MEKK2 and MEKK3 are two closely related mitogen-activated protein kinase (MAPK) kinase kinases. The kinase domains of MEKK2 and MEKK3 are nearly identical, although their N-terminal regulatory domains are significantly divergent. By yeast two-hybrid library screening, we have identified MEK5, the MAPK kinase in the big mitogen-activated protein kinase 1 (BMK1)/ERK5 pathway, as a binding partner for MEKK2. MEKK2 expression stimulates BMK1/ERK5 activity, the downstream substrate for MEK5. Compared with MEKK3, MEKK2 activated BMK1/ERK5 to a greater extent, which might correlate with a higher affinity MEKK2-MEK5 interaction. A dominant negative form of MEK5 blocked the activation of BMK1/ERK5 by MEKK2, whereas activation of c-Jun N-terminal kinase (JNK) was unaffected, showing that MEK5 is a specific downstream effector of MEKK2 in the BMK1/ERK5 pathway. Activation of BMK1/ERK5 by epidermal growth factor and H2O2 in Cos7 and HEK293 cells was completely blocked by a kinaseinactive MEKK3 (MEKK3kin−), whereas MEKK2kin− had no effect. However, in D10 T cells, expression of MEKK2kin− but not MEKK3kin− inhibited BMK1/ERK5 activity. Two-hybrid screening also identified Lck-associated adapter/Rlk- and Itk-binding protein (Lad/RIBP), a T cell adapter protein, as a binding partner for MEKK2. MEKK2 and Lad/RIBP colocalize at the T cell contact site with antigen-loaded presenting cells, demonstrating cotranslocation of MEKK2 and Lad/RIBP during T cell activation. MEKK3 neither binds Lad/RIBP nor is recruited to the T cell contact with antigen presenting cell. MEKK2 and MEKK3 are differentially associated with signaling from specific upstream receptor systems, whereas both activate the MEK5-BMK1/ERK5 pathway.

The mitogen-activated protein kinase (MAPK) pathways transduce various extracellular stimuli into distinct intracellular responses. The core component of such a MAPK module is a set of three sequential kinases that are evolutionarily conserved in eukaryotes from unicellular yeast to plants and animals. In mammalian cells, three distinguishable MAPK modules have been well described; they are the extracellular signal-regulated kinases 1 and 2 (ERK1/2), the c-Jun N-terminal kinase (JNK), and the p38 pathways. The MAPK pathways regulate cell growth, differentiation, adaptation to the environment, and apoptosis in response to a great number of stimuli, including cytokines and various stresses (1–3). The MAPKs also control numerous regulatory processes during development and homeostasis (4–6).

Our laboratory previously cloned two MAPK kinase kinases, designated MEKK2 and MEKK3 (7). MEKK2 and MEKK3 are extremely homologous (94% conserved) in their catalytic domains, but their regulatory N-terminal sequences are quite divergent, with only 65% homology, predicting that they perform similar as well as different cellular tasks. In this regard, Schaefer et al. (8) showed that T cell MEKK2 but not MEKK3 is activated and translocates to the plasma membrane at the contact with antigen-loaded presenting cells. Thus, although MEKK2 and MEKK3 are both mediators of signal transduction to the MAPK pathways, they are subject to stimulus- and cell type-specific regulation. A differential involvement of MEKK2 and MEKK3 in cellular signaling has also been demonstrated by our recent finding that MEKK2 but not MEKK3 regulates the activity of the protein kinase C-related kinase PRK2 (9).

Big mitogen-activated protein kinase 1 (BMK1)/ERK5 was recently cloned as a novel member of the MAPK family (10, 11). Like ERK1/2, BMK1/ERK5 has a TEF sequence in its dual phosphorylation motif; however, other structural features such as a large regulatory C terminus and a unique loop 12 domain distinguish BMK1/ERK5 from ERK1/2 and other MAP kinases (10). This predicts that the regulation and function of BMK1/ERK5 are distinct from those of other MAPKs. In this regard, Zhou et al. (11) showed that BMK1/ERK5 interacts specifically with MEK5 but not its closely related MAPK kinases MEK1 and MEK2, suggesting that MEK5/BMK1 represents a separate signaling module. Indeed, MEK5 selectively phosphorylates and activates BMK1/ERK5 (12, 13), and its activity is required for the activation of BMK1/ERK5 induced by extracellular signals, including growth factors, serum, oxidative stress, and hyperosmolarity (13, 14). Consistent with the notion that MEK5/BMK1 lies in a signal transduction pathway.
parallel to the classical MAPK cascades. MEK5 is not significantly phosphorylated by Raf-1, c-Mos, or MEK1, specific MAPK kinase kinases of the ERK and JNK pathways (12).

The best characterized downstream targets of BMK1/ERK5 are the MADS-box myocyte enhancer factor 2 (MEF2) transcription factors MEF2A and MEF2C, which are also effectors of the p38 pathway (13, 15–19). Although MEF2 family members, which currently also include MEF2B and MEF2D, were initially thought to be muscle-restricted, they are also expressed at high levels in a variety of other cell types (20). MEF2 proteins activate expression from many muscle-specific genes and play an essential role in controlling myogenesis (20, 21). Phosphorylation of MEF2A and MEF2C by BMK1/ERK5 and p38 potently enhances their transcriptional activation potentials (13, 15–19), linking these kinases to the regulation of muscle development. Furthermore, all four MEF2 members can bind to the proto-oncogene c-jun promoter (22, 23); in fact, it has been shown that c-jun transcription is subject to regulation by BMK1/ERK5 and p38 (16, 18, 19, 24). BMK1/ERK5 activity is required for EGF-induced cell proliferation and cell cycle progression (14). It is not clear whether this involves a requirement for BMK1/ERK5-mediated expression of c-jun. Nonetheless, in fibroblasts c-Jun appears to be necessary for normal cell cycle progression and cell growth (25, 26).

Here we report that MEKK2, like MEKK3, binds MEK5 and activates the BMK1/ERK5 pathway. Using two-hybrid analysis and comminoprecipitation, we show that MEKK2 but not MEKK3 binds the adapter protein Lad/RIBP. Lad/RIBP is an adapter protein that contains several protein-interaction motifs including an SH2 domain; binds Lck, Rlk, and Itk; and is involved in T cell receptor-mediated antigen activation of the T cell (27, 28). In D10 T cells activated by antigen presentation, MEKK2 and Lad/RIBP colocalize at the T cell contact with the antigen presenting cell. We have also shown that MEKK3 is not regulated by antigen and does not localize to the T cell contact with antigen presenting cell (8). In contrast, MEKK3 appears to mediate EGF receptor activation of the BMK1/ERK5 pathway. Our findings show that MEKK2 and MEKK3 are differentially associated in signaling by specific receptor tyrosine kinase systems, and both regulate the BMK1/ERK5 pathway.

MATERIALS AND METHODS

DNA Constructs—The dominant negative mutants of MEKK2 and MEKK3 have been described previously (8, 29). The dominant negative mutant of MEK5 (MEK5AA) was generated by replacing the c-Jun-binding phosphorylation residues (Ser-311 and Thr-315) with alanine.

Yeast Two-hybrid Screen and Interaction Analyses—MEKK2 amino acids 1–188 or 228–360 was fused in-frame to the C terminus of the bacterial DNA-binding protein LexA in vector pBTM116 (30). The construct was used to transform yeast strain L40 (31), together with mouse T cell lymphoma library cDNA cloned C-terminally to the LexA domain of Gal4 (GAD) in plasmid pACT (32) (purchased from CLONTECH), essentially as described previously (9).

For two-hybrid interaction analysis, transformant L40 cells were streaked on SC-His + 3-AT plates after first growth on plates with histidine. A second two-hybrid system was also used in the study; in this case, yeast CG1945 (CLONTECH) was used as the host strain, and MEK5 was fused to the Gal4 DNA binding domain (GBD) in vector pAS2–1 (CLONTECH) as “bait.” In these two systems, the “prey” proteins were either fused to the GAD in pACT or the VP16 activation domain (VAD) in plasmid pVP16 (31). Quantitation of β-galactosidase (β-gal) activity was assayed on liquid cultures using o-nitrophenol-β-D-galactopyranoside as substrate.

Mammalian Cell Culture and Transfection—HEK293 and Cos7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum for 36–40 h. For analysis of BMK1/ERK5 activation induced by extracellular stimuli, 36–40 h posttransfection cells were incubated with low serum medium (0.4% bovine calf serum) for a further 24 h and then treated with 1 ng/ml EGF for 15 min or 500 μM H2O2 for 30 min. To investigate the requirement of MEKK2 for BMK1/ERK5 activity in T cells, parental D10 cells or D10→MEKK2kin−/−→GFP or MEKK3kin−/−→YFP infectants (8) were interleukin-2- and serum-starved in Click’s (Eagle’s Hank’s amino acids medium) ( Irvine Scientific) with 1% fetal bovine serum for 15 h. Cells were harvested and lysed in a lysis buffer consisting of 50 mM Tris–Cl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM Na3P2O7, 1 mM Na2VO4, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μM phenylmethylsulfonyl fluoride, and 2 μg/ml aprotinin. Cell lysates were subjected to immunoprecipitation or directly to SDS-polyacrylamide gel electrophoresis (PAGE), followed by Western blotting.

Communoprecipitation and Western Blot Analysis—293 cells were transfected with T-FLAG-MEK5 or mock vector, together with HA-MEKK2 or V5-epitope-tagged MEKK3. After lysis, 400 μg of cell lysates were incubated with anti-Flag M5 antibody (Sigma) with gentle rocking at 4 °C for 2 h, followed by incubation with rec-protein G-Sepharose 4B beads (Zymed Laboratories Inc.) for 1 h. The beads were washed three times with lysis buffer and after heat denaturation were subjected to SDS-PAGE. The antibodies used for Western blotting were as follows: monoclonal anti-HA 12CA5 antibody from Roche Molecular Biochemicals, monoclonal anti-V5 antibody from Invitrogen, and anti-BMK1/ERK5 antiserum from Sigma. Immunoactive bands were detected using the enhanced chemiluminescence reagents (Perkin Elmer Life Sciences). To investigate MEKK2/Lad/RIBP interaction, lysates of cells transfected with Flag–Lad and HA–MEKK2 or empty vector were precipitated with 12CA5 and Western-blotted with M5.

Immunofluorescence and Cell Imaging Analysis—The T cell clone D10 and the D10-derived clones expressing MEKK2-GFP and Lad-YFP were maintained as described previously (8). D10 T cell clones were mixed with antigen presenting cells (APCs) loaded with no antigen or with 500 μg/ml conalbumin. After 10 min, conjugates were bound to coverslips and analyzed by immunofluorescence microscopy. For immunocytochemistry, cells were fixed for 10 min with 3% paraformaldehyde, rinsed, and permeabilized for 5 min on ice with 0.2% Triton X-100 in phosphate-buffered saline. Conjugates were stained with antibodies or analyzed directly for GFP/YFP fluorescence using a Leica DMXRA epifluorescence microscope. Data was acquired with a Cooke Corp. SensiCam CCD camera and was digitally deconvolved using a neighbor algorithm with Slidebook software from Intelligent Imaging Corp. Conjugate contact analysis involved acquising stacks of images in 0.2-μm steps throughout the contact volume (8).

In Vitro Kinase Assay—for analyzing activation of MEK5 by MEKK2 in cells, HEK293 cells were transfected with Flag-tagged MEK5 alone or together with MEKK2. Cell lysates were immunoprecipitated with anti-Flag M5 antibody and rec-protein G beads in the above lysis buffer with 150 mM NaCl and 1% Triton X-100. After washing beads were incubated with 20 μg of myelin basic protein (MBP) (Upstate Biotechnology) in a 50-μl kinase buffer (20 mM HEPES, pH 7.5, 10 mM MgCl2, 10 mM MnCl2, and 5 mM CaCl2) with 90 nM [γ-32P]ATP (4500 Ci/mmol) at 30 °C for 20 min.

For analysis of endogenous JNK activity, 400 μg of lysates of transfected 293 or Cos7 cells were incubated with 50 μg of bacterially expressed and purified glutathione S-transferase–c-Jun(1–79) bound to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech), at 4 °C for 3 h. The beads were washed and suspended in 50 μl of kinase buffer. In vitro phosphorylation of c-Jun(1–79) by endogenous JNK was analyzed as described above.

RESULTS

MEKK2 Binds MEK5—The regulatory sequence outside the MEKK2 kinase domain is located in the N-terminal 360 residues (MEKK2aa1–360). However, no known signature domain can be found for this region. In fact, data base searches show that it is homologous only to the corresponding sequence of MEKK3. In attempts to identify the BMK1/ERK5 phosphorylation sites, we performed yeast two-hybrid analyses using MEKK2aa1–360 as bait. Unfortunately, this fragment of MEKK2 by itself turned out to have strong transcriptional activation activity and was thus not suitable for the purpose of library screening. Analysis of the MEKK2 protein indicates that residues from 189 to 227 are particularly rich in

Phosphorylation of MEF2A and MEF2C by BMK1/ERK5 and p38 potently enhances their transcriptional activation potentials (13, 15–19), linking these kinases to the regulation of muscle development. Furthermore, all four MEF2 members can bind to the proto-oncogene c-jun promoter (22, 23); in fact, it has been shown that c-jun transcription is subject to regulation by BMK1/ERK5 and p38 (16, 18, 19, 24). BMK1/ERK5 activity is required for EGF-induced cell proliferation and cell cycle progression (14). It is not clear whether this involves a requirement for BMK1/ERK5-mediated expression of c-jun. Nonetheless, in fibroblasts c-Jun appears to be necessary for normal cell cycle progression and cell growth (25, 26).
MEKK2 Binds Lad/RIBP and MEK5

Fig. 1. Association of MEKK2 and MEKK3 with MEK5. A–D, yeast two-hybrid analyses. A, full-length MEKK2 fused to LexA in pBTM116 was used to transform yeast L40 cells, together with a plasmid encoding a GAD fusion of MEK5. Binding of MEK5 to MEKK2 was tested by streaking the transformant cells on SC-His plates supplemented with 15 mM 3-AT. The plates were incubated at 30 °C for 2 days. B, β-gal activities of cells in A using o-nitrophenyl-β-D-galactopyranoside as substrate. C, binding was analyzed in yeast host strain CG1945 using MEK5 fused to the GBD as bait. The prey proteins were constructed as VAD fusions in vector pVP16. Transformant cells were streaked and grown on SC-His + 3 mM 3-AT plates for 3.5 days. D, β-gal activities. The cells assayed (a–g) corresponded to those shown in C except that LexA-MEK5 (or the negative control, LexA-Lamin) was used as bait, and the host was L40. E, MEK5 binds MEKK2 and MEKK3 in mammalian cells. HEK293 cells were transfected with mock vector or Flag-tagged MEKK2, together with a plasmid expressing V5-tagged MEKK3 (right panel). Cell lysates were immunoprecipitated with anti-Flag M5 antibody and Western-blotted with anti-HA 12CA5 (to detect MEKK2) or anti-V5 antibody (to detect MEKK3) after SDS-PAGE.

Acidic residues, which have been shown to be an essential characteristic of many transcription factors (33, 34). We therefore fused amino acids 1–188 of MEK2 to the bacterial DNA-binding protein LexA (LexA-MEKK2aa1–188) and used it as bait to screen a mouse T-cell lymphoma cDNA library in the yeast reporter host strain L40 (31). One positive clone encoded the open reading frame of MEK5 except for the first 4 amino acids. Analysis of the His3 and LacZ reporter genes of yeast L40 transformed with LexA-MEKK2aa1–188 showed that LexA-MEK5 interacted with MEK5 in the yeast two-hybrid system (Fig. 1, A and B). To further validate the binding partnership between MEKK2 and MEK5, we switched the bait and prey constructs and tested their interaction in a different yeast two-hybrid system. MEK5 was fused to the Gal4 DNA-binding domain (GBD) and MEKK2 to the activation domain of the herpes simplex virus protein VP16. As expected, yeast CG1945 cells could grow on synthetic complete plates Lacking histidine (SC-His) and containing 3 mM 3-AT only when they were provided with both GBD-MEK5 and VAD-MEKK2 (Fig. 1C). The results also showed that MEK5 binds full-length MEKK3 in the two-hybrid analysis, confirming the findings of Chao et al. (35), in which they identified a fragment of MEKK3 (amino acids 213–626) as a binding sequence for MEK5. This region of MEKK3 corresponds to residues 200–618 of MEK2, which does not overlap with MEKK2aa1–188, which we had shown binds to MEK5. These results suggested that MEK5 interacts with at least two separate regions within the MEKK2 protein. To test this possibility, MEKK2 truncated of the first 188 amino acids (MEKK2Δ1–188) was fused to the VAD, and its interaction with MEK5 was examined by the two-hybrid analysis. Yeast cells transformed with GBD-MEK5 and VAD-MEKK2Δ1–188 were histidine prototrophic (Fig. 1C), showing that MEKK2Δ1–188 binds MEK5.

Because the LacZ gene in CG1945 is driven by a weak promoter and does not allow for quantitative β-gal assays, quantitation of the strength of interaction with MEK5 was performed using the LexA-based yeast strain L40 as the host (Fig. 1D). Cells transformed with LexA-MEKK5 and VAD-MEKK2 exhibited a β-gal activity ~6 times that of cells with LexA-MEK5 and VAD-MEKK3. Cells containing LexA-MEK5 and VAD-MEKK2Δ1–188 had a basal level of β-gal activity as control cells having LexA-MEK5 and the vector VAD. However, cell lysates from yeast CG1945 transformed with GBD-MEK5 and VAD-MEKK2Δ1–188 were able to convert the substrate o-nitrophenyl-β-D-galactopyranoside into slightly yellow substance when the incubations were allowed to proceed overnight, whereas the control reactions remained colorless (data not shown). These results may suggest that MEK5 interacts more strongly with MEKK2 than with MEKK3, and the major binding site for MEK5 in MEKK2 is located in the first 188 amino acids. Alternatively, significant interaction between MEK5 and MEKK2aa189–619 requires prior binding to, or a structure maintained by, MEKK2aa1–188. It should be cautioned, however, that studies have found that the β-gal activity does not always correlate with the strength of an interaction (36), and analysis of the interactions using recombiant purified proteins will probably need to be completed. Future work will investigate the differential contributions and potential synergy of the two sites in the MEKK2 protein sequence that interact with MEK5.

To substantiate the yeast two-hybrid findings in mammalian cells, HEK293 cells were transfected with Flag-tagged MEK5 or mock expression vector, plus HA-tagged MEKK2 or V5-epitope-tagged MEKK3. Cell lysates were then immunoprecipitated with anti-Flag antibody and Western-blotted with anti-HA 12CA5 (to detect MEKK2) or anti-V5 antibody (to detect MEKK3) after SDS-PAGE.

MEKK2 and MEKK3 Activate the MEK5-BMK1/ERK5 Pathway—We asked whether MEKK2 could stimulate the kinase activity of MEK5 in vitro. To investigate this, HEK293 cells were transfected with MEK5 alone or together with MEKK2. Equal amounts of MEK5 were immunoprecipitated from cell lysates and its kinase activity was analyzed by an in vitro kinase reaction using MBP as substrate (Fig. 2A). MEK5 by itself had background level of activity (compare lanes 1 and 4). Cotransfection with MEKK2 gave a reproducible 2-fold increase in MEK5 activity (lane 2). The increased phosphorylation of MBP was due to activation of MEK5 by MEKK2 because immunoprecipitate from lysates of cells cotransfected with MEKK2 and a dominant negative mutant (MEK5AA) had a similar background MBP phosphorylation activity (Fig. 2A, lane 3).

Because MEK5 is a direct upstream regulator of BMK1/

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MEKKs are activated by dual phosphorylation of BMK1/ERK5—kinases of the MEK5-BMK1/ERK5 pathway. Less is known whether this further modified form of BMK1/ERK5 results in sustained signaling.

Western blotting with anti-BMK1/ERK5 antibody. ERK5. Cells lysates were then subjected to SDS-PAGE followed by Western blotting with anti-BMK1/ERK5 antibody. Bottom panel, equal amounts of lysates were blotted with anti-VEG antibody to detect the expression levels of MEKK2 and MEKK3.

ERK5 (11, 13), we next examined whether MEKK2 could activate BMK1/ERK5 by a transient transfection assay. Previous studies have established that the migration of phosphorylated and activated BMK1/ERK5 in a SDS-polyacrylamide gel is significantly retarded compared with the inactive protein (13, 14, 37). Therefore, activation of BMK1/ERK5 can be assayed by Western blotting (Fig. 2B, top panel). Transfection with MEKK3 did not activate BMK1/ERK5 (lane 5), consistent with the report by Chao et al. (35). In contrast, expression of MEKK2 at similar or even lower protein levels (Fig. 2B, bottom panel) led to the appearance of a phosphorylated BMK1/ERK5 band with altered electrophoretic mobility (lane 3). The data suggest that MEKK2 is more potent than MEKK3 in promoting the activation of BMK1/ERK5. Expression of MEK5 alone did not activate BMK1/ERK5 (lane 2). However, in contrast to the results of Chao et al. (35), we found that full-length MEKK3 was able to activate BMK1/ERK5 when cotransfected with MEK5 (lane 6). The greater ability for MEKK2 to activate BMK1/ERK5 was again seen in cells cotransfected with MEK5 (lane 4), in which an almost complete gel shift of BMK1/ERK5 was detected. In both cases, cotransfection with MEK5 resulted in a second phospho-BMK1/ERK5 band, the mobility of which was even more retarded (lanes 4 and 6). It is currently not known whether this further modified form of BMK1/ERK5 represents the fully activated state of the protein. Nevertheless, inclusion of MEK5 in the transfections clearly enhanced the abilities of MEKK2 and MEKK3 to activate BMK1/ERK5, supporting the conclusion that MEKK2 and MEKK3 are MEK kinases of the MEK5-BMK1/ERK5 pathway.

**Dominant Negative MEK5 Suppresses MEKK2 Activation of BMK1/ERK5**—MEK5s are activated by dual phosphorylation of two conserved serine/threonine residues located between subdomains VII and VIII (11, 38–40). The Raf-1 phosphorylation motif of MEK1, S185XXS222 (38, 39), is conserved as S11SXXT115 in MEK5. Previous investigations have shown that a MEK5 mutant with Ser-311 and Thr-315 changed to Ala (MEK5AA) is dominant inhibitory to BMK1/ERK5 activation stimulated by several mitogens in various cell types (13, 16). To further demonstrate MEK5 functions downstream of MEKK2, we examined whether MEK5AA would inhibit MEKK2-mediated BMK1/ERK5 activation (Fig. 3). The lower phosphorylated BMK1/ERK5 band seen in MEKK2-transfected cells (Fig. 3A, lane 2) was nearly completely blocked when MEK5AA was cotransfected (lane 4). Moreover, the upper phospho-BMK1/ERK5 band resulting from cotransfection with MEK5 and wild-type MEK5 (lane 3) was not detected when the MEK5AA mutant was used instead (lane 4), indicating that its presence requires an active MEK5. Analysis of the same cell lysates showed that MEKK2-mediated JNK activation was not significantly altered by wild-type MEK5 or MEK5AA (Fig. 3B). Therefore we conclude that MEK5 is a specific target of MEKK2 in the BMK1/ERK5 pathway.

**BMK1/ERK5 Activation in Response to EGF and Oxygen Radical Formation Is Regulated by MEKK3 but not MEKK2**—BMK1/ERK5 is activated in response to a number of extracellular stimuli, including H2O2 and EGF (13, 14, 16, 37, 41, 42). We set out to determine whether MEKK2 and MEKK3 were mediators of these responses. As shown in Fig. 4A, BMK1/ERK5 was activated by treatment of Cos7 cells with H2O2 or EGF (lanes 1 and 3, respectively). However, transient expression of kinase-inactive MEKK2 (MEKK2kin-) did not alter the BMK1/ERK5 activation profile (lanes 2 and 4), suggesting that MEKK2 is not required for H2O2 and EGF stimulation of BMK1/ERK5 activity. In contrast, in agreement with the report by Chao et al. (35) using HeLa cells, activation of BMK1/ERK5 in response to EGF was completely blocked by MEKK3kin− (Fig. 4B, lanes 3 and 4). In addition, we also observed that MEKK3kin− blocked H2O2 activation of BMK1/ERK5 (Fig. 4B, lanes 1 and 2). Results identical to those shown here with Cos7 were obtained in HEK293 cells (data not shown). Search for extracellular stimuli in fibroblasts that activate BMK1/ERK5 in a MEK2 dependent manner has not been successful to date in our studies. Therefore, we used two-hybrid screening to identify additional MEKK2 binding partners that could identify receptor systems controlling MEKK2.

**MEK2 Binds Lad/RIBP whereas MEKK3 Does Not**—Using a different region of the MEKK2 N terminus (aa 228–360) as bait, we have also identified several positive clones that are of interest as binding partners for MEKK2. One clone that showed strong interaction with MEKK2aa228–360 was the adapter protein Lad (27), also known as RIBP (28).

To investigate whether full-length MEK2 bound Lad/RIBP in the two-hybrid system, L40 yeast cells were cotransformed...
MEKK2 and by the extracellular stimuli. H$_2$O$_2$- and EGF-induced activation of BMK1/ERK5.

With LexA-MEKK2 and GAD fusion of Lad/RIBP (GAD-Lad). The constructs were tested for their ability to allow growth of cells on SC-His plates supplemented with 10 mM 3-AT (Fig. 5A). The results showed that activation of the His$_3$ gene required the presence of both MEKK2 and Lad/RIBP, indicating specific interaction between the two proteins. This conclusion was further supported by quantitative analysis of the LacZ reporter gene (Fig. 5B). The strong growth on histidine-minus plates and the high level of β-gal expression indicate that MEKK2 binds Lad/RIBP with high affinity. In contrast, MEKK3 was not able to interact with Lad/RIBP in the histidine prototrophy assay (but it bound MEK5 strongly, indicating that it was expressed and nucleus-localized) (Fig. 5A).

To demonstrate the selective interaction between MEKK2 and Lad/RIBP in mammalian cells, MEKK2 and Lad/RIBP were expressed in HEK293 cells as HA- and Flag-tagged proteins, respectively. Western blotting analysis revealed that detection of Lad/RIBP in the anti-HA immunoprecipitations of cell lysates depended on the coexpression of MEKK2 (Fig. 5C), showing that MEKK2 was complexed with Lad/RIBP in cells. Transfection of MEKK2 caused an electrophoretic mobility shift of Lad (Fig. 5, C and D), presumably resulting from phosphorylation by MEKK2. Significantly, MEKK2 appeared to bind only the fastest-migrating, unmodified form of Lad/RIBP. Blotting the cell lysates (without immunoprecipitation) with anti-Lad antibody showed that cotransfection with MEKK2 resulted in the appearance of Lad/RIBP. MEKK2 did not bind Lad, nor did it affect the gel mobility of Lad/RIBP.

Fig. 4. Kinase-inactive MEKK3, but not MEKK2, suppresses H$_2$O$_2$- and EGF-induced activation of BMK1/ERK5. Cos7 cells were transfected with kinase-inactive MEKK2kin or MEKK3kin, or empty vector (lanes 1–4). Cells were treated with 500 μM H$_2$O$_2$ for 30 min or 1 ng/ml EGF for 15 min. Activation of BMK1/ERK5 was analyzed by Western blotting with anti-BMK1/ERK5 antibody. Lanes 5 and 6 in A serve as controls to compare activation of BMK1/ERK5 by MEKK2 and by the extracellular stimuli.

FIG. 4.

With LexA-MEKK2 and GAD fusion of Lad/RIBP (GAD-Lad). The constructs were tested for their ability to allow growth of cells on SC-His plates supplemented with 10 mM 3-AT (Fig. 5A). The results showed that activation of the His$_3$ gene required the presence of both MEKK2 and Lad/RIBP, indicating specific interaction between the two proteins. This conclusion was further supported by quantitative analysis of the LacZ reporter gene (Fig. 5B). The strong growth on histidine-minus plates and the high level of β-gal expression indicate that MEKK2 binds Lad/RIBP with high affinity. In contrast, MEKK3 was not able to interact with Lad/RIBP in the histidine prototrophy assay (but it bound MEK5 strongly, indicating that it was expressed and nucleus-localized) (Fig. 5A).

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MEKK2 and Lad/RIBP Localize to the T Cell Contact Site with Antigen-loaded APCs—The finding that Lad/RIBP interacted with MEKK2 prompted examination of their respective localization in D10 T cells. We have previously shown that MEKK2 is involved in T cell activation by antigen (8), and MEKK2 and Lad can interact and are involved in the T cell activation complex that translocates to the immune synapse (8). These findings are consistent with the biochemical findings that MEKK2 but not MEKK3 binds Lad/RIBP. We have attempted to assay BMK1/ERK5 activity in response to T cell receptor activation. D10 T cells appear to have a high basal BMK1/ERK5 activity that is apparent in the slower migrating BMK1/ERK5 band shown in Fig. 8, lanes 1 and 3. Stable expression of MEKK2kin<sup>−</sup> (lane 2) but not MEKK3kin<sup>−</sup> (lane contact site with the APCs. Lad-YFP can also be shown to be at the contact site peripheral to the MTOC of the D10 T cell conjugated to the antigen-loaded APCs. A significant amount of Lad-YFP remains cytoplasmic even with antigen presentation by APCs. In contrast to the colocalization of Lad-YFP and γ-tubulin, Lad-YFP is not significantly associated with α-tubulin in D10 T cells. Fig. 6, bottom panels, shows the localization of MEKK2-GFP at the D10 T cell contact site with antigen-loaded APCs. It is clear that MEKK2-GFP is localized at the contact site involved in antigen activation of the T cell. MEKK2-GFP, similar to a fraction of the Lad-YFP, is localized peripheral to the MTOC at the contact site of D10 T cells with the antigen-loaded APCs.

Fig. 7 clearly shows that MEKK2 and Lad-YFP are colocalized at the T cell cytoplasmic surface at the contact of conjugates with antigen-loaded APCs. Shown are two representative D10 T cell conjugates with antigen-loaded APCs. The fluorescence images represent deconvolved 0.2 μm sections showing the D10 T cell/antigen-loaded APC contact site. The colocalization of MEKK2 and Lad/RIBP at the T cell receptor signaling complex at the conjugate interface supports the finding that MEKK2 and Lad can interact and are involved in the T cell activation complex that translocates to the immune synapse formed with the antigen-loaded APCs (8).

Our previous work showed that MEKK2 was activated during conjugate formation and localized to the contact site (8). Furthermore, MEKK3 does not localize to the conjugate contact site (8). These findings are consistent with the biochemical findings that MEKK2 but not MEKK3 binds Lad/RIBP. We have attempted to assay BMK1/ERK5 activity in response to T cell receptor activation. D10 T cells appear to have a high basal BMK1/ERK5 activity that is apparent in the slower migrating BMK1/ERK5 band shown in Fig. 8, lanes 1 and 3. Stable expression of MEKK2kin<sup>−</sup> (lane 2) but not MEKK3kin<sup>−</sup> (lane contact site with the APCs. Lad-YFP can also be shown to be at the contact site peripheral to the MTOC of the D10 T cell conjugated to the antigen-loaded APCs. A significant amount of Lad-YFP remains cytoplasmic even with antigen presentation by APCs. In contrast to the colocalization of Lad-YFP and γ-tubulin, Lad-YFP is not significantly associated with α-tubulin in D10 T cells. Fig. 6, bottom panels, shows the localization of MEKK2-GFP at the D10 T cell contact site with antigen-loaded APCs. It is clear that MEKK2-GFP is localized at the contact site involved in antigen activation of the T cell. MEKK2-GFP, similar to a fraction of the Lad-YFP, is localized peripheral to the MTOC at the contact site of D10 T cells with the antigen-loaded APCs.

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**FIG. 6.** Localization of D10 T cell adapter protein, Lad/RIBP. A Lad-YFP retrovirus was used to introduce a Lad-YFP fusion protein into D10 T cells. Top panels, fluorescence images of Lad-YFP showing its cytoplasmic and MTOC localization. An antibody to γ-tubulin was used to specifically stain the MTOC. Middle panels, D10/Lad-YFP T cells were mixed with equal numbers of conalbumin (500 μg/ml)-loaded APCs. After 10 min, conjugates were fixed in paraformaldehyde and stained with either anti-γ-tubulin or anti-α-tubulin antibody. Images were then acquired for α- or γ-tubulin staining and Lad-YFP. Middle left panel shows the redistribution of the MTOC following conjugate formation. The D10 T cell is shown on the right side of each image. Middle center panel shows the localization of the T cell Lad-YFP in conjugates with a concentrated localization of the adapter protein at the conjugate interface. Middle right panel shows that α-tubulin is largely absent from the contact interface where Lad-YFP is found. Bottom panels, D10/MEKK2-GFP T cells were used for conjugate formation with conalbumin-loaded APCs similar to that for D10/Lad-YFP T cells in the middle panels (T cell shown on the right side of each image). Conjugates were fixed and stained with anti-α-tubulin antibody and imaged for localization of MEKK2-GFP and α-tubulin. T cell MEKK2-GFP is shown to localize at the conjugate interface. Similar to Lad-YFP, MEKK2-GFP is localized proximal to the plasma membrane relative to α-tubulin. Bar in bottom right panel represents 5 μm.

4) in D10 cells at similar protein levels (8) caused a loss of the gel-shifted BMK1/ERK5. Thus, in D10 cells, kinase-inactive MEKK2 but not kinase-inactive MEKK3 suppresses the stimulus that is activating the BMK1/ERK5 pathway. This contrasts with the findings in Cos7 and HEK293 cells, in which MEKK3 kinase- but not MEKK2 kinase- expression inhibited EGF and H2O2 stimulated BMK1/ERK5 activation.

**FIG. 7.** Colocalization of Lad and MEKK2 at the D10 T cell/APC interface. D10 T cells expressing Lad-YFP were mixed for 10 min at 37 °C with conalbumin (500 μg/ml)-loaded APCs. Conjugates were placed on coverslips, fixed and stained with anti-MEKK2 polyclonal rabbit antisera. Conjugates were then imaged in 0.2 μm steps through the entire cell volume, and sections were deconvolved to remove out-of-focus fluorescence. Shown are images of a 0.2 μm section through the middle of the contact site of the T cell/APC conjugate. Top right panel is a Nomarski image of the conjugate with the T cell on the top right side of image with the same orientation for the fluorescence images. Top left panel shows Lad-YFP in the same conjugate as the Nomarski image. Bottom left panel shows the colocalization of MEKK2 and Lad-YFP at the T cell contact site with the antigen-loaded APCs. Bottom right panel shows the staining of MEKK2 at the cytoplasmic surface of the T cell plasma membrane in the conjugate interface. MEKK2 staining is also evident in the B cell.

**FIG. 8.** MEKK2, but not MEKK3, is required for the BMK1/ERK5 activity in resting D10 T cells. Cell lysates of D10 T cells stably infected without or with kinase-inactive MEKK2 or MEKK3 were Western-blotted with anti-BMK1/ERK5 antibody.

**DISCUSSION**

We demonstrate for the first time that MEKK2 activates the MEK5-BMK1/ERK5 pathway. Expressed MEKK2 activated BMK1/ERK5 to a greater extent than MEKK3. The differential BMK1/ERK5 activation profile correlated with the stronger interaction of MEK5 with MEKK2 than with MEKK3. We found that the MEKK2-MEK5 interaction gave a considerably higher (6-fold) β-gal activity in the yeast two-hybrid analysis relative to MEKK3-MEK5 interaction.

EGF- and H2O2-induced activation of BMK1/ERK5 was blocked by kinase-inactive MEKK3 in Cos7 and HEK293 cells but was not affected by the corresponding kinase-inactive MEKK2 mutant. The same kinase-inactive MEKK2 mutant inhibits T cell receptor signaling in D10 T cells (8), as well as suppressing the basal BMK1/ERK5 activity in this cell line. MEKK3 clearly is not regulated in the same way as MEKK2 in D10 T cells. Thus, the data are consistent with MEKK2 and MEKK3 being targets of different upstream regulatory pathways. We have recently reported that MEKK2 but not MEKK3 regulates the activity of the PKC-related kinase, PRK2 (9). Thus, not only are MEKK2 and MEKK3 regulated by different upstream receptor signaling systems but they appear to have overlapping but distinct downstream effectors as well. This is consistent with the genetic study by Yang et al. (47) that showed MEKK2 is not able to compensate for the defects in early embryonic cardiovascular development caused by targeted disruption of the MEKK3 gene.

It has increasingly been recognized that scaffolding is an important mechanism to control signal strength and specificity. In this regard, scaffolding molecules can be classified into two groups: those with catalytic activities and those without. Like the prototype scaffold protein STE5 in yeast (48–50), two newly identified mammalian proteins, MP-1 (51) and JIP-1
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(52), were shown to organize the ERK and JNK modules. MEKK2 binds to and activates PRK2 independent of its enzymatic activity, suggesting that MEKK2 may also play a scaffolding role reminiscent of KSR in regulating the activity of Raf (53–57). Binding of PRK2 requires both the regulatory and the kinase domains of MEKK2, but the N-terminal regulatory sequence of MEKK2 is sufficient for binding Lad/RIBP and MEK5. This agrees with the selective MEKK2-PRK2 and MEKK2-Lad/RIBP interactions and the apparent difference in the binding affinities of MEKK2-MEK5 and MEKK3-MEK5, because the kinase domains of MEKK2 and MEK3 are almost identical. Interestingly, MEK5 can also associate with BMK1/ERK5 (11), raising the possibility that a tripartite MEKK2-MEK5-BMK1/ERK5 complex organized by MEK5 may exist in cells. Similarly, JNK kinase 1 can interact with both MEKK1 and JNK1 (58), and MEKK1 has been shown to directly bind JNK1 (59), suggesting MEKK1 functions as a scaffold for the JNK pathway (60). English et al. (61) also reported that another MAPK kinase kinase, Raf-1, could bind BMK1/ERK5 and contribute to Ras activation of BMK1/ERK5 independent of Raf-1 kinase activity. Thus, MEK5-BMK1/ERK5 may be part of different MAPK modules for selective regulation by multiple upstream stimuli.

Our finding that MEK5 binds the N terminus of MEKK2 may have significant implications for MEKK2-mediated signaling. Cheng et al. (62) showed that formation of the MEKK2-JNK kinase 2-JNK1 module requires only the C-terminal kinase domain of MEKK2. This would suggest that MEKK2 might organize and activate the BMK1/ERK5 and JNK cascades simultaneously, rather than competitively. Consistent with this idea, neither wild-type nor kinase-inactive MEK5 significantly interferes with MEKK2 activation of JNK.

Our findings demonstrate the interaction of MEKK2 but not MEK3 with the adapter protein, Lad/RIBP. Lad/RIBP was shown to be tyrosine phosphorylated and to bind Lek (27) and the Tec family tyrosine kinases Itk and Rlk (28) that are requisite for activated T cell responses (63, 64). Further characterization of the Lad/RIBP sequence defined several tyrosine phosphorylation sites, an SH2 domain, a proline-rich SH3-binding domain, and a zinc finger motif. Expression of Lad/RIBP is rapidly induced after T cell activation (28). Furthermore, Lad/RIBP-deficient T cells showed significant impairment of proliferation and a pronounced decrease in interleukin-2 and interferon-γ production in response to anti-CD3 stimulation (28). These studies indicate that Lad/RIBP plays a critical role in T cell receptor-mediated T cell signaling. We have shown that MEKK2 is critical in T cell signaling and that it is activated and translocates to the T cell plasma membrane interface in conjugates formed with antigen-loaded APCs. MEKK2 and a fraction of Lad/RIBP colocalize at the T cell contact interface in T cell/APC conjugates consistent with their involvement in T cell receptor signaling. MEK3 is not regulated in this T cell system and fails to translocate to the contact site in conjugates. These findings are consistent with Lad/RIBP interacting with MEKK2 but not MEK3 during T cell activation. Our findings indicate MEKK2 is part of the immune synapse complex but not the MTOC signaling complex.

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