figure 2

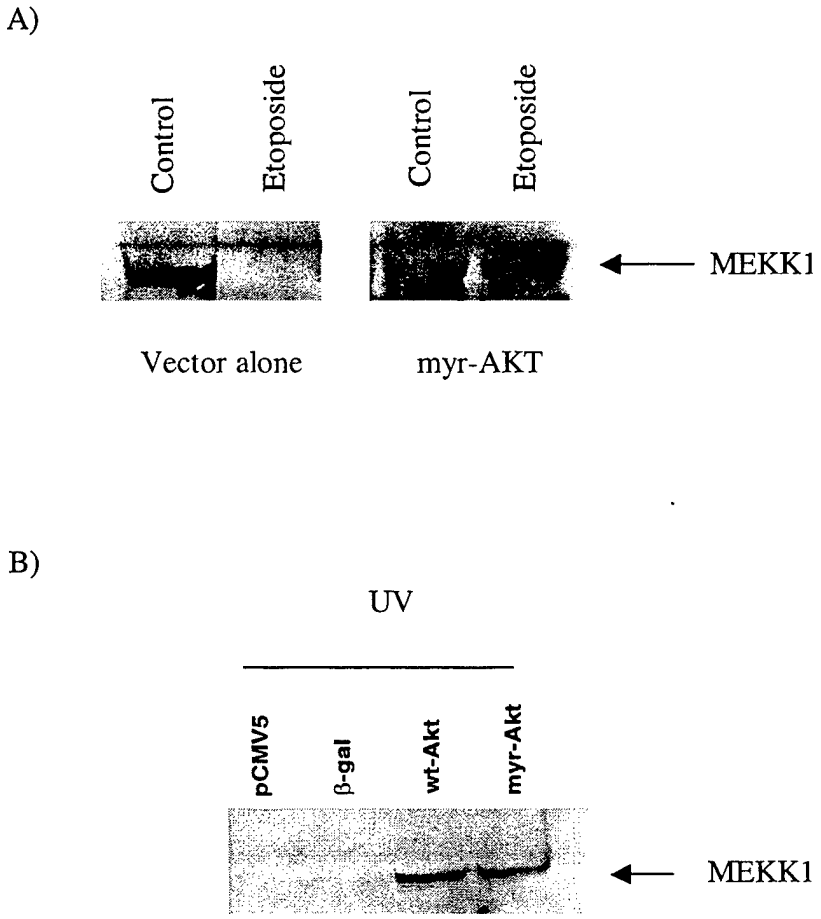

FIG. 2. Cleavage of endogenous MEKK1 following etoposide or ultraviolet radiation in HEK 293 cells expressing Akt. HEK 293 cells were treated with 100μM etoposide or ultraviolet radiation (UV) for 48 hours. The cells were then lysed as described in Material and Methods and western blotted for endogenous MEKK1 A) HEK 293 cells stably expressing vector alone or myr-Akt were treated with etoposide for 48 hours and western blotted with MEKK1. B) HEK 293 cells were transiently transfected with vector alone, wt-Akt or myr-Akt and treated with 40 J/m^2 UV. The cells were lysed and western blotted for endogenous MEKK1.

Figure 3 A)

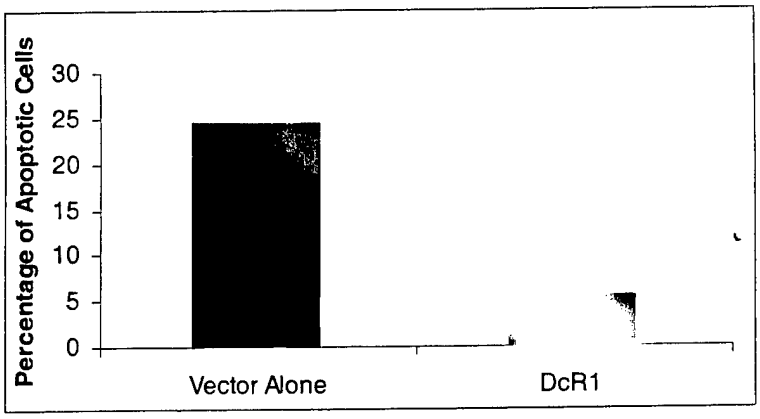

Figure 3 B)

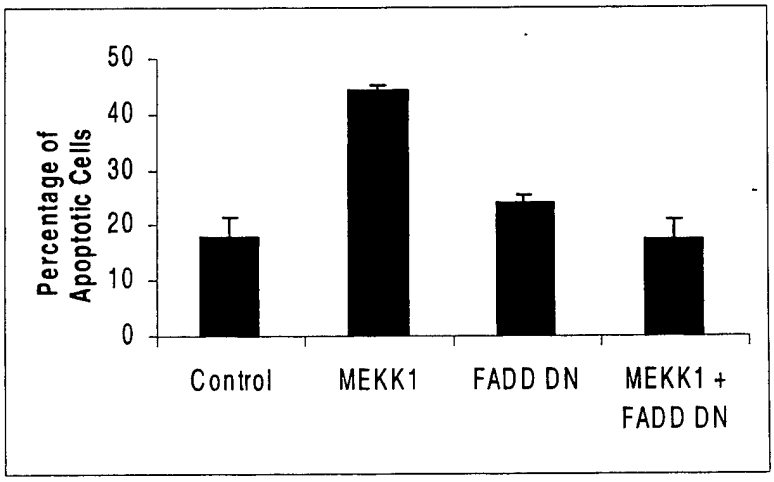

FIG. 3 MEKK1-induced apoptosis in HEK293 cells over expression of DcR1 and FADD DN. A) HEK 293 stably expressing DcR1 and vector alone were transiently transfected with MEKK1 for 48 hours. The cells were then stained for MEKK1 and TdT. Number of TdT positive cells expressing MEKK1 determined the amount of apoptosis. B) HEK 293 cells were transiently transfected with MEKK1 in the presence or absence of FADD DN. The amount of apoptosis was determined by acridine orange staining. Control cells were untransfected or only tranfected with FADD DN. These experiments were repeated three times.

Figure 4 A)

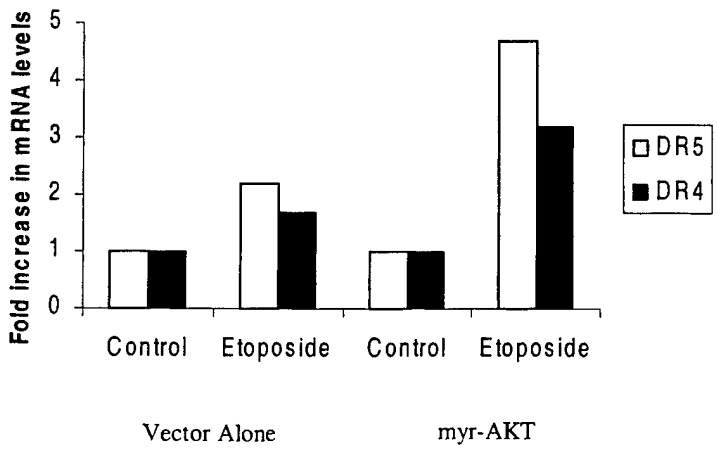

Figure 4 B)

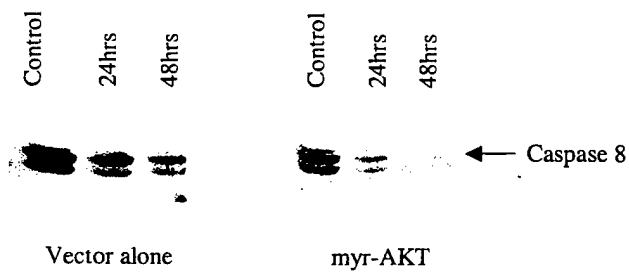

Figure 4 C)

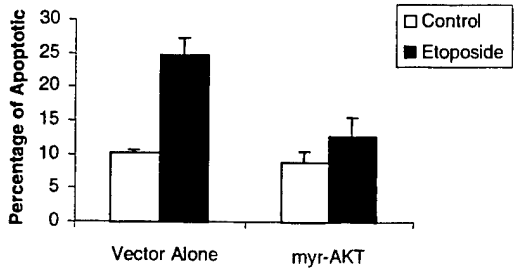

FIG. 4. DR4 and DR5 activation following etoposide treatment in HEK 293 cells A) HEK 293 cells expressing vector alone or myr-Akt were treated with 100μM of etoposide for 24 hours. mRNA was isolated from the cells and an RNase protection assay was preformed as described in the methods section. The expression levels of DR4 and DR5 were determined using a phosphorimager system and the fold increase of mRNA over untreated controls was determined. These results are indicative of trends observed in three separate experiments. B) HEK 293 cells expressing vector alone or myr-Akt were also treated with 100μM etoposide for 24 and 48 hours. The cells were lysed and western blotted for the full-length inactive form of caspase 8 as described in methods section. C) HEK 293 cells expressing vector alone or myr-Akt were treated with 100μM etoposide for 24 hours and the amount of apoptosis was determined as described in Materials and Method section. The standard deviation was determined by four separate experiments.

Figure 5 A) i)

Control 91kDa MEKK1

tBID

Figure 5 A) ii)

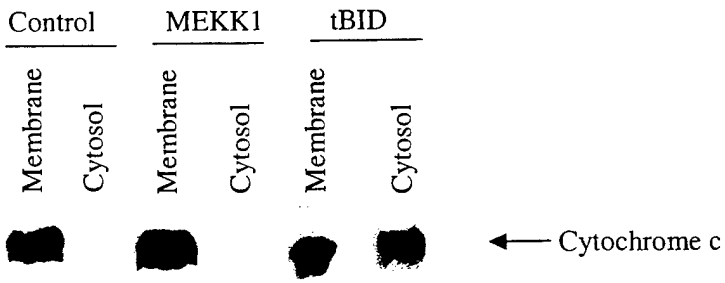

FIG. 5. Cytochrome c release from the mitochondria and JNK activation following expression of MEKK1 in HEK293 cells. A) i) HEK 293 cells were either transiently transfected with vector alone (control), with cleaved 91kDa MEKK1 form (91kDa MEKK1) or with truncated BID (tBID). The cells were then incubated for 48 hours and stained for the presence of cytochrome c (red) and for either 91kDa MEKK1 or tBID (green). The arrow denotes the presence of mitochondrial cytochrome c in 91kDa MEKK1 transfected cells. This experiment was repeated three separate times. ii) HEK 293 cells were transfected with full length MEKK1 or tBID for 48 hours. The cells were lysed and cytosolic and membrane fractions were separated. The fractions were western blotted for cytochrome c as described in materials and methods. B) HEK 293 cells were transfected with MEKK1 with either pCMV5 or myr-Akt, and pCMV5 with or without myr-Akt and analyzed for JNK activity in a GST-c-Jun assay. Kinase activity was quantitated by phosphorimaging.