Direct Activation of the Stress-activated Protein Kinase (SAPK) and Extracellular Signal-regulated Protein Kinase (ERK) Pathways by an Inducible Mitogen-activated Protein Kinase/ERK Kinase Kinase 3 (MEKK) Derivative*

Heidrun Ellinger-Ziegelbauer, Keith Brown, Kathy Kelly, and Ulrich Siebenlist‡

From the Laboratory of Immunoregulation, NIAID, National Institutes of Health, Bethesda, Maryland 20892-1876

The extracellular signal-regulated kinase (ERK) pathway, the stress-activated protein kinase (SAPK) pathway, and the p38 pathway are three major mitogen-activated protein kinase (MAPK) cascades known to participate in the regulation of cellular responses to a variety of extracellular signals. Upstream regulatory components of these kinase cascades, the MAPK/ERK kinase kinases (MEKK), have been described in several systems. We have isolated a cDNA encoding human MEKK3. Transfected MEKK3 has the ability to activate both SAPK and ERK pathways, but does not induce p38 activity, in agreement with a previous report on murine MEKK3 (Blank, J. L., Gerwins, P., Elliott, E. M., Sather, S., and Johnson, G. L. (1996) J. Biol. Chem. 271, 5361–5368). We now demonstrate that MEKK3 activates SEK and MEK, the known kinases targeting SAPK and ERK, respectively. Utilizing an estrogen ligand-activated MEKK3 derivative, we furthermore demonstrate that MEKK3 regulates the SAPK and the ERK pathway directly. Consistent with the fact that several SAPK-inducing agents activate the transcription factor NFκB, we now show that MEKK3 also enhances transcription from an NFκB-dependent reporter gene in cotransfection assays. The ability of MEKK3 to simultaneously activate the SAPK and ERK pathways is remarkable, given that they have divergent roles in cellular homeostasis.

One of the major signaling systems by which cells transduce extracellular signals into intracellular responses are the mitogen-activated protein kinase (MAPK) cascades. These have been characterized in a variety of organisms including yeast, the nematode Caenorhabditis elegans, Drosophila, and mammalian cells, revealing substantial evolutionary conservation (for reviews, see Refs. 1–6). The core components of these cascades are three sequential kinases. The MAPKs are activated by dual phosphorylation on threonine (T) and tyrosine (Y) by upstream MAPK kinases (MAPKK). MAPKs themselves are phosphorylated and thereby activated by serine/threonine-specific MAPKK kinases (MAPKKK), a subset of which is often referred to as MEK kinases (MEKK). In yeast several parallel MAPK cascades have been characterized, mostly by genetic screens (1, 2, 4, 6). Each kinase cascade in yeast appears to function as a linear unit which transduces a distinct signal.

In mammalian cells, three MAPK subfamilies have so far been clearly identified, although more are believed to exist. These are (i) the extracellular signal regulated kinases ERK1 and ERK2 (also referred to as p44/42MAPK) (9); (ii) the c-Jun-N-terminal/stress-activated cytokine proteins (JNKK/SEK) for the JNK/SAPK, including human JNKK/rat SAPKγ, human JNKK/rat SAPKα, and human JNK3, probably the human homologue of rat SAPKβ (p54β) (10–12); and (iii) the p38 kinase, which is similar to the yeast high osmolarity glycerol response (HOG) kinase (14, 15). The corresponding MAPKKs acting directly upstream in the cascades are (i) MEK1 or 2 (MAPK/ERK kinase) for ERK1/2 (16, 17); (ii) JNK kinase (JNKK)/SAPK kinase (SEK) for the JNKs/SAPKs (18, 19); and (iii) MKK3 or MKK6 for p38 (20–22). In yeasts, multiple MEKs have been identified, each associated with a distinct MAPK cascade (2, 4, 5). In mammals, MEKK1 (7, 23–26) and Raf-1 (3, 6) have been demonstrated to act as MAPKKs in the SAPK and ERK pathways, respectively. A MEKK for the p38 pathway has not yet been identified, although evidence suggests that it may be related to the transforming growth factor-β-activated kinase 1 (27, 28).

A diverse array of signals and factors activating mammalian MAPK cascades have been described (10, 13–15). ERKs are often activated by growth signals, such as epidermal growth factor (EGF) or platelet-derived growth factor (29), and the molecular steps by which these factors activate ERKs have been well worked out (6, 30–32). The growth factors effect tyrosine phosphorylation of their receptors, which in turn initiates the formation of well defined signaling complexes at the membrane. One component of this complex is the small G protein Ras, which upon GDP/GTP exchange recruits Raf-1. Raf-1 then becomes activated through phosphorylations to effect the mitogenic response to growth factor stimulation via the ERK pathway. ERK activity, however, can also be induced through receptors with seven transmembrane helices coupled to trimeric G proteins (33). Depending on the cell type, ERK activation results in either proliferation or differentiation (34). The SAPK and p38 pathways are both regulated by stress signals such as UV light or by proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) or interleukin-1 (IL-1). MEKK1 appears to mediate activation of SAPK in response to...
some of the SAPK-inducing signals (7, 23, 25, 26). For other
SAPK activity-inducing signals and for the p38 pathway, the upstream MAPKKKs are unknown. To transduce the multiplicity
of MAPK-activating signals into appropriate responses, it is
reasonable to postulate additional MEKKs.
Using a PCR strategy to identify mammalian homologues of
a yeast MEKK, a strategy which first led to the identification
of MEKK1 (7, 23, 35), Blank et al. (36) recently cloned additional
murine MEKKs, namely MEKK2 and MEKK3. We independ-
ently cloned and characterized human (h) MEKK3 and we
report here on novel functional aspects of this kinase. Expres-
sion of hMEKK3 in transfected cells potently activates
the SAPK and the ERK pathway, in agreement with Blank et al.
(36). Extending these observations, we show that hMEKK3
regulates SAPKs and ERKs via SEK and MEK1, respectively.
Furthermore, we demonstrate that hMEKK3 directly activates
these pathways. This rules out the possibility that hMEKK3
activates the SAPK and/or ERK pathways via induction of a
factor, such as a cytokine. This critical result was obtained with
the use of an inducible derivative of hMEKK3, which allowed
for a kinetic analysis of MAPK activation. Finally we provide
evidence that MEKK3 (in addition to MEKK1) is able to induce
NFB-dependent transcription. This is consistent with the fact
that many of the agents which activate SAPKs and/or ERKs,
such as TNF-α, IL-1, or serum, also activate the transcription
factor NFB (37), and suggests that multiple MEKKs can par-
ticipate in activation of NFB.

MATERIALS AND METHODS
Plasmids—A cDNA encoding amino acids 12–626 of human MEKK3 was
cloned as a fusion with an unrelated cDNA during screening of a
zAPZI library of activated peripheral blood T-lymphocytes (38) with
probes that had a Kozak sequence (40). Further analysis of the
differential display products, which were cloned with a 5' rapid amplification
of cDNA ends kit (Life Technologies, Inc.) and fused to the original cDNA via overlap extension
PCR (39). This extended cDNA encoded an in-frame methionine in the
case of a Kozak sequence (40) and an in-frame stop codon located
further 5'. It was cloned into pBluescript SK (Strategene) to yield BS
hMEKK3-F. For expression in eukaryotic cells, the complete open read-
ing frame was cut out from BS hMEKK3-F into EcoRI/XhoI fragments, the ends were filled in with Klenow fragment and ligated into the
Klenow-filled EcoRI site of PMT2TMC8, a modified PMT2T vector (41), to yield PMT2T hMEKK3-F. To construct PMT2T hMEKK3-CD, the
C-terminal part of the catalytic domain of the Sea/EcoRI fragment from
BSK hMEKK3-CD-F was ligated to the N-terminal part obtained as a PCR
fragment with an artificial Xhol site at the 5' end and a restricted Sval
site at the 3' end; the ligation product was then cloned into PPT2TXXE, opened at the Xhol and EcoRI sites. PPT2TXXE is a modified PPT2T vector
that supplies an N-terminal in-frame methionine (in the context of a Kozak sequence), which, upon insertion of the catalytic
domain, results in the addition of amino acids MASTN N-terminal to
the catalytic domain, a region which is quite
aminoacid level over the entire sequence, including the region
N-terminal to the catalytic domain, a region which is quite

Rosette affinity chromatography (43).

Cloning of Human MEKK3—A cDNA encoding a previously
unknown human kinase was cloned from a cDNA library made
from activated peripheral blood T lymphocytes, as described
under “Materials and Methods.” Comparison of the sequence of this
clone with the GenBank™ data base (Blast Fileserver, National Center for Biotechnology Information) revealed high
homology to MEKKs, especially to Byr2 of fission yeast and
Ste11 of budding yeast. We therefore investigated the relationship
of this putative MEKK3 to known mammalian MAPK path-
ways. During preparation of this manuscript, the cloning of two
highly related murine MEKKs, MEKK2 and MEKK3, was
reported by Blank et al. (36). Since our clone has highest homol-
y to murine MEKK3 (see below) it appears to be the human
homologue, and we refer to it hereafter as human (h) MEKK3.

Human and murine MEKK3 exhibit 96.5% identity at the
amino acid level over the entire sequence, including the region
N-terminal to the catalytic domain, a region which is quite

ATF2 (amino acids 1–96 of ATF2), and GST HA-JNK1 (full-length
JNK1 with an HA-tag at the N terminus), were used to express recom-
binant GST fusion proteins that were purified by glutathione-Sepha-
rose affinity chromatography (43).

Cell Culture and Transfection—Con6 and NIH3T3 cells were cul-
tured in Dulbecco's modified Eagle's medium supplemented with 1 mM
glutamine, 10% fetal bovine serum and antibiotics. Lipofectamine-me-
diated transfections were performed according to the manufacturer's
instructions (Life Technologies, Inc.), with the indicated amounts of
plasmid DNA per 100-mm plate. One day after transfection, cells were
serum-starved overnight in Dulbecco's modified Eagle's medium con-
taining 0.5% fetal bovine serum. Two days after transfection, cells were
stimulated as indicated and harvested. For stimulation with estrogen
(Sigma), cells were not serum-starved. For CAT reporter assays,
NIH3T3 cells were transfected by a calcium phosphate precipitation
protocol as described previously (41), except that the cells were se-
rum-starved as indicated, and then either left untreated or stimu-
lated for an additional 7 h with 2000 units/ml murine TNF-α (Boeh-
gringer Mannheim).

Immunoprecipitation and Western Blot Analysis—After washing
twice with cold phosphate-buffered saline, cells from one 100-mm plate
were lysed in 1 ml of Triton xylene buffer (25 mM HEPES, pH 7.5, 150 mM
NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 2 mM dithiothreitol,
25 mM β-glycerophosphate, 1 mM sodium vanadate, 50 mM sodium
fluoride, and a μl Pepstain). Further purification of immunoprecipi-
tates or GST-SEK bound to glutathione-Sepharose beads was per-
formed by a column chromatography step. The following antibodies,
using 2 μg of substrate, 10 μCi of [γ-32P]ATP and 50 μM ATP (SAPK,
ERK) or 5 μM ATP (p38) in total 20 μl of kinase buffer. The substrates
were used as GST c-Jun (1–79) for SAPK, myelin basic protein (Life
Technologies, Inc.) for ERK, GST ATP2 (1–96) for p38, GST HA-JNK1
for SEK, and GST-ERK[K71A] (Upstate Biotechnology Inc.) for MEK.
The reactions were terminated by boiling in SDS sample buffer and the
products resolved on 12.5% SDS-polyacrylamide gels and electrophoresis.
After staining of the gel with Comassie stain and drying, the phospho-
rylated proteins were visualized by autoradiography. Phosphorylation
signals were quantitated by PhosphoImager analysis (Molecular Dy-
namics, Inc.).

RESULTS

Cloning of Human MEKK3—A cDNA encoding a previously
unknown human kinase was cloned from a cDNA library made
from activated peripheral blood T lymphocytes, as described
under “Materials and Methods.” Comparison of the sequence of this
close to the GenBank™ data base (Blast Fileserver, National Center for Biotechnology Information) revealed high
homology to MEKKs, especially to Byr2 of fission yeast and
Ste11 of budding yeast. We therefore investigated the relation-
ship of this putative MEKK3 to known mammalian MAPK path-
ways. During preparation of this manuscript, the cloning of two
highly related murine MEKKs, MEKK2 and MEKK3, was
reported by Blank et al. (36). Since our clone has highest homol-
y to murine MEKK3 (see below) it appears to be the human
homologue, and we refer to it hereafter as human (h) MEKK3.

Human and murine MEKK3 exhibit 96.5% identity at the
amino acid level over the entire sequence, including the region
N-terminal to the catalytic domain, a region which is quite
divergent among distinct MEKKs. Alignment of the catalytic domainsofMEKKsclonedinmammals,yeast,andthetobacco plant (Fig. 1) revealsthatMEKK3 is morecloselyrelatedtothe plant MEKK (N.t. NPK) and Ste11 offission yeast (S.c. Ste11), than to mammalian MEKK1, except MEKK2. RT PCR and Northern blot analyses with RNA from various human and murine cell lines and murine tissues revealed an approximately 4.6-kilobase transcript, which appears to be ubiquitously expressed at low levels (data not shown).

MEKK3 Activates the SAPK and ERK Pathway, but Not the High Osmolarity Glycerol/p38 Pathway—

To investigate which of the known MAPK cascades are regulated by MEKK3, we cotransfected NIH3T3 (Fig. 2), Cos7, and HeLa cells (data not shown) with expression vectors for MEKK3 together with HA epitope-tagged versions of SAPK p54\[\text{b}\], ERK2, or p38. The epitope-tagged MAPKs were then immunoprecipitated using the anti-HA antibody and tested for activity in an immunocomplex kinase assay with their preferred substrates (Fig. 2, A–C). For comparison, MEKK1 and a constitutively active form of human Raf-1, RafBXB (44), both cloned into the same expression vector as MEKK3, were also transfected with the different MAPKs. Initially we expressed either the MEKK3 catalytic domain (CD) or the full-length protein, considering the possibility that full-length MEKK3 might be inactive as observed for Raf-1 (44). But like MEKK1 (23), both the N-terminal truncation and the full-length forms of MEKK3 behaved similarly (Fig. 2, and data not shown). MEKK3 activated SAPK activity as strongly as did MEKK1 (shown in Fig. 2A with two different concentrations of expression vectors). An additional titration from 25 to 1000 ng/plate of the MEKK3 or MEKK1 expression plasmids in Cos7 cells further supported this conclusion (data not shown). Surprisingly, both MEKK3 and MEKK1 are more efficient activators in this experiment than the known SAPK inducers anisomycin or TNF (compare lanes 4–7 with lanes 2 and 3). As expected, RafBXB did not effect SAPK p54\[\text{b}\], even with high amounts of expression vector (Fig. 2A, and data not shown).

In contrast to the very similar activities of MEKK1 and MEKK3 toward SAPK, ERK is highly activated by MEKK3, but only marginally by MEKK1 (Fig. 2C). This difference with respect to ERK is unlikely to be due to a significant difference in expression of the two MEKKs, since both have equally high activity with respect to SAPK. By comparison, the constitutively active form of Raf-1 (RafBXB), a well known activator of ERK in response to growth factors (6, 30–32), was a weaker activator of ERK than was MEKK3 in these transfection experiments; nevertheless, it still appeared to be 5–10 times more potent than MEKK1 (Fig. 2C). It was not possible to determine precise levels of activity of these kinases, since quantitation of the amounts of expressed protein was not feasible, p38, which can be induced by various stress signals such as exposure to arsenite (Fig. 2B, lane 2), was not significantly activated after
transfection of MEKK3. As expected, RafBXB had no influence on p38 activity, whereas MEKK1 induced it, as observed by others (45).

As a further test of SAPK and ERK activation, the effect of activation on the electrophoretic mobility of these proteins was investigated. Mobility shifts induced upon activation reflect phosphorylation of threonine and tyrosine in the conserved XXY motif by the upstream MAPKKs (see Introduction). Immunoblot analysis clearly demonstrated that MEKK3 expression vectors for MEKK3, MEKK1, and RafBXB, or HA-p38, were cotransfected with the indicated amounts (μg) of PMT2T expression vectors for MEKK3, MEKK1, and RafBXB, or stimulated with 10 μg/ml anisomycin (An) for 30 min, 2000 units/ml TNF-α for 15 min, 25 ng/ml EGF for 5 min, or 0.5 mM arsenite (Ars) for 30 min. Cells were serum-starved overnight in medium containing 0.5% fetal bovine serum the day before harvest. SAPK (A, p38, and ERK2 (C) assay was as described under “Materials and Methods” with the indicated substrates. Numbers at the bottom indicate fold activation relative to unstimulated cells, which were transfected only with the HA-tagged MAPK homologue. D, additionally, extracts were immunoblotted with a polyclonal anti-JNK/SAPK antibody (left panel) or monoclonal anti-HA antibody (right panel) to show activation of the SAPK p54β and ERK2 by a phosphorylation-dependent mobility shift. The phosphorylated forms are indicated with the prefix pp.

SAPK strongly and p38 more weakly, and in contrast also to Raf-1, which appears to be specific for ERK. These results regarding the apparently dual activities of MEKK3 are essentially in agreement with Blank et al. (36), who also demonstrated that MEKK2 behaves similarly (MEKK3 and MEKK2 are 86.6% identical in their catalytic domains). Our work adds the direct comparisons with Raf and MEKK1 as well as the MEKK3-induced changes in the phosphorylation of SAPK and ERK. In the following we investigate by what intermediate steps MEKK3 activates both ERK and SAPK, a question which was not previously resolved.

MEKK3 Activates the SAPK and ERK Pathways via SEK and MEK—Presently known MAPKKs for SAPKs and ERKs are SEK and MEK1/2, respectively. After coexpression of tagged SEK and MEK1 with MEKK3, MEKK1, or RafBXB, tagged SEK (Fig. 3A) and MEK1 (Fig. 3B) were immunoprecipitated and incubated in an in vitro kinase assay with their matching downstream substrates. MEKK3 activated both SEK (Fig. 3A) and MEK1 (Fig. 3B), and its strength of activation appeared similar to that of MEKK1 toward SEK and to that of RafBXB toward MEK1. Surprisingly, the MEKK1 control also activated MEK1 in these assays, despite the fact that ERK activity was only marginally increased (Fig. 2C). The reason for this latter observation is unknown but the data are in agreement with those reported by Xu et al. (46). In summary, our results establish that MEKK3 expression activates both SEK and MEK1, which in turn activate SAPK and ERK, respectively. Blank et al. (36) were unable to establish a connection between MEKK3 and SEK or MEK1, possibly because the recombinant MEKK3 protein with which they attempted to demonstrate this in vitro may not have been fully functional. An alternative hypothetical explanation of their data is that MEKK3 may regulate the SAPK and ERK pathways only indirectly, an important question addressed below.

MEKK3 Is a Direct Activator of the SAPK and ERK Pathways—In the experiments described above, the MAPKs and MAPKKs were cotransfected with constitutively active MEKK3. It is thus possible that continuous expression of MEKK3 during the relatively long time course of a transfection experiment may have induced a factor (via an unknown pathway), such as a secreted cytokine, which in turn may have been the true inducer of SAPK and/or ERK activity. An example of this scenario is the delayed activation of SAPK activity by Raf-1 in HOla cells (23), which occurs via the Raf-1-induced synthesis of the heparin-binding EGF, the actual signal for activation of SAPK (47).

To resolve how MEKK3 activates the MAPK and SAPK pathways, we generated a MEKK3 whose activity could be regulated so that immediate responses to its induced activation could be investigated. We constructed an inducible MEKK3...
MEKK3 Regulates Two MAPK Cascades

**FIG. 4. MEKK3 is a direct regulator of the SAPK and ERK pathways.** NIH3T3 cells were cotransfected with 2 μg pCEV MEKK3 CD-ERLBD (expressing estrogen-inducible MEKK3) and 6 μg of PMT2T HA-p54β (A, left panel; B) or with 3 μg of pCEV MEKK3 CD-ERLBD and 3 μg of pcDNA HA-ERK2 (A, right panel). Before harvesting, cells were incubated with 1 μM estrogen (E$_2$) for the indicated times or with an equal amount of ethanol, which served as a control for the solvent (lanes 1 in A; B, lane 5 in A). A, immunocomplex kinase assay revealing estrogen-inducible SAPK and ERK activity. B, anti-JNK/SAPK immunoblot demonstrating estrogen-dependent phosphorylation of SAPK (ppSAPK) in the extracts used for the kinase assays shown in the left panel of A.

derivative by fusing the estrogen receptor ligand binding domain (ERLBD) to the MEKK3 catalytic domain. This approach has been previously applied to reversibly induce the activity of a variety of proteins (48–52). HA-p54β or HA-ERK2 were transiently transfected into NIH3T3 cells together with the MEKK3 CD-ERLBD chimera. Before harvest, cells were treated for periods up to one hour with estrogen. Both SAPK and ERK activity were induced by 15 min post treatment, as demonstrated by an in vitro kinase assay (Fig. 4A), and also by Western blot analysis in the case of SAPK (Fig. 4B). In the absence of estrogen, MAPK activity was hardly detectable (Fig. 4A, lanes 1 and 5), although the MEKK3 chimera was expressed in all samples, as verified by Western analysis with an antibody against the estrogen receptor (data not shown). This demonstrates the tight negative control on kinase activity exerted by the estrogen ligand domain. The hyperphosphorylated forms of SAPK were also clearly evident by 15 min upon addition of estrogen (Fig. 4B, lane 2). These data demonstrate that MEKK3 is a direct activator of both the SAPK and ERK pathways. In contrast, what was observed in cells cotransfected with constitutively active MEKK3 (see Fig. 2D), the total amount of SAPK did not visibly decrease during 1 hour of stimulation with the inducible MEKK3 derivative. Therefore, the decrease in the level of SAPK appears to be triggered by long term activation of either MEKK3 or SAPK itself.

**Both MEKK1 and MEKK3 Activate NFκB—**Since many of the agents which induce SAPK and ERK activity are also known to activate the transcription factor NFκB (37), we investigated whether MEKK3 or MEKK1 could induce transcription of an NFκB-dependent reporter gene. NIH3T3 cells were transiently cotransfected with a κB site-dependent reporter construct (HIV-κB-CAT) and an MEKK expression vector. RafBXB was transfected for comparison, as it has been shown previously to increase NFκB activity (53). Both MEKK3-CD and MEKK1 (and to a slightly lesser degree full-length MEKK3) induced NFκB activity about 10-fold, 4-fold more than RafBXB in these transfections (Fig. 5). During preparation of this manuscript, MEKK1-induced activation of NFκB was also reported by two other groups (54, 55). Our findings suggest that MEKK3- and MEKK1-activated MAPK cascades may participate in transducing signals leading to activation of NFκB, although actual intervening molecular steps are unknown since none of the kinases in these cascades are likely to phosphorylate IκBα directly (56).

**DISCUSSION**

The biochemical characterization of human MEKK3 presented here clearly demonstrates the ability of MEKK3 to directly regulate two of the three currently identified mammalian MAPK cascades, namely the SAPK and the ERK pathways. In contrast, MEKK3 does not regulate the p38/High osmolarity glycerol pathway. MEKK3 activates SAPK via SEK and it activates ERK via MEK1/2. SEK is also the target for MEKK1-dependent SAPK activation and MEK1/2 is also the target for Raf-1-dependent ERK activation. In agreement with our results, Blank et al. (36) recently demonstrated the ability of MEKK3 (as well as MEKK2) to activate the two MAPK
pathways upon exogenous expression, but the activation of neither SEK nor MEK1/2 by MEKK3 was demonstrated, nor was the question of a direct versus an indirect activation addressed. An indirect activation might have involved MEKK3-induced synthesis of a factor, possibly a secreted cytokine, which in turn could have been the true activator of one or both of the MAPK pathways. A precedent for this exists in the case of a transfected Raf-1 kinase, which can activate SAPK via an induced cytokine in HeLa cells (23), but such a scenario was ruled out here by the rapid activation of the two MAPK pathways when MEKK3 kinase activity was selectively induced in cells. To generate an inducible MEKK3, the estrogen receptor ligand binding domain was fused to the catalytic domain of MEKK3, converting the constitutively active MEKK3 into an estrogen-dependent kinase. We thus conclude that MEKK3 can directly activate the MAPKs ERK and SAPK via MEK1/2 and SEK, respectively.

The demonstration that both pathways are direct targets of one kinase is particularly significant in light of the fact that the ERK and SAPK pathways have been generally associated with quite divergent biologic responses. The ERK pathway is clearly associated with growth and differentiation responses, while the SAPK pathway is associated with stress responses and apoptosis (3, 23, 34). Physiologic situations in which both pathways are activated include stimulation of T-cells through both the T-cell antigen receptor and the CD28 auxiliary receptor (27). Indeed, the kinases which channel a signal to activate a particular MAPK are quite distinct in their apparent biologic effects suggesting a complexity in signal transduction which has not previously been recognized and for which there is as yet no precedent in yeast. MEKKs identified in yeast to date appear to function in a linear cascade in which each is generally responsive to a specific signal and in which each is dedicated to one MAPK module (5).

Indicated, the kinases which channel a signal to activate a particular MAPK may be held together in a physical complex, as exemplified by the pheromone response pathway in budding yeast. In this pathway, the Ste5 protein functions as a scaffold for a multikinase complex by simultaneously binding Ste11 (a MEKK), Ste7 (a MAPKK) and Fus3 or Kss1 (MAPKs) (57). Although similar arrangements are likely in mammals, previous data as well as the data presented here show that in mammals the same MEKK can be used for different MAPKs, suggesting more versatility and a more complex regulation pattern.

Adduced complexity in mammalian signaling systems may also be indicated by the diversity of stimuli which regulate a particular MAPK, sometimes in a cell type-dependent manner. Agents which can activate SAPK include proinflammatory cytokines such as IL-1 and TNF-α (11–13), stress conditions such as UV irradiation and treatment with protein synthesis inhibitors (10, 11, 13), and growth factors such as EGF and nerve growth factor (13, 58). Whereas TNF-α and UV irradiation activate SAPK in a variety of cell types, EGF activates SAPK potently in HeLa cells (23, 58), but only marginally in certain fibroblast cell lines (13, 58) (data not shown). ERK activity is primarily induced by growth factors via a Ras-dependent pathway (23, 59, 60), but can also be induced by TNF-α in certain cell types (13, 58). Known or suggested components upstream of the MEKKs comprise a growing family of STE20-like kinases (4, 61), low molecular weight GTP-binding proteins including Ras, Rac and Cdc42Hs (8), and trimeric G proteins (33, 62, 63). It is thus possible that MEKK3 (and possibly MEKK2) could be involved when a broader spectrum of activities is called for, although the exact connection to upstream signals may differ with the cell type. Alternatively, cells may be able to regulate MEKK2 and MEKK3 in such a way that they activate either the SAPK or the ERK pathway, but not both. Depending on the signal, multikinase complexes containing either the SAPK or the ERK pathway components may be formed, possibly mediated by accessory proteins (57). Which extracellular signals and intermediaries ultimately regulate MEKK3 remain to be shown.

The transcription factor NFκB appears to at least one possible downstream target of MEKK3. NFκB plays a pivotal role in regulating genes which encode functions relevant to the immune and stress responses, as well as growth and differentiation (37). Raf-1, MEKK1 and now also MEKK3 could be intermediates in at least some of the situations in which NFκB becomes activated. Elucidation of the steps linking these MEKKs and NFκB activation may reveal potential targets for drug intervention in the case of pathological activation of this transcription factor in inflammatory diseases.

Acknowledgments—We thank J. R. Woodgett, A. Fauci for continued support and review of the manuscript.

REFERENCES

1. Blumer, K. J., and Johnson, G. L. (1994) Trends Biochem. Sci. 19, 236–240
2. Davis, R. J. (1994) Trends Biochem. Sci. 19, 470–473
3. Marshall, C. J. (1995) Cell 80, 179–185
4. Herskowitz, I. (1995) Cell 80, 187–197
5. Cano, E., and Mahadevan, L. C. (1995) Trends Biochem. Sci. 20, 117–122
6. Seger, R., and Krebs, E. G. (1995) FASEB J 9, 726–735
7. Lange-Carter, C. A., and Johnson, G. L. (1994) Science 265, 1458–1461
8. Vojtek, A. B., and Cohen, P. (1995) Cell 82, 527–537
9. Cobb, M. H., Robbins, D. J., and Boulton, T. G. (1991) Curr. Opin. Cell Biol. 3, 1025–1032
10. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Xu, D., Tosh, K., and Davis, R. J. (1994) Cell 78, 1025–1037
11. Kallunki, T., Xu, D., Tsigelny, I., Shuss, H. K., Derijard, B., Moore, G., Davis, R., and Karin, M. (1994) Genes Dev. 8, 2096–2107
12. Gupta, S., Barret, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B., and Davis, R. J. (1996) EMBO J 15, 2760–2770
13. Kyrkias, D. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994) Nature 369, 156–160
14. Han, J., Lee, J. D., Bibbs, L., and Ulevitch, R. J. (1994) Science 265, 808–811
15. Freshney, N. W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuang, J., and Saklatvala, J. (1994) Cell 79, 1039–1049
16. Crews, C. M., Alessandria, A., and Erikson, E. (1992) Science 258, 478–480
17. Zheng, C.-F., and Guan, K.-L. (1995) J. Biol. Chem. 270, 11345–11349
18. Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyrkias, D. M., and Zon, L. I. (1995) J. Biol. Chem. 270, 784–788
19. Yan, M., Dai, T., Teak, J. C., Kyrkias, D. M., Zon, L. I., Woodgett, J. R., and Templeton, D. J. (1994) Nature 372, 798–800
20. Han, J., Lee, J. D., Jiang, Y., Li, Z., Feng, L., and Ulevitch, R. J. (1996) J. Biol. Chem. 271, 2886–2891
21. Raingeaud, J., Whitmarsh, A. J., Barrett, T., Derijard, B., and Davis, R. J. (1996) Mol. Cell. Biol. 16, 1247–1255
22. Stein, B., Brady, H., Yang, M. X., Young, D. B., and Barbasoa, M. S. (1996) J. Biol. Chem. 271, 11427–11433
23. Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R. J., Johnson, G. L., and Karin, M. (1994) Science 265, 1739–1743
24. Russell, M., Lange-Carter, C. A., and Johnson, G. L. (1995) J. Biol. Chem. 270, 11757–11760
25. Xiao, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Grellmann, M. E. (1995) Science 270, 1326–1331
26. Johnson, N. L., Gardner, A. M., Diener, K. M., Lange-Carter, C. A., Glevay, J., Jarpe, M. B., Minden, A., Karin, M., Zon, L. I., and Johnson, G. L. (1996) J. Biol. Chem. 271, 3269–3277
27. Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Tanigushi, T., Nishida, E., and Matsumoto, K. (1993) Science 260, 1458–1461
28. Labinios, M., Meier, K. E., Smith, E. A., Gause, K. C., LeRoy, E. C., and Trojanowski, J. (1994) J. Biol. Chem. 269, 9822–9825
29. Stokoe, D., MacDonald, S. G., Cawdallader, K., Symons, M., and Hancock, J. F. (1994) Science 264, 1463–1467
30. Kazlauskas, A. (1994) Curr. Opin. Genet. Dev. 4, 5–14
31. Claasson-Welsh, L. (1994) J. Biol. Chem. 269, 32023–32026
32. Crespo, P., Xu, N., Simonds, W. F., and Gutkind, J. S. (1994) Nature 369,
MEKK3 Regulates Two MAPK Cascades

418–420

34. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) *Cell* 77, 841–852
35. Lange-Carter, C. A., Pleiman, C. M., Gardiner, A. M., Blumer, K. J., and Johnson, G. L. (1993) *Science* 260, 315–319
36. Blank, J. L., Gerwins, P., Elliott, E. M., Sather, S., and Johnson, G. J. (1996) *J. Biol. Chem.* 271, 5361–5368
37. Siebenlist, U., Franzoso, G., and Brown, K. (1994) *Annu. Rev. Cell Biol.* 10, 405–455
38. Zipfel, P. F., Irving, S. G., Kelly, K., and Siebenlist, U. (1989) *Mol. Cell. Biol.* 9, 1041–1048
39. Horton, R. M., Cai, Z., Ho, S. N., and Pease, L. R. (1990) *BioTechniques* 8, 530–535
40. Bours, V., Burd, P. R., Brown, K., Villalobos, J., Park, S., Ryseck, R.-P., Bravo, R., Kelly, K., and Siebenlist, U. (1992) *Mol. Cell. Biol.* 12, 685–695
41. Miki, T., Fleming, T. P., Crescenzi, M., Molloy, C. J., Blam, S. B., Reynolds, S. H., and Arumunon, S. A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 5167–5171
42. Smith, D. B., and Johnson, K. S. (1986) *Cell* 44, 283–292
43. Bruder, J. T., Heidecker, G., and Rapp, U. R (1992) *Genes Dev.* 6, 545–556
44. Lin, A., Minden, A., Martinetto, H., Claret, F.-X., Lange-Carter, C., Mercorio, F., Johnson, G. L., and Karin, M. (1994) *Science* 268, 286–290
45. Xu, S., Robbins, D., Frost, J., Dang, A., Lange-Carter, C., and Cobb, M. H. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 6608–6612
46. Cosso, O. A., Chiariello, M., Kalinec, G., Kyriakis, J. M., Woodgett, J., and Gutkind, J. S. (1995) *Cell* 81, 1317–1326
47. Manser, E., Leung, T., Saihuddin, H., Zhan, Z., and Lim, L. (1994) *Nature* 376, 40–46
48. Owaki, H., Varma, R., Gillis, B., Bruder, J. T., Rapp, U. R., Davis, L. S., and Geppert, T. D. (1993) *EMBO J.* 12, 4367–4373