In Vitro Enhancement of p38 Mitogen-activated Protein Kinase Activity by Phosphorylated Glia Maturation Factor*

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We previously demonstrated that glia maturation factor (GMF), a 17-kDa brain protein, can be phosphorylated in test tube by several protein kinases, and that endogenous GMF is rapidly phosphorylated upon stimulation of astrocytes by phorbol 12-myristate 13-acetate. We further observed that protein kinase A (PKA)-phosphorylated GMF is a potent inhibitor (IC₅₀ = 3 nM) of the ERK1/ERK2 (p44/p42) subfamily of mitogen-activated protein (MAP) kinase. We now report that, by contrast, PKA-phosphorylated GMF strongly enhances the activity of a related but distinct subfamily of MAP kinase, the p38 MAP kinase, showing an increase of 60-fold over baseline and an EC₅₀ of 7 nM. Non-phosphorylated GMF or GMF phosphorylated by other kinases exhibits only minimal effect. The intracellular interaction of PKA, GMF, and p38 is supported by the phosphorylation of GMF upon cellular stimulation by forskolin (blocked by PKA inhibitor) and by the co-immunoprecipitation of p38 with GMF from cell lysates. Withdrawal of nerve growth factor from PC12 leads to increased GMF phosphorylation with a time course similar to that reported for p38 activation. The results correlate well with a previous report that ERK and p38 carry out opposing functions and implicate GMF as a regulator of major cellular events.

Glia maturation factor (GMF) is a 17-kDa brain protein purified, sequenced, and cloned by us and our co-workers (1–3).

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Preparation of PKA-phosphorylated GMF—Recombinant GMF was phosphorylated with the catalytic subunit of PKA in an overnight incubation at room temperature in 40 μl of reaction mixture containing the following: 2 μg of GMF, 30 units of PKA, 25 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 10 μM okadaic acid, 0.02% sodium azide, and 10 mM ATP (non-radioactive). After the reaction, the amount of ATP was reduced by repeated (three cycles) dialution and centrifugation through an Amicon Microcon-10 membrane filter (10-kDa cut-off). The kinase assay buffer (see below) was used as the diluent. The volume after the last centrifugation was 20 μl. To this, an equal volume of PKI was added to inactivate the PKA. At this stage, the final concentration of PKI was 100 μM and that of ATP was 50 μM (200-fold reduction from initial concentration). Where “mock GMF-P” is indicated, the complete procedure (including incubation with PKA) was carried out in the absence of GMF. When non-phosphorylated GMF was used for comparative study, the GMF sample went through a simulated procedure for the preparation of GMF-P as above except for the omission of PKA.

Preparation of PKC-, RSK-, and CKII-phosphorylated GMF—Recombinant GMF was phosphorylated in a 40-μl reaction volume in the presence of either PKC (100 ng), RSK (2 μg), or CKII (0.5 milliunit) as a growth/differentiation factor, the lack of a leading peptide sequence and the strict intracellular localization of the protein leave room for speculation of an intracellular function. We recently demonstrated that recombinant GMF can be phosphorylated in test-tube by PKA, PKC, and CKII at the serine residue, and by RSK at the threonine residue (5). Endogenous GMF in astrocytes is phosphorylated at the serine and threonine residues within 15 min after stimulation by phorbol 12-myristate 13-acetate (5), suggesting an involvement in signal transduction. In an in vitro kinase assay, PKA-phosphorylated GMF but not the non-phosphorylated GMF shows a strong inhibitory effect on the activity of ERK1/ERK2 (p44/p42) MAP kinase, with a Kᵢ of 3 nM, making it the most potent ERK inhibitor ever reported (6). The question then arises as to whether phosphorylated GMF also affects the other subfamilies of MAP kinase, such as the JNK and the p38 varieties. Here we report that PKA-phosphorylated GMF is a strong enhancer of p38 kinase activity, exhibiting a stimulation of over 60-fold above the base-line value, and showing an EC₅₀ of 7 nM.
p38 Enhancement by GMF-P

described for PKA except for the following differences. When carrying out phosphorylation with PKC, the reaction mixture also contained 0.6 mM CaCl₂, 40 μg/ml phosphatidyl serine, and 0.5 μg/ml dioctanoylger- erol, while EGTA was omitted; after reaction with PKC, the PKC inhibitor peptide (4 μM) was added in place of PKI. When carrying out phosphorylation with RSK, the reaction mixture also contained 4 μM PKC inhibitor peptide, 0.4 mM PKI, and 4 μM calmidazolium. No inhibitor was added following incubation with either RSK or CKII. Where "mock GMF-P" is indicated, the incubation with the respective kinases was carried out in the absence of GMF.

Immune Complex Kinase Assay for ERK, JNK, and p38—The assay for kinase activity was carried out at 30 °C for 15 min in a 40-μl reaction volume containing the kinase assay buffer and the following additions: 10 μl of an immune complex containing either ERK1, JNK1, or p38 (prepared from C6 cell lysate as described under "Immunoprecipitation"); 2.5 μg of kinase substrate (either MBP, GST-c-Jun, or GST-ATF-2, respectively); 100 μl of an immune complex containing either ERK1, JNK1, or p38 (prepared from C6 cell lysate as described under "Immunoprecipitation"); 2.5 μg of kinase substrate (either MBP, GST-c-Jun, or GST-ATF-2, respectively); 100 μl of a 5% [32P]ATP (ATP 2000 Ci/mmol) solution, and various amounts of GMF or GMF-P as indicated. The amount of immune complex enzyme was equivalent to an extract of 1 mg of kinase substrate (either MBP, GST-c-Jun, or GST-ATF-2, respectively) and 100 μl of an immune complex containing either ERK1, JNK1, or p38 (prepared from C6 cell lysate as described under "Immunoprecipitation"); 2.5 μg of kinase substrate (either MBP, GST-c-Jun, or GST-ATF-2, respectively); 100 μl of a 5% [32P]ATP (ATP 2000 Ci/mmol) solution, and various amounts of GMF or GMF-P as indicated. The amount of immune complex enzyme was equivalent to an extract of 1 mg of kinase substrate (either MBP, GST-c-Jun, or GST-ATF-2, respectively) and 100 μl of an immune complex containing either ERK1, JNK1, or p38.

The residual amount of unlabelled ATP carried over from the earlier steps (see "Preparation of PKA-phosphorylated GMF") was less than 5% of the [32P]ATP. The standard kinase assay buffer consisted of: 100 mM Tris-HCl (pH 7.0), 40 mM EGTA, 40 mM sodium orthovanadate, 40 mM magnesium acetate, 1 mM dithiothreitol, 10 μM okadaic acid, and 30 μM calmidazolium. After incubation for 15 min, the reaction mixture was centrifuged to remove the immune complex containing ERK, JNK, or p38. The supernatant, which contained the kinase substrate along with a Hewlett Packard ScanJet II CX/T and analyzed with a Jandel Scientific software (Mocha).

RESULTS AND DISCUSSION

The three subfamilies of MAP kinase were studied using the immune complex kinase assay. To this end, C6 cell lysates were immunoprecipitated with rabbit polyclonal antibodies directed against ERK1, JNK1, and p38. The protein G-immobilized antibody/enzyme complex was then assayed for kinase activity using their respective kinase substrates: MBP for ERK1, c-Jun for JNK1, and ATF-2 for p38. When the assays were conducted in the presence of different amounts of GMF-P, we noticed that while GMF-P inhibited the activity of ERK1 (confirming our previous results), GMF-P strongly stimulated the activity of p38 (Fig. 1). A minor suppressive effect was observed with GMF-P on JNK.

We next obtained dose-response curves for the effects of PKA-phosphorylated GMF and non-phosphorylated GMF on p38. Figs. 2 and 3 show that increasing concentrations of GMF-P increased the activity of p38. At 22 nM GMF-P, a stimulation of 66-fold was detected relative to baseline level (absence of GMF-P). A half-maximal activity was observed at about 7 nM (EC₅₀), which is comparable to the IC₅₀ of 3 nM for its inhibitory effect on ERK (6). On the other hand, non-phosphorylated GMF had a detectable but much weaker enhancing effect. At 22 nM GMF-P and GMF at the 22 nM point was about 21-fold.

In order to ascertain whether the enhancing effect on p38 was specific to PKA phosphorylation of GMF, we conducted an experiment where recombinant GMF was separately phosphorylated by PKA, PKC, RSK, and CKII (the four enzymes known to phosphorylate GMF) in a comparable manner. Fig. 4 shows that none of the other enzymes could mimic the effect of PKA. In order to find out whether endogenous GMF is actually phosphorylated by PKA in the intact cell, we stimulated C6 cells with forskolin, a compound that raises cyclic AMP level. Fig. 5 shows that forskolin led to increased phosphorylation of intracellular GMF. That the increase was indeed mediated by PKA was evident in the abrogation of the change in the pres-
In Fig. 3, we show the coprecipitation of p38 with GMF when C6 cell lysate was immunoprecipitated with the antibody against GMF, implying the intracellular interaction of the two proteins. The relation of GMF with p38 was further strengthened when we demonstrated that withdrawal of NGF from PC12 cells stimulated the phosphorylation of GMF (Fig. 7).

In the absence of p38, none of the kinases (PKC, RSK, CKII, and PKA) was without appreciable direct effect on p38 activity under the experimental conditions. PKC phosphorylation corresponds to that previously reported for the activation of p38 in NGF-withdrawn PC12 cells (10). The MAP kinase superfamily is a highly conserved signal transducer present in both unicellular and multicellular eukaryotes (11, 12). In the mammalian system, the major subfamily of MAP kinase, the JNK/SAPK (stress-activated protein kinase), and the p38/RK isoforms, the homology among the three being about 45–50% (13). All the three subfamilies are serine/threonine kinases and are activated by dual phosphorylation on threonine and tyrosine residues, sharing the “Thr-Pro-Tyr” and “Thr-Gly-Tyr” motifs, respectively. The upstream transduction chain of ERK is ERK1/ERK2, which is located at a pivotal point in the signal transduction cascades, as it regulates by phosphorylation a vast number of cytoplasmic and nuclear targets. Other than ERK, the mammalian system possesses at least two other subfamilies of MAP kinase, the JNK/SAPK (stress-activated protein kinase), and the p38/RK isoforms, the homology among the three being about 45–50% (13). All the three subfamilies are serine/threonine kinases and are activated by dual phosphorylation on threonine and tyrosine residues, sharing the “Thr-Pro-Tyr” and “Thr-Gly-Tyr” motifs, respectively. The upstream transduction chain of ERK is well known, and consists of RPTK (receptor protein-tyrosine phosphorylation) kinases.
kinase) → GRB2 → SOS → RAS → RAF1 → MEK1/2. In addition, the ERK pathway is activated by PKC and receptor-associated G protein (14). On the other hand, the upstream transducers of JNK and p38 are less well understood, although they are known to be activated by pro-inflammatory cytokines and environmental stress (hyperosmolarity, heat, and UV) (15–17). The immediate upstream transducers for JNK and p38 are MEKK → MKK4, and MEKK → MKK3/4, respectively (18). Further up, the transduction chain for JNK involves Rac and Cdc42, two members of the Rho family of small GTPases, rather than Ras as in the case of ERK (19–22). Despite multiple targets they each have, the three MAP kinase subfamilies are in common in being able to phosphorylate specific transcription factors (with some overlap) following nuclear translocation. The nuclear substrate for ERK is ELK-1; those for JNK are c-Jun, ATF-2, and ELK-1; and that for p38 is ATF-2 (18, 23, 24).

A new light has recently been shed on the differential roles of the three subfamilies of MAP kinase, in that it has been reported that ERK on the one hand and JNK and p38 on the other exhibit opposing functions during growth factor-withdrawal apoptosis in PC12 cells. While the activation of ERK is correlated with the inhibition of apoptosis, the activation of JNK and p38 is correlated with its enhancement (10). These data are interpreted to mean that the ERK pathway is involved in cell survival while the JNK and p38 pathways are involved in cell death. In light of this finding, our observation that PKA-phosphorylated GMF regulates ERK and p38 in opposite directions, with the respective IC50 and EC50 in the same order of magnitude, implicates a role for intracellular GMF in the life/death decisions of a cell. One should be cautioned, however, that the bulk of the present work is carried out in vitro, and a definitive conclusion regarding the intracellular function of GMF must await further extensive experimentations. Nevertheless, the information presented in this paper opens up new avenues of inquiry that may eventually establish GMF as a major player in the modulation of signal transduction.

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REFERENCES
1. Lim, R., Miller, J. F., and Zaheer, A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3901–3905
2. Lim, R., Zaheer, A., and Lane, W. S. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5223–5227
3. Kaplan, R., Zaheer, A., Jay, M., and Lim, R. (1991) J. Neurochem. 57, 483–490
4. Zaheer, A., Fink, B. D., and Lim, R. (1993) J. Neurochem. 60, 914–920
5. Lim, R., and Zaheer, A. (1995) Biochem. Biophys. Res. Commun. 211, 928–934
6. Zaheer, A., and Lim, R. (1996) Biochemistry 35, 6283–6288
7. Chen, Y.-R., Meyer, C. P., and Tan, T.-H. (1996) J. Biol. Chem. 271, 631–634
8. Meyer, C. F., Wang, X., Chang, C., Templeton, D., and Tan, T.-H. (1996) J. Biol. Chem. 271, 8971–8976
9. Kase, H., Iwahashi, K., Nakanishi, S., Matuda, Y., Yamada, K., Takahashi, M., Murakata, C., Sato, A., and Kaneko, M. (1987) Biochem. Biophys. Res. Commun. 142, 436–440
10. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995) Science 270, 1326–1331
11. Davis, R. J. (1995) J. Biol. Chem. 268, 14553–14556
12. Seger, R., and Krebs, E. G. (1995) FASEB J. 9, 726–735
13. Davis, R. J. (1994) Trends Biochem. Sci. 19, 470–473
14. Daum, G., Eisenman-Tappe, I., Fries, H.-W., Troppmair, J., and Rapp, U. R. (1994) Trends Biochem. Sci. 19, 474–480
15. Han, J., Lee, J.-D., Tobias, P. S., and Ulevitch, R. J. (1993) J. Biol. Chem. 268, 25009–25014
16. Han, J., Lee, J.-D., Bibbs, L., and Ulevitch, R. J. (1994) Science 265, 808–811
17. Derijard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) Cell 76, 1025–1037
18. Derijard, B., Raingeaud, J., Barrett, T., Wu, I.-H., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) Science 267, 682–685
19. Olson, M. F., Ashworth, A., and Hall, A. (1995) Science 269, 1270–1272
20. Coss, O. A., Chiariello, M., Yu, J.-C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) Cell 81, 1137–1146
21. Minden, A., Lin, A., Claret, F.-X., Abo, A., and Karin, M. (1995) Cell 81, 1147–1157
22. Vojtek, A. B., and Cooper, J. A. (1995) Cell 82, 527–529
23. Gupta, S., Campbell, D., Derijard, B., and Davis, R. J. (1995) Science 267, 389–393
24. Whitmarsh, A. J., Shore, P., Sharrocks, A. D., and Davis, R. J. (1995) Science 269, 403–407

Fig. 7. Phosphorylation of endogenous GMF in PC12 cells following NGF withdrawal. PC12 cells were grown in RPMI 1640 supplemented with 10% horse serum and 5% fetal bovine serum in the presence of NGF (50 ng/ml). One week later, when the cells were fully differentiated in terms of neurite outgrowth, the cells were switched to phosphate-free, serum-free medium containing 2 μCi of [32P]orthophosphoric acid for a total of 8 h and harvested. Preceding the harvest, the cells were deprived of NGF for various times indicated in the graph. GMF was immunoprecipitated from cell lysate and subjected to SDS-PAGE and autoradiography as described in legend to Fig. 5. Relative densitometric values of the radioactive bands are shown. Results are representative of three experiments.
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