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Signal Transduction Pathways Regulated by Mitogen-activated/Extracellular Response 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 kinase (MAPK), including members of the J un kinase (J NK)/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-J un kinase (J NK)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 J NK/SAPK pathway. In contrast, specific cytokines and stresses to the cell appear to preferentially acti-

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† The abbreviations used are: J NK, J un kinase; J NKK, J NK 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; X-gal, bromo-4-chloro-3-indoly b-galactoside; b-gal, b-galactosidase; FITC, fluorescein isothiocyanate; IPTG, isopropyl-1-thio-b-D-galactoside.

vate 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-J un, Elk-1, and ATF-2 (1, 2, 4, 6, 7). Similar to the ERK members of the MAPK family, J NK/SAPK is a component of a sequential protein kinase pathway (1, 4, 8–13). J NK/SAPK is phosphorylated, resulting in its activation, by J NK kinase (J NK)/stress-activated Erk kinase (SEK-1) (9, 10, 14, 15). J NKK/SEK-1 is itself regulated by phosphorylation by an upstream kinase referred to as MEK kinase (MEKK) (13). The MEKK-regulated J NK/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 [TNF-α] 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 (26, 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 oncogenes 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-J un (30),

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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, JNK/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, and CD95 (33, 41, 42). This observation is consistent with the idea that these kinases mediate a cell death response characterized by cytoplasmic condensation, nuclear condensation, and DNA fragmentation. MEKK-mediated cell death appears independent of JNK/SAPK activation and has properties characteristic of apoptosis.

Protein Kinase Assays

JNK/SAPK—Activity was measured using glutathione S-transferase-c-jun unphosphorylated bound to glutathione-Sepharose 4B (2). Cells expressing MEKK1 (MEKK1COOH) or control cell lines were lysed in 0.5% Nonidet P-40 in 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 × g for 10 min to remove nuclei and supernatants (25 μg of protein) mixed with 10 μl of glutathione S-transferase-c-jun unphosphorylated. 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 MgCl2, 20 μM β-glycerophosphate, 10 mM dithiothreitol, 50 μM sodium vanadate). Beads were suspended in 40 μl of kinase buffer with 10 μCi of [γ-32P]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 specificity 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 unphosphorylated protein.

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 plated in Dulbecco’s modified Eagle’s medium, 0.1% calf serum for overnight incubation prior to injection and used for injection at 50–70% confluency. Cells were microinjected with an expression plasmid encoding an activated form of MEKK-1. 3230 MEK Kinase-induced Cell Death

RESULTS

Expression of Activated MEKK Induces Cell Death—Repeated attempts to isolate stable transfectants expressing MEKK1COOH in several fibroblast lines failed. The findings suggested that expression of activated MEKK1 induced 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 MEKK1COOH, 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. J arpe, A. Minden, M. Kain, L. L. Zan, and G. L. J ohnson, unpublished observation.
MEKK\textsubscript{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 \( \beta \)-gal plasmid alone are similar in morphology to un.injected cells. Of the six MEKK-injected cells in the field, three are highly condensed and the condensed chromatin is distinct from the cytoplasm. It is also apparent that the cytoplasm has become highly condensed and there is clear condensation, and cell death, suggesting that growth factors and cytokines have some influence on the onset of the response induced by MEKK\textsubscript{COOH} but high serum could not prevent MEKK\textsubscript{COOH} induced cell death. Greater than 80% of MEKK\textsubscript{COOH}-expressing cells had a cytoplasmic and nuclear morphology characteristic of apoptosis 18 h post-injection.

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 \( \beta \)-gal, MEKK\textsubscript{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 \( \beta \)-gal-expressing cells (panel D).

Quantitation of microinjection experiments demonstrated that expression of MEKK\textsubscript{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 \( \beta \)-gal. Approximately 84% of all MEKK\textsubscript{COOH}-injected cells had a highly condensed cellular morphology 17 h after injection. This count actually understimates 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 \( \beta \)-gal-injected had an altered morphology even after 48–72 h post-injection.

Cell death resulting from MEKK\textsubscript{COOH} expression required the kinase activity of the enzyme; the kinase inactive mutant of MEKK\textsubscript{COOH} was without effect (Table I). The apoptotic-like cell death was also dependent on the MEKK\textsubscript{COOH} concentration as measured by serial dilution (0–100 ng/\( \mu \)l) of the expression plasmid used for microinjection. Maintenance of the MEKK\textsubscript{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\textsubscript{COOH} but high serum could not prevent MEKK\textsubscript{COOH} induced cell death. Greater than 80% of MEKK\textsubscript{COOH}-expressing cells had a cytoplasmic and nuclear morphology characteristic of apoptosis 18 h post-injection.

Fig. 1 demonstrates in more detail the dramatic morphological changes in Swiss 3T3 cells resulting from expression of MEKK\textsubscript{COOH}. Cytoplasmic shrinkage is evident from the \( \beta \)-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 \( \beta \)-gal. A similar dramatic cytoplasmic shrinkage and nuclear condensation was observed with MEKK\textsubscript{COOH} expression in REF52 cells (Fig. 3B), where BxBRaf again had no effect on cytoplasmic and nuclear integrity. To assess if DNA fragmentation was induced by MEKK\textsubscript{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\textsubscript{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\textsubscript{COOH} did not incorporate dUTP into their DNA. Thus, expression of MEKK\textsubscript{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 \( \beta \)-gal-expressing cells or to un injected cells (Fig. 3, A and B). Transient
MEK Kinase-induced Cell Death

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 their properties analyzed in the presence or absence of IPTG-coverslip were scored as positive for cell death when they were highly condensed, small round cells as shown in Figs. 1 & 3.

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, pCMV5-BxBRaf, or pCMV/β-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 (YPDYVPDYA) at its NH2 terminus and was detected using the mouse monoclonal 12CAS 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).

| 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 inductive 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. 6A) and resulted in an increase in cells having the condensed morphology relative to the control IPTG-treated LacR+ clone (Fig. 6B). 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. 6C). 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. 7A) but did not activate either ERK or p38/Hog1 activity (Fig. 7B). 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. 7C). This result was unexpected since MEKK_COOH transient expression stimulated Gal4/Jun activity (Fig. 7C), indicating...
that transient expression of MEKK$_{\text{COOH}}$ was capable of trans-
activating c-Jun function in Swiss 3T3 cells. In addition, the
JNK/SAPK activity stimulated by IPTG-induction of
MEKK$_{\text{COOH}}$ correlated with the characterized JNK/SAPK en-
zyme by fractionation on Mono Q FPLC. Thus, MEKK$_{\text{COOH}}$
expression in stable clones achieved with IPTG induction se-
lectively regulated Gal4/Myc and Gal4/Elk-1 but not Gal4/Jun,
even though JNK/SAPK was activated.

The failure of IPTG-induced MEKK$_{\text{COOH}}$ expression to acti-

FIG. 3. Expression of MEKK$_{\text{COOH}}$ induces cytoplasmic shrink-
age and nuclear condensation. Swiss 3T3 cells (A) and REF52 cells
(B) were microinjected with the pCMV5 expression plasmid encoding
$\beta$-gal in the absence (Control) or the presence of pCMV5 expression
plasmids for MEKK$_{\text{COOH}}$ or BxBRaf. Seventeen h after injection, Swiss
3T3 cells were fixed and stained by indirect fluorescence for $\beta$-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$_{\text{COOH}}$ caused dra-
matic cytoplasmic shrinkage and nuclear condensation. One of the two
REF52 cells expressing MEKK$_{\text{COOH}}$ 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$_{\text{COOH}}$. Cells were microinjected with the expression plasmids
for $\beta$-gal and MEKK$_{\text{COOH}}$. 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-$\beta$-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$_{\text{COOH}}$-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 $\beta$-gal staining is in
the condensed cytoplasm. Cells microinjected with pCMV5-$\beta$-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 pres-
ence of expression plasmids encoding Gal4/Elk-1 and Gal4-TK-
luciferase as described under “Materials and Methods.” Forty-
eight h post-transfection, cells were lysed and assayed for luciferase
activity.

The failure of IPTG-induced MEKK$_{\text{COOH}}$ expression to acti-

vate Gal4/J un may be related to the multiple c-j un NH$_2$-ter-
minal phosphorylation sites involved in regulating c-j un trans-
activation. Serines 63 and 73 and threonines 91 and 93 are
apparent regulatory phosphorylation sites in c-j un (1, 54–56).
Both clusters are proposed to be sites of phosphorylation for
Erks and JNK/SAPKs (56). Transient transfection of
MEKKCOOH activates JNK/SAPK (9) but also activates ERKs (13). In contrast, IPTG induction of MEKKCOOH 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 MEKKCOOH. Cumulatively, the findings demonstrate that induction of MEKKCOOH expression enhances cell death independent of ERK, p38/Hog1, 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 MEKKCOOH, 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 kinaseSEK-1 enzyme (1, 4, 9, 14, 15). In transient transfection assays, expression of JNK/SAPK (APF) inhibited approxi-
MEKKCOOH-induced Cell Death  3235

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 pCMV5MEKKCOOH (3 μg) and pSRαJ NK/SAPK (9 μg) or pSRαJ NK/SAPK (9 μg). All of the dishes were transfected with the Gal4/Jun-luciferase (9 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.

Fig. 9. Expression of JNK/SAPK(APF) does not affect the MEKKCOOH-induced cellular condensation response. Swiss 3T3 cells were microinjected with expression plasmid encoding JNK/SAPK(APF) (150 ng/m) in the absence (panels A and B) or presence of pCMV5 expression plasmids encoding MEKKCOOH (5 ng/m) (panels C and D). This is 20-fold less MEKKCOOH expression plasmid than that used in Figs. 1–3. Each injection condition also included 50 ng/ml 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 NH2 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 MEKKCOOH expression in Swiss 3T3 cells indicates that MEKK-regulated signal transduction pathways integrate with the apoptotic response system. MEKKCOOH-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 MEKKCOOH. 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 un could protect neurons from serum deprivation-induced apoptosis (37). It was proposed that the dominant negative c-Jun inactivated c-Jun un 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 un was believed to maintain the neurons in stringent growth arrest. At first glance, the protective effect of dominant negative c-Jun un 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 un is not significantly activated in MEKK COOH-expressing cells. These findings are provocative because they indicate that MEKK-stimulated JNK/SAPK activation preferentially regulates Elk-1 and not c-Jun un. 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/signal 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 posttranslationally 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. Similarly, TNFα stimulates 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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MEK Kinase-induced Cell Death

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