MEK Kinase 1 (MEKK1) Transduces c-Jun NH$_2$-terminal Kinase Activation in Response to Changes in the Microtubule Cytoskeleton*

(Received for publication, January 25, 1999, and in revised form, February 22, 1999)

Toshiaki Yujiri‡, Gary R. Fanger‡, Timothy P. Garrington‡, Thomas K. Schlesinger‡, Spencer Gibson¶,§, and Gary L. Johnson¶,‡

From the ¶Program in Molecular Signal Transduction, Division of Basic Sciences, National Jewish Medical and Research Center, Denver, Colorado 80206 and the §Department of Pharmacology, University of Colorado Medical School, Denver, Colorado 80262

Cell shape change and the restructuring of the cytoskeleton are important regulatory responses that influence the growth, differentiation, and commitment to apoptosis of different cell types. MEK kinase 1 (MEKK1) activates the c-Jun NH$_2$-terminal kinase (JNK) pathway in response to exposure of cells to microtubule toxins, including taxol. MEKK1 expression is elevated 3-fold in mitosis and microtubule toxin-treated cells accumulated at G$_2$/M of the cell cycle. Targeted disruption of MEKK1 expression in embryonic stem cells resulted in the loss of JNK activation and increased apoptosis in response to taxol. Targeted disruption of the MEK kinase 2 gene had no effect on activation of the JNK pathway in response to microtubule toxins demonstrating a specific role of MEKK1 in this response. Cytochalasin D-mediated disruption of actin fibers activates JNK and stimulates apoptosis similarly in MEKK1−/− and wild type cells. The results show that MEKK1 is required for JNK activation in response to microtubule but not actin fiber toxins in embryonic stem cells. MEKK1 activation can protect cells from apoptosis in response to change in the integrity of the microtubule cytoskeleton.

Restructuring of the microtubule cytoskeleton occurs in response to a variety of events such as immune cell infiltration and invasion, interaction of cells with the extracellular matrix, and during the mitotic phase of the cell cycle. Cell shape change and the underlying restructuring of the cytoskeleton regulates gene expression and contributes to the commitment of cells to grow, undergo apoptosis, or differentiate. The importance of the cytoarchitecture and its regulation was realized when it was described that a reduction in cell spreading resulting in a spherical versus flat cell shape had an inhibitory effect on DNA synthesis (1, 2). More recently, it was demonstrated that cytoarchitecture determines whether a cell will grow or undergo apoptosis, as decreased cell spreading, which results in cell rounding using micropatterned substrates of various dimensions, induced an apoptotic response whereas cell spreading allowed survival and proliferation (3). The underlying restructuring of the cytoskeleton during these responses has been shown to influence gene expression. For example, microtubule disruption induces expression of the urokinase-type plasminogen activator and interleukin 1-β but inhibits tubulin synthesis (4–6). The promoter region for the B chain of platelet-derived growth factor was shown to contain a cis-acting response element that was regulated by shear stress induced by changes in cytoarchitecture (7). The collagenase-1 gene, which encodes a matrix metalloproteinase important for cell migration and invasion, is also regulated in response to cytochalasin D disruption of the actin cytoskeleton (8).

Reorganization of the cytoarchitecture regulates signaling pathways including the mobilization of intracellular calcium, activation of tyrosine kinases, Ras, extracellular signal-regulated kinase (ERK), and c-Jun NH$_2$-terminal kinase (JNK) (9–12). Consistent with the activation of signal pathways, specific transcription factors are activated by cytoskeletal restructuring (8, 13). In this report, we show that targeted disruption of MEKK1 expression selectively inhibits JNK activation in response to microtubule toxins. Functionally, MEKK1 is the transducer for the specific regulation of the JNK pathway and promotes cell survival during changes in microtubule integrity.

EXPERIMENTAL PROCEDURES

Cell Culture—Cells were maintained in a humidified 7.0% CO$_2$ environment in Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc.). Medium for T47D human breast adenocarcinoma cells was supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Medium for MEKK1−/− and MEKK1+/− mouse embryonic stem (ES) cells was supplemented with 15% heat-inactivated fetal bovine serum (Summit Biotechnology, CO), 144 μM monothioglycerol (Sigma), and 1% leukemia inhibitory factor Chinese hamster ovary cell-conditioned medium.

Analysis of Kinase Activity—MEKK1 and JNK activities were measured as described previously (14), except 1 μg of recombinant purified kinase-inactive glutathione 5-transferase-c-Jun NH$_2$-terminal kinase kinase (GST-JNKK$_{5k}$) was used as a substrate to assay MEKK1 activity. To determine ERK and p38 activity, cell lysates were Western blotted with either an anti-phospho-ERK or anti-phospho-p38 antibody (New England Biolabs, MA) as a measure of kinase activity.

Apoptosis Assay—Apoptosis was measured using acridine orange/ethidium bromide staining. Apoptotic cell death was verified using DNA ladder formation assays.

Microscopy—Cells were plated onto uncoated glass coverslips 2 days before being fixed in a solution containing 3% paraformaldehyde and 3% sucrose in phosphate-buffered saline (pH 7.4). Cells were permeabi-

* This work was supported by National Institutes of Health Grants DK73871, DK48845, GM30324, and CA58157. 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.

‡ Leukemia Society Fellow.

§ To whom correspondence should be addressed: Program in Molecular Signal Transduction, Division of Basic Sciences, National Jewish Medical and Research Center, 1400 Jackson St., Denver, CO 80206. Tel.: 303-398-1504; Fax: 303-398-1225; E-mail: johnsong@njc.org.

1 The abbreviations used are: ERK, extracellular signal-regulated kinase; MEKK, MEK kinase; JNK, c-Jun NH$_2$-terminal kinase; MAPK, mitogen-activated protein kinase; ES, embryonic stem; JNKK, JNK kinase; GST, glutathione S-transferase; JNKK$_{5k}$, recombinant kinase-inactive JNKK; DAPI, 4′,6-diamidino-2-phenylindole.
MEKK1 is Activated in Response to Stimuli That Alter Microtubules—MEKKs regulate the JNK pathway (15–17), suggesting that a specific MEKK could mediate the JNK activation in response to microtubule reorganization of the cytoarchitecture. We found that MEKK1 is activated by stimuli that alter microtubule dynamics. Nocodazole disrupts microtubules in T47D cells (Fig. 2A) and activates MEKK1 in a concentration- and time-dependent manner (Fig. 2B and C). Taxol treatment of T47D cells, which stabilizes microtubule structures (Fig. 2A), activates MEKK1 (Fig. 2D). Thus, the microtubule toxins, nocodazole and taxol, activate MEKK1.

MEKK1 Protein Expression Is Increased during the M Phase of the Cell Cycle—During the cell cycle the greatest change in microtubule structure occurs in mitosis. Analysis of T47D breast carcinoma cells proliferating in normal growth conditions demonstrated that the expression of MEKK1 is increased in the M phase of the cell cycle. Fig. 3A shows T47D cells, co-stained using an antibody recognizing MEKK1 and the DNA stain, DAPI. Immunofluorescence microscopy readily demonstrated that mitotic cells have significantly higher levels of MEKK1 expression than non-mitotic cells. To quantitate differences in anti-MEKK1 immunofluorescence in mitotic versus non-mitotic cells, deconvolved confocal three-dimensional images were constructed. Images of mitotic and non-mitotic cells were quantitated for anti-MEKK1 immunofluorescence. Fig. 3B shows that mitotic T47D cells express approximately 3-fold higher MEKK1 protein levels than non-mitotic cells. Similar results have been observed in other cell types (not shown).

To biochemically confirm the immunofluorescence analysis, T47D cells were treated with microtubule toxins to arrest cells at G2/M in the cell cycle. The G2/M block of treated cells was confirmed by cell cycle analysis using flow cytometry (not shown). Immunoblotting with the anti-MEKK1 antibody used for immunofluorescence demonstrated that MEKK1 protein levels were increased in drug-treated cells accumulated at G2/M, compared with untreated cell populations having cells randomly in all phases of the cell cycle (Fig. 3C, upper left panel). As a control, treatment of cells with etoposide, which induces DNA damage in S phase and blocks cells in G2/M of the
cell cycle, did not increase MEKK1 expression (Fig. 3C, upper right panel). In fact, etoposide, which acutely activates MEKK1, induces a loss of MEKK1 expression in T47D cells after several hours of drug exposure, resulting from the induction of caspase 3 cleavage of the 196-kDa MEKK1 protein (18, 19). We have found that taxol and nocodazole treatment of cells does not induce the cleavage of MEKK1 like that observed for DNA-damaging drugs. Thus, the regulation of MEKK1 protein levels and caspase cleavage in response to microtubule toxins and DNA-damaging drugs is different. Consistent with the increased levels of MEKK1 in T47D cells blocked at G2/M, the total MEKK1 activity is also increased by the microtubule toxin-induced block at G2/M (Fig. 3C, lower panel). In contrast, hydroxyurea and etoposide, which act in S phase of the cell cycle do not cause an increase in MEKK1 activity following prolonged cellular exposure to these drugs. Cumulatively, the findings demonstrate increased MEKK1 expression at the G2/M phase of the cell cycle and that microtubule toxins activate MEKK1. Thus, MEKK1 regulation is responsive to changes in microtubule organization in the cell.

**Targeted Disruption of MEKK1 Expression Causes Loss of JNK Activation in Response to Microtubule Reorganization**—We have targeted the disruption of MEKK1 expression by homologous recombination (20). MEKK1−/− ES cells do not express the MEKK1 protein. Treatment of wild type ES cells with either taxol or nocodazole activates MEKK1 measured by immunoprecipitation and *in vitro* kinase assay (Fig. 4, A and B). Importantly, no kinase activity is observed in immunoprecipitates from lysates of MEKK1−/− ES cells treated with nocodazole (Fig. 4B). This finding unequivocally demonstrates that our antibodies selectively immunoprecipitate MEKK1 and the summed MEKK1 immunofluorescence of G1/S versus M phase T47D cells. The difference in immunofluorescence between G1/S and M phase cells is significant at a p < 0.05 using the Student’s t test. C, upper panels, T47D cells were incubated for 20 h in 10% serum without (control) or with 0.05 μg/ml nocodazole, 1 μM taxol, 1 μM colchimide, or 0.1 μM etoposide. Flow cytometry analysis verified that taxol, nocodazole, and colchimide arrested cells at G2/M and etoposide-treated cells were blocked in G1/S. Cells were harvested and analyzed by immunoblotting for MEKK1 expression (left) in cells exposed to microtubule toxins. Lower panel, MEKK1 was immunoprecipitated and assayed from lysates prepared from T47D cells treated with 1 mM hydroxyurea, 0.1 mM etoposide, 1 μM taxol, 0.05 μg/ml nocodazole, or no drug for 20 h. MEKK1 activity was assayed using JNKKk-r in an *in vitro* kinase assay.
that MEKK1 is responsible for the JNKK phosphorylation in the in vitro kinase assay. As predicted from this result, JNK activation in response to the microtubule toxins nocodazole and taxol is lost in two independent MEKK1−/− clones (Fig. 5A). Interestingly, the disruption of the actin cytoskeleton with cytochalasin D activates the JNK pathway in MEKK1−/− and MEKK1+/+ ES cells (Fig. 5A). Thus, MEKK1 is absolutely required for JNK activation in response to microtubule but not actin fiber disruption. Re-expression of the 196-kDa MEKK1 protein by stable transfection of MEKK1+/+ ES cells with 5 μM taxol for 1 h, lysed, and assayed for JNK activity. C, immunoblot of MEKK2 protein by stable transfection of MEKK1−/−, MEKK2−/−, and MEKK2−/− cells, showing the loss of MEKK2 expression in ES cells having targeted disruption of the MEKK2 gene. D, cytochalasin D (2 μM) and nocodazole (0.5 μM/ml) were incubated with wild type (+/+), and MEKK2−/− (−/−) ES cells for 2 h. Cells were lysed and assayed for JNK activity using GST-c-Jun as substrate.

Targeted Disruption of MEKK1 Expression Increases Apoptosis in Response to Microtubule Toxins—We have shown that MEKK1−/− ES cells do not activate JNK following treatment with nocodazole or taxol. Prolonged shape change and microtubule disruption induce apoptosis in many cell types (3, 20). Fig. 6A shows that MEKK1−/− ES cells have a significantly greater apoptotic index in response to taxol, relative to MEKK1+/+ ES cells. Fig. 6B shows that temporally MEKK1−/− cells become apoptotic more rapidly than MEKK1+/+ cells. At a significantly slower rate, MEKK1+/+ cells will reach the same apoptotic index as taxol-treated MEKK1−/− cells. Thus, MEKK1 activation has a protective function in response to microtubule poisoning. To prove this fact, the two MEKK1−/− ES cell lines having MEKK1 expression re-established by stable transfection of a MEKK1 expression plasmid were tested for their sensitivity to taxol-induced apoptosis (Fig. 6A). Just as in the reconstitution of JNK activation, the re-expression of MEKK1 expression rescued the survival of cells exposed to taxol similar to wild type ES cells.

Cytochalasin D disrupts the actin cytoskeleton but has little effect on microtubule integrity. Cytochalasin D treatment of T47D cells strongly stimulates MEKK1 and JNK activities (Fig. 7A). Similarly, cytochalasin D treatment of wild type ES cells activates MEKK1 (Fig. 7B) and strongly activates JNK in both MEKK1+/+ and MEKK1−/− ES cells (Fig. 5A). The activation of JNK, independent of MEKK1 in ES cells, allowed the question of whether MEKK1 or JNK mediates the cell survival response to actin cytoskeleton poisoning. Fig. 7, C and D, shows that the sensitivity of ES cells to undergo cytochalasin D-induced apoptosis is virtually identical in a MEKK1−/− or MEKK1+/+ background. The increased apoptotic response of MEKK1−/− cells is specific to microtubule toxins and not actin cytoskeleton toxins. Thus, the enhanced sensitivity of MEKK1−/− cells to undergo apoptosis in response to microtu-

---

3 T. P. Garrington, T. Yujiri, S. Gibson, and G. L. Johnson, manuscript in preparation.
MEKK1 quantitated by acridine orange staining. The results represent the phosphorylation following exposure of ES cells to cytochalasin D. Cells were lysed and MEKK1-immunoprecipitated with anti-MEKK1 antibody. Immunoprecipitates were incubated with recombinant JNKK-1 and [γ-32P]ATP, resolved by SDS-polyacrylamide gel electrophoresis, and analyzed by autoradiography. Autophosphorylation of MEKK1 and phosphorylation of JNKK-1 is shown by arrows. For assaying JNK activity, GST-c-Jun was added to aliquots of the same cell lysates used for immunoprecipitation. B, wild type ES cells were incubated with 8 μg/ml cytochalasin D for the indicated times. Cells were lysed, MEKK1 antibody was added to lysates for immunoprecipitation, and MEKK1 activity was assayed as described in A. C and D, wild type (+/+ ) and MEKK1−/− (−/−) ES cells were incubated without or with 2 μg/ml cytochalasin D (CD) for 24 h (C) or 20 or 24 h (D). Apoptotic cells were quantitated by acridine orange staining. The results represent the mean ± S.E. of triplicate determinations and are representative of three independent experiments.

bule toxins may involve the loss of JNK activation regulated by MEKK1.

DISCUSSION

Changes in cytoskeletal dynamics stimulate signal transduction pathways. Several studies indicate that the microtubules play an integral part in regulating signaling pathways. We have shown that JNK is the dominant MAPK activated by microtubule toxins, including taxol and nocodazole. MEKK1 is absolutely required for JNK activation when cells are exposed to nocodazole and taxol. MEKK1 is similarly required for JNK activation in response to mild hyperosmolarity and cold stress, of cancer and other diseases. JNK pathway might define new drug targets for the treatment of cancer and other diseases.

REFERENCES

1. Folkman, J., and Hochberg, M. (1973) J. Exp. Med. 138, 745–753
2. Maroudas, N. G. (1973) Exp. Cell Res. 1, 104–110
3. Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M., and Ingber, D. E. (1997) Science 276, 1425–1428
4. Cleveland, D. W., and Sullivan, K. F. (1985) Annu. Rev. Biochem. 54, 331–365
5. Ferrua, B., Manie, S., Boglio, A., Shaw, A., Sontichon, N., Limouze, M., and Sacher, I. (1999) Cell. Immunol. 131, 391–397
6. Manie, S., Schmid-Aliana, A., Kubar, J., Furrus, B., and Rossi, B. (1993) J. Biol. Chem. 268, 13675–13681
7. Resnick, N., Collins, T., Atkinson, W., Bonthron, D., Dewey, C. F., Jr., and Gimbrone, A., Jr. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4591–4595
8. Kheradmand, P., Werner, E., Tremble, P., Agmon, M., and Werb, Z. (1998) Science 280, 899–902
9. Malek, A. M., and Izumo, S. (1990) J. Cell Sci. 100, 713–726
10. Jalali, S., Li, Y. S., Sotoodeh, M., Yuan, S., Li, S., Chien, S., and Shyy, J. Y. (1998) Arterioscler. Thromb. Vasc. Biol. 18, 227–234
Regulation of MEKK1 by the Cytoskeleton

12. Li, Y. S., Shyy, J. Y., Li, S., Lee, J., Su, B., Karin, M., and Chien, S. (1996) *Mol. Cell. Biol.* 16, 5947–5954
13. Rosette, C., and Karin, M. (1995) *J. Cell Biol.* 128, 1111–1119
14. Fanger, G. R., Johnson, N. L., and Johnson, G. L. (1997) *EMBO J.* 16, 4961–4972
15. Blank, J. L., Gerwins, P., Elliott, E. M., Sather, S., and Johnson, G. L. (1996) *J. Biol. Chem.* 271, 5361–5368
16. Gerwins, P., Blank, J. L., and Johnson, G. L. (1997) *J. Biol. Chem.* 272, 8288–8295
17. Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R., Johnson, G., and Karin, M. (1994) *Science* 266, 1719–1722
18. Widmann, C., Gerwins, P., Johnson, N. L., Jarpe, M. B., and Johnson, G. L. (1998) *Mol. Cell. Biol.* 18, 2416–2429
19. Widmann, C., Gibson, S., and Johnson, G. L. (1998) *J. Biol. Chem.* 273, 7141–7147
20. Yujiri, T., Sather, A., Fanger, G. R., and Johnson, G. L. (1998) *Science* 282, 1911–1914
21. Ridley, A. J., and Hall, A. (1994) *EMBO J.* 13, 260–261
22. Best, A., Ahmed, S., Kozma, R., and Lim, L. (1996) *J. Biol. Chem.* 271, 3756–3762
23. Coso, O. A., Chieariello, M., Yu, J., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) *Cell* 81, 1137–1146
24. Minden, A., Lin, A., Clare, F. X., Abo, A., and Karin, M. (1995) *Cell* 81, 1147–1157
25. Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G., and Frisch, S. M. (1997) *Cell* 80, 315–323