MEKK1 is a mitogen-activated protein kinase kinase (MAP3K) that can regulate the c-Jun amino-terminal kinase (JNK) MAP kinase cascade. MEKK1 is comprised of a kinase domain and a long amino-terminal regulatory domain. This amino-terminal domain has a scaffold function in that it can assemble modules of the JNK and ERK MAP kinase cascades. Recently, we have demonstrated that MEKK1 binds to p115 Rho GTPase-activating protein, which has GTPase-activating protein activity toward RhoA. Thus, we tested whether Rho GTPases interact with the regulatory domain of MEKK1. RhoA, but not Rac or Cdc42, binds to a site in the amino-terminal one-third of MEKK1, which includes its PHD domain. The interaction is prevented by mutation of the essential cysteine in the MEKK1 PHD domain. Rho-GTP stimulates the kinase activity of full-length MEKK1 as much as 10-fold toward MEK4 but does not appear to be ubiquitinated by MEKK1 under conditions that result in modification of ERK2. In summary, we have characterized a novel point at which Rho GTPases impinge upon the regulation and function of MEKK1.

**RhoA Binds to the Amino Terminus of MEKK1 and Regulates Its Kinase Activity**

MAP/ERK\(^1\) kinase 1 (MEKK1), a MAP3 kinase, participates in a diverse array of responses to cellular stress (1, 2). It is sensitive to numerous stimuli, including cold shock, UV light, lysophosphatidic acid, osmotic stress, and microtubule-disrupting drugs. In vitro MEKK1 activates the ERK and JNK MAP kinase pathways by phosphorylation of the relevant MAP2 kinases (3, 4). Overexpression of MEKK1 demonstrates that it may function not only in these MAP kinase pathways but also in regulation of NF-kB (5). MEKK1 plays a role in disease pathology. Notable is its binding to the human T cell lymphotropic virus Tax protein, which enhances NF-kB activation (6). Findings in cells from animals in which the MEKK1 gene has been disrupted further support a role in regulating the JNK pathway in certain settings (7, 8).

With nearly 1500 residues, MEKK1 is the longest of four related mammalian enzymes (9–13). Binding partners identified with its amino-terminal domain include the MAP kinases JNK and ERK2, the MAP2 kinases MEK7 and MEKK1, the MAP3 kinase Raf-1, and the MAP4 kinases Nck-interacting kinase and germlinal center kinase (14–18). Thus, MEKK1 can assemble elements of signaling modules, in a manner reminiscent of the yeast scaffold Ste5p (19–21). Through its PHD domain, MEKK1 can also ubiquitinate and promote the degradation of ERK2 (22). Additional interactions with proteins such as α-actinin may impact MEKK1 localization, and with 14-3-3, reflect phosphorylation-dependent interactions (23, 24). The small G proteins Ras, Rac, and Cdc42 have also been shown to interact with the kinase domain of MEKK1 (25, 26). Perhaps the clearest of functional changes as a result of these interactions is the activation of MEKK1 by the MAP4 kinase germlinal center kinase elucidated by Kyriakis and co-workers (18).

Rho family GTPases, including RhoA, B, C, Rac1, 2, and Cdc42, control cellular morphology and motility and MAP kinase signaling pathways and influence other processes, such as the cell cycle (27). Many of these effects derive directly or indirectly from their ability to regulate the cytoskeleton.

The most distinct effects of these GTPases were noted in Swiss 3T3 cells in which RhoA induces stress fiber formation and Rac1 induces membrane ruffling and lamellipodia, whereas Cdc42 induces spike-like filopodia (28).

The connections between MEKK1 and the cytoskeleton are striking. Cells cultured from MEKK1−/− mice exhibit defects in cellular morphology and motility, suggesting that MEKK1 regulates cytoskeletal function (8, 29). Drugs that target the cytoskeleton are among the most powerful activators of MEKK1 (16, 30). MEKK1 is localized not only to actin stress fibers and focal adhesions but also to microtubules, intermediate filaments, and around and in the nucleus (23, 26). As is the case for other signaling molecules, it is expected that its localization will have a major impact on its function (23, 31).

We recently found that MEKK1 recruits p115 RhoGAP to its amino terminus (32). In view of the association of p115 RhoGAP with MEKK1, the importance of Rho to regulation of the cytoskeleton, and the linkage of MEKK1 to the cytoskeleton, we examined the possible interaction of Rho and other small GTPases with the regulatory amino-terminal portion of MEKK1.

**Materials and Methods**

**Cell Culture, Transfection, Plasmids, and Proteins—HEK 293 and Madin–Darby canine kidney cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. GST-MEK1 221–559 (PHD domain) and GST-ERK2 (K52R) were produced in *Escherichia coli.* Full-length recombinant MEKK1, RhoA, and other small G proteins were produced in Sf9 cells (33, 34). Transient trans-

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* The abbreviations used are: MAP, mitogen-activated protein; MAP3K, mitogen-activated protein kinase kinase kinase; MAP2K, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun amino-terminal kinase; MEK, MAP kinase/ERK kinase; MEKK, MEK kinase; GAP, GTPase-activating protein; Hek, human embryonic kidney; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase.
Antibodies and Immunoprecipitation—Antibodies were as follows: MEKK1 (Santa Cruz Biotechnology, 1–9C-2A and C-22), RhoA (Santa Cruz Biotechnology, 26C4,119-G and 119-R), Myc monoclonal 9E10 (National Cell Culture Center). HER 293 cells were lysed, and immunoprecipitation was performed from solubile extracts using 20 μl of the C-22 rabbit polyclonal antibody, in the presence or absence of the C-22-P peptide (Santa Cruz Biotechnology), and 50 μl of protein A-Sepharose CL-4B (Amersham Biosciences) as described in detail previously (16). Immunoprecipitates resolved by SDS-PAGE were immunoblotted as described (16).

In Vitro Ubiquitination Assays—Approximately 3 μg of the wild type MEKK1 PHD domain (OST-MEK1 221–559 (22)), His6-RhoA-GTP, and GST-ERK (GSTERK5 (GST5R2)) were incubated with 50–500 nm His6-E1, 0.5–5 μM His6-E2, 10 μM ubiquitin, and 2 mM ATP in reaction buffer (50 mM Tris-HCl, pH 7.5, 2.5 mM MgCl2, and 0.5 mM dithiothreitol). Anti-ERK polyclonal antibody (Y691) and anti-RhoA (Santa Cruz Biotechnology, 28C4) were used for immunoblotting analysis. His6-E1, His6-E2, and ubiquitin were kind gifts from Dr. Zhijian J. Chen (University of Texas Southwestern Medical Center) (36).

Kinase Assays—All protein kinase reactions were performed in kinase assay buffer (20 mM Heps, pH 7.8, 10 μM ATP, and 10 mM MgCl2), supplemented with 5 μCi of [γ-32P]ATP as described previously (16). Reactions were incubated at 30 °C for the indicated times and resolved by SDS-PAGE. Gels were stained with Coomassie Blue, destained overnight with 10% glacial acetic acid and 20% methanol, dried, and then visualized by autoradiography.

Yeast Two-hybrid Assays—Yeast two-hybrid analysis was performed essentially as described previously (32). The Saccharomyces cerevisiae Y190 strain was transformed with the following constructs: pAS1CYH2 MEKK1 (1–132), pAS1CYH2 MEKK1 (149–347), pAS1CYH2 MEKK1 (149–636), pAS1CYH2 MEKK1 (630–772), pAS1CYH2/MEKK (766–1173), pAS1CYH2/MEKK (1174–1493), pAS1CYH2 SNF1, pAS1CYH2/MEKK (1–179), pACT2 RhoA wild type, pACT2 RhoA leucine 63, pACT2 RhoA asparagine 19, pACT2 Rac1, pACT2 Cdc42, pACT2 Ras, pACT2 WNK1 1–555, pACT2 JNK2, pACT2 SNF1, pACT2 α-actinin, and pACT2 5d (a fragment of p115 RhoGAP). Final growth selection was carried out on His−, Leu−, Trp− plates supplemented with X-gal.

RESULTS AND DISCUSSION

RhoA Interacts with MEKK1 via Its Amino Terminus—Previously, we found that p115 RhoGAP binds to a central region of MEKK1 located amino-terminal to its kinase domain (32). Therefore, we determined whether Rho GTPases might also interact with MEKK1 outside of its kinase domain. Using a yeast two-hybrid assay, we found that a fragment of MEKK1 containing residues 1–719 interacted with wild type RhoA, but not with Cdc42, Rac1, or H-Ras (Table I). None of these GTPases were found to interact with RhoA to residues 149–636, but not with the negative control SNF1 (Fig. 1B). In contrast, the RhoA mutant N19, which has a much reduced affinity for GTP and exists either bound to GDP or nucleotide-free, was unable to bind any of the MEKK1 fragments. The RhoA L63 mutant, which is constitutively in its GTP-bound form, also bound residues 149–636, but not any of the other MEKK1 fragments or the negative control SNF1. These findings are consistent with the idea that MEKK1 interacts most strongly with Rho in its GTP-ligated state but do not rule out the possibility that Rho-GDP may also bind to MEKK1. For comparison, JNK2 bound only to residues 1–132, a fragment that we have shown contains a JNK-selective docking or D domain (Fig. 1B) (14, 33).

Hanging a narrow region of the MEKK1 that interacts with RhoA to residues 149–636, we examined the amino acid sequence of this fragment to identify possible RhoA binding motifs (Fig. 2A). Domains of target proteins that interact with the...
GTPases Rac1 and Cdc42 are readily identifiable by primary sequence motifs within the target proteins. Best defined is the CRIB motif found in a number of effectors of these small G proteins (38). Primary sequence motifs have been much more difficult to discern in RhoA targets by sequence inspection, mutagenesis, or through truncations. However, one motif termed the Rho effector motif is present in the Rho-binding domains of the targets PKN/PRK1, PRK2, Rhotekin, and Rhophilin (39–41). A region of MEKK1, residues 437–456, displays some sequence similarity to the Rho effector motif found in RhoA-binding proteins (Fig. 2A) and is highly conserved among mammalian species.

To test whether this region of the MEKK1 fragment contained a functional Rho effector motif, we introduced several mutations in residues that might be involved in Rho binding (Fig. 2B and C, and Table II). Only a triple mutation, G452C, R454C, N455D, lost all detectable interaction with RhoA. However, mutating Gly-452 and Arg-454 to alanine instead of cysteine was not sufficient to destroy binding (data not shown), suggesting an artifactual effect of the tandem cysteine residues. That observation, coupled with the overlap of the putative Rho binding site with the PHD domain of MEKK1, caused us to test whether mutation of the key cysteine residue required for the E3 ubiquitin ligase activity of the MEKK1 PHD domain (22), Cys-433, might influence Rho binding. Interestingly, the mutant C433A also failed to bind RhoA (Fig. 2C and Table II).

**In Situ Complexes Formed between MEKK1 and RhoA in Mammalian Cells**—To test whether MEKK1 associates with RhoA in cells, we examined MEKK1 immunoprecipitates for the presence of RhoA. We found that endogenous RhoA co-immunoprecipitated with endogenous MEKK1 (Fig. 3). The specificity of the MEKK1-RhoA interaction was tested by using the antigenic peptide to block MEKK1 immunoprecipitation.
from HEK 293 cell lysates. The peptide blocked both the immunoprecipitation of MEKK1 and the co-immunoprecipitation of RhoA. Similarly, we found that MEKK1 associates with endogenous RhoA in extracts prepared from Madin-Darby canine kidney cells (not shown). A control experiment failed to reveal a similar interaction with another MAP3K, Raf-1 (not shown); this MAP3K has been demonstrated to bind specifically to Ras (42, 43).

**MEKK1 Does Not Ubiquitinate RhoA in Vitro**—The failure of MEKK1 C433A to bind to RhoA suggests that the binding of RhoA to the PHD domain of MEKK1 may facilitate its ubiquitination by MEKK1. To determine whether this can occur, we used the isolated PHD domain of MEKK1 in an *in vitro* ubiquitination assay with RhoA. As a positive control, we used GST-ERK2, which was shown previously to be ubiquitinated by MEKK1 in *vitro* and in cells (22). A band migrating between the 48- and 58-kDa protein standards, which is the expected size for mono-ubiquitin ERK2, was detected upon immunoblotting the reactions with an anti-ERK antibody (Fig. 4, top panel). Under the same conditions with RhoA-GTP in place of ERK2 as the substrate, no modified forms of RhoA were detected by immunoblotting with an anti-Rho antibody (Fig. 4, bottom panel). These results suggest that RhoA is not a good ubiquitination substrate of the MEKK1 PHD domain. Because the E2 protein in the ubiquitination reaction also contributes to specificity, it is possible that some other E2 might cause the reaction to proceed. Whether RhoA is a substrate under other circumstances or not, it may competitively inhibit the ability of MEKK1 to ubiquitinate other proteins in cells by binding to the MEKK1 PHD domain. If this is so, activation of Rho would be expected to stabilize proteins that might otherwise be ubiquitinated by MEKK1. In the future, we hope to test this possibility.

**RhoA Stimulates the Kinase Activity of MEKK1 in Vitro and in Vivo**—Given that MEKK1 appears to interact selectively with RhoA-GTP, we determined whether this interaction alters the kinase activity of native MEKK1. Endogenous MEKK1 was immunoprecipitated from HEK 293 cells after transfection with Myc-tagged RhoA, RhoA L63, and RhoA N19 constructs (Fig. 5). Equal amounts of the Rho proteins were expressed in the cells (top panel), and roughly equal amounts of MEKK1 were immunoprecipitated (third panel). Expression of wild type RhoA or RhoA N19 produced little activation of MEKK1 as compared with vector alone. In contrast, expression of RhoA L63 in HEK 293 cells stimulated the activity of endogenous MEKK1 toward MEK4 K131M nearly 10-fold (bottom panels). To establish whether the activation mechanism could be recapitulated in *vitro*, full-length recombinant MEKK1 expressed in SF9 cells was treated with phosphoprotein phosphatase 2a to reduce its activity (33). MEKK1 expressed in SF9 cells has very high activity, in part due to autophosphorylation. The dephosphorylated protein was mixed in kinase assays with GST-MEK4 K131M as a substrate. An autoradiogram of the kinase assay and the quantitation of the 32P incorporation into MEK4 are shown in the bottom two panels. One of three similar experiments is shown. CMV, cytomegalovirus.
In vitro Effect of RhoA on MEKK1 kinase activity. MEKK1 was purified from S9 lysates and pretreated with P22a to remove phosphate present on the protein as it was isolated. Kinase reactions were then performed as described under “Materials and Methods” following the addition of various forms of small GTPases. A, incubation of MEKK1 with Rho-GTP, Rho-GDP or boiled Rho prior to assay with MEK4 K131M. Fold activation is shown above the lanes. One of four similar experiments is shown. B, incubation of MEKK1 with Rac1, Cdc42 or RhoA in GTP or GDP bound forms prior to assay with MEK4 K131M. One of two similar experiments is shown.

Rho-GTP. For comparison, MEKK1 was incubated with Cdc42 or Rac1 loaded with GDP or GTP (Fig. 6B). Rac1 bound to either GDP or GTP caused little increase in activity; RhoA-GTP increased activity as expected. Interestingly, GTP-ligated Cdc42 also increased MEKK1 activity to approximately the same extent as RhoA-GTP.

We have been unable to show binding of Cdc42 to the amino-terminal domains of MEKK1. A previous study used pull-down assays to demonstrate that Rac1 and Cdc42 can bind to the MEKK1 kinase domain (26), leading us to suggest that the change in activity we observe may be due to interaction of Cdc42 with the kinase domain, a site distinct from the one identified here. If this is so, it is puzzling that MEKK1 distinguished between Cdc42 and Rac1.

The GTP-bound form of RhoA increases the activity of several other protein kinases, including PRK/PKN and Rho kinase (39–41, 44, 45). Although the magnitude of activation of MEKK1 induced by RhoA is smaller than that of p21-activated kinases by GTP-bound Cdc42 or Rac1 (46,47), the activation of MEKK1 by RhoA is of a similar magnitude to that of these other kinases regulated by RhoA. Thus, MEKK1 shows activity changes in keeping with those displayed by other Rho targets. We observed a nearly additive effect of the combination of MEK1 and RhoA on activation of a serum response element promoter SRE.L (not shown), suggesting a potential readout for Rho effects on MEKK1. LIM kinases have previously been proposed to mediate activation of this reporter by Rho (48). However, in the case of MEKK1, we have been unable to demonstrate the relationship between its kinase activity and activation of the reporter; a kinase-dead mutant of MEKK1 displays the same effect on reporter activity in combination with Rho as does the wild type kinase. Likewise, JNK activation by MEKK1 is not absolutely dependent on MEKK1 kinase activity (14). Thus, the potential cellular consequences of increased MEKK1 activity induced by Rho-GTP have not yet been identified.

The capacity of MEKK1 to bind both RhoA and a Rho-selective RhoGAP suggests that Rho bound to MEKK1 will be rapidly inactivated by the associated GAP. This might cause only transient activation of MEKK1 due to rapid inactivation of Rho. In addition, the population of Rho in the vicinity of MEKK1 may have a different active lifetime than Rho in distinct locations due to the proximity of the GAP. MEKK1 scaffolds members of the JNK and ERK MAP kinase cascades (14–16). The current findings suggest that it also scaffolds elements of Rho signaling pathways. We hope to identify the properties of these MEKK1 complexes and to determine whether they represent functionally distinct entities.

In summary, we have defined a novel point at which Rho GTPases can impinge on the function of MEKK1. Given that MEKK1 localizes to focal adhesion and stress fibers, is activated by drugs that impact cytoskeletal structure, and is required for cell motility, MEKK1 must perform a critical role in the regulation of the function of the cytoskeleton. It seems likely that interactions with Rho signaling pathways will contribute to these properties of MEKK1.

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