Ubiquitylation of MEKK1 Inhibits Its Phosphorylation of MKK1 and MKK4 and Activation of the ERK1/2 and JNK Pathways*

Received for publication, November 4, 2002, and in revised form, November 25, 2002 Published, JBC Papers in Press, November 26, 2002, DOI 10.1074/jbc.C200616200

James A. Witowsky and Gary L. Johnson‡
From the Department of Pharmacology, University of Colorado Health Sciences Center and University of Colorado Cancer Center, Denver, Colorado 80262

MEKK1 is a MAPK kinase kinase that is activated in response to stimuli that alter the cytoskeleton and cell shape. MEKK1 phosphorylates and activates MKK1 and MKK4, leading to ERK1/2 and JNK activation. MEKK1 has a plant homeobox domain (PHD) that has been shown to have E3 ligase activity. (Lu, Z., Xu, S., Joazeiro, C., Cobb, M. H., and Hunter, T. (2002) Mol. Cell 9, 945–956). MEKK1 kinase activity is required for ubiquitylation of MEKK1. MEKK1 ubiquitylation is inhibited by mutation of cysteine 441 to alanine (C441A) within the PHD. The functional consequence of MEKK1 ubiquitylation is the inhibition of MEKK1 catalyzed phosphorylation of MKK1 and MKK4 resulting in inhibition of ERK1/2 and JNK activation. The C441A mutation within the PHD of MEKK1 prevents ubiquitylation and preserves the ability of MEKK1 to catalyze MKK1 and MKK4 phosphorylation. MEKK1 ubiquitylation represents a mechanism for inhibiting the ability of a protein kinase to phosphorylate substrates and regulate downstream signaling pathways.

Ubiquitin is a highly conserved 76-amino acid globular protein that was identified as the first protein to act as a covalent modifier. Ubiquitylation is the process of attachment of ubiquitin to a target protein and is a multistep process that involves the action of at least three classes of enzyme: the ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3), which assists in substrate recognition and the transfer of ubiquitin to the target protein. There are two main types of E3 enzyme, those that contain a homology to E6 C terminus (HECT) domain and those that have a really interesting new gene (RING finger or RING-finger-like) domain (1). The plant homeobox domain (PHD) is a RING finger-like domain defined by a series of cysteine and histidine residues with a characteristic spacing that mediates coordination of two zinc ions in a cross-brace structure (2). Disruption of a metal coordinating residue within the RING finger or PHD has been shown to inactivate the ligase properties of the enzyme (3).

Classically, ubiquitin is involved in the homeostasis of cellular proteins by removing unnecessary, deleterious, or misfolded proteins primarily through the ubiquitin-proteasome degradation pathway (4). Ubiquitin has been shown to function in many cellular processes including cell cycle progression, apoptosis, cell differentiation, and DNA repair (5). Recently, ubiquitylation of specific proteins has been shown to have functions independent of proteasome-mediated degradation (6). For example, vesicular sorting and TRAF6 organization of the TAK1 signal transduction complex has been shown to involve ubiquitylation of proteins for the control of protein-protein interactions (7–9).

MEKK1 is a 196-kDa serine/threonine MAPK kinase kinase that can regulate both the extracellular-regulated kinase (ERK1/2) and the c-Jun NH2-terminal kinase (JNK) pathways in response to specific stimuli (10). We and others have shown that MEKK1 is activated by a variety of stimuli that alter cell shape and is required for normal cell motility (11, 12). MEKK1 is a complex protein containing multiple identified domains that may serve to regulate function. The E3 activity of the PHD of MEKK1 was recently described by Lu et al. (13). Here we demonstrate that full-length MEKK1 requires an intact PHD for its own ubiquitylation, and this ubiquitylation impairs its ability to phosphorylate MKK1 and MKK4.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The cysteine to alanine mutation at position 441 of MEKK1 was made in pCMV5 using a PCR strategy. The 5’ oligonucleotide CAG ATG TGT CCG ATC GCC TTG CTG GGC that encodes the mutation and a PvuI restriction site was used in conjunc-

Purification of Ubiquitylated Proteins—Lysates expressing the indicated proteins were prepared, and the protein concentration was determined by the Bradford method. 0.5–1.0 mg of total protein was brought to a volume of 500 μl with lysis buffer and rotated end-over-end at 4 °C with 30 μl of Ni-NTA beads (PharMingen). After the 2–4-h incubation, the beads were washed three times with lysis buffer, and the beads were eluted in 100 μl of 150 mM imidazole.

MEKK1 Kinase Assays—50 μg of total protein from lysates was im-
munoprecipitated with antibodies to the NH2-terminal epitope tag of the expressed proteins. Complexes were washed twice in lysis buffer and once in kinase buffer (20 mM HEPES, pH 7.5, 10 mM MgCl2, 5 mM β-ni-

This work was supported by National Institutes of Health Grant DK37871. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Pharmacology, University of Colorado Health Sciences Center, 4200 East Ninth Ave., Denver, CO 80262. Fax: 303-315-1022; E-mail: gary.johnson@uchsc.edu.

The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; C441A, cysteine 441 to alanine mutation; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; HA, hemagglutinin epitope tag; HECT, homology to E6 COOH terminus; Hs, human; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MEKK1, mitogen-activated protein kinase kinase 1; MKK1, mitogen-activated protein kinase kinase 1; MKK4, mitogen-activated protein...
FIG. 1. MEKK1 stimulates ubiquitylation in a kinase-dependent manner and requires an intact full-length protein. A, the relative positions of known protein binding domains, truncations, and point mutations of MEKK1 are indicated. HEK293 cells were transfected with either empty vector (EV), MEKK1, or a kinase-inactive mutant of MEKK1 (MEKK1K). B, cells were treated with 20 μM proteasome inhibitor, MG-132, and the cell lysates were separated on 10% SDS-PAGE and Western-blotted with 9E10, a monoclonal anti-Myc antibody. C, in addition to empty vector (EV) and MEKK1, the NH2-terminal 719 amino acids (1–719) and the 91-kDa COOH-terminal fragment were expressed separately or together in the presence or absence of His6-Myc-ubiquitin and probed as in B. Ub, ubiquitin.

50 μl of kinase buffer supplemented with 10 μCi of [γ-32P]ATP and 1 μg of either GST-MEK1 or GST-MEK4 (Upstate Biotechnology Inc). Endogenous JNK activity was determined as described previously (14).

RESULTS

Expression of MEKK1 Stimulates Ubiquitylation—Within the MEKK1 protein sequence, multiple protein binding regions and domains have been identified that may regulate its cellular localization and activity (Fig. 1A). The COOH-terminal region contains the kinase domain, two ubiquitin interaction motifs (UIMs), a region of interaction with small GTPases, and a caspase 3-like cleavage site. Cleavage of MEKK1 by caspases generates a catalytically active 91-kDa COOH-terminal fragment and a 105-kDa NH2-terminal fragment containing the zinc finger-like PHD. To determine whether MEKK1 stimulates ubiquitylation in vivo, we expressed HA-tagged wild type and kinase-inactive MEKK1 in cells in the presence or absence of His6-Myc-tagged ubiquitin (Fig. 1B). Cell lysates were separated on SDS-PAGE and Western blotted with the anti-Myc antibody 9E10 to recognize ubiquitin-conjugated proteins. A dramatic increase in immunoreactivity is seen in the lysates co-expressed with wild type MEKK1 and ubiquitin. The smear of immunoreactivity seen is characteristic of ubiquitin modifications of multiple proteins in the cell lysate. Therefore, MEKK1 stimulates covalent modification of proteins with ubiquitin (Fig. 1B). The stimulation is a specific function of MEKK1, and Myc immunoreactivity is not seen in the control lysate from cells transfected with empty vector with ubiquitin. The kinase activity of MEKK1 is required for the dramatic increase in protein ubiquitylation. Expression of kinase-inactive MEKK1 shows only a modest increase of Myc immunoreactivity over the background. Quantitation of the autoradiogram indicates that ubiquitylation stimulated by kinase-inactive MEKK1 is less than 10% of the ubiquitylation seen with wild type MEKK1. Consistent with our findings, the PHD of MEKK1 was recently reported to possess a ubiquitin ligase activity that was more active in wild type than in kinase inactive MEKK1 (13).

Our laboratory has shown expression of the 91-kDa COOH-terminal kinase fragment of MEKK1 activates ERK1/2 and JNK pathways (15). We sought to determine whether ubiquitin ligase activity observed with MEKK1 expression required a functional kinase domain within the full-length MEKK1 protein. To test this, we compared the full-length MEKK1 protein to expression of the PHD containing amino-terminal fragment (1–719 amino acids) and the 91-kDa kinase-active carboxy-terminal fragment alone or in combination in the presence or absence of His6-Myc-ubiquitin (Fig. 1C). Total cell lysates were Western blotted with anti-Myc antibody. An intense smear of immunoreactivity is observed with full-length kinase-active MEKK1 in the presence of ubiquitin. Overexposure of the nitrocellulose membrane reveals that expression of the NH2-terminal fragment behaves similar to the kinase-inactive full-length protein, as there is only a modest increase of immunoreactivity over the control lysates. Expression of the COOH-terminal kinase fragment alone or in combination with the PHD-containing NH2 terminus stimulates ubiquitylation at a modest but higher level than observed with the NH2 terminus alone (Fig. 1C). Co-expression of the NH2- and COOH-terminal regions of MEKK1 did not enhance protein ubiquitylation above that observed with the kinase domain alone. These experiments demonstrate a requirement for full-length kinase-active MEKK1 for significant stimulation of ubiquitylation.

MEKK1 Ubiquitylation Requires a Functional PHD—To determine whether a functional PHD is required for the ubiquitylation of MEKK1 in the full-length protein, we mutated cysteines 441 to alanine. Cysteine 441 is predicted to be a critical zinc-coordinating residue in the RING finger-related PHD based on similar mutation made in the RING finger of BRAC1 (3). Wild type and C441A MEKK1 were transfected in the presence or absence of His6-Myc-ubiquitin. The cell lysates were Western blotted for expression of the proteins with the anti-MEKK1 antibody, C22 (Fig. 2A). In the absence of ubiquitin, MEKK1 and C441A are expressed at similar levels. Co-expression of ubiquitin and wild type MEKK1 results in the ubiquitylation of full-length MEKK1 protein that migrates as larger molecular weight species. C441A is not significantly
ubiquitylated, and slower migrating bands are not observed relative to C441A MEKK1 expressed in the absence of ubiquitin. The 91-kDa COOH-terminal fragment is generated from both wild type and C441A MEKK1 proteins. In the presence of ubiquitin, the 91-kDa COOH-terminal fragment derived by proteolytic cleavage of wild type MEKK1 (16) forms a ladder in ~8-kDa increments consistent with the fragments being multiubiquitylated. The COOH-terminal fragment generated from C441A MEKK1 is largely distributed among three bands of reactivity with the most prominent at 91 kDa and represents a mono- and diubiquitylated COOH-terminal fragments, respectively. Affinity precipitation of the lysates with NTA-Ni beads followed by immunodetection of MEKK1 confirmed that full-length MEKK1, but not the C441A MEKK1 PHD mutant, is ubiquitylated (Fig. 2B). The amount of COOH-terminal fragment eluted from the Ni²⁺ beads may simply be contamination in the bead pull down or due to the presence of two overlapping UIMs. The UIMs could possibly associate with ubiquitylated proteins including MEKK1 in the lysate. A band of ~100 kDa in the wild type MEKK1 lane correlates with the 91-kDa MEKK1 fragment that is monoubiquitylated with endogenous ubiquitin, suggesting that C441A MEKK1, possibly through the UIM, has a limited ability to be ubiquitylated.

**MEKK1 Ubiquitylation Inhibits in Vitro Substrate Phosphorylation.**—To determine whether ubiquitylation modulated MEKK1 kinase activity, we performed in vitro kinase assays of MEKK1 isolated from cells using M KK1 and M KK4 as substrates (Fig. 3). A ladder of ubiquitylated MEKK1 is seen in the presence of co-transfected ubiquitin. Strikingly, ubiquitylation of MEKK1 leads to a significant inhibition of substrate phosphorylation. In contrast, M KK1 and M KK4 phosphorylation by C441A is unaffected by expression of ubiquitin. These results indicate that PHD-dependent ubiquitylation of MEKK1 inhibits the ability of MEKK1 to phosphorylate M KK1 and M KK4 in vitro.

**MEKK1 Ubiquitylation Inhibits ERK1/2 and JNK Activation in Cells.**—The ability of MEKK1 and C441A MEKK1 to activate ERK1/2 and JNK pathways in cells was investigated (Fig. 4). Activation of the ERK1/2 MAPK pathway was assessed by a phosphospecific antibody to ERK1/2 that specifically recognizes phosphorylation of the activating residues in the T-loop of the kinase (Fig. 4B). An antibody that cross-reacts with both ERK1 and ERK2 was used to detect total ERK1/2 protein (Fig. 4C). Expression of MEKK1 (Fig. 4A) activated the ERK1/2 pathway, and this stimulation was lost with the co-expression of ubiquitin (Fig. 4B). Additionally, there is an apparent increase in the total
amount of MEKK1 protein with ubiquitin co-expression. The C441A mutant in the presence or absence of ubiquitin activated the ERK1/2 pathway to a greater extent than wild type MEKK1. These results are consistent with ubiquitylation of MEKK1 being a negative regulator of MEKK1 activation of the ERK1/2 pathway.

A similar inhibitory effect of MEKK1 ubiquitylation was observed for the JNK pathway. Cells were treated as described previously, and lysates were prepared. JNK activity was measured by affinity precipitation of activated JNK with GST-c-Jun (amino acids 1–79) (Fig. 4E). Total JNK and MEKK1 were visualized by immunodetection with their respective antibodies shown in Fig. 4, D and F. To achieve comparable protein expression levels for the wild type MEKK1 protein in the presence and absence of ubiquitin, the total amount of DNA transfected was reduced to 100 ng per dish. This low amount of DNA results in a somewhat modest JNK activation with the wild type MEKK1 that is significantly reduced with co-expression of ubiquitin. The activity of C441A MEKK1 is maintained in the presence of expressed ubiquitin compared with C441A alone.

Ubiquitylated MEKK1 Is Expressed in the Absence of Proteasome Inhibitor—Because ubiquitylation of proteins is often a signal for degradation by the proteasome, we determined whether MEKK1-stimulated ubiquitylation of MEKK1 caused the loss of MEKK1 protein in cells. MEKK1 or C441A MEKK1 was expressed in the presence or absence of His6-my-ubiquitin. The cells were treated with M6SO or the protease inhibitor MG-132, and the clarified lysates were separated by SDS-PAGE. Immunodetection of MEKK1, ERK1/2, and JNK revealed little difference between cells treated with MG-132 or vehicle (Fig. 5A).

DISCUSSION

MEKK1 is unique among MAPK kinase kinases in that it encodes a PHD in its NH2 terminus. The MEKK1 PHD has E3 ligase activity (13) that is dependent on the kinase activity of the full-length 196-kDa MEKK1 protein. Lu et al. (13) demonstrated that a GST fusion of the MEKK1 PHD had E3 ligase activity in vitro. Our work demonstrates that a functional PHD is required for MEKK1 poly/multiubiquitylation. Thus, activation of MEKK1 not only stimulates its ability to phosphorylate and activate MKK1 and MKK4 in the ERK1/2 and JNK pathways but also leads to the ubiquitylation of MEKK1 itself. This ubiquitylation consequently inhibits MEKK1-catalyzed phosphorylation of MKK1 and MKK4, thereby down-regulating the MEKK1 activation of the ERK1/2 and JNK pathways. The inhibition of MEKK1 phosphorylation activity is independent of proteasome-dependent degradation of MEKK1. In fact, ubiquitylated MEKK1 seems to be a stable protein in cells. Although it is difficult to quantitate because of the poly/multiubiquitylation of MEKK1 and the consequent scaffolding in SDS-PAGE, it appears that ubiquitylated MEKK1 actually accumulates in cells and is not degraded significantly by the proteasome. We propose that the ubiquitylation-dependent uncoupling of MEKK1-catalyzed phosphorylation of MKK1 and MKK4 represents a novel function for protein ubiquitylation.

It appears that the C441A MEKK1 protein has greater activity toward MKK1 and MKK4 than wild type MEKK1, based on the relative protein levels in the immunoprecipitates. This is consistent with the PHD having a negative regulatory function that correlates with its E3 ligase activity. Certainly, other proteins that interact with MEKK1 may be ubiquitylated by the PHD E3 ligase activity of MEKK1. The E3 ligase function of MEKK1 is likely to down-regulate MEKK1 activation of ERK1/2 and JNK and also ubiquitylate other proteins to regulate their function and/or targeting to the proteasome for degradation.

How could MEKK1 ubiquitylation inhibit MKK1 and MKK4 phosphorylation? The ubiquitin modification of MEKK1 may simply block binding of MKK1 and MKK4. Adjacent to the COOH-terminal kinase domain are two UIMs that could interact with ubiquitins covalently bound to MEKK1 and induce a steric hindrance for substrate interaction. The fact that a full-length 196-kDa MEKK1 is required for MEKK1 ubiquitylation suggests that an intramolecular interaction is required for regulation of the MEKK1 E3 ligase activity. It should be noted that our studies were done using transfection. We must still define the role of ubiquitylation of endogenous MEKK1 in cells.

The rapidly expanding functions for ubiquitin modification of proteins indicates that ubiquitin is used to regulate protein targeting, scaffolding, and activity. In regard to positive regulation of signal transduction, the polyubiquitylation of TRAF6 is involved in the organization of p38 and NFkB signaling complexes (8, 9). In contrast, activation of the E3 ligase activity of c-Cbl by Src kinase phosphorylation of c-Cbl (17, 18) results in the ubiquitylation of Src and the receptors for EGF (19–21) and PDGF (22). Thus, c-Cbl down-regulates tyrosine kinase signaling. It has also been shown in yeast that pheromone activation of the mating pathway involving the MAPK, Fus3, stimulates a feedback ubiquitylation and degradation of Ste11, a MAPK kinase kinase (23). Our results demonstrate that the PHD of MEKK1 is required for inhibition of ERK1/2 and JNK activation by MEKK1. The C441A MEKK1 mutant protein stimulates the ERK1/2 and JNK pathways, and this activation is not down-regulated as is seen with wild type MEKK1, showing the requirement of the PHD of MEKK1 for ubiquitin-dependent inhibition of MEKK1 substrate phosphorylation. The regulation of Ste11 and MEKK1, both MAPK kinase kinases, by ubiquitylation appears different. Ste11 is degraded and MEKK1 substrate phosphorylation is inhibited when each is modified with ubiquitin. The function of the MEKK1 PHD is a previously unrecognized mechanism for controlling the activity of a MAPK kinase kinase and the turn-off of MAPK signaling in cells.

REFERENCES

1. Glickman, M. H., and Ciechanover, A. (2002) Physiol. Rev. 82, 373–428
2. Pascual, J., Martinez-Yamout, M., Dyson, H. J., and Wright, P. E. (2000) J. Mol. Biol. 304, 723–729
3. Hashizume, R., Fukuda, M., Maeda, I., Nishikawa, H., Oyake, D., Yabuki, Y., Ogata, H., and Ohta, T. (2001) J. Biol. Chem. 276, 14531–14540
4. Jennis, H. P. (1995) Eur. J. Biochem. 231, 1–39
5. Naujokat, C., and Hoffmann, S. (2000) Lab. Invest. 82, 985–980
6. Marx, J. (2002) Science 297, 1792–1794
7. Katzmann, D. J., Bahat, M., and Emr, S. D. (2001) Cell 106, 145–155
8. Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z. J. (2000) Cell 103, 351–361
9. Wang, C., Deng, L., Heng, M., Akkaraju, R. R., Inoue, J., and Chen, Z. J. (2001) Nature 412, 346–351
10. Yujiri, T., Sather, S., Fanger, G. R., and Johnson, G. L. (1998) Science 282, 1911–1914
11. Yujiri, T., Ware, M., Widmann, C., Oyer, R., Russell, D., Chan, E., Zaitsev, Y., Clarke, P., Tyler, K., Oya, Y., Fanger, G. R., Henson, P., and Johnson, G. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7272–7277
12. Xia, Y., Makris, C., Su, B., Li, E., Yang, J., Nemerow, G. R., and Karin, M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5243–5248
13. Lu, Z., Xu, X., Joazeiro, C., Cobb, M. H., and Hunter, T. (2002) Mol. Cell, 9, 945–956
14. Heasley, L. E., Storey, B., Fanger, G. R., Butterfeld, L., Zamarripa, J., Blumberg, D., and Maue, R. A. (1996) Mol. Cell. Biol. 16, 648–656
15. Widmann, C., Gibson, S., and Johnson, G. L. (1998) J. Biol. Chem. 273, 7141–7147
16. Widmann, C., Gerwins, P., Johnson, N. L., Jarpe, M. B., and Johnson, G. L. (1998) Mol. Cell. Biol. 18, 2416–2429
17. Yokouchi, M., Kondo, T., Sanayji, A., Houghton, A., Yoshimura, A., Komiyai, S., Zhang, H., and Baron, R. (2001) J. Biol. Chem. 276, 35185–35193
18. Kassenbrock, C. K., Hunter, S., Garl, P., Johnson, G. L., and Anderson, S. M. (2002) J. Biol. Chem. 277, 34977–34975
19. Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998) Genes Dev. 12, 3663–3674
20. Yokouchi, M., Kondo, T., Houghton, A., Bartkiewicz, M., Horne, W. C., Zhang, H., Yoshimura, A., and Baron, R. (1999) J. Biol. Chem. 274, 31707–31712
21. Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsengkavan, A. Y., Abrey, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) Mol. Cell 4, 1099–1104
22. Rosenkranz, S., Ikuno, Y., Leong, F. L., Klingshofer, R. A., Miyake, S., Band, H., and Kuzalska, A. (2000) J. Biol. Chem. 275, 9620–9627
23. Esch, R. K., and Errede, B. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9160–9165