in vivo activation of SEK1 by SAPK agonists and the ability of dominant-negative SEK1 to inhibit SAPK activation by extracellular agonists, we propose that SEK1 is a physiological activator of the SAPKs *in vivo*. We chose the name SEK1 (for SAPK/ERK kinase) because other extracellularly regulated kinases (ERKs) have been isolated[17–19] and we do not know yet if these are physiological substrates of SEK1. The inability of Ras and Ras-coupled agonists to activate SEK1 or the SAPKs strongly *in vivo*, coupled with the inability of SEK1(K→R) to block Ras activation of MAPKs and the inability of Raf-1 to activate the SAPKs[20], indicate that SEK1 and the SAPKs lie on a signalling pathway that is largely distinct from the Ras/Raf/MEK/MAPK pathway. In an accompanying paper, we demonstrate that SEK1 is phosphorylated and activated in intact cells not by Raf-1, but by MEK-kinase-1 (ref. 20), a mammalian homologue of the STE11 kinase from *Saccharomyces cerevisiae*[21]. These results support the contention that at least two signal transduction cascades exist in mammals with distinct functions: the MEKK/SEK/SAPK-mediated stress response and the Raf/MEK/MAPK-mediated mitogenic response. The specificity of SEK1 and the MEKs defines the segregation of the stress and mitogenic pathways. Thus, earlier work in yeast, demonstrating multiple independent but homologous signalling pathways (reviewed in ref. 22) can now be extended to mammalian systems. □

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Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1

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A KINASE distinct from the MEK activator Raf[1–3], termed MEK kinase-1 (MEKK), was originally identified by virtue of its homology to kinases involved in yeast mating signal cascades[4]. Like Raf, MEKK is capable of activating MEK *in vitro*[4,5]. High-level expression of MEKK in COS-7 cells[4] or using vaccinia virus vectors[5] also activates MEK and MAPK, indicating that MEKK and Raf provide alternative means of activating the MAPK signalling pathway. We have derived NIH3T3 cell sublines that can be induced to express active MEKK. Here we show that induction of MEKK does not result in the activation of MAPK, but instead stimulates the stress-activated protein kinases (SAPKs)[6–8] which are identical to a Jun amino-terminal kinase[9,10]. We find that MEKK regulates a new signalling cascade by phosphorylating an SAPK activator, SEK1 which in turn phosphorylates and activates SAPK.

Stably transfected NIH3T3 subclones express MEKK in response to isopropyl-β-D-thiogalactosidase (IPTG) (Fig. 1*a*) but MAPK activity remains unchanged (Fig. 1*b*). In contrast, SAPK activity is increased six- to eightfold in MEKK-inducible cell lines but not in the parent NIH3T3 cells. These MEKK-expressing cells are able to activate MAPK in response to some mitogenic signals, because treatment with phorbol ester increases MAPK activity in each of these clones (as well as in NIH3T3 cells), whereas SAPK activity is unaffected (Fig. 1*c*). Increased SAPK activity is evident by 3 hours and maximal after 12 hours of induction; MAPK activity is unchanged throughout the 23-hour incubation with inducer (Fig. 1*d*). Together these results indicate that, in contrast to the presumed role of MEKK in activating MEK and MAPK, MEKK acts instead to activate SAPKs. Expression of truncated ΔMEKK in these clones resulted in six- to eightfold inhibition of growth rate compared with parental NIH3T3 cells.

We modelled activation of SAPK by MEKK using cloned genes and purified proteins expressed using vaccinia virus vectors[11,12]. MEKK induced electrophoretic retardation of SAPK, which was suggestive of quantitative phosphorylation (Fig. 2*a*), and also increased the amount of phosphotyrosine in SAPK and activated its Jun N-terminal kinase activity. Thus, in this overexpression model as well as in the inducible cell line, MEKK expression results in activation of the SAPK pathway.

We considered the possibility that activation of SAPK occurred as a consequence of activation of the MEK and MAPK cascade. To stimulate MAPK independently of MEKK, we used activated Raf and a constitutively active allele of MEK1 termed MEK 2E (ref. 5). Both Raf and MEK 2E were able to induce phosphorylation of coexpressed MAPK (Fig. 2*b*). Neither of these MAPK activators induced phosphorylation of SAPK, indicating that the SAPK activation pathway is effectively insulated from the MAPK pathway.

MEKK was unable to phosphorylate SAPK *in vitro* (below). We therefore tested whether MEKK activated the newly identified SAPK activator, SEK1 (ref. 13), whose sequence is similar to MEK1. Immunopurified MEKK (but not the inactive mutant

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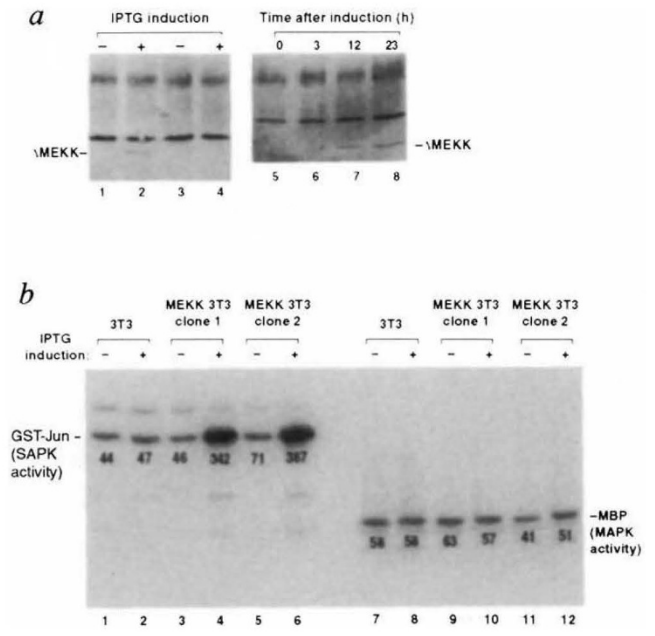

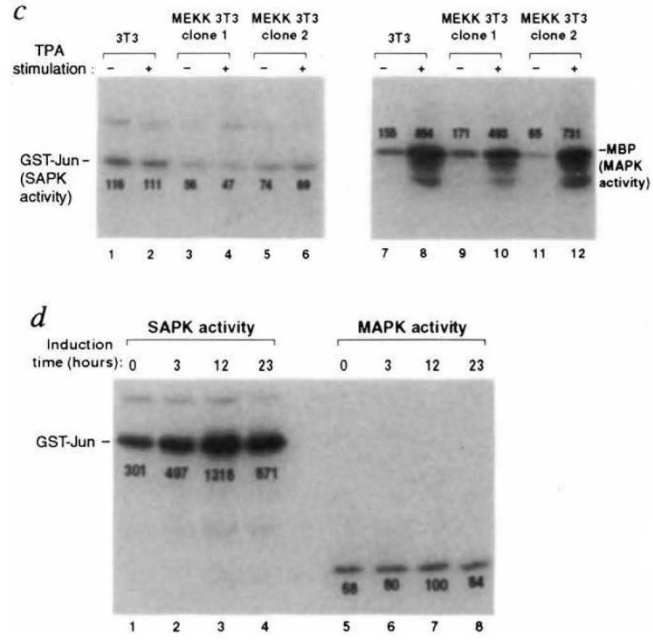

FIG. 1 *a*, MEKK expression in NIH3T3 cells. Epitope-tagged truncated MEKK (ΔMEKK) was detected in MEKK clone 1 cells (lanes 1 and 2) or in MEKK clone 2 cells (lanes 5–8) but not in a control clone (lanes 3 and 4) treated with IPTG for 24 h (lanes 2 and 4) or at the indicated times (lanes 6–8). *b*, MAPK and SAPK activity in MEKK-inducible cell lines. Numbers below labelled bands indicate c.p.m. of radioactivity in substrates. SAPK activity, but not MAPK activity, was increased in response to MEKK expression. *c*, Functional MAPK signalling in NIH3T3 cells and MEKK-expressing subclones after stimulation (+) with 250 ng ml^{-1} TPA. *d*, Time course of induction of SAPK activity in MEKK 3T3 clone 2 cells after IPTG treatment. MAPK activity throughout this period remained unchanged, whereas SAPK activity was increased even at the 3-h time point, when MEKK expression could not yet be detected. MEKK expression in cells used here is shown in *a*, lanes 5–8.

METHODS. The EE epitope-tagged[5] C-terminal 320 amino acids of MEKK1 (ΔMEKK) was expressed in NIH3T3 cells using the lacSwitch promoter (Stratagene). ΔMEKK was induced in cell clones with 1 mM IPTG and detected by immunoprecipitation and immunoblotting using the anti-EE monoclonal antibody (mAb). MAPK and SAPK activity was determined using polyclonal antibodies recognizing a C-terminal peptide of p42MAPK or a p54SAPK–GST fusion protein. Immune complexes containing protein from 10^5 cells were reacted with 0.5 μg GST–Jun (amino acids 5–89; ref. 8) for SAPK, or MBP (Sigma) for MAPK in 20-μl reactions (50 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 15 μM ATP, 5 μCi [^{32}P-γ]ATP), for 30 min at room temperature. Radioactivity was quantified using an AMBIS β-detector.

MEKK(K → R) rapidly phosphorylated a glutathione-*S*-transferase(GST)–SEK fusion protein on serine and threonine residues (Fig. 3*a*) but failed to phosphorylate a SEK mutant in which the two residues equivalent to the sites of activation in MEK were mutated. Phosphorylation of GST–SEK activated the SAPK activity of GST–SEK. Thus, SEK is a substrate of MEKK and phosphorylation by MEKK is sufficient to activate SEK. MEKK expression also activates SEK *in vivo* (Fig. 3*b*). Activation of SAPK by MEKK requires functional SEK because coexpression of a dominant inhibitory allele of SEK blocks activation of SAPK by MEKK (Fig. 3*c*).

Coexpression of full-length MEKK protein is able to effect phosphorylation of SEK and activation of SAPK (Fig. 4*a*), similar to the activation induced by truncated MEKK. The high activity of full-length MEKK protein during overexpression suggests that a cellular activity might regulate natural MEKK expressed at lower levels. This result is in contrast to Raf, which displays low levels of kinase activity unless truncated[14].

Our results demonstrate complete reconstitution of a kinase cascade, beginning with MEKK, that phosphorylates and activates SEK, which subsequently phosphorylates and activates SAPK. Each component of this cascade is functionally parallel

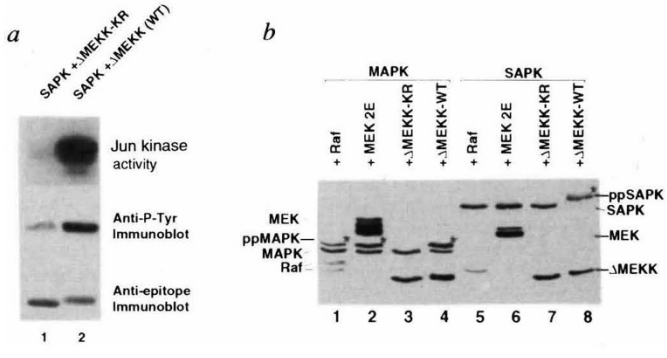

FIG. 2 Activation of SEK–SAPK pathway by coexpression of ΔMEKK using vaccinia virus vectors. *a*, Epitope-tagged SAPK was expressed with either the untagged inactive K → R mutant of ΔMEKK (lane 1) or wild-type ΔMEKK (lane 2). Coexpression of active MEKK resulted in mobility shift of SAPK detected by anti-epitope immunoblotting (bottom panel) and also increased tyrosine phosphorylation of SAPK detected in anti-epitope immunoprecipitates (middle panel). SAPK activity was also strongly elevated, reflected by phosphorylation of GST–Jun(5–89) using anti-epitope immunoprecipitates (top panel). *b*, epitope-tagged MAPK (lanes 1–4) or SAPK (lanes 5–8) was expressed with epitope-tagged forms of truncated active Raf (lanes 1, 5), constitutively active MEK 2E (lanes 2, 6), ΔMEKK(K → R) mutant (lanes 3, 7) or ΔMEKK wild type (lanes 4, 8), and detected in whole cell lysates using anti-epitope western blot. Activation of both MAPK and SAPK is identifiable by the appearance of bands with delayed mobility, indicated by stars. pp prefix, phosphorylated protein forms. Raf and active MEK 2E are able to activate MAPK, but not SAPK, thus the SAPK pathway is insulated from the MAPK pathway. MEKK is able to activate MAPK in this overexpression system, though it is not when expressed at lower levels (Fig. 1). Of the kinases tested, only MEKK is able to activate SAPK.

METHODS. The N-terminal EE-epitope-tagged p54SAPKα1, and untagged ΔMEKK were expressed using the vaccinia virus expression system and the plasmid pTM1 (ref. 12). Phosphotyrosine was detected using mAb 4G10 (UBI) to probe anti-EE immunoprecipitates. Jun kinase was assayed as for Fig. 1. Kinase expression and blotting has been described[5].

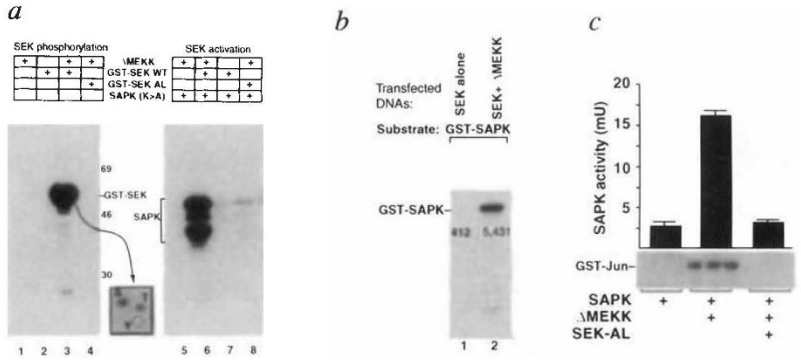

FIG. 3 *a*, MEKK phosphorylates and activates of SEK *in vitro*. Immunopurified ΔMEKK phosphorylated wild-type GST–SEK (lane 3) on serine and threonine (see phosphoaminoacid analysis, inset), but not mutant GST–SEK protein lacking the two phosphorylation sites (lane 4). GST–SEK1 phosphorylated by MEKK *in vitro* acquired SAPK kinase activity, as shown after secondary reaction with inactive (K>A) mutant thrombin-cleaved GST–SAPK with radioactive ATP (lane 6). Mutant GST–SEK protein lacking phosphorylation sites (lane 8) or reactions without either MEKK or SEK did not allow phosphorylation of SAPK. *b*, MEKK activates SEK1 *in vivo*. Epitope-tagged SEK expressed in CV1 cells using a CMV expression vector (lane 1) became activated by coexpression of ΔMEKK lacking the epitope tag (lane 2). Anti-epitope immunocomplexes were assayed for SEK activity using GST–SAPK as substrate (see Fig. 1 legend). *c*, SAPK activation by MEKK requires SEK1. Epitope-tagged SAPK expressed using SV40-based vectors was activated by coexpression with ΔMEKK. This activation was reversed by triple coexpression of a dominant inhibitory mutant of SEK1 containing (S220A, T224L; SEK AL). METHODS. Epitope-tagged ΔMEKK was expressed, immunopurified and eluted using excess EE peptide[5]. Bacterial SEK–GST fusion protein was purified and reacted *in situ* on glutathione–agarose beads. Activated MEKK was separated from GST–SEK by washing the glutathione beads, and subsequently incubated with SAPK substrate (cleaved from GST by thrombin, and containing an inactivating K55A mutation) in kinase reactions containing [^{32}P]ATP (20 mM ATP total concentration). Inhibition of HA-epitope-tagged SAPK by SEK-S220A, T224L (SEK-AL) was tested in L929 cells using the SV40-based pMT2 vector; 7 μg SAPK, 7 μg ΔMEKK and 15 μg SEK-AL expression plasmids were transfected together with empty pMT2 vector DNA to equalize plasmid mass. SAPK assays represent triplicate measurements using GST–Jun substrate as described[13].

to a component of the MAPK activation pathway: SAPK is analogous to MAPK, SEK analogous to MEK, and MEKK analogous to Raf (Fig. 4*b*). Other evidence suggests that SAPK signalling in response to ultraviolet irradiation[10,15] and tumour-necrosis factor-α[16] lies downstream of Ras. Additionally, dominant inhibitory Ras reduces the activity of MEKK[17] and the MEKK homologue Byr2 associates with Ras1 in yeast[18]. Thus, in parallel to Raf–MEK–MAPK, the MEKK–SEK–SAPK pathway most probably lies downstream of Ras. Cofactors for Ras must exist that contribute specifically to either the mitogenic or stress-response pathways.

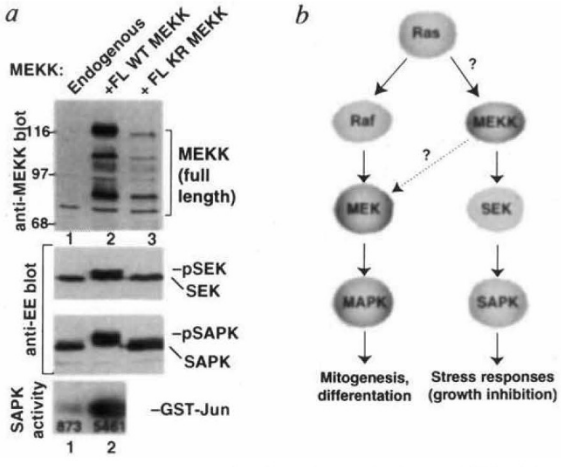

FIG. 4 *a*, Activation of SEK and SAPK *in vivo* by full-length MEKK. Using the vaccinia virus expression system in CV1 cells, SEK1 or SAPK were separately expressed alone (lane 1) or with vectors encoding full-length wild-type MEKK1 (lane 2) or a kinase-inactive (K447R) mutant allele of MEKK1 (lane 3). Expression of several bands related to full-length MEKK (top panel) was detected using chicken polyclonal antibodies raised against bacterially expressed ΔMEKK. Endogenous proteins of 70K, 90K and 110K were also detected (lane 1). Epitope-tagged SEK1 and SAPK1 (middle panels) were detected by immunoblotting. Electrophoretically retarded bands arising from phosphorylation of both SEK and SAPK were observed from coexpression with full-length MEKK, indicating that MEKK thus expressed is constitutively active. SAPK activity was activated by full-length MEKK (bottom panel). *b*, Diagram of separate pathways emanating from Raf and MEKK. Both Raf and MEKK appear to be dependent upon the function of Ras, as described in the text. The Raf–MEK–MAPK pathway is functionally analogous to the MEKK–SEK–SAPK pathway, although the result of stimulation of each pathway is distinct. A dotted arrow from MEKK to MEK reflects the ability of MEKK to phosphorylate MEK *in vitro* and during high-level cell expression. In stable inducible cell lines, MAPK activation by MEKK is not seen, drawing the physiological significance of this path into question. The opposing nature of two signalling pathways both emanating from Ras suggests an important role for factors cooperating with Ras to provide specificity for stimulation of one path versus the other.

Is MEKK able to activate MEK, as originally proposed? When tested *in vitro* or during overexpression[4,5], MEKK is able to phosphorylate and activate MEK. But with the stable MEKK-inducible NIH3T3 cells studied here, MEKK, through SEK, activates SAPK and not MAPK, even though these cells express an intact MAPK activation pathway. MEKK might stimulate MAPK in other cell types, or transiently, although we detected no MAPK activity as early as 3 hours, at which time SAPK activity was raised and MEKK was undetectable. Additionally, activated *Drosophila* MEK (Dsor1) rescues D-Raf null mutants in both the Torso and R7 photoreceptor pathways[19], implicating Raf as the major physiological MEK activator.

Perhaps it is appropriate to consider MEKK versus RAF signalling as analogue rather than binary cell regulation. Depending on the interplay between kinase activities, substrate availability and the intracellular milieu, MEKK might activate SAPK in our experimental systems; other cellular conditions might translate MEKK activity into a variety of mixed signals involving other homologues of MAPK[20], including SAPK. □

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