Stress-induced Inhibition of ERK1 and ERK2 by Direct Interaction with p38 MAP Kinase*

Received for publication, December 26, 2000, and in revised form,
Published, JBC Papers in Press, January 18, 2001,
DOI 10.1074/jbc.C000917200

Hong Zhang†, Xiaqing Shi‡, Maggie Hampong‡, Litsa Blanis§, and Steven Pelech‡§¶

From the 3Department of Medicine,
Koerner Pavilion, University of British Columbia,
Vancouver, British Columbia V6T 1Z3
and the 3Kinesux Bioinformatics Corporation,
Vancouver, British Columbia V6T 1Z4, Canada

We have identified a direct physical interaction between the stress signaling p38α MAP kinase and the mitogen-activated protein kinases ERK1 and ERK2 by affinity chromatography and coimmunoprecipitation studies. Phosphorylation and activation of p38α enhanced its interaction with ERK1/2, and this correlated with inhibition of ERK1/2 phosphotransferase activity. The loss of epidermal growth factor-induced activation and phosphorylation of ERK1/2 but not of their direct activator MEK1 in HeLa cells transfected with the p38α activator MK670(E) indicated that activated p38α may sequester ERK1/2 and sterically block their phosphorylation by MEK1.

Mitogen-activated protein (MAP)1 kinase modules are involved in the signal transduction of a wide variety of cellular responses in all eukaryotic organisms including proliferation, differentiation, and apoptosis (1). At least four distinct and parallel MAP kinase cascades have been identified, including extracellular signal-regulated kinases 1 and 2 (ERK1/2), the c-Jun N-terminal or stress-activated protein kinases (JNK/SAPK), and ERK5/big MAP kinase 1 (BMK1). It is well established that ERK1/2 are typically stimulated by growth-related stimuli through the Raf1/B-MEK1/2-ERK1/2 protein kinase cascade. The JNK and p38 MAP kinases are primarily activated by stress-related signals such as heat and osmotic shock, UV irradiation, and proinflammatory cytokines by means of the MAP kinase kinases, MKK3, -4, -6, and -7 (2–4). Whereas the selective activation of distinct MAP kinase pathways in response to different extra cellular stimuli has been extensively documented, there is increasing evidence for cross-talk between different MAP kinase pathways. A p38-dependent ERK1/2 activation was observed in several mammalian cell lines including the human embryonic kidney cell line HEK293 upon arsenite treatment (5). It was also found that inactivation of p38α by SB202190 treatment resulted in a delayed and prolonged activation of ERK1/2 in the human hepatoma cell line HepG2 (6). In both cases, MEK1 was implicated in the activation of ERK1/2. Here we report that in HeLa and HEK293 cells, stress stimuli lead to an inhibition of ERK1/2 via p38α. Phosphorylated p38α is capable of forming a complex with ERK1/2, and it prevents their phosphorylation by MEK1/2.

EXPERIMENTAL PROCEDURES

Construction of pGEX-p38α—The pGEX-p38α wild-type and p38α (AF) dominant negative mutant were constructed by subcloning the respective cDNA sequences into pGEX-4T vector (Amersham Pharmacia Biotech). The human p38α full-length sequences were obtained by digesting pcDNA3-Faggp38α wild-type and p38α (AF) plasmids, a generous gift of Dr. J. Han (the Scripp Research Institute, La Jolla, CA). pGEX-ERK1 was obtained by subcloning human ERK1 cdna full-length sequence into pGEX-2T vector (Amersham Pharmacia Biotech). The resulting GST fusion protein constructs were verified by DNA sequencing (7).

Expression of GST Fusion Proteins in Bacteria—GST fusion protein plasmids were transformed into DH5α bacteria. Expression of GST fusion proteins was induced by 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 3 h. GST fusion proteins were purified as described previously on glutathione-agarose (Sigma) and eluted from beads with reduced glutathione; GST tag was cleaved with thrombin when necessary (8).

GST Fusion Protein Pull-down—Two mg of rat brain lysate or 1 mg of HeLa cell lysate was mixed with 20 μg of immobilized GST-ERK1, GST-p38α, or GST alone on glutathione-agarose beads at 4 °C with rotation. After a 2-h incubation, beads were washed three times with 50 mM Tris (pH 8.0), 150 mM NaCl, and 1% Nonidet P-40 followed by separation of bound proteins by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to nitrocellulose membrane, and immunoblotting was performed with either anti-p38α or anti-ERK-1CT antibody (StressGen, Victoria, British Columbia, Canada).

In Vivo Association of ERK1 and p38α—HeLa cells cultured in Dulbecco’s minimum essential medium (DMEM, Life Technologies, Inc.) containing 10% fetal bovine serum were grown to 50–60% confluence and transfected with pcDNA3-p38α using SuperFect reagent (Qiagen, Mississauga, Ontario, Canada) as per the manufacturer’s instructions. For each 100-mm dish, 5 μg of plasmid DNA were introduced into cells using 30 μl of SuperFect reagent in serum-free medium. After a 3-h incubation, cells were starved in fresh serum-free DMEM. 24 h after transfection, cells were stimulated with anisomycin (Sigma) or arsenite (Sigma) alone or in combination with SB203580 (Calbiochem) in serum-free DMEM as indicated in the figure legends. Cells were lysed in 500 μl of lysis buffer (150 mM NaCl, 20 mM Tris pH 8.0, 0.5% (w/v) Nonidet P-40, 1 mM diithiothreitol (DTT), 20 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and sonicated for 30 s. Cell debris was removed by centrifugation at 13,000 rpm for 15 min at 4 °C. Protein concentration was determined by the Bradford assay (9). Cell lysates were precleared with 40 μl of protein A-Sepharose (Amersham Pharmacia Biotech) beads and then incubated with anti-ERK1-CT antibody (1 μg/ml of lysate) at 4 °C overnight. 40 μl of protein A-Sepharose beads were then added to precipitate immune complexes and washed with lysis buffer three times; immunoprecipitates were analyzed by immunoblotting with anti-p38α antibody.

MBP Kinase Assay—Anti-ERK1 immunoprecipitates bound to protein A beads were further washed twice in assay dilution buffer (25 mM
**Inhibition of ERK1/2 by p38 MAP Kinase**

**RESULTS AND DISCUSSION**

**Evidence for the Inhibition of ERK1/2 by p38**—By using antibody specific for the phosphorylated (activated) form of ERK1/2 in Western blotting studies, we observed that anisomycin treatment resulted in decreased ERK1/2 phosphorylation. By contrast, SB203580, a specific p38 MAP kinase inhibitor, increased ERK1/2 phosphorylation in HeLa cells similar to that observed with epidermal growth factor (EGF) exposure (Fig. 1A). These findings are consistent with the results in HepG2 cells (6) and indicate that p38 MAP kinase somehow exerts an inhibitory effect on ERK1/2 activation. Surprisingly, an increase of myelin basic protein (MBP) phosphotransferase activity associated with anti-ERK1 immunoprecipitates was detected upon anisomycin treatment, which could be inhibited by including SB203580 in kinase assay reactions (Fig. 1B). This observation indicated that the increase of MBP phosphotransferase activity precipitated by anti-ERK1 antibody upon anisomycin treatment was at least partially due to p38 MAP kinases. The apparent contradiction between the ERK1/2 phosphorylation state in Western blotting and the MBP phosphotransferase activity associated with anti-ERK1 immunoprecipitates could be potentially reconciled if p38 is coprecipitated with ERK1/2 in response to anisomycin treatment. Previously, we reported that ERK1 was found in immunoprecipitates of a p38 homologue in immature sea star oocytes (11). Based on these observations, we postulated that the direct interaction between p38 and ERK1/2 MAP kinases might play a role in coordinating the regulation of these two distinct MAP kinase pathways.

**Interaction of ERK1/2 and p38 in Vitro**—To examine whether a physical interaction occurs between p38 and ERK1, we expressed glutathione-S-transferase (GST) fusion proteins of the full-length human ERK1 and p38α in bacteria and used them to affinity purify proteins from a rat brain lysate. Immunoblotting with anti-p38α antibody revealed the presence of p38α protein on the GST-ERK1 beads at a level well above that bound to GST alone (Fig. 2A). Under similar conditions, GST-p38α was able to pull down both ERK1 and ERK2 proteins (Fig. 2B). These results indicated a specific, direct interaction between ERK1/2 and p38α.

**Interaction of ERK1/2 and p38 Is p38 Activity-dependent**—We next examined the interaction between ERK1 and p38α in HeLa cells treated with stimuli that specifically activate either the ERK1/2 or p38 MAP kinase pathways. The treatment of anisomycin resulted in a significant increase of p38α that was bound by GST-ERK1 (data not shown). No apparent difference of the amount of p38α protein purified by GST-ERK1 fusion protein between EGF-treated and untreated control HeLa cells was observed. Treatment of a similar time course as that of ERK1, p38α in control HeLa cells showed no apparent difference in the amount of p38α protein purified by GST-ERK1 fusion protein between EGF-treated and untreated control HeLa cells was observed. Treatment of HeLa cells with SB203580 prior to anisomycin stimulation diminished the association of ERK1 with p38α. The correlation of the enhancement of the binding of ERK1 and p38α with p38 kinase activation indicated that the interaction between these two MAP kinases was dependent upon the p38 but not the ERK1 activity status.

We further monitored the physical interaction of these two MAP kinases in mammalian cells by coimmunoprecipitation. HeLa cells were transfected with human p38α full-length DNA and then treated with anisomycin 24 h later. Cell lysate was prepared and precipitated with anti-ERK1-CT antibody. Anti-ERK1 immunoprecipitates were washed and immunoblotted with anti-p38α antibody in Western blot analysis for coprecipitated p38α. As shown in Fig. 3A, a much higher level of p38α protein was detected in the anti-ERK1 precipitates from anisomycin-treated HeLa cells than in those from untreated control cells. This finding was consistent with the GST fusion protein pull-down results and further confirmed the requirement of p38 kinase activity for the interaction between ERK1 and p38α.

---

**FIG. 1. Effects of anisomycin and SB203580 on the ERK1/2 phosphorylation and MBF kinase activity associated with anti-ERK1 immunoprecipitates.** A, HeLa cells were treated with anisomycin (10 μg/ml, 30 min), SB203580 (5 μM, 20 min), and EGF (100 ng/ml, 15 min; Calbiochem), respectively, and the phosphorylation of ERK1/2 was determined by Western blot analysis with anti-active ERK1 antibody (Santa Cruz Biotechnology). B, MBF kinase activity was determined in anti-ERK1 immunoprecipitates from anisomycin-, SB203580-, or EGF-treated HeLa cells. The MBF kinase activity associated with anti-ERK1 immunoprecipitates from anisomycin-treated cells could be inhibited by including 5 μM SB203580 in the kinase assay reaction. The data shown are the means ± S.E. of 4 experiments.

**FIG. 2. Interaction of ERK1 and p38α in rat brain lysate revealed by GST fusion protein pull-down experiments.** A, after incubation with rat brain lysate, GST-ERK1 and GST beads were washed and immunoblotted with anti-p38α antibody. B, ERK1/2 were found on GST-p38α beads after incubation with rat brain lysate.
Inhibition of ERK1/2 by p38 MAP Kinase

Figure 3. Coimmunoprecipitation of ERK1 and p38α in human cell lines in a p38 kinase activity-dependent manner. A, after transfection with pcDNA3-p38α and treatment with anisomycin (10 μg/ml, 30 min), HeLa cells were lysed, and the lysate was precipitated by anti-ERK1-CT antibody. Anti-ERK1 precipitates were immunoblotted with anti-p38α antibody. B, HEK293 cells were transfected with pcDNA3-p38α 24 h prior to treatment with arsenite (0.5 μM, 4 h) or a combination of SB203580 (5 μM, 60 min prior to arsenite) and arsenite (SB = Arsenite). Cell lysates were prepared and immunoprecipitation was performed with anti-ERK1-CT antibody. Immunoprecipitates were probed with anti-p38α antibody. p38α was present in higher levels in arsenite-treated cells than found in SB203580-treated and untreated control cells.

Similarly, interaction between ERK1 and p38α in HEK293 cells transfected with p38α was observed in a p38 activity-dependent manner (Fig. 3B). Compared with untreated control cells, an increasing level of p38α protein precipitated by anti-ERK1-CT antibody was found in arsenite-treated HEK293 cells. Pretreating HEK293 cells with SB203580 prior to arsenite reduced the level of p38α precipitated by anti-ERK1-CT to that of untreated control. These results confirmed a p38 activity-dependent association of p38α with endogenous ERK1 in mammalian cells.

Figure 4. Direct interaction between bacterially expressed ERK1 and p38α in vitro. A, following incubating with 20 μg of MKK6(E), 50 μg of eluted GST-p38α wild-type (WT) or GST-p38α (AF) was precipitated with glutathione-agarose beads. 50 μg of thrombin-digested GST-ERK1 was added to GST-p38α beads, and ERK1 that bound to p38α was detected by Western blotting with anti-ERK1-CT antibody. B, 50 μg of thrombin-digested p38α wild-type or p38α (AF) was first incubated with MKK6(E) and then incubated with 50 μg of GST-ERK1 immobilized on glutathione-agarose beads. GST-ERK1-bound p38α was detected by Western blotting with anti-p38α antibody. C, a similar blot to that shown in B was probed with anti-active p38α antibody (New England Biolabs, Beverly, MA).

Figure 5. p38α suppresses ERK1 activation by EGF without affecting MEK1 phosphorylation. HeLa cells were transfected either with pcDNA3-MKK6(E) (lane MMK6) or with pcDNA3-CAT (Control). 24 h later, both MKK6- and CAT-transfected cells were treated with EGF (100 ng/ml, 15 min). A, activation of ERK1/2 was determined by Western blotting with anti-active ERK1/2 antibody. B, phosphorylation of MEK1 was monitored by Western blotting with anti-active MEK1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

In summary, our study provides the first experimental evidence for the direct interaction between two MAP kinases lying in two distinct signaling cascades, raising the possibility that the direct interaction between p38α and ERK1 may play a role in coordinating activation of these two distinct MAP kinase pathways. These findings differ from those revealed by previous studies in that the cross-talk between the p38 and ERK1/2 signaling pathways was believed to mediate through upstream activating kinases of the ERK1/2 cascade. Moreover, the necessity of p38, but not ERK, phosphotransferase activity for the interaction between ERK1 and p38α indicates the cross-talk between these two MAP kinase pathways is a one-way process.

Combined with previous studies, our results indicate that the communication between p38 and the ERK1/2 pathways may act through two distinct modes. Active p38 may suppress ERK1/2 phosphotransferase activity either through inhibition of upstream activating kinases of ERK1/2 or through direct interaction between p38 and ERK1/2. Based on our observation of the direct association between p38α and ERK1 and the inhibitory effect of p38 on ERK1/2 phosphotransferase activity independent of MEK1 phosphorylation, we hypothesize that activated p38 may sequester ERK1/2 and sterically block phosphorylation of these MAP kinases by MEK1/2. The ability of activated p38 to regulate another protein kinase allosterically is not restricted to ERK1/2. We have recently reported that activated p38 can also form a complex with casein kinase CK2 in HeLa cells, but in that instance p38 activates CK2 (13).

Acknowledgments—We thank Dr. J. Han, the Scripps Research Institute (La Jolla, CA) for pcDNA3-flag-p38α wild-type and p38α (AF) dominant negative mutant. Constructs of constitutively active MKK6(E) mutants, pGEX-MKK6(E) and pcDNA3-MKK6(E), were provided by Dr. C. Glembocksi, San Diego State University and Dr. R. Davis, University of Massachusetts, respectively.
REFERENCES

1. English, J., Pearson, G., Wilsbacher, J., Swantek, J., Karandikar, M., Xu, S., and Cobb, M. H. (1999) Exp. Cell Res. 253, 255–270
2. Ono, K., and Han, J. (2000) Cell. Signal. 12, 1–13
3. Ichijo, H. (1999) Oncogene 18, 6087–6093
4. Leppa, S., and Bohmann, D. (1999) Oncogene 18, 6158–6162
5. Ludwig, S., Hoffmeyer, A., Goebeler, M., Killan, K., Hafner, H., Neufeld, B., Han, J., and Rapp, U. R. (1998) J. Biol. Chem. 273, 1917–1922
6. Singh, R. P., Dhanaw, P., Golden, C., Kapoor, G. S., and Mehta, K. D. (1999) J. Biol. Chem. 274, 19593–19600
7. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
8. Smith, D. B., and Johnson, K. S. (1988) Gene 67, 31–40
9. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
10. Laemmli, U. K. (1970) Nature 277, 680–685
11. Morrison, D. L., Yee, A., Paddon, H. B., Aebersold, R., and Pelech, S. L. (2000) J. Biol. Chem. 275, 34236–34244
12. Raingeaud, J., Whitmarsh, A. J., Barrett, T., Derijard, B., and Davis, R. J. (1996) Mol. Cell. Biol. 16, 1247–1255
13. Sayed, M., Kim, S. O., Salh, B. S., Issinger, O. G., and Pelech, S. L. (2000) J. Biol. Chem. 275, 16569–16573