MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase (MEKKs)) regulate c-Jun N-terminal kinase and extracellular signal-regulated kinase pathways. The 14-3-3ζ and 14-3-3ε isoforms were isolated in a two-hybrid screen for proteins interacting with the N-terminal regulatory domain of MEKK3. 14-3-3 proteins bound both the N-terminal regulatory and C-terminal kinase domains of MEKK3. The binding affinity of 14-3-3 for the MEKK3 N terminus was 90 nM, demonstrating a high affinity interaction. 14-3-3 proteins also interacted with MEKK1 and MEKK2, but not MEKK4. Endogenous 14-3-3 protein and MEKK1 and MEKK2 were similarly distributed in the cell, consistent with their in vitro interactions. MEKK1 and 14-3-3 proteins colocalized using two-color digital confocal immunofluorescence. Binding of 14-3-3 proteins mapped to the N-terminal 393 residues of 196-kDa MEKK1. Unlike MEKK2 and MEKK3, the C-terminal kinase domain of MEKK1 demonstrated little or no ability to interact with 14-3-3 proteins. MEKK1, but not MEKK2, -3 or -4, is a caspase-3 substrate that when cleaved releases the kinase domain from the N-terminal regulatory domain. Functionally, caspase-3 cleavage of MEKK1 releases the kinase domain from the N-terminal 14-3-3-binding region, demonstrating that caspases can selectively alter protein kinase interactions with regulatory proteins. With regard to MEKK1, -2 and -3, 14-3-3 proteins do not appear to directly influence activity, but rather function as "scaffolds" for protein-protein interactions.

We have cloned four MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase (MEKKs)), referred to as MEKK1, -2, -3, and -4 (1–3). Each is capable of activating the JNK pathway, and MEKK1, -2, and -3 are also capable of regulating the ERK pathway (1–5). MEKK1 and MEKK4 bind Cdc42 and Rac, whereas MEKK2 and MEKK3 do not; the subcellular localization of the MEKKs is different (6). MEKK1 is capable of inducing apoptosis (7, 8) and is a substrate for proteolytic cleavage by caspase-3 (9). Caspase-3 cleavage of MEKK1 releases the 91-kDa kinase domain from the N-terminal region, which encodes a proline-rich sequence and a predicted pleckstrin homology domain (for review, see Ref. 10). The cleavage of MEKK1 enhances its ability to induce apoptosis, and mutation of the MEKK1 cleavage site suppresses loss of adherence-induced apoptosis (9). MEKK2, -3, and -4 are not caspase substrates, and they are not very effective at inducing apoptosis (10).

14-3-3 proteins were first isolated as highly abundant acidic proteins in brain extracts, and at least seven highly conserved 14-3-3 isoforms have been identified (for review, see Ref. 13). 14-3-3 proteins associate with a number of different signaling proteins and have been proposed to be important in controlling mitogenic signaling pathways (Refs. 14–19; for review, see Ref. 20). Relevant to the regulation of signal transduction pathways, 14-3-3 proteins have been shown to interact with Raf-1, Ber-Abl, polymyoma middle tumor antigen, KSR (kinase suppressor of Ras), the Bcl family member BAD, the platelet adhesion receptor, glycoprotein Ib-IX, insulin receptor substrate-1, and protein-tyrosine phosphatase H1 (14–17, 19, 21–24). The biological consequence of binding of 14-3-3 proteins is controversial, but the importance of 14-3-3 proteins in controlling signal transduction pathways is beginning to emerge. For Raf-1, 14-3-3 binding has been reported to enhance, suppress, or play no role in regulating kinase activity (Refs. 14, 17–19, and 25; for review, see Ref. 26). Furthermore, 14-3-3 binding has been shown to protect phosphoserine residues from phosphatases and function as a scaffold to promote association with other proteins (27–29). Expression of 14-3-3 proteins in Xenopus oocytes activates the ERK pathway, and 14-3-3 proteins can potentiate ERK activation by KSR (22). In Drosophila, D14-3-3 is necessary for photoreceptor development and genetically maps upstream of Raf and downstream of Ras (30, 31). In the fission yeast Schizosaccharomyces pombe, 14-3-3 proteins regulate a DNA-damage checkpoint (32), and in the budding yeast Saccharomyces cerevisiae, 14-3-3 proteins are essential for regulation of the Ras/mitogen-activated protein kinase pathway during pseudohyphal development (33). Structurally, 14-3-3 proteins dimerize with each subunit having a cleft that could function as a binding site for proteins and could induce the close proximity of different proteins to alter their interaction.

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and regulation (34). Thus, 14-3-3 proteins are essential for regulated signal transduction and may not directly activate or inhibit kinases, but rather behave as a "scaffold" or "anchor" to localize protein kinase activity.

In this report, we demonstrate that 14-3-3 proteins interact with MEKK1, -2, and -3, but not MEKK4. These findings implicate 14-3-3 proteins in the control of MEKK proteins as "scaffold-like" proteins since association does not directly influence kinase activity. In addition, we demonstrate that caspase-3 cleavage of MEKK1 releases the active kinase domain from the N-terminal 14-3-3-binding region, defining a new function for caspases, namely the release of active kinase from its cellular scaffold.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Analysis—Yeast two-hybrid interaction analysis was performed as essentially described by Vojtek and Hollenberg (35). Plasmids pVP16, pBTH116, and pBTH116-lamin and the L40 yeast strain were kindly provided by Drs. Anne B. Vojtek and Jonathan A. Cooper. The N-terminal coding region of MEKK3 (amino acids 4–361) was subcloned into pBTH116 (pBTH116-MEKK3(NH2)), and sequence was confirmed using an Applied Biosystems Model 377 automated DNA sequencer. pBTH116-MEKK3(NH2) was transformed into the L40 yeast strain as described under the appropriate selection, and expression of the fusion protein (LexA-MEKK3) was determined in protein extract preparations (36) by immunoblotting with a LexA monoclonal antibody (kindly provided by Drs. John A. Printen and George F. Sprague, Jr.). The L40 yeast strain containing pBTH116-MEKK3(NH2) was transformed with a murine day 9.5 and 10.5 embryonic library in buffer containing 0.5% Triton X-100, 10 mM Tris, 5 mM EDTA, 50 mM plasmids encoding the indicated MEKK family member were lysed in

Buffer containing 100 units/ml penicillin and 100 μg/ml aprotinin and 1 mg/ml cytos Georgian peptide as well as with full-length MEKK3. Identification of binding sites in both the MEKK3 N-terminal regulatory and C-terminal domains was confirmed using an Applied Biosystems Model 377 automated DNA sequencing program. MEKK3(NH2) was also used as an antigen to generate rabbit polyclonal antibody (kindly provided by Drs. John A. Printen and George F. Sprague, Jr.). The L40 yeast strain containing pBTH116-MEKK3(NH2) was transformed with a murine day 9.5 and 10.5 embryonic library in pVP16 (kindly provided by Dr. Stanley M. Hollenberg). Histidine prototrophy was determined on plates containing either 1 or 5 mM 3-aminotriazole to screen for proteins with lower and higher affinities for MEKK3(NH2). MEKK3(NH2) was also used as an antigen to generate rabbit polyclonal antibody (kindly provided by Drs. John A. Printen and George F. Sprague, Jr.). The L40 yeast strain containing pBTH116-MEKK3(NH2) was transformed with a murine day 9.5 and 10.5 embryonic library in pVP16 (kindly provided by Dr. Stanley M. Hollenberg). Histidine prototrophy was determined on plates containing either 1 or 5 mM 3-aminotriazole to screen for proteins with lower and higher affinities for MEKK3(NH2). β-Galactosidase activity was utilized as a secondary screen. Clones that tested positive for both interaction screens were sequenced and identified using the BLAST algorithm to search the nucleotide data base at the National Library of Medicine (37).

Cell Culture and Transfection—Cells were maintained in a humidified CO2 environment in Dulbecco's modified Eagle's medium supplemented with 100 μl penicillin 5% newborn calf serum and 100 μg/ml streptomycin (Life Technologies, Inc.). PC12 cells were cultured in medium containing 10% fetal bovine serum and 5% horse serum. COS cell growth medium contained 5% newborn calf serum and 5% calf serum. HEK293 cells were cultured in medium containing 10% fetal bovine serum and 5% horse serum. Where indicated, cells were transfected with LipotectAMINE (Life Technologies, Inc.).

Affinity Precipitation—Full-length 14-3-3ζ cDNA was obtained by polymerase chain reaction from a mouse brain cdNA library (CLONTECH, Palo Alto, CA) using primers encompassing the 5′-start site and the 3′-stop site. Nucleotide sequence was confirmed by comparison with the reported sequence for 14-3-3ζ (GenBank™ accession number Z19599). 14-3-3ζ was subcloned into pGEX-5X-1 (Pharmacia, Uppsala, Sweden), expressed in Echerichia coli strain JM109, and purified with glutathione-Sepharose beads according to the manufacturer's instructions. Either untransfected PC12 cells or HEK293 cells transfected with plasmids encoding the indicated MEKK family member were lysed in buffer containing 0.5% Triton X-100, 10 mM Tris, 5 mM EDTA, 50 mM sodium fluoride, 50 mM sodium chloride, and 20 μg/ml aprotinin and incubated with 10 μg of immobilized GST or GST-14-3-3ζ at 4 °C for 3 h. Following washing of each reaction, the samples were run on an SDS-polyacrylamide gel, transferred to nitrocellulose, and Western-blotted with the indicated antibody.

Bacterially Expressed MEKK3(NH2)—The N-terminal portion of MEKK3 (amino acids 4–361) was subcloned into the maltose-binding protein (MBP) fusion vector pMAL-c2 (New England Biolabs Inc., Beverly, MA). MBP-MEKK3(NH2) was purified from bacteria using amylose affinity chromatography as described by the manufacturer. MBP-MEKK3(NH2) was also used as an antigen to generate rabbit polyclonal antibodies. MEKK3(NH2), produced from bacterially expressed MBP-MEKK3(NH2) that was cleared with Factor Xa, was conjugated to cyanogen bromide-activated Sepharose 4B (Pharmacia) and used to affinity-purify specific MEKK3 antibodies.

Caspase Cleavage Assay—35S-Labeled 196-kDa MEKK1 was generated using a coupled reticulocyte lysate expression system (Promega, Madison, WI). Equal amounts of 196-kDa MEKK1 were incubated for 1 h in cleavage buffer (50 mM Tris (pH 7.4), 1 mM EDTA, and 10 mM EGTA) with 1 μg of bacterially expressed and purified GST or GST-14-3-3 eluted from the glutathione-Sepharose beads. Jurkat cells were used as a source of caspase activity when 6 μg of lystate derived from cells that were either unstimulated or stimulated with Pas was added, and the reaction was incubated for 2 h at 37 °C. Samples were separated by SDS-PAGE, and bands were visualized by autoradiography.

Surface Plasmon Resonance Analysis—The Biacore 2000 was utilized to analyze the association of recombinant MEKK3 and 14-3-3 protein expressed in and purified from bacteria (for review, see Ref. 38). GST or GST-14-3-3ζ was immobilized on a CM5 sensor chip via an anti-GST monoclonal antibody (Biacore, Inc., Piscataway, NJ). For the immobilized protein, concentration and flow rate were assayed to yield consistent results. 500–1000 resonance counts per second were obtained with each experiment. Recombinant MBP-MEKK3(NH2) binding was assayed at a flow rate of 20 μl/min at concentrations ranging from 10 to 1000 nm. Data were collected on a NEC Powermate V100 using the Biacore control software program. The relative affinity of MEKK3(NH2) for 14-3-3 was determined using the BIAevaluation 2.0 software program by determining the Kd and Kd′ rates for this interaction by nonlinear curve-fitting methods.

Confocal Immunofluorescence—Cells were fixed with phosphate-buffered saline (pH 7.4) containing 3% parafomaldehyde and 3% sucrose and then permeabilized with 0.2% Triton X-100. The cells were incubated with rabbit polyclonal antibodies raised against peptides corresponding to MEKK1, MEKK2, 14-3-3ζ (Santa Cruz Biotechnology, Santa Cruz, CA), and 14-3-3ζ (Santa Cruz Biotechnology) for 1 h, washed, incubated with 1.5 mg/ml Cy2-conjugated affinity-purified donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and mounted onto slides with 20 mg/ml o-phenylenediamine 1 mg Triton X-195 (pH 8.5). To determine colocalization with 14-3-3 proteins, endogenous MEKK1 was localized with a monoclonal antibody raised against the C terminus (Santa Cruz Biotechnology). The colocalization index was calculated using deconvolution and segmentation analysis with slidebook imaging software (Intelligent Imaging Innovations Inc., Denver, CO) by determining the number of components that stained positive for either MEKK1 or 14-3-3 and calculating the percentage that stained for both molecules. Cells were visualized by digital confocal immunofluorescence, and images were captured with a cooled CCD camera mounted on a Leica DMRXA microscope using a 63× Plan Neofluo objective.

RESULTS

Yeast two-hybrid interaction analysis (39) was used to identify proteins that interact with MEKKs. In one set of screens, the N-terminal regulatory domain of MEKK3 (amino acids 4–361) was fused in frame to the DNA-binding domain of LexA (pBTH116) and used to screen a mouse embryonic library subcloned into pVP16. Histidine prototrophy in the presence of 1 or 5 mM 3-aminotriazole and β-galactosidase activity were used for selection of proteins interacting with the N terminus of MEKK3. 1.7 × 106 transformants were screened. Of the 100 clones selected for sequence analysis, one 14-3-3ζ clone and six 14-3-3ζ clones were identified by comparison with the nucleotide data base at the National Library of Medicine using the BLAST algorithm. Four of the 14-3-3ζ clones and the 14-3-3ζ clone were obtained from plates containing 5 mM 3-aminotriazole, suggesting a high affinity interaction between these proteins and the N terminus of MEKK3. Fig. 1 (A and B) shows that 14-3-3ζ interacted with both the N- and C-terminal moieties as well as with full-length MEKK3. Identification of binding sites in both the MEKK3 N-terminal regulatory and C-
The putative N-terminal regulatory domain of MEKK3 can be readily expressed in bacteria and purified as a fusion protein with MBP to produce MBP-MEKK3(NH$_2$). Surface plasmon resonance analysis demonstrated a $K_D$ of 90 nM for the interaction of MBP-MEKK3(NH$_2$) with immobilized GST-14-3-3e (Fig. 2). Unfortunately, we have not succeeded in preparing functional full-length or C-terminal kinase domain of MEKK3 for similar analysis, but the high affinity interaction of 14-3-3 protein with the N terminus of MEKK3 suggests that this is an interaction relevant to the regulation of MEKK3 by 14-3-3 proteins in cells.

To determine if 14-3-3 proteins associated with other MEKK proteins, full-length MEKK1, -2, -3, and -4 with the HA tag at their N termini were expressed in HEK293 cells. Immunoblotting with the HA tag antibody (12CA5) was used to normalize for expression of each MEKK (Fig. 3). MEKK2 and MEKK3 migrate as ~80-kDa proteins, and MEKK4 is ~180 kDa. Full-length MEKK1 is 196 kDa, but, when expressed in HEK293 cells, is partially proteoyeized to generate two large N-terminal fragments of ~134 kDa (fragment A) and 113 kDa (fragment B). We have demonstrated that the 113-kDa fragment B is the result of cleavage of MEKK1 by caspase-3 at Asp-874, which also generates a 91-kDa C-terminal activated kinase domain.

**Regulation of MEKKs**

One role of 14-3-3 proteins is to interact with phosphorylated amino acids, resulting in protection from dephosphorylation by phosphatases and potential alterations in overall protein activity (27, 28, 40). Although the phosphorylation sites of full-length MEKK1, -2, and -3 have not been identified, phosphorylation sites have been mapped to the C-terminal catalytic domain of MEKK1 (41). These observations prompted us to focus on the kinase domains of MEKK1, -2, and -3 as potential sites for interaction with 14-3-3 proteins. The kinase domains of MEKK1, -2, and -3 show dramatically different abilities to bind to 14-3-3 protein. In several experiments, no binding of MEKK4 to 14-3-3 protein could be detected, although functional MEKK4 protein was expressed as determined by its stimulation of JNK activity (data not shown). The failure of MEKK4 to bind 14-3-3 proteins demonstrates a difference in the regulation of MEKK1, -2, and -3 relative to MEKK4. In this regard, transfection-mediated expression of MEKK1, -2, and -3 results in constitutively activated MEKK proteins that can strongly activate JNK. In contrast, overexpression of full-length MEKK4 has a modest ability to activate the JNK pathway, whereas the MEKK4 catalytic domain strongly activates JNK (3). Cumulatively, these results indicate that the regulatory properties of MEKK4 are different from those of the other MEKKs.

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** Both the N-terminal regulatory and C-terminal kinase domains of MEKK3 interact with 14-3-3 proteins. A, the L40 yeast strain was cotransformed with a LexA DNA-binding domain fusion plasmid (pBTM116) expressing the amino-terminal regulatory domain of MEKK3 (MEKK3$_{NH2}$), full-length MEKK3 (MEKK3$_{FL}$), or lamin as a negative control in combination with either an empty plasmid (pVP16) expressing the activation domain alone or pVP16 expressing full-length 14-3-3. Following selection on leucine/trytophan-negative plates, five separate colonies were isolated and assayed for $\beta$-galactosidase activity. Error bars represent S.D. B, HEK293 cell lysates recombinantly expressing the kinase domain of MEKK3 (MEKK3c) were incubated with Sepharose beads conjugated to either GST alone as a negative control or GST-14-3-3e. Precipitates were analyzed by Western blot analysis with an anti-hemagglutinin antibody. C, wild-type PC12 cells were stimulated with epidermal growth factor (EGF) for 10 min prior to being lysed. Lysates were incubated with either GST-14-3-3e or GST alone immobilized on Sepharose beads. Western blot analysis was performed with affinity-purified rabbit polyclonal antibodies raised against MEKK3(NH$_2$) as described under “Experimental Procedures.” In B and C, representative examples of three separate experiments are shown for each panel. Immunoreactive proteins were visualized by enhanced chemiluminescence and autoradiography.

terminal kinase domains was similar to that for Raf-1, which has 14-3-3 interaction sequences in the regulatory and catalytic domains (14, 19).

GST-14-3-3e associated with endogenous MEKK3 in lysates from PC12 cells (Fig. 1C). When cells were first stimulated with epidermal growth factor, there was an ~2-fold increase in MEKK3 binding to GST-14-3-3e-Sepharose beads. MEKK3 becomes serine/threonine-phosphorylated when cells are stimulated by growth factor, and this could increase its interaction with 14-3-3 proteins.

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4 R. R. Vaillancourt and G. L. Johnson, unpublished observations.
The inability of the kinase domain of MEKK1 to bind 14-3-3 proteins prompted us to determine if the 91-kDa C-terminal kinase domain (Fig. 5A), created by caspase-3 cleavage, was able to bind 14-3-3. Fig. 5B shows that, even though they were expressed at similar levels, the 196-kDa full-length MEKK1 protein associated with 14-3-3 unlike the 91-kDa C-terminal MEKK1 protein, which displayed little associative properties for 14-3-3. This result is consistent with the inability of the kinase domain (see Fig. 4) and the first 300 amino acids (data not shown) of the 91-kDa kinase domain of MEKK1 to bind 14-3-3. Fig. 5C shows that multiple deletion products encoding different regions of the N terminus of 196-kDa MEKK1 bind to 14-3-3. Minimally, the N-terminal 393 amino acids of MEKK1 are sufficient to bind 14-3-3. This region is upstream of the predicted pleckstrin homology domain encoded in the MEKK1 sequence. Therefore, although 196-kDa MEKK1 binds to 14-3-3 proteins via a domain that is located in the first 393 amino acids, the 91-kDa C-terminal kinase domain does not associate with 14-3-3 proteins.

Since 14-3-3 proteins associate with MEKK1 and caspase cleavage of MEKK1 is an important component of the apoptotic
response (9), we next determined whether association of 14-3-3 protein would influence caspase cleavage in vivo and in vitro. [$^{35}$S]Methionine-labeled MEKK1 was recombinantly expressed in rabbit reticulocyte lysate and combined with either GST or GST-14-3-3e or without any recombinant protein (NONE). Lysates isolated from Jurkat cells either unstimulated (−Fas) or stimulated with Fas (+Fas) were incubated with the binding reactions prior to separation by SDS-PAGE analysis. Fragments were visualized by autoradiography. A, HEK293 cells were transfected with plasmids expressing the indicated genes. Following incubation for 48 h, cells were lysed, and to visualize proteolytic generation of the 91-kDa form of MEKK1, samples were separated by SDS-PAGE and subjected to Western blot analysis.

![Image](http://www.jbc.org/content/278/13/3480/F6.large.jpg)

**Fig. 6.** In vivo and in vitro analyses of the effects of 14-3-3 association on caspase-mediated cleavage of 196-kDa MEKK1. A, $^{35}$S-labeled 196-kDa MEKK1 was prepared using rabbit reticulocyte lysate and incubated with either GST or GST-14-3-3e or without any recombinant protein (NONE). Lysates isolated from Jurkat cells either unstimulated (−Fas) or stimulated with Fas (+Fas) were incubated with the binding reactions prior to separation by SDS-PAGE analysis. Fragments were visualized by autoradiography. B, HEK293 cells were transfected with plasmids expressing the indicated genes. Following incubation for 48 h, cells were lysed, and to visualize proteolytic generation of the 91-kDa form of MEKK1, samples were separated by SDS-PAGE and subjected to Western blot analysis.

and ERK activation and determined whether in vitro association of 14-3-3 protein with MEKK1, -2, or -3 influenced kinase activity. As illustrated in Fig. 7A, overexpression of 14-3-3 alone was neither sufficient to stimulate JNK activation nor capable of influencing JNK activation that was induced by overexpression of MEKK1, -2, or -3. Furthermore, 14-3-3 protein overexpression did not alter ERK activation by MEKK1, -2, or -3, and it did not elicit ERK activation (data not shown), as has been reported in Xenopus oocytes (22). As determined by in vitro kinase assays, association of 14-3-3 protein with MEKK1 or MEKK2 did not alter their ability to phosphorylate a kinase-inactive form of JNK kinase (JNKK) was utilized as a substrate.

![Image](http://www.jbc.org/content/278/13/3480/F7.large.jpg)

**Fig. 7.** Association of 14-3-3 does not influence MEKK activity. A, HEK293 cells were transfected with either empty vector or vector expressing 14-3-3e protein. Where indicated, plasmid expressing either MEKK1, -2, or -3 was included in the transfection, and MEKK activity was evaluated in intact cells by immunoprecipitating an HA-tagged form of JNK and measuring its ability to phosphorylate c-Jun in an in vitro kinase assay. B, recombinantly expressed 14-3-3e was analyzed by Western blotting. C, cells were transfected with either empty plasmid or plasmid expressing either MEKK1 or MEKK2. Following immunoprecipitation with an anti-HA antibody, either GST alone or GST-14-3-3e was allowed to associate with the precipitation reaction, and MEKK activity was measured in an in vitro kinase assay in which a kinase-inactive form of JNK kinase (JNKK) was utilized as a substrate.

Digital confocal immunofluorescence was used to localize 14-3-3 proteins in cells relative to the distribution of MEKK1 and MEKK2 (Fig. 8). MEKK1 and MEKK2 have been previously characterized for subcellular distribution (6). MEKK1 has a nuclear and punctate cytoplasmic distribution, whereas MEKK2 has a punctate cytoplasmic and Golgi localization. Indirect immunofluorescence with both antibodies for 14-3-3e (specific for this isoform) indicated that 14-3-3 proteins have a Golgi and punctate cytoplasmic distribution and that at least 14-3-3e was, in addition, localized in the nucleus. Thus, staining of 14-3-3 proteins shows a similar subcellular distribution of MEKK1 and MEKK2, consistent with an ability of 14-3-3 proteins to interact with MEKK proteins in cells. We do not have antibodies that selectively stain MEKK3 using indi-
FIG. 8. MEKK family members and 14-3-3 proteins display similar intracellular localization. Digital confocal immunofluorescence was utilized to determine the subcellular localization of 14-3-3 and MEKK proteins in COS-7 cells. The 14-3-3β antibody (A) recognizes multiple 14-3-3 isoforms, whereas the 14-3-3ε antibody (C) is specific for this isoform. As previously published (6), the anti-MEKK2 (B) and anti-MEKK1 (D) antibodies, both raised using peptides corresponding to divergent C-terminal regions of each protein, are specific for these isoforms and do not recognize other MEKK family members. Images were deconvolved to remove out-of-focus immunofluorescence.

FIG. 9. Subcellular colocalization of endogenously expressed 14-3-3 and MEKK. Shown are the results of digital confocal image analysis of MEKK1 and 14-3-3 protein in COS cells costained with antibodies directed against MEKK1 (red) and 14-3-3 protein (green). Overlap of both MEKK1 and 14-3-3ε is indicated by yellow, particularly in the punctate structures located in the cytoplasm. Quantitative analysis indicated that the overlap index of the punctate structures located in the cytoplasm was 29.1 ± 4.3%, a value determined by scoring the number of components labeled as green (14-3-3) and as red (MEKK1) compared with those components that were colocalized (yellow). In comparison, quantitative analysis of staining for the p85 subunit of phosphatidylinositol 3-kinase, a protein that does not biochemically interact with MEKK1, resulted in an average colocalization index of 3.8 ± 0.8%. Thus, endogenous MEKK and 14-3-3 proteins interact in vivo. The combined two-hybrid interaction and biochemical and subcellular colocalization analysis indicates that 14-3-3 binding to MEKK1, -2, and -3 is a biologically relevant high affinity interaction.

DISCUSSION

In this report, we characterize the discovery that 14-3-3 proteins associate with specific members of the MEKK family of kinases. Our results indicate that MEKK1, -2, and -3 must be added to the growing list of signal transduction proteins that bind 14-3-3 proteins. Unlike MEKK1, -2, and -3, MEKK4 was unable to associate with 14-3-3 proteins and thus highlights the specificity inherent to 14-3-3 interactions. Furthermore, these results provide additional evidence that MEKK4 is regulated by mechanisms distinct from those of the other MEKK family members, a discovery that was only assumed by the dramatically different sequence of the N-terminal regulatory domains (for review, see Ref. 11). Additional binding specificity is apparent, as the kinase domain of MEKK1 was unable to bind to 14-3-3 proteins, unlike MEKK2 and MEKK3. Thus, although a large number of proteins have been described to interact with 14-3-3 proteins, results presented herein confirm that 14-3-3 proteins display a significant level of binding specificity.

Yeast two-hybrid analysis was used to initially discover the interaction of 14-3-3 with the N terminus of MEKK3. Approximately 7% of the clones that were capable of stimulating β-galactosidase activity and mediating histidine prototrophy represented 14-3-3 genes and were the largest population of interacting clones. By comparison, no 14-3-3 isoforms were isolated by two-hybrid analysis when the same library was screened with the C-terminal kinase domain of MEKK1, even though a similar number of positively interacting clones were sequenced. Thus, by two-hybrid analysis, a distinct level of binding specificity was apparent between these two different MEKK regions, highlighting the unique ability of the two-hybrid system to discern between binding partners.

It has been difficult to define the biological role of 14-3-3 protein association (for review, see Ref. 20). One of the most extensively studied kinases with regard to 14-3-3 protein bind-
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The MEKKs are most intriguing. Unlike MEKK2 and MEKK3, the kinase domain of MEKK1 are most intriguing. Unlike MEKK2 and MEKK3, the kinase domain strongly induces apoptosis, whereas a caspase cleavage-resistant MEKK1 mutant expressed in cells suppresses apoptotic responses to external stimuli. Our findings indicate that caspase-3 cleavage of MEKK1 results in the kinase domain being released and no longer tethered to 14-3-3 proteins, potentially allowing for it to interact with and regulate proteins. Interestingly, MEKK2 and MEKK3 induce little or no apoptosis, and unlike MEKK1, their catalytic domains strongly associate with 14-3-3 proteins. Thus, we propose that one function for caspase-3 cleavage of MEKK1 is the release of the MEKK1 kinase domain from 14-3-3 association so that it may interact with new substrates and participate in the apoptotic process. In contrast, when full-length MEKK1 is associated with 14-3-3 proteins, it functions positively to regulate cell processes in response to growth factors and cytokines (6). The association with 14-3-3 proteins, the activity of caspase-3, and cleavage of MEKK1 will therefore regulate the function of MEKK1 in cells.

REFERENCES
1. Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J., and Johnson, G. L. (1993) Science 260, 315–319
2. Blank, J. L., Gerwina, P., A., Pleiman, C. M., Gardner, A. M., Sather, S., and Johnson, G. L. (1996) J. Biol. Chem. 271, 5361–5368
3. Gerwina, P., Blank, J. L., and Johnson, G. L. (1997) J. Biol. Chem. 272, 2826–2829
4. Yan, M., Dal, T., Deal, J. C., Kiyakis, J. M., Zon, L. I., Woodgett, J. R., and Templeton, D. J. (1994) Nature 372, 798–800
5. Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R. J., Johnson, G. L., and Karin, M. (1994) Science 266, 1719–1723
6. Fanger, G. R., Johnson, N. L., and Johnson, G. L. (1997) EMBO J. 16, 4961–4972
7. Xia, Z., Dickens, M., Rainezaudaj, J., Davis, R. J., and Greenberg, M. E. (1995) Science 270, 1520–1521
8. Johnson, N. L., Gardner, A. M., Diener, K., Lange-Carter, C. A., Gleave, J., Jarpe, M. B., Minden, A., Karin, M., Zon, L. I., and Johnson, G. L. (1996) J. Biol. Chem. 271, 3229–3237
9. Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G. L., and Frisch, S. M. (1997) Cell 90, 315–323
10. Robson, M. D., and Shaw, A. S. (1994) Curr. Biol. 4, 533–535
11. Kockel, L., Vorbruggen, G., Jackle, H., and Bohmann, D. (1997) Cell 90, 1140–1147
12. Fukui, H., Xia, K., Pallas, D. C., Cui, C., Conroy, K., Narsimhan, R. P., Mamon, H., and Johnson, G. L. (1997) Curr. Opin. Genet. & Dev. 7, 67–74
13. Russell, M., Lange-Carter, C. A., and Johnson, G. L. (1995) J. Biol. Chem. 270, 11757–11760
14. Aitken, A., Collinge, D. B., van Heusden, B. P. H., Isoke, T., Roseboom, P. H., and Rosenfeld, G., and Soll, J. (1992) Trends Biochem. Sci. 17, 498–501
15. Fu, H., Xia, K., Pallas, D. C., Cui, C., Conroy, K., Narasimhan, R. P., Mamon, H., Collier, R. J., and Roseboom, P. H. (1994) Science 266, 126–128
16. Reuther, G. W., Fu, H., Cripe, L. D., Collier, R. J., and Pendergast, A. M. (1994) Science 266, 129–133
17. Irie, K., Gotoh, Y., Yashar, B. M., Errede, B., Nishida, E., and Matsumoto, K. (1995) Science 265, 1716–1719
18. Fantl, W. J., Muslin, A. J., Kikuchi, M., Artzt, J. A., MacNicol, A. M., Gross, R. W., and Williams, L. T. (1994) Nature 371, 612–614
19. Freed, E., Symons, M., Macdonald, S. G., McCormick, F., and Ruggieri, R. (1994) Science 265, 171–175
20. Morrison, D. (1994) Science 265, 56–57
21. Du, X., Fox, J. E., and Pei, S. (1996) J. Biol. Chem. 271, 7362–7367
22. Xing, H., Komenda, K., and Muslin, A. J. (1997) Curr. Biol. 7, 294–300
23. Ogihara, T., Isobe, T., Ichimura, T., Taoka, M., Funaki, M., Sakoda, H., Onishi, Y., Inukai, K., Anai, M., Fukushima, Y., Kikuchi, M., Yazaki, Y., Oka, Y., and Asano, T. (1997) J. Biol. Chem. 272, 25267–25274
24. Zhang, S. H., Kobayashi, R., Graves, P. R., Piwnica-Worms, H., and Tonks, N. K. (1997) J. Biol. Chem. 272, 27281–27287
25. Clark, G. J., Drugan, J. K., Rossman, K. L., Carpenter, J. W., Rogers-Graham, K., Fu, H., Der, C. J., and Campbell, S. L. (1997) J. Biol. Chem. 272, 20990–20993
26. Morrison, D. K. and Cutler, R. E. (1997) Curr. Opin. Cell Biol. 9, 174–179
27. Dent, P., Jelinek, T., Morrison, D. K., Weber, M. J., and Sturgill, T. W. (1995) Science 268, 1090–1092
28. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) Cell 84, 889–897
29. Banik, U., Wang, G., Wagner, P. D., and Kaufman, S. (1996) J. Biol. Chem. 272, 26219–26225
30. Chang, H. C., and Rubin, G. M. (1997) Genes Dev. 11, 1132–1139
31. Kieckel, L., Vorbruggen, G., Jackle, H., Moldzik, M., and Bohmann, D. (1997) Genes Dev. 11, 1140–1147
32. Ford, J. C., Al-Khodaity, F., Fotou, E., Sheldrick, K. S., Griffiths, D. J. F., and Carr, A. M. (1994) Science 265, 533–535
33. Roberts, R. L., Mosch, H. U., and Fink, G. R. (1997) Cell 89, 1055–1065
34. Xiao, B., Smerdon, S. J., Jones, D. H., Dados, G. G., Soll, J., Aitken, A., and Gamblin, S. J. (1995) Nature 376, 188–191
35. Vojtek, A. B., and Hellenberg, S. M. (1995) Methods Enzymol. 255, 331–342
36. Printen, J. A., and Sprague, G. F. (1994) Genes Chromosom. Cancer 13, 609–619
37. Altschul, S. F., Gish, W., Miller, W., Meyers, E. W., and Lipman, D. J. (1990) 
   *J. Mol. Biol.* **215**, 403–410
38. Raghavan, M., and Bjorkman, P. J. (1995) *Structure* **3**, 331–333
39. Fields, S., and Song, O. (1989) *Nature* **340**, 245–247
40. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) *Cell* **87**, 619–628
41. Siow, Y. W., Kalmar, G. B., Sanghera, J. S., Tai, G., Oh, S. S., and Pelech, S. L. (1997) *J. Biol. Chem* **272**, 7586–7594
42. Moorhead, G., Douglas, P., Morrice, N., Scarrabel, M., Aitken, A., and MacKintosh, C. (1996) *Curr. Biol.* **6**, 1104–1113
43. Michaud, N. R., Fabian, J. R., Mathes, K. D., and Morrison, D. K. (1995) *Mol. Cell. Biol.* **15**, 3380–3397
44. Inouye, C., Dhillon, N., and Thorner, J. (1997) *Science* **278**, 103–106
45. Pawson, T., and Gish, G. D. (1992) *Cell* **71**, 359–362
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