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Signal Transduction Pathways Regulated by Mitogen-activated/Extracellular Response Kinase Kinase Kinase Induce Cell Death*

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Mitogen-activated/extracellular response kinase kinase (MEK) kinase (MEKK) is a serine-threonine kinase that regulates sequential protein phosphorylation pathways, leading to the activation of mitogen-activated protein kinases (MAPK), including members of the Jun kinase (JNK)/stress-activated protein kinase (SAPK) family. In Swiss 3T3 and REF52 fibroblasts, activated MEKK induces cell death involving cytoplasmic shrinkage, nuclear condensation, and DNA fragmentation characteristic of apoptosis. Expression of activated MEKK enhanced the apoptotic response to ultraviolet irradiation, indicating that MEKK-regulated pathways sensitize cells to apoptotic stimuli. Inducible expression of activated MEKK stimulated the transactivation of c-Myc and Elk-1. Activated Raf, the serine-threonine protein kinase that activates the ERK members of the MAPK family, stimulated Elk-1 transactivation but not c-Myc; expression of activated Raf does not induce any of the cellular changes associated with MEKK-mediated cell death. Thus, MEKK selectively regulates signal transduction pathways that contribute to the apoptotic response.

The c-Jun kinase (JNK)[1]/stress-activated protein kinase (SAPK) has been shown to be activated by diverse stimuli, including growth factors, cytokines, gamma and ultraviolet irradiation, and protein synthesis inhibitors (1–3). Growth factors activate the extracellular response kinase pathway and may also activate the JNK/SAPK pathway. In contrast, specific cytokines and stresses to the cell appear to preferentially activate the JNK/SAPK pathway (1–5). Several of the cytokines and stresses that activate the JNK/SAPK pathway also induce cell death characteristic of apoptosis. The JNK/SAPKs have been shown to phosphorylate and regulate the activity of several transcription factors including c-Jun, Elk-1, and ATF-2 (1, 2, 4, 6, 7). Similar to the ERK members of the MAPK family, JNK/SAPK is a component of a sequential protein kinase pathway (1, 4, 8–13). JNK/SAPK is phosphorylated, resulting in its activation, by JNK kinase (JNKK)/stress-activated Erk kinase (SEK-1) (9, 10, 14, 15). JNKK/SEK-1 is itself regulated by phosphorylation by an upstream kinase referred to as MEK kinase (MEKK) (13). The MEKK-regulated JNK/SAPK sequential protein kinase pathway is parallel to the Raf/Erk pathway.

Apoptosis is a regulated cell death process characterized by cytoplasmic shrinkage, nuclear condensation, and DNA fragmentation (16). Apoptosis can be induced by signaling from specific cell surface receptors (*i.e.* tumor necrosis factor (TNFα) and Fas) (17, 18), expression of viral proteins such as E1A (19–21), and anticancer agents including chemotherapeutic drugs and ionizing radiation (22). It is known that specific growth factors and cytokines can protect cells against apoptosis (23–25), Bcl-2 and Bcl-x are protective against apoptosis (23, 25), a wild-type p53 protein is often involved in the nuclear events mediating apoptosis (20, 27, 28), and c-Myc can be required for apoptosis (24, 30). Despite the identification of proteins involved in mediating or protecting against apoptosis, biochemical pathways involved in apoptosis are poorly defined.

Receptor-stimulated apoptosis must involve signal transduction pathways that transfer cell surface events to cytoplasmic and nuclear proteins that initiate the cell death program. Signal transduction pathways involved in apoptosis appear to overlap with those that mediate growth and differentiation (31, 32). The integration of signal transduction pathways regulated by growth factor and cytokine receptors commits a cell either to proliferation or apoptosis (33). Cellular stress such as DNA damage can stimulate signal transduction pathways and alter their integration so that the cell commits to a pathway of apoptosis (34). Several checkpoints exist in the pathways leading to apoptosis that involve proteins such as Bcl-2 and p53. Overexpression of Bcl-2 can collaborate with oncoproteins to suppress apoptosis and increase tumor cell growth. Similarly, mutational inactivation of p53 can inhibit apoptosis and contribute to the resistance of tumor cells to chemotherapeutic agents and irradiation (28).

To date, candidate molecules involved in signaling apoptosis include ceramide (35), Ras (31), Rho (36), c-Myc (36), c-Jun (30),

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[1] The abbreviations used are: JNK, Jun kinase; JNKK, JNK kinase; SAPK, stress-activated protein kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular response kinase; MEK, mitogen-activated/extracellular response kinase kinase; MEKK, mitogen-activated/extracellular response kinase kinase kinase; SEK, stress-activated protein kinase kinase; X-gal, bromo-4-chloro-3-indoyl β-D-galactoside; β-gal, β-galactosidase; FITC, fluorescein isothiocyanate; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

and the proteins associated with the TNFα receptor (38) and Fas (39). The interleukin converting enzyme-like proteases appear to be frequent end point mediators of apoptosis (40). Members of the MAPK family may be predicted to be involved in apoptotic signaling because Ras can regulate their activity and may be involved in ceramide-mediated apoptosis (31). MAPKs are also involved in the regulation of transcription factors including c-Myc and c-Jun, which have been shown to influence apoptosis. The regulation of MAPKs including the ERKs, JNK/SAPK and p38/Hog1 involves sequential phosphorylation pathways. For ERKs the pathway is Raf-MEK-ERK, which is strongly implicated in the regulation of growth and differentiation of different cell types. For JNK/SAPK and p38/Hog1 the proposed pathway involves a MEKK, JNKK/SEK, JNK/SAPK, or p38/Hog1. The exact number and selectivity of members of these kinase pathways is still being defined. Members of the TNF receptor family including the TNFα receptor (4), Fas,[2] and CD40 (5) activate JNK/SAPK pathways. The TNFα receptor and Fas can mediate apoptosis, but CD40 actually can be protective against apoptosis in B cells (33, 41, 42). Even though JNK/SAPK activation is a common signal response to these receptors, their influence on cell function can be quite different. Thus, JNK/SAPK activation *per se* would not be predicted to be the primary mediator of apoptotic signaling by TNF receptor family members. Signal transduction pathways in addition to or other than JNK/SAPK activation must therefore be involved in mediating receptor-stimulated apoptosis. In this report, we demonstrate that expression of activated MEKK mediates a cell death response characterized by cytoplasmic shrinkage, nuclear condensation, and DNA fragmentation. MEKK-mediated cell death appears independent of JNK/SAPK activation and has properties characteristic of apoptosis.

MATERIALS AND METHODS

Microinjection of Swiss 3T3 and REF52 Cells

For microinjection Swiss 3T3 and REF52 cells were plated on acid-washed glass coverslips in Dulbecco's modified Eagle's medium and 10% bovine calf serum or newborn calf serum (NCS). Cells were placed in Dulbecco's modified Eagle's medium, 0.1% calf serum for overnight incubation prior to injection and used for injection at 50–70% confluence. Injections were performed with an Eppendorf automated microinjection system with needles pulled from glass capillaries on a vertical pipette puller (Kopf, Tujunga, CA). Cells were injected with pCMVβ-gal in the presence or absence of pCMV5MEKK$_{COOH}$ or pCMV5BxBRaf at 20–100 ng/μl for each expression plasmid in 100 mM KCl, 5 mM $NaPO_4$, pH 7.3. Following injection cells were placed in 1% NCS for 12–18 h (Swiss 3T3) or 42 h (REF52) prior to fixation with paraformaldehyde and staining. Similar results were obtained when cells were placed in 10% NCS after microinjection. Propidium iodide (5 pg/ml) was used to stain DNA. X-Gal reactions were performed for 6 h.

Swiss 3T3 cells were microinjected with 100 ng/μl pCMVβ-gal and 20 ng/μl pCMV5MEKK$_{COOH}$. To label free DNA ends, fixed and rehydrated cells were incubated with terminal deoxytransferase (TDT) and 10 nM biotin-dUTP following the manufacturer's instructions (Boehringer Mannheim). Cells were stained with FITC-streptavidin to label DNA fragments. β-Galactosidase (β-gal) was detected using rabbit anti-β-gal antibody (Cappel Laboratories) and a rhodamine-labeled goat anti-rabbit antibody (Cappel Laboratories).

Transactivation Analysis

Swiss 3T3 cells were transfected using calcium phosphate or Lipofectamine with the reporter plasmid Gal4-TK-luciferase, which contains four Gal4 binding sites (43) adjacent to a minimal thymidine kinase promoter that controls expression of luciferase, in the presence or absence of activator plasmids encoding Gal4$_{(1–147)}$/Myc$_{(7–101)}$ (44), Gal4$_{(1–147)}$/Elk-1$_{(83–428)}$ (45) or Gal4$_{(1–147)}$/c-Jun$_{(1–233)}$ (2). Transfections included pCMV5 without a cDNA insert (basal control), pCMV5MEKK$_{COOH}$ and in some experiments pCMV5BxBRaf. Cells were incubated for 24–48 h after transfection, lysed, and assayed for luciferase activity. Values were normalized to equivalent amounts of protein (μg) for all experiments.

Protein Kinase Assays

JNK/SAPK—Activity was measured using glutathione *S*-transferase-c-Jun$_{(1–79)}$ bound to glutathione-Sepharose 4B (2). Cells expressing MEKK$_{COOH}$ or control cells were lysed in 0.5% Nonidet P-40, 20 mM Tris-HCl, pH 7.6, 0.25 NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 20 μg/ml aprotinin, and 5 μg/ml leupeptin. Lysates were centrifuged at 15,000 $\times$ *g* for 10 min to remove nuclei and supernatants (25 μg of protein) mixed with 10 μl of glutathione *S*-transferase-c-Jun$_{(1–79)}$-Sepharose (3–5 μg of glutathione *S*-transferase-c-Jun$_{(1–79)}$). The mixture was rotated at 4 °C for 1 h, washed twice in lysis buffer, and washed once in kinase buffer (20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 20 mM β-glycerophosphate, 10 mM *p*-nitrophenyl phosphate, 1 mM dithiothreitol, 50 μM sodium vanadate). Beads were suspended in 40 μl of kinase buffer with 10 μCi of [γ-^{32}P]ATP and incubated at 30 °C for 20 min. Samples were boiled in Laemmli buffer and phosphorylated proteins resolved on 10% SDS-polyacrylamide gels. To verify the selectivity of the JNK/SAPK assay, cell lysates were fractionated by Mono Q ion exchange chromatography and each fraction assayed as described above. Fractions were immunoblotted with a rabbit antiserum recognizing JNK/SAPK. Only fractions containing immunoreactive JNK/SAPK phosphorylated the glutathione *S*-transferase-c-Jun$_{(1–79)}$ protein.

p42/44 ERK MAPK—ERK activity was assayed after fractionation of cell lysates on DEAE-Sephacel (46). Alternatively, ERK activity was assayed following Mono Q ion exchange chromatography as described previously and characterized (47). The epidermal growth factor receptor 662–681 peptide was used as a selective substrate for measuring ERK activity (48).

p38/Hog-1—Cells were lysed in 1% Triton X-100, 0.5% Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM NaF, 0.2 mM sodium vanadate, 1 mM EDTA, 1 mM EGTA, 5 mM phenylmethylsulfonyl fluoride. Nuclei were removed by centrifugation at 15,000 $\times$ *g* for 5 min. Supernatants (200 μg of protein) were used for immunoprecipitation of p38/Hog-1 using rabbit antiserum raised against the COOH-terminal peptide sequence of p38/Hog-1 (CFVPPPLDQEEMES) (49) and protein A-Sepharose. Immunoprecipitates were washed once in lysis buffer, once in assay buffer (25 mM Hepes, pH 7.4, 25 mM β-glycerophosphate, 25 mM $NaCl_2$, 2 mM dithiothreitol, 0.1 mM sodium vanadate), resuspended in kinase assay buffer with 20–50 ng of a recombinant NH_2-terminal fragment of ATF-2 as substrate and 20 μCi of [γ-^{32}P]ATP (50). For verification of the immunoprecipitation assay, lysates were fractionated by Mono Q ion exchange chromatography and each fraction assayed for ATF-2 kinase activity and immunoblotted with anti-p38 antibody. The results demonstrated that p38/Hog-1 containing fractions selectively phosphorylated the recombinant ATF-2 protein.

Competitive Inhibitory Mutant JNK/SAPK and JNKK/SEK-1

The competitive inhibitory JNK/SAPK mutant referred to as JNK/SAPK(APF) had the amino acids threonine 183 and tyrosine 185 mutated to alanine and phenylalanine, respectively (14). These are the sites phosphorylated by JNKK/SEK-1 and required for activation of the JNK/SAPK kinase activity (14, 15). Competitive inhibitory JNKK/SEK-1 was made by mutation of the active site lysine at residue 116 mutated to an arginine (K116R) rendering the protein kinase inactive (14).

RESULTS

Expression of Activated MEKK Induces Cell Death—Repeated attempts to isolate stable transfectants expressing MEKK$_{COOH}$ in several fibroblast lines failed. The findings suggested that expression of activated MEKK inhibited clonal expansion of transfected cells. For this reason, we characterized the functional consequence of expressing activated MEKK in Swiss 3T3 and REF52 cells using nuclear microinjection of an expression plasmid encoding an activated form of MEKK-1. Cells were microinjected with an expression plasmid encoding β-gal in the presence or the absence of the expression plasmid encoding MEKK$_{COOH}$, a truncated activated form of MEKK-1 (8, 13). Fig. 1 shows a field of Swiss 3T3 cells microinjected with expression plasmids for β-gal alone (control) or β-gal plus

[2] N. L. Johnson, A. M. Gardner, K. M. Diener, C. A. Lange-Carter, J. Gleavy, M. B. Jarpe, A. Minden, M. Karin, L. I. Zon, and G. L. Johnson, unpublished observation.

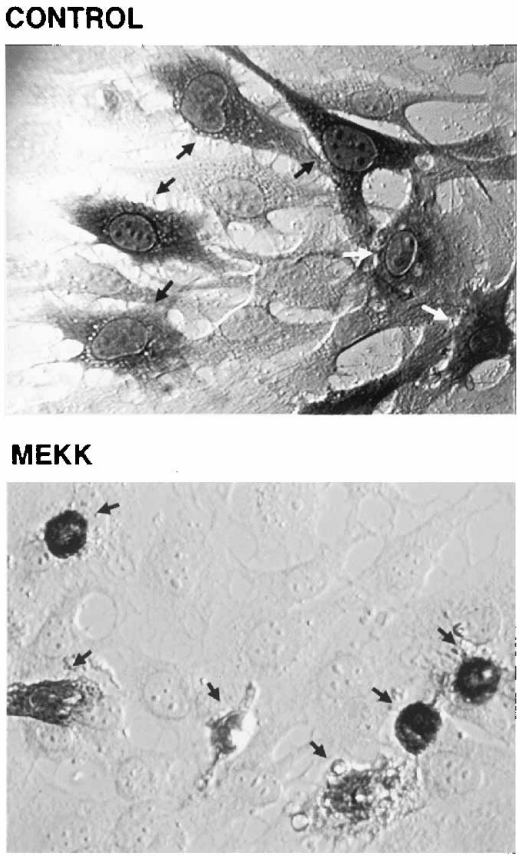

FIG. 1. **Morphological changes induced by expression of activated MEKK ($MEKK_{COOH}$).** Swiss 3T3 cells were microinjected with pCMV5β-gal alone (*Control*) or in the presence of pCMV5$MEKK_{COOH}$ (*MEKK*). At 17–18 h post-injection, cells were fixed and stained with X-gal to identify injected cells. The control panel shows six injected cells denoted by *arrows*, all of which have a normal flattened morphology similar to the adjacent non-injected cells. The MEKK panel shows six injected cells denoted by *arrows* and identified by X-gal staining, all of which have an altered morphology. Three of the $MEKK_{COOH}$-expressing cells have a highly condensed cytoplasm and the three cells in the bottom part of the panel are at earlier stages of cytoplasmic shrinkage and cellular condensation. None of the uninjected cells in the panel have an altered morphology and are similar to the control cells. The panels are representative of 10–12 different experiments and 3–4 independent plasmid preparations.

$MEKK_{COOH}$. It is readily apparent that expression of the activated MEKK-1 induced a strong morphological change of the cells. In contrast, cells microinjected with the β-gal plasmid alone are similar in morphology to uninjected cells. Of the six MEKK-injected cells in the field, three are highly condensed with a very dark staining of the cytoplasm that has dramatically shrunken relative to the flattened morphology of the cells injected with β-gal alone. The other three cells are in an earlier stage of cytoplasmic shrinkage, and, based on independent experiments with longer post-injection incubation, it is clear they would have eventually assumed a highly condensed morphology. The results indicated $MEKK_{COOH}$ expression resulted in death of the cells.

For further analysis and comparison cells were microinjected with BxBRaf, a truncated activated form of Raf-1 (51) that selectively activates the ERK pathway (12). In microinjected cells, expression of β-gal, $MEKK_{COOH}$, or BxBRaf was demonstrated by indirect immunofluorescence using specific antibodies recognizing each protein (Fig. 2). Swiss 3T3 cells (*panels A* and *B*) and REF 52 cells (*panels C* and *D*) microinjected with the indicated expression plasmid were fixed and stained only 8 h post-injection to demonstrate that each protein was being expressed in the cytoplasm of the cells. Since the cells shown in Fig. 2 were fixed and stained soon after injection, they were not yet undergoing significant cellular condensation as observed in Fig. 1. However, it is apparent with the REF 52 cells expressing MEKK (*panel C*) that they have begun to undergo a morphological change relative to β-gal-expressing cells (*panel D*).

Quantitation of microinjection experiments demonstrated that expression of $MEKK_{COOH}$ resulted in significant cell death characterized by the dramatic morphological condensation (Table I). In contrast, BxBRaf expression did not affect cell viability relative to control cells expressing only β-gal. Approximately 84% of all $MEKK_{COOH}$-injected cells had a highly condensed cellular morphology 17 h after injection. This count actually underestimates the number of condensed cells because Swiss 3T3 cells in advanced stages of the cell death response were often nonadherent to coverslips. Some of the nonadherent highly condensed cells could be found to be released from the coverslip into the culture medium, but were not scored in the quantitation. In contrast, fewer than 3% of BxBRaf and 1% of control β-gal-injected cells had an altered morphology even after 48–72 h post-injection.

Cell death resulting from $MEKK_{COOH}$ expression required the kinase activity of the enzyme; the kinase inactive mutant of $MEKK_{COOH}$ was without effect (Table I). The apoptotic-like cell death was also dependent on the $MEKK_{COOH}$ concentration as measured by serial dilution (0–100 ng/μl) of the expression plasmid used for microinjection. Maintenance of the $MEKK_{COOH}$-expressing cells in 10% serum slightly prolonged the time required for induction of cytoplasmic shrinkage, nuclear condensation, and cell death, suggesting that growth factors and cytokines had some influence on the onset of the response induced by $MEKK_{COOH}$ but high serum could not prevent $MEKK_{COOH}$ induced cell death. Greater than 80% of $MEKK_{COOH}$-expressing cells had a cytoplasmic and nuclear morphology characteristic of apoptosis 18 h post-injection.

Fig. 3*A* demonstrates in more detail the dramatic morphological changes in Swiss 3T3 cells resulting from expression of $MEKK_{COOH}$. Cytoplasmic shrinkage is evident from the β-gal staining and nuclear condensation is obvious in MEKK-1-expressing cells stained with propidium iodide. In contrast, cells expressing BxBRaf do not demonstrate any detectable morphological difference from control cells expressing only β-gal. A similar dramatic cytoplasmic shrinkage and nuclear condensation was observed with $MEKK_{COOH}$ expression in REF52 cells (Fig. 3*B*), where BxBRaf again had no effect on cytoplasmic and nuclear integrity. To assess if DNA fragmentation was induced by $MEKK_{COOH}$ expression, TDT was used to covalently transfer biotin-dUTP to the ends of DNA breaks *in situ* (Fig. 4). Streptavidin-FITC was then used for detection of dUTP incorporated into cellular DNA. Even though Swiss 3T3 cells do not undergo significant DNA degradation and laddering at the nucleosomal level, they do generate larger DNA fragments when stimulated to undergo apoptosis (32). The condensed nuclei of $MEKK_{COOH}$-injected cells were highly fluorescent, indicating significant DNA fragmentation (Fig. 4, *B*, *D*, and *F*). It is also apparent that the cytoplasm has become highly condensed and the condensed chromatin is distinct from the cytoplasm. Microinjected cells not yet undergoing cytoplasmic and nuclear condensation in response to $MEKK_{COOH}$ did not incorporate dUTP into their DNA. Thus, expression of $MEKK_{COOH}$ induced all the hallmarks of apoptosis, including cytoplasmic shrinkage, nuclear condensation, and DNA fragmentation.

Expression of BxBRaf did not induce a response measured by any of the criteria mentioned above. BxBRaf-expressing cells displayed a normal flattened morphology similar to β-gal-expressing cells or to uninjected cells (Fig. 3, *A* and *B*). Transient

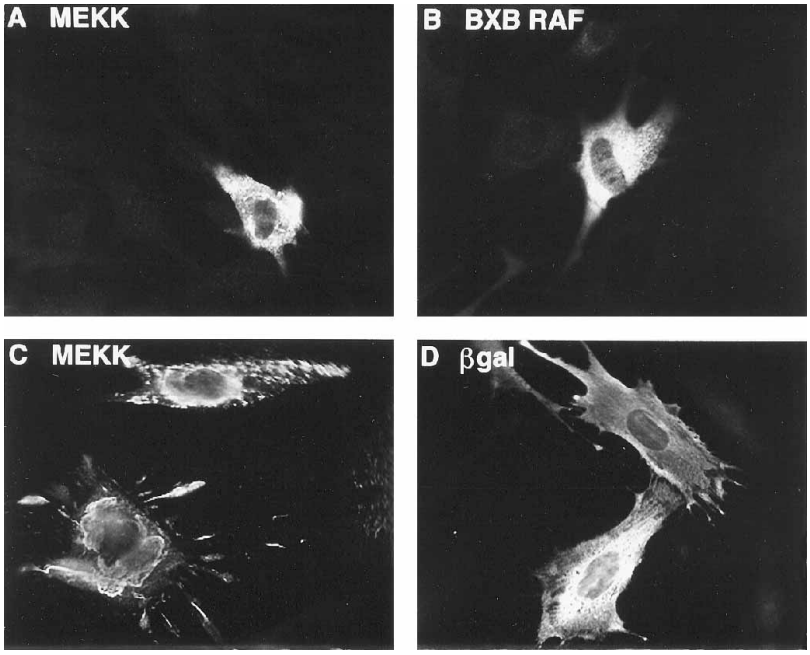

FIG. 2. **Expression $MEKK_{COOH}$ and BxBRaf in Swiss 3T3 and REF52 cells.** Swiss 3T3 cells (*panels A* and *B*) and REF52 cells (*panels C* and *D*) were microinjected with pCMV5$MEKK_{COOH}$, pCMV5BxBRaf, or pCMVβ-gal as indicated. Eight hours after injection, cells were fixed and stained by indirect immunofluorescence for expression of each protein. The $MEKK_{COOH}$ protein has the 9-amino acid hemagglutinin tag (YPYDVPDYA) at its NH_2 terminus and was detected using the mouse monoclonal 12CA5 antibody and a secondary FITC-rabbit anti-mouse antibody. BxBRaf was detected using a rabbit anti-Raf antibody recognizing the COOH terminus of Raf-1, and a rhodamine-donkey anti-rabbit secondary antibody. β-Gal was detected using a mouse anti-β-gal monoclonal antibody, and the FITC-rabbit anti-mouse secondary antibody. At 8 h post-injection, the morphological changes in the $MEKK_{COOH}$-expressing REF52 cells (*panel C*) are beginning to be apparent while the changes in Swiss 3T3 cells are not yet apparent (compare *panels A* and *B*).

TABLE I
Quantitation of $MEKK_{COOH}$-induced cell death

Swiss 3T3 cells were injected with solutions containing 100 ng/μl CMV-βgal in the presence or absence of 100 ng/μl of pCMV5-BxBRaf, pCMV5-$MEKK_{COOH}$ or pCMV5-$Kin^{-}MEKK_{COOH}$ (kinase inactive mutant; 13). Seventeen hours after injection cells were fixed and stained for β-galactosidase activity with X-Gal. Injected cells attached to the coverslip were scored as positive for cell death when they were highly condensed, small round cells as shown in Figs. 1 & 3.

DNA Injected	Cells Injected	Condensed Cells
β-gal	336	4 (1%)
β-gal^{+} BxBRaf	175	5 (3%)
β-gal^{+} $MEKK_{COOH}$	200	167 (84%)
β-gal^{+} $Kin^{-}MEKK_{COOH}$	50	0 (0%)

BxBRaf expression in Swiss 3T3 cells stimulated ERK activity (data not shown), and the transactivation function of the Gal4/Elk-1 chimeric transcription factor (Fig. 5) whose activation is dependent on phosphorylation by Erk members of the MAPK family (45, 52, 53). Cumulatively, the results indicate that activation of the Raf/ERK pathway does not induce the cytoplasmic and nuclear changes observed with MEKK.

Induction of Activated MEKK Sensitizes Swiss 3T3 Cells to UV-induced Apoptosis—Because stable expression of $MEKK_{COOH}$ appeared to inhibit clonal expansion of Swiss 3T3 cells during G418 drug selection, clones were isolated having inducible expression of the kinase. The LacSwitch expression system (Stratagene) was used to control the expression of $MEKK_{COOH}$. Several independent clones were isolated and their properties analyzed in the presence or absence of IPTG-induced expression of $MEKK_{COOH}$. The parental $LacR^{+}$ clone expressing only the Lac repressor was used as the control. Clones expressing inducible $MEKK_{COOH}$ showed a small increase in the number of cells having a condensed cytoplasmic and nuclear morphology relative to control cells even in the absence of IPTG-induced $MEKK_{COOH}$. This is probably due to a basal level of $MEKK_{COOH}$ expression in uninduced cells. Addition of IPTG culture medium induced the expression of $MEKK_{COOH}$ (Fig. 6*A*) and resulted in an increase in cells having the condensed morphology relative to the control IPTG-treated $LacR^{+}$ clone (Fig. 6*B*). However, $MEKK_{COOH}$-expressing cells did not growth arrest, and only a fraction of the cells assumed a condensed morphology as dramatic as what was observed with microinjection of the $MEKK_{COOH}$ expression plasmid. This may be related to selection of cells during the cloning procedure that adapted to a low, constitutive level of $MEKK_{COOH}$ expression. Interestingly, no clones were isolated from a total of 150 that had a significant constitutive $MEKK_{COOH}$ expression measured by immunoblotting. In addition, the level of $MEKK_{COOH}$ expression following IPTG induction is certainly less than that achieved with nuclear microinjection.

It was found that IPTG-induced $MEKK_{COOH}$ expression stimulated signal transduction pathways that made the cells significantly more sensitive to stresses that induce cell death. For example, cells expressing $MEKK_{COOH}$ were highly sensitive to ultraviolet irradiation (Fig. 6*C*). Two hours after exposure to ultraviolet irradiation greater than 30% of the $MEKK_{COOH}$-expressing cells became morphologically highly condensed and appeared apoptotic. In contrast, the population of uninduced cells showed no increase in condensed apoptotic-like cells at this time point. Thus, overnight induction of $MEKK_{COOH}$ expression modestly increased the basal index of morphologically condensed cells and primed the cells for apoptosis in response to UV irradiation. The results indicate that MEKK-regulated signal transduction pathways enhance apoptotic responses to external stimuli.

Expression of $MEKK_{COOH}$ Stimulates JNK/SAPK and the Transactivation of c-Myc and Elk-1—The ability of $MEKK_{COOH}$ but not BxBRaf expression to induce cell death indicates that each kinase regulates different sequential protein kinase pathways. Induction of $MEKK_{COOH}$ expression in Swiss 3T3 cells, as predicted, stimulated JNK/SAPK activity (Fig. 7*A*) but did not activate either ERK or p38/Hog1 activity (Fig. 7*B*). Because known substrates for JNK/SAPK are transcription factors, we assayed $MEKK_{COOH}$ inducible clones for transactivation of specific gene transcription. Chimeric transcription factors having the Gal4 DNA binding domain and the transactivation domain of c-Myc, Elk-1, or c-Jun were used for assay of $MEKK_{COOH}$ signaling using a Gal4 promoter-luciferase reporter gene (2, 43–45). Surprisingly, IPTG-induced stable expression of $MEKK_{COOH}$ markedly activated the transactivation function of c-Myc and Elk-1 but had little effect on Gal4/Jun activity (Fig. 7*C*). This result was unexpected since $MEKK_{COOH}$ transient expression stimulated Gal4/Jun activity (Fig. 7*C*), indicating

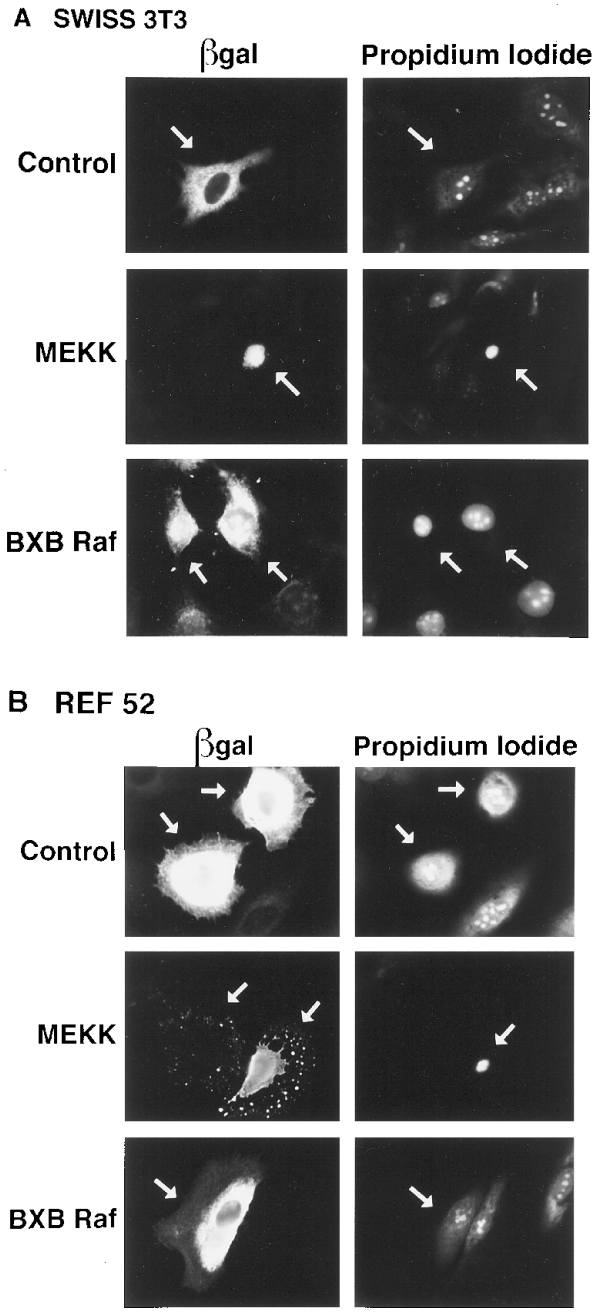

FIG. 3. **Expression of $MEKK_{COOH}$ induces cytoplasmic shrinkage and nuclear condensation.** Swiss 3T3 cells (*A*) and REF52 cells (*B*) were microinjected with the pCMV5 expression plasmid encoding β-gal in the absence (*Control*) or the presence of pCMV5 expression plasmids for $MEKK_{COOH}$ or BxBRaf. Seventeen h after injection, Swiss 3T3 cells were fixed and stained by indirect fluorescence for β-gal expression to detect injected cells. REF52 cells were fixed 42 h after injection. Cells were also stained with propidium iodide for detection of DNA and nuclear morphology. Expression of $MEKK_{COOH}$ caused dramatic cytoplasmic shrinkage and nuclear condensation. One of the two REF52 cells expressing $MEKK_{COOH}$ has lost its nucleus, and only the cytoplasmic blebs remain on the coverslip.

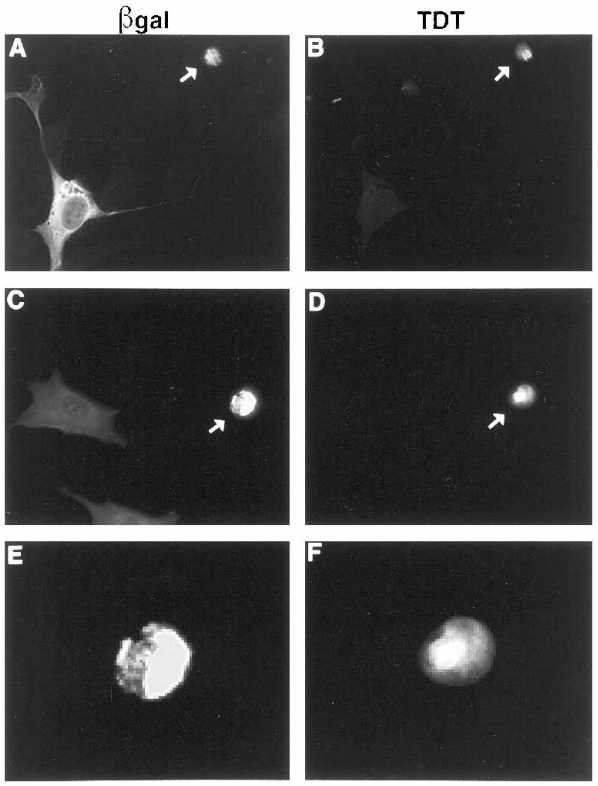

FIG. 4. **Detection of DNA fragments in Swiss 3T3 cells expressing $MEKK_{COOH}$.** Cells were microinjected with the expression plasmids for β-gal and $MEKK_{COOH}$. Eighteen h after injection, cells were fixed and incubated with TDT and 10 nM biotin-dUTP to label the ends of DNA fragments. The fixed cells were then washed and incubated with a rabbit anti-β-gal antibody and detected with a rhodamine-goat anti-rabbit secondary antibody. Biotin-dUTP was stained with FITC-streptavidin. *Panels A–D* represent two different fields showing an $MEKK_{COOH}$-expressing cell having a highly condensed cell morphology and TDT-positive labeling. In the same field are injected cells that have not yet undergone the morphological changes and are TDT-negative in their nuclear staining. *Panels E* and *F* are 3-fold magnifications of the apoptotic cell in *panels C* and *D*. The TDT reaction clearly labels DNA that is highly condensed in the nucleus, whereas the β-gal staining is in the condensed cytoplasm. Cells microinjected with pCMV5β-gal alone were normal, had a flattened morphology, and were TDT-negative.

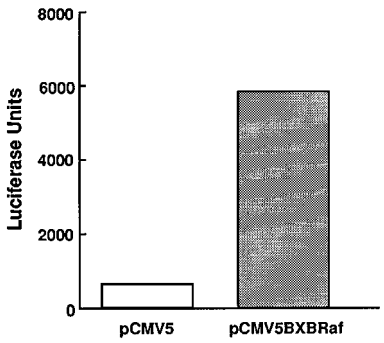

FIG. 5. **Wild-type Swiss 3T3 cells were transfected with pCMV5BXBRaf or pCMV5 without a cDNA insert in the presence of expression plasmids encoding Gal4/Elk-1 and Gal4-TK-luciferase as described under "Materials and Methods."** Forty-eight h post-tranfection, cells were lysed and assayed for luciferase activity.

that transient expression of $MEKK_{COOH}$ was capable of transactivating c-Jun function in Swiss 3T3 cells. In addition, the JNK/SAPK activity stimulated by IPTG-induction of $MEKK_{COOH}$ correlated with the characterized JNK/SAPK enzyme by fractionation on Mono Q FPLC. Thus, $MEKK_{COOH}$ expression in stable clones achieved with IPTG induction selectively regulated Gal4/Myc and Gal4/Elk-1 but not Gal4/Jun, even though JNK/SAPK was activated.

The failure of IPTG-induced $MEKK_{COOH}$ expression to activate Gal4/Jun may be related to the multiple c-Jun NH_2-terminal phosphorylation sites involved in regulating c-Jun transactivation. Serines 63 and 73 and threonines 91 and 93 are apparent regulatory phosphorylation sites in c-Jun (1, 54–56). Both clusters are proposed to be sites of phosphorylation for Erks and JNK/SAPKs (56). Transient transfection of

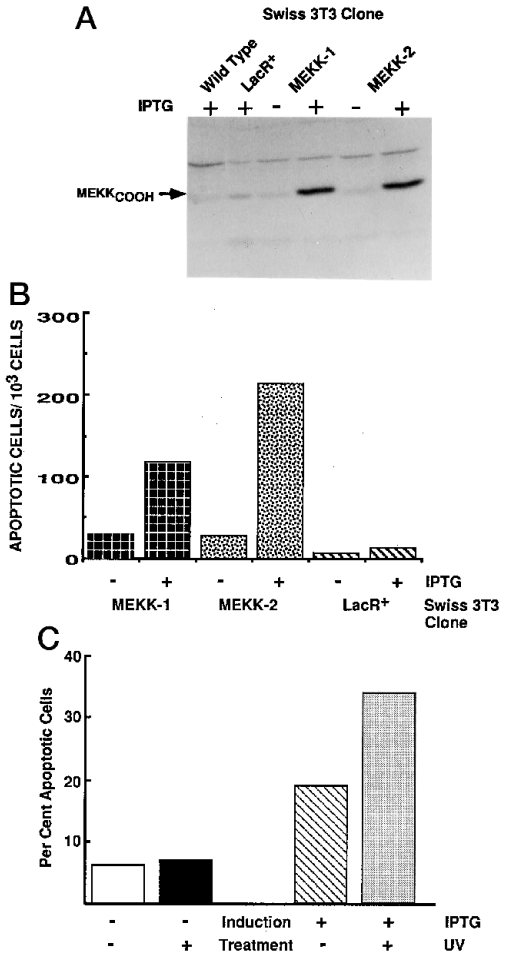

FIG. 6. **Induction of MEKK$_{COOH}$ expression in Swiss 3T3 cells increases the number of condensed cells.** *A*, Swiss 3T3 cells having an inducible MEKK$_{COOH}$ under the control of the *lac* repressor were isolated using the LacSwitch inducible mammalian expression system (Stratagene). IPTG (5 mM) induced MEKK$_{COOH}$ expression in two independent clones (MEKK-1 and MEKK-2). MEKK$_{COOH}$ protein expression was detected using an antibody recognizing the extreme COOH terminus of MEKK (13). *B*, parental LacR$^+$, MEKK-1, and MEKK-2 cells were incubated in the presence or absence of 5 mM IPTG for 48 h. Cells were stained with acridine orange and condensed cells quantitated per 1000 cells counted per coverslip. Results are representative of three independent experiments with each clone. *C*, Swiss 3T3 cells having inducible MEKK$_{COOH}$ were incubated in the presence or absence of 5 mM IPTG for 17 h. The indicated cells were then exposed to UV-C irradiation 39 J/M^2). Two h after irradiation, cells were fixed and stained with propidium iodide. Condensed cells having a morphology similar to that shown in Fig. 1 were quantitated for each condition. Results are representative of three independent experiments.

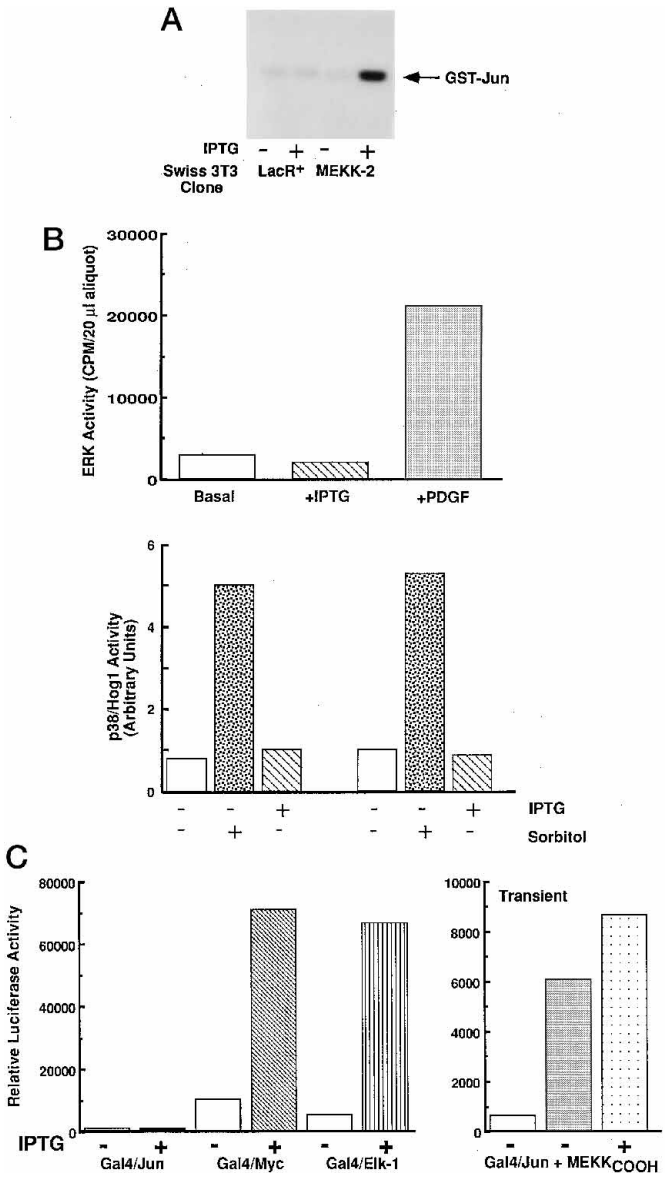

FIG. 7. **Assay of MEKK-regulated protein kinases in Swiss 3T3 cells.** *A*, the parental LacR$^+$ or MEKK-2 Swiss 3T3 cells were incubated for 17 h in the absence or presence of 5 mM IPTG and assayed for JNK/SAPK activity. The LacR$^+$ clone lacks the MEKK$_{COOH}$ expression plasmid. Induction of MEKK$_{COOH}$ results in the activation of JNK/SAPK, which phosphorylates glutathione *S*-transferase-c-Jun. *B*, induction of MEKK$_{COOH}$ does not activate ERK (p42/44 MAPK) or p38/Hog1 activity in the MEKK-2 Swiss 3T3 clone. Treatment of MEKK-2 Swiss 3T3 cells with PDGF (10 ng/ml, 10 min) or sorbitol (400 mM, 20 min) activated ERK and p38/Hog1 activity, respectively, demonstrating these response pathways were functional. *C*, regulation of specific transactivation by MEKK$_{COOH}$. *Left panel*, the MEKK-2 Swiss 3T3 clone was used for measurement of Gal4/Jun$_{(1–233)}$, Gal4/Myc$_{(7–101)}$ and Gal4/Elk-1$_{(83–428)}$ transactivation in response to IPTG-induced MEKK$_{COOH}$ expression. Induction of MEKK$_{COOH}$ expression did not significantly increase Gal4/Jun transactivation. *Right panel*, transient transfection of MEKK$_{COOH}$ resulted in increased Gal4/Jun transactivation in the MEKK-2 Swiss 3T3 cell clone. A similar result was seen with or without IPTG treatment. The condition shown is with IPTG incubation.

MEKK$_{COOH}$ activates JNK/SAPK (9) but also activates ERKs (13). In contrast IPTG induction of MEKK$_{COOH}$ results in the activation of JNK/SAPK but not Erks. The difference in regulation of c-Jun transactivation may be related to the differential phosphorylation of these sites by JNK/SAPK and ERKs. Further studies will be required to address this question.

Expression of activated Raf in Swiss 3T3 cells stimulated Elk-1 transactivation (Fig. 5) but not c-Myc or c-Jun transactivation (not shown). This result indicates that Elk-1 transactivation alone does not mediate the cell death response in fibroblasts observed with MEKK$_{COOH}$. Cumulatively, the findings demonstrate that induction of MEKK$_{COOH}$ expression enhances cell death independent of ERK, p38/Hog-1, or c-Jun transactivation in Swiss 3T3 cells and may involve c-Myc transactivation.

Inhibitory JNK/SAPK Does Not Attenuate MEKK-stimulated c-Myc Transactivation or Cell Condensation—To determine if JNK/SAPK activation was required for c-Myc transactivation in response to MEKK$_{COOH}$, Gal4/Myc activation was assayed in the presence or absence of JNK/SAPK(APF) (Fig. 8). The JNK/SAPK(APF) was used as a competitive inhibitor of JNK/SAPK for activation by the immediate upstream JNK kinase/SEK-1 enzyme (1, 4, 9, 14, 15). In transient transfection assays, expression of JNK/SAPK(APF) inhibited approxi-

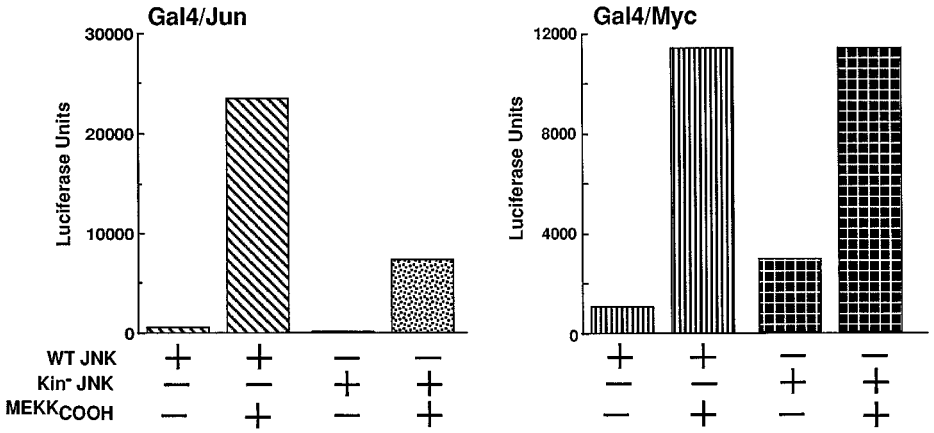

FIG. 8. **Competitive inhibitory JNK/SAPK(APF) attenuates Gal4/Jun but not Gal4/Myc activation.** Swiss 3T3 cells were transfected using the calcium phosphate procedure with pCMV5 with no cDNA (3 μg) or pCMV5MEKK$_{COOH}$ (3 μg) and pSRαJNK/SAPK (9 μg) or pSR$\alpha\Delta$JNK/SAPK (9 μg). All of the dishes were transfected with the Gal4/Jun$_{(1-233)}$ (3 μg) and Gal4-TK-luciferase (9 μg) reporter constructs. Cells were harvested 42 h post-transfection and assayed for luciferase activity. The results are representative of three independent experiments where a 3-fold excess of JNK/SAPK(APF) inhibited approximately 65% of Gal4/Jun activation with no effect on Gal4/Myc activation.

mately 65% of the Gal4/Jun activation in response to MEKK$_{COOH}$. In contrast, expression of JNK/SAPK(APF) had no effect on MEKK$_{COOH}$ activation of Gal4/Myc induction of luciferase activity. Thus, c-Jun transactivation appears to be independent of the MEKK$_{COOH}$-stimulated pathway leading to c-Myc transactivation. Similarly, JNK/SAPK activation can be significantly inhibited with no effect on c-Myc transactivation.

The cell death response to MEKK$_{COOH}$ also appeared to be largely independent of JNK/SAPK. In several experiments, expression of JNK/SAPK(APF) alone had no demonstrative effect on Swiss 3T3 cells (Fig. 9, *A* and *B*). The expressed JNK/SAPK(APF) was localized in both the cytoplasm and nucleus, while β-gal expression was restricted to the cytoplasm. Co-expression of JNK/SAPK(APF) with MEKK$_{COOH}$ did not block MEKK$_{COOH}$-induced cytoplasmic shrinkage and cellular condensation. As shown in Fig. 9*C*, a 20-fold lower concentration of MEKK$_{COOH}$ than that used in Figs. 1–3 still induced the cytoplasmic shrinkage characteristic of apoptosis in microinjected Swiss 3T3 cells. Co-microinjection of a 30-fold greater concentration of JNK/SAPK(APF) plasmid relative to the MEKK$_{COOH}$ plasmid did not affect the MEKK$_{COOH}$-mediated cell death response (Fig. 9*D*). *Panels C* and *D* show the cells undergoing a dramatic cytoplasmic shrinkage. Because of the low amount of MEKK$_{COOH}$ expression plasmid used, the cell condensation response was slower in onset than cells in Figs. 1–3. The percentage of MEKK$_{COOH}$-microinjected cells committed to cytoplasmic shrinkage and cellular condensation and the timing of this response was the same in the presence or absence of JNK/SAPK(APF). In addition, the competitive inhibitory mutant K116RJNKK/SEK-1, the kinase immediately upstream of JNK/SAPK which phosphorylates and activates JNK/SAPK (14, 15), was also unable to attenuate MEKK$_{COOH}$ induced cell death. Expression of JNK/SAPK(APF) or K116RJNKK/SEK-1 alone had no measurable effect on the morphology of Swiss 3T3 cells (data not shown). Thus, MEKK$_{COOH}$ induces cell death via the regulation of signal pathways that appear largely independent of JNK/SAPK regulation and c-Jun transactivation. Finally, BxBRaf neither induced cell death nor activated c-Myc (not shown), indicating that MEKK$_{COOH}$-regulated responses were not mediated by the Erk1 and 2 proteins (p42/p44 MAP kinases), consistent with the lack of ERK activation in the inducible MEKK$_{COOH}$ Swiss 3T3 cells.

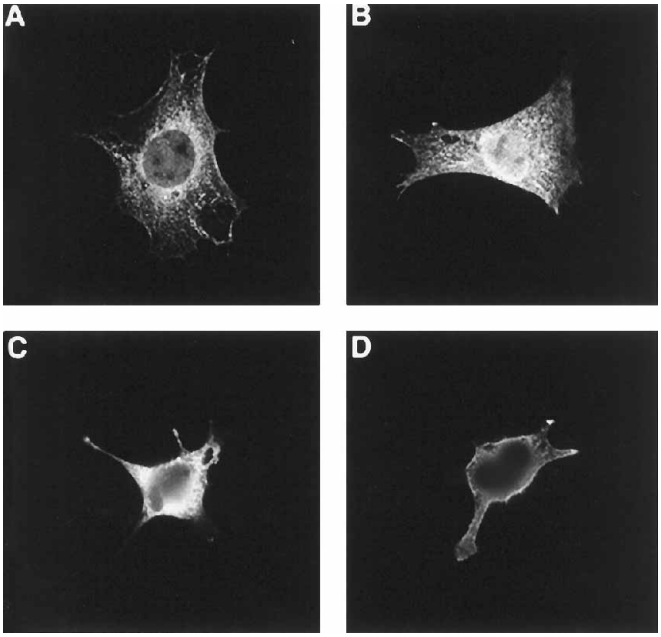

FIG. 9. **Expression of JNK/SAPK(APF) does not affect the MEKK$_{COOH}$-induced cellular condensation response.** Swiss 3T3 cells were microinjected with expression plasmid encoding JNK/SAPK(APF) (150 ng/μl) in the absence (*panels A* and *B*) or presence of pCMV5 expression plasmids encoding MEKK$_{COOH}$ (5 ng/μl) (*panels C* and *D*). This is 20-fold less MEKK$_{COOH}$ expression plasmid than that used in Figs. 1–3. Each injection condition also included 50 ng/μl pCMVβ-gal expression plasmid. *Panels A*, *C*, and *D* are stained for β-gal expression. *Panel B* is stained for JNK(APF) expression where the construct was tagged at the NH_2 terminus with the 9-amino acid HA sequence and detected with the 12CA5 monoclonal antibody. Experiments were also conducted with the competitive inhibitory K116RJNKK/SEK-1 kinase inactive mutant with similar results (not shown). Data are representative of five to six experiments each for JNK(APF) and K116RJNKK/SEK-1 inhibitory mutants.

DISCUSSION

Our results demonstrate, for the first time, a role for MEKK in mediating a cell death response characteristic of apoptosis. Receptors such as the cytotoxic TNFα receptor and Fas must be capable of regulating signal transduction pathways controlling cytoplasmic and nuclear events involved in apoptosis. The enhanced apoptosis to ultraviolet irradiation observed with MEKK$_{COOH}$ expression in Swiss 3T3 cells indicates that MEKK-regulated signal transduction pathways integrate with the apoptotic response system. MEKK$_{COOH}$-expressing cells have a higher basal apoptotic index and are primed to undergo apoptosis in response to a stress stimulation. The short time required to observe the enhanced apoptosis (2 h) suggests that cell cycle traverse, DNA synthesis, or significant transcription/translation is not required for the enhanced cell death in response to ultraviolet irradiation in cells expressing MEKK$_{COOH}$. This finding is striking and suggests that genetic or pharmacological manipulation of MEKK activity could be used to sensitize cells to irradiation-induced death.

The ability to dissociate c-Jun transactivation from $MEKK_{COOH}$-stimulated cell death argues that the JNK/SAPK activity achieved in the inducible Swiss 3T3 cell clones is insufficient alone to activate c-Jun transactivation or induce cell death. It is more likely that the JNK/SAPK activity we have measured is involved in stimulating a protective program in response to potentially lethal stimuli as previously proposed (34). Protective responses could involve changes in metabolism or alterations in the activity of proteins such as Bcl-2 (23, 25). This prediction is consistent with the activation of JNK/SAPK mediated by CD40 ligation in B cells, which protects against rather than stimulates apoptosis (5, 33, 41, 42).

Recently, it was shown that dominant negative c-Jun could protect neurons from serum deprivation-induced apoptosis (37). It was proposed that the dominant negative c-Jun inactivated c-Jun and prevented an attempt by the post-mitotic neurons to enter an abortive cell cycle progression that triggered a cell death program. Thus, dominant negative c-Jun was believed to maintain the neurons in stringent growth arrest. At first glance, the protective effect of dominant negative c-Jun seems contradictory to our results that JNK/SAPK and c-Jun transactivation are not involved in MEKK-induced cell death. Our results demonstrate that the dramatic cytoplasmic shrinkage, nuclear condensation, and onset of cell death induced by $MEKK_{COOH}$ are largely independent of JNK or c-Jun transactivation. Importantly, $MEKK_{COOH}$-induced cell death occurs in high serum where growth factor and cytokine stimulation of the cells is normal. We have also determined that expression of $MEKK_{COOH}$ in Swiss 3T3 cells does not significantly inhibit or alter cell cycle progression. Thus, an abnormal cell cycle event that may occur with serum deprivation does not appear to account for MEKK-induced cell death.

Expression of $MEKK_{COOH}$ increased the transactivation of c-Myc and Elk-1 in Swiss 3T3 cells. c-Myc has been shown to be required for apoptosis in lymphocytes (57–59), to induce apoptosis when overexpressed in growth factor-deprived fibroblasts (24, 29, 30), and to enhance TNF-mediated apoptosis (60). The requirement of c-Myc for apoptosis is not understood mechanistically, but c-Myc is proposed to transcriptionally activate an apoptotic pathway (24, 29, 30, 58, 59). The activation of Elk-1 by $MEKK_{COOH}$ induction in Swiss 3T3 cells correlates best with the stimulation of JNK/SAPK. Recently, it was found that JNK/SAPK in addition to Erks phosphorylated and activated Elk-1 consistent with our findings (7). In contrast, we demonstrate that c-Jun is not significantly activated in $MEKK_{COOH}$-expressing cells. These findings are provocative because they indicate that MEKK-stimulated JNK/SAPK activation preferentially regulated Elk-1 and not c-Jun. A second signal in addition to JNK/SAPK may be required for c-Jun transactivation in cells (56). We are unaware of any proposed role for Elk-1 in inducing an apoptotic response, but serum deprivation-induced apoptosis of Swiss 3T3 cells results in the increased expression of early cell cycle genes consistent with an increased serum response factor/serum response element activity associated with elevated Elk-1 activity (32). The induction of apoptosis in several cell types does not appear to require transcription, but our inducible cell lines and plasmid microinjection experiments do not allow us to test whether $MEKK_{COOH}$ can induce cell death in the absence of transcription. We are currently attempting to make active recombinant $MEKK_{COOH}$ to test this possibility. In cells where transcription is not necessary for the induction of apoptosis, it is likely that proteins required for apoptosis are already expressed and may be post-translationally regulated by sequential protein kinase pathways involving MEKK. For example, the phosphorylation of nuclear proteins could alter their activity independent of transcription and contribute to a cell death response.

In Jurkat cells, a human T cell line, Fas-induced apoptosis has been proposed to involve a ceramide-stimulated, Ras-dependent signaling pathway (31). We recently demonstrated that MEKK activity can be stimulated by Ras and that MEKK1 physically binds to Ras in a GTP-dependent manner (61, 62). The ability of MEKK to regulate an apoptotic-like cell death response suggests it is a candidate component for the ceramide-regulated apoptotic pathway.

The importance of our observations describing the involvement of MEKK regulated sequential protein kinase pathways in physiologically relevant signaling leading to cell death is supported by several findings. First, $MEKK_{COOH}$ induces or enhances a cell death response in the presence of 10% calf serum, indicating that growth factor deprivation is not a prerequisite for MEKK-induced cell death. This is similar to TNFα, Fas, and ceramide-mediated apoptosis, which proceeds in high serum. Thus, the involvement of MEKK in cell death responses is not simply to activate a subset of growth factor-stimulated signaling events causing an aborted cell cycle-induced apoptosis that would normally be prevented by serum factors. Second, the enhanced cell death to ultraviolet irradiation indicates that expression of $MEKK_{COOH}$ may activate signals that potentiate stresses to the cell. This finding indicates that MEKK-regulated signal transduction pathways integrate with cellular responses involved in mediating apoptosis, that ultraviolet irradiation likely activates additional pathways, and that $MEKK_{COOH}$-mediated signaling synergizes with the ultraviolet response to accelerate apoptosis. Third, MEKK stimulated sequential protein kinase pathways independent of ERK, JNK/SAPK, p38/Hog1, and c-Jun transactivation that can stimulate c-Myc transactivation. These results indicate that MEKK-regulated pathways traverse the cytoplasm to regulate as yet undefined protein kinases that activate c-Myc in the nucleus. The regulation of c-Myc activity is a unique function of MEKK signaling and one that we postulate is likely to contribute to the cell death response. Serum deprivation significantly induces JNK/SAPK activation in several cell types including Swiss 3T3 cells.[2] Similarly, TNFα stimulates a JNK/SAPK pathway (9), and we have recently demonstrated TNFα stimulation of MEKK activity in mouse macrophages (63). c-Myc overexpression has been shown to enhance TNFα receptor stimulation of apoptosis (21). These findings are consistent with a linkage between TNFα receptor signaling, MEKK, and c-Myc. Cumulatively, the findings define MEKK as a potentially important component in the regulation of signal transduction pathways involved in apoptosis.

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Signal Transduction Pathways Regulated by Mitogen-activated/Extracellular Response Kinase Kinase Kinase Induce Cell Death

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