In Vitro Inhibition of MAP Kinase (ERK1/ERK2) Activity by Phosphorylated Glia Maturation Factor (GMF)†

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ABSTRACT: We report that recombinant glia maturation factor (GMF), a 17-kDa brain protein, inhibits the activity of mitogen-activated protein (MAP) kinase in the test tube assay, in particular the ERK1/ERK2 isoforms. A preliminary phosphorylation of GMF by protein kinase A (PKA) dramatically increases its inhibitory effect by over 600-fold ($K_i \sim 3$ nM), making it the most potent MAP kinase inhibitor ever reported. Immunoprecipitation of GMF from cell extracts using its specific antibody coprecipitates ERK (and *vice versa*), suggesting the association of the two proteins in the cell. The inhibitory effect of PKA-phosphorylated GMF is specific, as it does not suppress the activity of cdc2 kinase, another proline-directed kinase. Nor does it inhibit MAP kinase kinase (MEK) and MAP kinase-activated protein (MAPKAP) kinase-2, the two enzymes immediately upstream and downstream, respectively, of ERK. Of the other three enzymes that can phosphorylate GMF, only p90 ribosomal S6 kinase (RSK) enhances the inhibitory function of GMF on ERK; protein kinase C (PKC) and casein kinase II (CKII) are without effect. The inhibition of ERK by PKA-phosphorylated GMF suggests that GMF could be one of the mediators of the suppressive effect of the PKA pathway on the MAP kinase pathway. On the other hand, that RSK-phosphorylated GMF also inhibits ERK implies a negative feedback loop in the regulation of MAP kinase activity.

Although it is well established that a cascade of protein phosphorylation is responsible for signal transduction, the mechanism of regulation of the phosphorylation steps is poorly understood. We previously reported that the 17-kDa brain protein glia maturation factor (GMF)[1] can be phosphorylated in a test tube by a number of serine/threonine protein kinases, including protein kinase C (PKC), protein kinase A (PKA), casein kinase II (CKII), and p90 ribosomal S6 kinase (RSK) (Zaheer & Lim, 1992; Lim & Zaheer, 1995); and that in cultured astrocytes, phorbol ester stimulates a rapid and transient phosphorylation of endogenous GMF in the serine and threonine residues, implying a role in signal transduction (Lim & Zaheer, 1995). Although GMF is not a substrate for mitogen-activated protein (MAP) kinase, we now found that GMF inhibits the activity of ERK isoforms of the MAPK family and that the inhibition is enhanced over 600-fold after phosphorylation of GMF by PKA. In fact, the potency of phospho-GMF is 1 million times that of another MAPK inhibitor, a pseudosubstrate derived from myelin basic protein (Clark-Lewis et al., 1991).

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[1] Abbreviations: cdc, cell division cycle-dependent protein kinase; CK, casein kinase; ERK, extracellular signal-regulated kinase; GMF, glia maturation factor; GMF-P, phosphorylated GMF; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAP kinase, MAP kinase-activated protein kinase; MBP, myelin basic protein; MEK, MAPK/ERK kinase; PKA, protein kinase A; PKC, protein kinase C; PKI, PKA inhibitor peptide; RSK, ribosomal S6 kinase (p90); SAPK, stress-activated protein kinase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Materials. PKA (catalytic subunit from bovine heart) was purchased from Promega Corp. The following materials were from Upstate Biotechnology Incorporated (UBI): myelin basic protein (MBP); okadaic acid; ERK1 (MAP kinase p44) in the form of agarose-conjugated glutathione *S*-transferase (GST) fusion protein; RSK (RSK-2, p90 S6 kinase) and MAPKAP kinase-2, both from rabbit skeletal muscle; MAP kinase peptide substrate (APRTPGGRR) derived from MBP; MAPKAP kinase-2 peptide substrate (KKLNRTLSVA) derived from glycogen synthase; PKA inhibitor peptide PKI (TYADFIASGRTGRRNAI); PKC inhibitor peptide (RFARKGALRQKNV). The following were obtained from Santa Cruz Biotech: ERK2 (MAP kinase p42, *Xenopus*) and MEK-1 (human), both in the form of polyhistidine-tagged fusion protein produced in *Escherichia coli*; MEK-1 peptide substrate (ADPDHDHTGFLTEY-VATRWRR). PKC (from rat brain) was a product of Calbiochem. CKII was a recombinant human protein from Boehringer Mannheim. Inhibitor for calcium calmodulin-dependent protein kinase (compound R24571; calmidazolium) was purchased from Sigma Chemical Co. cdc2-like kinase (from bovine brain) and cdc2 peptide substrate (KTPKKAKKPKTPKKAKKL) derived from H1 histone were gifts of J. H. Wang (Lew et al., 1992). GMF was a recombinant human protein produced in *E. coli* (Kaplan et al., 1991). All the above materials were over 95% pure except MAPKAP kinase-2 which was 80% pure, and RSK which was 50% pure. Rabbit polyclonal antibody against ERK1 was from Santa Cruz Biotech. G2−09 was a monoclonal mouse antibody (IgG2a) against GMF and was affinity-purified with protein A. [γ-^{32}P]ATP (3000 Ci/mmo1) was purchased from NEN-Dupont.

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MAP Kinase Assay Using MBP as Substrate. The assay was carried out at room temperature for 10 min in a 100 μL reaction volume containing MAP kinase buffer and the following additions: 25 μg/ml agarose-conjugated MAP kinase (GST-ERK1), 250 μg/mL MBP, 6 μM PKI, 50 μM [γ-^{32}P]ATP (2000 cpm/pmol), and various amounts of GMF. MAP kinase buffer consisted of 100 mM Tris-HC1 (pH 7.0), 0.4 mM EGTA, 0.4 mM sodium orthovanadate, 40 mM magnesium acetate, 1 mM dithiothreitol (DTT), 10 μM okadaic acid, and 30 μM calmidazolium. The phosphorylated MBP was separated on SDS−PAGE and autoradiographed.

Preparation of PKA-Phosphorylated GMF. Recombinant GMF was phosphorylated with the catalytic subunit of PKA (Promega) in an overnight incubation at room temperature in 300 μL of reaction mixture containing the following: 15 μg of GMF, 1200 units of PKA, 25 mM Tris-HC1 (pH 7.5), 25 mM $MgCl_2$, 3.75 mM EGTA, 0.15 mM sodium vanadate, 1 mM DTT, 10 μM okadaic acid, 0.02% sodium azide, and 10 mM ATP (nonradioactive). In preparation for subsequent MAP kinase study, two further steps were taken. First, to reduce the amount of ATP, the reaction mixture was centrifuged (filtered) on an Amicon Microcon-10 membrane (10 kDa cutoff) and diluted with MAP kinase buffer. The filtration/dilution cycle was carried out 4 times. Each cycle was calculated to have a 10-fold reduction in the concentration of ATP, or an overall reduction of 10 000-fold. The final volume after this step was 30 μL. Second, to inactivate the PKA in the mixture, the sample was incubated with an equal volume (30 μL) of PKI (final concentration = 100 μM) for 15 min at room temperature before being used as a source of GMF-P in the MAP kinase assay.

Estimation of GMF on the SDS Gel. To determine the fraction of GMF that was actually phosphorylated after incubation with PKA, an aliquot of the reaction mixture was separated on SDS−PAGE and stained with Coomassie blue. Both GMF and GMF-P (slower mobility) bands were scanned with a Hewlett Packard ScanJet II CX/T and analyzed with Jandel Scientific software (Mocha). The areas under the densitometric curves were measured, and the relative value for each protein band was read against the linear region of a plot similarly prepared using serial dilutions of a known quantity of GMF. The same method was used to estimate the recovery of GMF after repeated Microcon filtrations during the elimination of the nonradioactive ATP (see above).

MAP Kinase Assay Using Peptide Substrate. The assay was carried out at 30 °C for 10 min in 100 μL of MAP kinase buffer containing a constant amount of ERK1, 125 μM [γ-^{32}P]ATP (2000 cpm/pmol), 100 μM MAP kinase peptide substrate, and various amounts of GMF or GMF-P as indicated. At the end of incubation, the reaction mixture was centrifuged once in Microcon-10 to separate the peptide substrate from the enzyme and GMF, and 25 μL of the filtrate was spotted on a piece of P81 phosphocellulose paper (Whatman). The paper was washed with two changes of 0.75% phosphoric acid for 1 h, air-dried, and counted in a liquid scintillation counter. Background radioactivity where MAP kinase substrate was omitted was subtracted.

Immunoprecipitation. Near-confluent cultures of C6 rat glioma cells were extracted with a lysis buffer (1 mL/T-75 flask) consisting of 1% Triton X-100, 50 mM Tris-HC1 (pH 7.5), 100 mM NaCl, 50 mM NaF, 0.1 mM sodium vanadate, 10 μM okadaic acid, 1 mM PMSF (phenylmethanesulfonyl flouride), 1 mM benzamidine, and 10 μg/mL each of the following: aprotinin, leupeptin, chymostatin, pepstatin A, and antipain. The supernatant from a 15-min centrifugation at 12 000 rpm was precleared with protein G−agarose and incubated at 4 °C overnight with 20 μg of the specific antibody per milliliter of extract. The sample was mixed and incubated at 4 °C for 1 h with 20 μL of a 50% suspension of protein G−agarose, and the insoluble immune complex was collected and washed 3× by brief centrifugations.

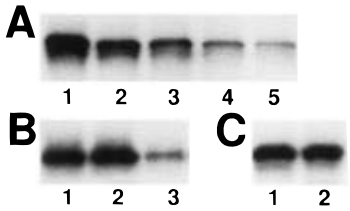

FIGURE 1: Inhibition of recombinant MAP kinase (ERK1) activity by recombinant GMF. MAP kinase (agarose-conjugated GST-ERK1) was assayed with MBP as described under Materials and Methods. Results presented are autoradiograms of ^{32}P-phosphorylated MBP after separation on SDS−PAGE. (A) Inhibition of kinase activity with respect to the amount of GMF. 1, No GMF; 2, 1 μg/mL; 3, 10 μg/mL; 4, 50 μg/mL; 5, 100 μg/mL. (B) Phosphatase activity ruled out. The assay was performed in the absence of GMF and phosphatase inhibitors (sodium vanadate and okadaic acid). After eliminating the agarose-conjugated enzyme by centrifugation, the supernatant containing the phosphorylated MBP was postincubated with GMF (50 μg/mL) for 30 min. 1, Complete reaction mixture except for omission of GMF (negative control, same as "A1"); 2, same as "B1" except for postincubation of phosphorylated MBP with GMF; 3, complete reaction mixture (positive control, same as "A4"). (C) Irreversible changes in MAP kinase ruled out. MAP kinase was preincubated with 50 μg/mL GMF in the kinase buffer for 15 min. After removing GMF by centrifugation and repeated washing, the pellet containing the agarose-conjugated enzyme was assayed with MBP in the absence of GMF. 1, Complete reaction mixture except for omission of GMF (negative control, same as "A1"); 2, same as "C1" except for preincubation of MAP kinase with GMF before using MAP kinase to phosphorylate MBP.

RESULTS

GMF Inhibits the Activity of MAP Kinase (ERK1). When recombinant human GMF was incubated with the recombinant MAP kinase isoform ERK1, in the presence of the MAP kinase substrate myelin basic protein (MBP), we observed a decrease in MBP phosphorylation in proportion to the amount of GMF present (Figure 1A). That this inhibition was not due to any phosphatase activity in GMF was verified by the absence of inhibition if MBP was incubated first with ERK1 and subsequently (in the absence of ERK1) with GMF (Figure 1B). We also ruled out the possibility of GMF imparting irreversible modifications on ERK1 by incubating ERK1 first with GMF and subsequently (in the absence of GMF) with MBP (Figure 1C).

Phosphorylation of GMF by PKA and Estimation of Phosphorylation Fraction. In order to study the effect of phosphorylated GMF on MAP kinase activity, we incubated recombinant GMF with the catalytic subunit of PKA in the presence of nonradioactive ATP according to the procedure specified under Materials and Methods and Table 1. As expected, the product appeared as a doublet in the SDS gel (Figure 2A) consisting of a band that retained the original mobility (17 kDa) and a slower-moving band which matched the radioactive band when [γ-^{32}P]ATP was used for phos-

Table 1: Conditions for Preparation of PKA-Phosphorylated GMF[a]

condition	GMF	PKA	ATP
(I) GMF-P	+	+	+
(II) mock GMF-P	−	+	+
(III) GMF	+	−	+
(IV) mock GMF	−	−	+

[a] This table shows the principal components used for the preparation of PKA-phosphorylated GMF (condition I: GMF-P) and its controls. For complete incubation mixtures and procedure, see Materials and Methods.

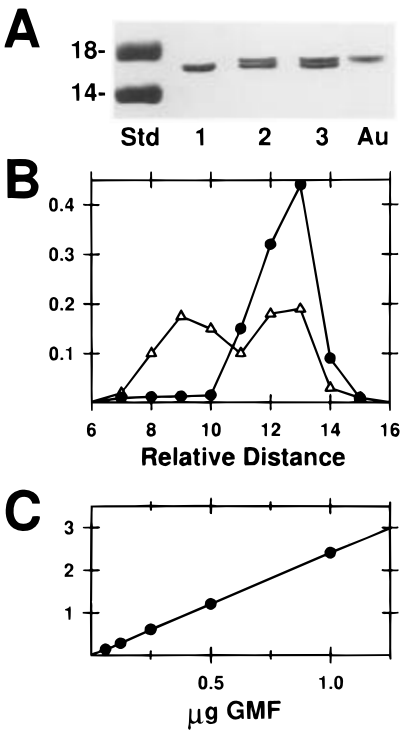

FIGURE 2: Phosphorylation of GMF by PKA. Recombinant GMF was incubated with PKA in the presence of nonradioactive ATP as described under Materials and Methods and in Table 1 (condition I). The product was analyzed by SDS−PAGE and densitometry for the determination of the phosphorylation fraction. (A) Coomassie blue pattern of SDS gel showing retarded mobility of phosphorylated GMF. Std, size standards with mass (in kDa) indicated on the left; 1, GMF not treated with PKA; 2 and 3 (duplicates), GMF treated with PKA showing phosphorylated (top band) as well as unphosphorylated (lower band) fractions (0.5 μg of initial GMF was used for each of lanes 1−3); Au, autoradiogram of ^{32}P-labeled PKA-phosphorylated GMF to denote position of GMF-P (matched to top band in lane 3). (B) Densitometric tracings (in arbitrary units) of gel pattern in "A". Filled circles correspond to lane 1 (single peak) while triangles correspond to lane 2. Integraded areas under the curves are shown in Table 2 for calculation of phosphorylation fraction. (C) Linear relationship between GMF standards and integrated areas when using GMF below 1 μg, confirming reliability of values in Table 2.

phorylation. Densitometric analysis of the protein bands showed a distribution of 54% and 46%, respectively, for phosphorylated and unphosphorylated GMF (Figure 2B and Table 2). The validity of the estimate was verified with a standard curve generated by untreated GMF samples applied to the gel (Figure 2C).

PKA Phosphorylation Enhances the Inhibitory Effect of GMF on ERK1. Endogenous MAP kinase was freshly immunoprecipitated from C6 cell extracts by means of anti-ERK1 antibody and assayed in the presence of phosphorylated GMF, prepared as described above. That the isolated

Table 2: Estimation of Phosphorylation Fraction of GMF after Treatment with PKA[a]

band position in SDS gel	lane 1[b] (untreated)	lane 2[b] (PKA-treated)	lane 3[b] (PKA-treated)
upper band (phosphorylated)	0.001 (0.1)	0.545 (54.1)	0.614 (54.7)
lower band (unphosphorylated)	1.157 (99.9)	0.463 (45.9)	0.508 (45.3)
total	1.158 (100)	1.008 (100)	1.122 (100)

[a] Recombinant GMF was incubated with PKA under conditions specified under Materials and Methods (see also condition I in Table 1) and analyzed with SDS−PAGE and densitometry. Values shown correspond to integrated areas under the curves from densitometric tracings (Figure 2B). Lanes 1−3 refer to GMF samples in the SDS gel (Figure 2A). Percentage distribution of phosphorylation fraction is indicated in parentheses. [b] Each lane contained an initial amount of 0.5 μg of GMF. Lanes 2 and 3 were duplicate samples.

MAP kinase was indeed the ERK1 isoform was verified by immunoblotting (Figure 3A). MAP kinase assay was conducted using the peptide substrate (derived from MBP). The results are presented in Tables 3 and 4and plotted in Figure 3B. As can be seen, the half-maximal inhibition concentration (IC_{50}) for PKA-phosphorylated GMF was 2.7 nM, compared with 1.8 μM for the nonphosphorylated GMF, showing an increase of 667-fold in potency. In Figure 3C, the double-reciprocal plot for phosphorylated GMF shows a family of curves consistent with a competitive inhibition, with a calculated K_i of 2.9 nM.

Association of MAP Kinase (ERK) with GMF Inside the Cell. In order to relate the in vitro observations to intracellular function, we demonstrated the coprecipitation of MAP kinase and GMF in cell extracts. Figure 4 shows that when C6 cell lysate was incubated with antibody against GMF, both GMF and MAP kinase (ERK) were immunoprecipitated. Likewise, when the same lysate was incubated with antibody against ERK1, both ERK1 and GMF were immunoprecipitated. The reciprocal precipitation between GMF and ERK provides strong evidence for their association inside the cell.

Specificity of MAP Kinase (ERK) Inhibition by Phosphorylated GMF. Table 5 demonstrates that the effect of PKA-phosphorylated GMF was target-specific, as it inhibited only ERK1 and ERK2, but not cdc2, MEK-1, and MAPKAP kinase-2. Table 6 shows that, of the four protein kinases that are known to phosphorylate GMF (Lim & Zaheer, 1995), only PKA and RSK strongly enhanced its inhibitory function on MAP kinase; PKC and CKII were without significant effect.

DISCUSSION

In this paper, we demonstrated that the 17-kDa brain protein GMF is an inhibitor of MAP kinase (ERK1/ERK2), and that the inhibition is substantially and dramatically enhanced by prior phosphorylation of GMF by PKA. In considering the validity of our results from which this conclusion was derived, we offer the following explanatory notes. First, to inactivate PKA after its phosphorylation of GMF (for GMF-P tubes), we added PKI at a concentration (100 μM) far exceeding its inhibition constant (K_i = 2 nM). The fact that the carried over PKA did not affect the MAP kinase assay is evident from data presented in Tables 3 and 4 (compare condition II with condition IV). Second, in order to study GMF and GMF-P under comparable conditions,

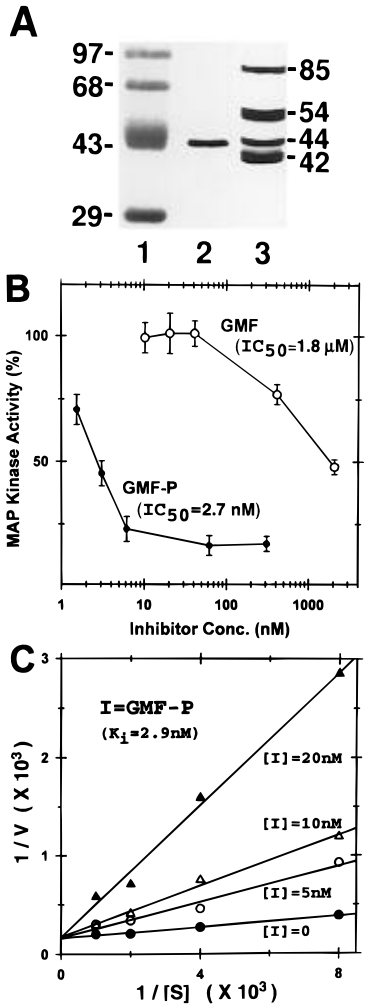

FIGURE 3: Inhibition of natural MAP kinase (ERK1) by PKA-phosphorylated recombinant GMF. (A) Western blot showing immunoprecipitated ERK1 for use in MAP kinase assay. ERK1 was precipitated from C6 cell lysate with anti-ERK1 antibody as described under Materials and Methods, and the immune complex was washed 3 times with MAP kinase buffer before assay. 1, Size standards; 2, anti-ERK1 precipitate; 3, cell extract not precipitated with antibody, showing all the ERK isoforms. Anti-pan-ERK (Transduction Labs) was used for immunoblotting. Molecular sizes (in kDa) are indicated on both sides. (B) Dose−response curves of nonphosphorylated and PKA-phosphorylated GMF (GMF-P) showing their inhibitory effects on MAP kinase (ERK1) activity. Error bars are SD of three determinations. MAP kinase was assayed with the peptide substrate as outlined under Materials and Methods, using ERK1 immune complex (see "A") as source of the enzyme, and using PKA-phosphorylated and nonphosphorylated GMF (see Figure 2) as source of the inhibitors. Each reaction mixture contained an amount of ERK1 equivalent to 1×10^6 cells. Concentration of GMF-P on *x*-axis represents the net amount of phosphorylated GMF, i.e., after correction for phosphorylation fraction (54%) and recovery from Microcon filtrations (70%) during the preparation steps. Concentration of GMF on *x*-axis is also corrected for recovery from Microcon filtrations (70%). MAP kinase activity on *y*-axis is expressed as percentage of base line activity by dividing cpm in "GMF-P tube" by cpm in paired "mock GMF-P tube" (i.e., condition I/condition II) for the GMF-P curve; and by dividing cpm in "GMF tube" by cpm in paired "mock GMF tube" (i.e., condition III/condition IV) for the GMF curve. (See Tables 1, 3, and 4 for details.) The small amount of activity contributed by GMF in the "GMF-P tubes" was not corrected for because of the huge difference between the effects of GMF and GMF-P. (C) Lineweaver−Burk plots showing competitive inhibition of MAP kinase (ERK1) activity by phosphorylated GMF. Assay conditions and calculations were as specified in "B". Substrate concentration [S] is expressed in μM; initial rate is expressed in terms of isotope incorporation (cpm) in the 10-min incubation.

Table 3: MAP Kinase (ERK1) Activity in the Presence of PKA-Phosphorylated GMF[a]

tube no.	GMF-P concentration (nM)[b]	condition I: GMF-P (cpm)	condition II: mock GMF-P (cpm)[c]
1	1.55	5660 ± 227	8007 ± 148
2	3.09	3684 ± 18	8148 ± 455
3	6.18	1637 ± 69	7091 ± 211
4	61.80	1303 ± 21	7888 ± 283
5	308.90	1251 ± 10	7283 ± 170

[a] MAP kinase (ERK1) was immunoprecipitated from C6 cells and assayed with its peptide substrate in the presence of PKA-treated and mock PKA-treated GMF (conditions I and II, respectively, as outlined in Table 1). Values are expressed as cpm per tube ± SD of three determinations. Each paired value was used to plot Figure 3B using the formula: % MAP kinase activity = (condition I/condition II) × 100. [b] Corrected for phosphorylation fraction (54%) and recovery from microcon filtrations (70%) during preliminary steps (see Materials and Methods). [c] Mean ± range = 7683 ± 465 (6%).

Table 4: MAP Kinase (ERK1) Activity in the Presence of Nonphosphorylated GMF[a]

tube no.	GMF concentration (nM)[b]	condition III: GMF (cpm)	condition IV: mock GMF (cpm)[c]
1	10.3	7635 ± 271	7690 ± 616
2	20.6	7846 ± 132	7360 ± 244
3	41.2	7393 ± 61	7316 ± 394
4	411.8	5603 ± 106	7200 ± 507
5	2059.0	3721 ± 84	7607 ± 295

[a] MAP kinase (ERK1) was immunoprecipitated from C6 cells and assayed with its peptide substrate in the presence of GMF and mock GMF (conditions III and IV, respectively, as outlined in Table 1). Values are expressed as cpm per tube ± SD of three determinations. Each paired value was used to plot Figure 3B using the formula: % MAP kinase activity = (condition III/condition IV) × 100 [b] Corrected for recovery from microcon filtrations (70%) during preliminary steps (see Materials and Methods). [c] Mean ± range = 7435 + 255 (3%).

GMF and also GMF-P tubes were treated with PKI and nonisotopic ATP. In a separate control experiment not presented under Results, we had determined that PKI even at the highest concentration present in our assay did not alter more than 5% of the MAP kinase activity. On the other hand, the residual nonisotopic ATP (from phosphorylation of GMF) carried over into MAP kinase assay did not amount to 1% of the radioactive ATP. Finally, the actual concentrations of GMF and GMF-P in the MAP kinase assays from which the IC_{50} and K_i values were obtained were carefully calculated using correction factors for phosphorylation fraction and for sample recovery during the preliminary steps. Whether or not the small amount of activity contributed by the unphosphorylated GMF in the GMF-P tubes was subtracted was immaterial in view of the huge difference in the inhibitory action of the two, and it would have amounted to less than 1% of the final values.

Among the signal transducers, MAP kinase occupies a unique and key position in that it is at the divergent point of several transduction pathways and because it affects downstream targets found in various subcellular compartments, both cytoplasmic and nuclear (Davis, 1993; Seger & Krebs, 1995). For such a strategically located enzyme, it is not unreasonable to assume that its activity is tightly controlled. Although specific phosphatases that can inactivate MAP kinase have been found (Ward et al., 1994; Hunter, 1995), endogenous compounds capable of directly enhancing or suppressing the activity of MAP kinase have not been previously reported. The closest examples one can cite are

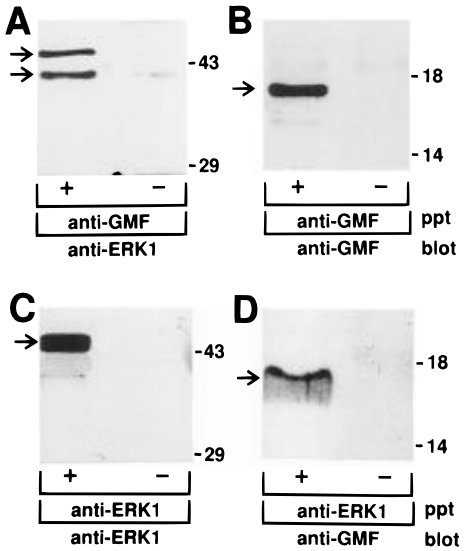

FIGURE 4: Coimmunoprecipitation of MAP kinase (ERK) and GMF from cell extracts. (A) C6 cell lysate immunoprecipitated with anti-GMF and subsequently immunoblotted with anti-ERK1, showing the presence of ERK1 and ERK2 (arrows) at 44 and 42kDa, respectively. (B) The same extract immunoprecipitated with anti-GMF and immunoblotted with anti-GMF, showing the presence of GMF (arrow) at 17 kDa. (C) The same extract immunoprecipitated with anti-ERK1 and immunoblotted with anti-ERK1, showing the presence of ERK1 (arrow) at 44 kDa. (D) The same extract immunoprecipitated with anti-ERK1 and immunoblotted with anti-GMF, showing the presence of GMF (arrow) at 17 kDa. A positive sign denotes the use of specific antibody for precipitation, and a negative sign denotes the use of nonimmuned IgG as a negative control. SDS−PAGE was carried out on a 14% gel. Molecular sizes (in kDa) are indicated on the right. Note that anti-ERK1 antibody blotted both ERK1 and ERK2, although it precipitated only ERK1.

Table 5: Effect of PKA-Phosphorylated GMF on Various Protein Kinases[a]

protein kinase (as target of inhibition)	kinase activity (% of base line)	
	GMF (as inhibitor)	GMF-P (as inhibitor)
ERK1	105 ± 16[b]	29 ± 10[b]
ERK2	95 ± 9	41 ± 11
cdc2	98 ± 4	103 ± 6
MEK-1	107 ± 2	103 ± 4
MAPKAP kinase-2	100 ± 7	100 ± 7

[a] PKA-phosphorylated GMF was prepared as described under Materials and Methods and tested for inhibitory effect on various kinases, using ERK1 as positive control, as detailed in Tables 1 through 4 for the study of MAP kinase activity. The kinase assay system was as described under Materials and Methods for MAP kinase using peptide as substrate. The following kinase/peptide substrate pairs were used as targets of GMF inhibition in a reaction volume of 100 μL: ERK1 (2.5 μg)/APRTPGGRR; ERK2 (1 μg)/APRTPGGRR; cdc2 (120 units)/KTPKKAKKPKTPKKAKKL; MEK-1 (1 μg)/ADPDHDHTGFLTEY-VATRWRR; MAPKAP kinase-2 (0.1 unit)/KKLNRTLSVA. All peptide substrates were used at 100 μM. All kinase and peptide concentrations were optimal as recommended by the suppliers. GMF and GMF-P were used at 20 nM (net concentration after correction for sample processing). None of the target kinases are known to phosphorylate GMF (Lim & Zaheer, 1995). [b] Mean ± SD of three determinations.

the inhibitory synthetic peptides (pseudosubstrates) modeled after myelin basic protein (Clark-Lewis et al., 1991). However, these peptides are not of physiologic significance as they are not naturally occurring materials, not to mention their high inhibition constant (about 3 mM). Our demonstration that GMF, a protein native to the cell and localized in the cytosol, is capable of inhibiting the two major and closely related isoforms of MAP kinase (ERK1 and EKR2) (Hanks & Hunter, 1995), with an inhibition constant in the range of 3 nM, is likely to be of biologic relevance. The potency of GMF is comparable to those of two other endogenous protein kinase inhibitors, namely, CIP1/WAF1, which inhibits cdk2 with a K_i of 8 nM (Harper et al., 1993), and PKI, which inhibits PKA with a K_i of 2 nM (Glass et al., 1989). Like GMF, neither of these inhibitors exhibits phosphatase activities.

Table 6: Comparing the Potency of Various Phospho-Isoforms of GMF on MAP Kinase Inhibition[a]

protein kinase (used to phosphorylate GMF)	MAP kinase (ERK1) activity (% of base line)
PKA	20 ± 3[b]
RSK-2[c]	25 ± 3
PKC[d]	92 ± 5
CKII	88 ± 7

[a] The experiment was carried out in a buffer consisting of 100 mM Tris-HC1, pH 7.0, 40 mM magnesium acetate, 0.4 mM sodium vanadate, 1 mM dithiothreitol, and 10 μM okadaic acid. The first step was conducted in 80 μL of the buffer containing 156 μM [γ-^{32}P]ATP (2000 cpm/pmol) with or without GMF (100 ng) and in the presence of one of the following enzymes: 100 ng of RSK-2, 2.5 ng of PKC, 100 microunits of CKII, and 8 units of PKA. (We have determined that all these enzymes phosphorylate GMF to the same extent.) After 10 min of incubation at 30 °C, the MAP kinase assay was initiated by the addition of 20 μL of the buffer containing 0.1 unit of MAP kinase (ERK1, UBI) and an amount of MAP kinase peptide substrate (APRTPGGRR) to make a final concentration of 100 μM in the 100 μL reaction volume. The MAP kinase assay was carried out for 15 min at 30 °C. At the end, the reaction mixture was transferred to a Microcon-10 membrane and centrifuged at 12 000 rpm for 15 min to separate the phosphorylated substrate peptide from the enzymes and GMF. An aliquot (25 μL) of the filterate was spotted on P81 paper, washed with two changes of 0.75% phosphoric acid for 1 h, air-dried, and quantitated by liquid scintillation counting of radioactivity. Results are expressed as a percentage with respect to the base line MAP kinase activity in the absence of GMF. None of the test enzymes showed an appreciable direct effect on the MAP kinase assay. [b] Mean ± SD of three determinations. [c] Because the commercial preparation of RSK-2 was only partially pure, the phosphorylation of GMF by RSK-2 was carried out in the presence of 0.5 μM PKA inhibitor peptide (PKI), 5 μM PKC inhibitor peptide (RFARKGALRQKNV), and 5 μM inhibitor for calcium calmodulin-dependent protein kinase (compound R24571). However, results were essentially the same when these inhibitors were omitted. [d] PKC reaction mixture also contained 0.6 mM $CaCl_2$, 40 μg/mL phosphatidylserine, and 0.8 μg/mL dioctanoylglycerol.

That the inhibitory action of PKA-phosphorylated GMF is target-specific is evident from the fact that it does not affect cdc2, another proline-directed kinase (Lew et al., 1992). Nor does it inhibit MEK-1 and MAPKAP kinase-2, the enzymes directly upstream and downstream, respectively, of ERK (Seger & Krebs, 1995; Stokoe et al., 1992). In the mammalian system, at least three parallel transduction pathways are formed by the MAP kinase superfamily (as characterized by the "TXY" motif): the ERK1/ERK2 pathway ("TEY" motif), the JNK/SAPK pathway ("TPY" motif), and the p38/RK pathway ("TGY" motif) (Davis, 1994). The current paper is confined to the study of ERK1/ERK2. Whether PKA-phosphorylated GMF also inhibits JNK/SAPK and p38/RK remains to be tested.

Of the four kinases known to phosphorylate GMF (Lim & Zaheer, 1995), only PKA and RSK, but not PKC and CKII, are able to strongly enhance its inhibition on MAP kinase. However, this does not preclude the possibility that PKC- and CKII-phosphorylated GMF may regulate other

signal transducers.

Our observation that PKA-phosphorylated GMF inhibits MAP kinase is in line with earlier reports that PKA inhibits (by phosphorylation) the function of Raf-1 (Schramm et al., 1994; Hafner et al., 1994), a signal transducer two steps upstream of MAP kinase, since both instances provide a mechanism by which the PKA pathway suppresses the MAP kinase pathway (Graves et al., 1993). Interestingly, RSK-phosphorylated GMF also inhibits MAP kinase. Since RSK is one step downstream of ERK (Seger & Krebs, 1995), this suggests a negative feedback loop for MAP kinase regulation: ERK→RSK→GMF→ERK. We should point out that the PKA-phosphorylated GMF and RSK-phosphorylated GMF are different phospho-isoforms, as we have determined that PKA phosphorylates a serine residue whereas RSK phosphorylates a threonine residue in GMF (Lim & Zaheer, 1995).

GMF was originally isolated as a growth/differentiation factor (Lim et al., 1989, 1990). However, its primarily cytosolic localization and the lack of a secretory peptide sequence leave room for the possibility of an intracellular function. Our earlier finding (Lim & Zaheer, 1995) that phorbol ester stimulates a rapid and transient intracellular phosphorylation of GMF and the current observation that certain forms of phosphorylated GMF strongly inhibit MAP kinase activity make this possibility all the more likely. At first sight, it may be difficult to understand that a protein can function both outside and inside the cell. However, precedents do exist. One example is fibroblast growth factor which, in addition to acting on the cell surface, was reported to have intranuclear function as well (Baldin et al., 1990). Another example is S-100 protein, a molecule that has long been known to be an intracellular calcium-binding protein but has recently been found to be also a neurotrophic factor (Kligman & Marshak, 1985). In contemplating the real biological function of GMF, two possibilities are considered. The first is that GMF exerts its action only inside the cell, and that the function observed by applying GMF to the extracellular environment is contingent on its uptake by, and entry into, the cell. This unified theory is attractive, but does not explain why some cell lines such as PC12 do not respond to exogenous GMF but rapidly phosphorylate endogenous GMF upon NGF stimulation (R. Lim and A. Zaheer, unpublished results). Further, many cell lines already have detectable amounts of GMF inside the cell, and the level may not alter significantly by the addition of GMF into the medium. The second possibility is that the two roles are independent. If so, it could well be that the normal function of GMF is limited to the inside of the cell, and that the extracellular role is an add-on function following cell injury and release of the protein (Nieto-Sampedro et al., 1988).

Lastly, it should be emphasized that the current study was based on test tube assay, using recombinant GMF and relatively pure enzymes and substrates under rigorously controlled conditions, and has come up with the conclusion that PKA- and RSK-phosphorylated GMFs are potent inhibitors of ERK1/ERK2. The extrapolation of these in vitro findings to the in vivo situation is as yet speculative, and will have to take into consideration such factors as the complexity of the transduction network, the multiple targets of MAP kinase, the possible existence of multiple phospho-isoforms of GMF and their compartmentation, the potential nuclear translocation of phosphorylated GMF, and the possibility that GMF may act at more than one transduction site. Likewise, the stoichiometry of interaction between GMF or GMF-P with MAP kinase cannot be meaningfully determined and interpreted without knowing these variables. Clearly, the total elucidation of the intracellular function of GMF is a challenging endeavor that will necessitate many more experiments in the future. We are ready to take on this challenge.

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