Protein Kinase A (PKA)- and Protein Kinase C-phosphorylated Glia Maturation Factor Promotes the Catalytic Activity of PKA*

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We observed previously that glia maturation factor (GMF), a 17-kDa brain protein, is rapidly phosphorylated in astrocytes following stimulation by phorbol ester, and that protein kinase A (PKA)-phosphorylated GMF is a potent inhibitor of extracellular signal-regulated kinase (ERK) and enhancer of p38; both are subfamilies of mitogen-activated protein (MAP) kinase, suggesting GMF as a bifunctional regulator of the MAP kinase cascades. In the current report, we present evidence that PKA-phosphorylated GMF also promotes (11-fold) the catalytic activity of PKA itself, resulting in a positive feedback loop. Furthermore, GMF phosphorylated by protein kinase C (PKC), but not by casein kinase II or p90 ribosomal S6 kinase, also activates PKA (7-fold). It appears that the mutual augmentation of GMF and PKA, and the stimulating effect of PKC, both serve to maximize the influence of PKA on the regulation of MAP kinase cascades by GMF. Using synthetic peptide fragments containing putative phosphorylation sites of GMF, we demonstrate that PKA is capable of phosphorylating threonine 26 and serine 82, whereas PKC, p90 ribosomal S6 kinase, and casein kinase II, can phosphorylate serine 71, threonine 26, and serine 52, respectively. The generation of various phospho-isoforams of GMF may explain its modulation of signal transduction at multiple locations.

Glia maturation factor (GMF) is a 17-kDa brain protein that was purified (1), sequenced (2), and cloned (3) in our laboratory. The highly conserved amino acid sequence of GMF contains several consensus phosphorylation sites, including sites for protein kinase A (PKA), protein kinase C (PKC), casein kinase II (CKII), and p90 ribosomal S6 kinase (RSK). In fact, we demonstrated previously that recombinant GMF can be phosphorylated by PKC, PKC, CKII, and RSK, whereas endogenous GMF is rapidly phosphorylated at both serine and threonine residues following stimulation of astrocytes by phorbol ester (4). Thus, it is possible that various phosphorylated isoforms of GMF may be generated inside the cell by kinases differentially stimulated by external stimuli. Although no isoform of GMF, phosphorylated or nonphosphorylated, possesses kinase or phosphatase activity (4, 5), we have shown recently that PKA-phosphorylated GMF is a potent inhibitor of ERK (5) and also an enhancer of p38 (6); both are subfamilies of MAP kinase, suggesting that GMF is a bifunctional regulator of the MAP kinase cascades. To find out any additional locations where GMF can regulate, we have tested the effect of GMF and GMF-P on PKA. In the present communication, we demonstrate the stimulatory effect of two phosphorylated isoforms of GMF, PKA- and PKC-phosphorylated GMF, on the catalytic activity of PKA.

EXPERIMENTAL PROCEDURES

Materials—PKA (catalytic subunit purified from bovine heart) was obtained from Promega Corp. PKC (from rat brain) was a product of Calbiochem. RSK (RSK-2), from rabbit skeletal muscle, was obtained from Upstate Biotech. Recombinant human CKII was from Boehringer Mannheim. PMA and kemptide (PKA substrate LRRASLG) were from Sigma. Monoclonal anti-PKA (catalytic subunit) antibody and polyclonal anti-Pan PKC antibody were from Transduction Labs and Upstate Biotech, respectively. GMF was a recombinant human protein (3) from Ershcherichia coli (over 98% pure). CT-11 was a mouse monoclonal antibody (IgG1) against a synthetic peptide corresponding to the C-terminal 11 amino acid residues of human GMF and was affinity purified with protein A. 125I-ATP (3000 Ci/mmol) was purchased from DuPont NEN. GMF peptides for phosphorylation experiments were custom synthesized by Genemed Biotech (South San Francisco, CA), except peptides I and IV, which were gifts of R. A. Copelend of DuPont Merck Pharmaceutical.

Preparation of Phosphorylated GMF—Recombinant GMF was phosphorylated by various protein kinases as follows. GMF (2 µg) was incubated overnight at room temperature in a 40-µl reaction mixture containing the following: 25 mM Tris-HCl, pH 7.5, 25 mM MgCl2, 3.75 mM EGTA, 0.15 mM sodium vanadate, 1 mM dithiothreitol, 10 µM okadac acid, 0.02% sodium azide, and 10 mM ATP (nonradioactive) in the presence of either PKA (80 units), PKC (100 ng), RSK (2 µg), or CKII (0.5 milliunit). (The concentrations of the kinases were adjusted to obtain comparable degrees of GMF phosphorylation, as determined by previous autoradiography.) The reaction mixture for PKA also contained 0.6 mM CaCl2, 40 µg/ml phosphatidyl serine, 0.8 µg/ml diocanoylglycerol, and no EGTA. The reaction mixture for RSK also contained 4 µM PKC inhibitor peptide (RFARKGALKRNAK), 0.4 µM PKA inhibitor peptide (TYADFIASGRTGRRNAI) and 4 µM calmidazolium. In control tubes (mock-GMF), the reaction was carried out in the absence of GMF. At the end of the overnight incubation, GMF was separated from ATP and the kinases by immunoprecipitation using the monoclonal anti-GMF antibody (CT-11). For immunoprecipitation, each reaction mixture was diluted with 1 ml of buffer A (see below) containing 1% Triton X-100. The incubation with CT-11 (10 µg/ml) was for 1 h at room temperature, followed by one additional hour of incubation with 20 µl of a 50% suspension of protein G-agarose. The immune complex containing GMF and GMF-P was collected and washed three times by brief centrifugations before being used in the PKA assay system. An aliquot of the immune complex was analyzed on SDS-polyacrylamide gel, stained with Coomassie Blue, and scanned with a Hewlett Packard ScanJet II CX/T to calculate the percentage of GMF phosphorylated by various kinases and the percentage of GMF recovered by immunoprecipitation, as described earlier (5).

PKA/Kemptide Assay—Assay for PKA activity was carried out in a 40-µl reaction volume containing 100 µM kemptide substrate, 0.1 unit PKA (Promega), various amounts of GMF or GMF-P (both as immune complex), in 25 mM Tris-HCl, pH 7.5, 25 mM MgCl2, and 125 µM phosphatase activity (4, 5), we have shown recently that PKA-phosphorylated GMF may be generated inside the cell by kinases differentially regulated by PKA (Promega), and veterans Affairs Medical Center, Iowa City, Iowa 52242.

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The abbreviations used are: GMF, glia maturation factor; GMF-P, GMF-phosphorylated GMF; PKA, protein kinase A; PKC, protein kinase C; CKII, casein kinase II; RSK, ribosomal S6 kinase (p90); ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; PMA, phorbol 12-myristate 13-acetate.

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near-confluent C6 cells were harvested into buffer A (1 ml/T-75 flask) and immunoprecipitation steps as for GMF-P (5).

Kinase Detection by "in-Gel" Assay—The analysis of kinases present in C6 cell extract capable of phosphorylating GMF was carried out by an "in-gel" kinase assay as described by others (7, 8). For this purpose, near-confluent C6 cells were harvested into buffer A (1 ml/T-75 flask) consisting of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 0.1 mM sodium vanadate, 10 μM okadaic acid, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of the following proteinase inhibitors: apro tinin, leupeptin, chymostatin, pepstatin A, and antipain. The cells were sonicated in the cold at 50 W for two bursts of 30 s each and then centrifuged at 4°C at 100,000 g. The supernatant was subjected to in situ denaturation-renaturation of protein kinases was carried out by first incubating the gels in a solution containing 6% guanidine-HCl in 50 mM Tris-HCl, pH 8, and 5 mM dithiothreitol for 1 h at room temperature, and then incubating overnight at 4°C in 50 mM Tris-HCl, pH 8, 5 mM dithiothreitol, and 0.04% Tween 20. The kinase assay was performed by incubating the gels in a kinase assay buffer (40 mM HEPES, pH 8, 2 mM dithiothreitol, 0.1 mM EGTA, and 5 mM magnesium acetate) containing 50 μM ATP and 50 μCi [γ-32P]ATP for 1 h at room temperature. The gels were washed extensively with several changes of 5% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate. The gels were dried and exposed to XAR film (Eastman Kodak Co.) with intensifying screen at −70°C for 1 day. The kinase activity was revealed by incorporation of 32P into matrix-bound GMF, and the size of the individual kinase was estimated from the known prestained protein markers (Bio-Rad) run in the same gel. A piece of control gel where GMF was omitted was processed in the above manner to detect autophosphorylation of the kinase bands.

Phosphorylation of GMF Peptides—A number of peptide fragments were synthesized according to the sequence of human GMF, each containing a single serine or threonine residue corresponding to the putative phosphorylation site of either PKA, PKC, RSK, or CKII. Phosphorylation studies were carried out in a reaction mixture of 40 μl containing 200 μM of a synthetic peptide, 125 μM [γ-32P]ATP (2000 cpm/pmol), and an appropriate amount of protein kinase as described above ("Preparation of Phosphorylated GMF"). The reaction was carried out at 30°C for 10 min. The kinase gels were sonicated in the cold at 50 W for two bursts of 30 s each and then centrifuged at 4°C at 100,000 g. The supernatant was subjected to in situ denaturation-renaturation of protein kinases was carried out by first incubating the gels in a solution containing 6% guanidine-HCl in 50 mM Tris-HCl, pH 8, and 5 mM dithiothreitol for 1 h at room temperature, and then incubating overnight at 4°C in 50 mM Tris-HCl, pH 8, 5 mM dithiothreitol, and 0.04% Tween 20. The kinase assay was performed by incubating the gels in a kinase assay buffer (40 mM HEPES, pH 8, 2 mM dithiothreitol, 0.1 mM EGTA, and 5 mM magnesium acetate) containing 50 μM ATP and 50 μCi [γ-32P]ATP for 1 h at room temperature. The gels were washed extensively with several changes of 5% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate. The gels were dried and exposed to XAR film (Eastman Kodak Co.) with intensifying screen at −70°C for 1 day. The kinase activity was revealed by incorporation of 32P into matrix-bound GMF, and the size of the individual kinase was estimated from the known prestained protein markers (Bio-Rad) run in the same gel. A piece of control gel where GMF was omitted was processed in the above manner to detect autophosphorylation of the kinase bands.

**RESULTS AND DISCUSSION**

In the present study, we surveyed the effects of various phosphorylated isoforms of GMF, obtained by the action of PKA, PKC, RSK, and CKII, on the activity of the catalytic subunit of cAMP-dependent protein kinase (PKA). Recombinant GMF was first reacted with each of the kinases to obtain GMF-P, which was then purified by precipitation with an anti-GMF monoclonal antibody. The immune complex in various amounts was then added to a test system consisting of PKA, [γ-32P]ATP, and the PKA-specific substrate kemptide. Results in Fig. 1 show significant increase in kemptide phosphorylation by PKA in the presence of GMF-P and PKC-phosphorylated GMF, but not GMF phosphorylated by RSK or CKII. Fig. 2 compares the dose-response curves of PKA- and PKC-phosphorylated forms of GMF, along with that of unmodified GMF. PKA-phosphorylated GMF showed a maximum stimulation of 11-fold over the baseline value (in the absence of any GMF); the corresponding value for PKC-phosphorylated GMF was 7-fold. On the other hand, nonphosphorylated GMF showed only a minor stimulatory effect, with a maximum stimulation of 1.6-fold. At 20 nM, the increase in activity over nonphosphorylated GMF was 6.7-fold for PKA-phosphorylated GMF and 4.1-fold for PKC-phosphorylated GMF. The half-maximal activity (EC50) for both phosphorylated forms of GMF was about 3 nM. In the next experiment, we tested the effects of multiple phosphorylation on the function of GMF. GMF was phosphorylated by a combination of PKA and one or more other kinases and subsequently tested in the PKA/kemptide assay. As shown
PKA Enhancement by GMF-P

FIG. 3. Stimulation of PKA activity by GMF phosphorylated by a combination of kinases. GMF was phosphorylated and tested as in Fig. 1 except that two or more kinases were included during the phosphorylation of GMF (PKA inhibitor and EGTA were omitted). Results are presented as in Fig. 1. Note the presence of potentiation when GMF was double-phosphorylated by PKA and PKC.

in Fig. 3, neither RSK nor CKII augmented or inhibited the action of PKA-phosphorylated GMF, although a small increase in function was noted when the three kinases were combined. However, a bigger change was seen when PKA was used together with PKC, showing an increment in GMF function larger than the sum of their individual effects, indicating the presence of synergism.

To find out which kinases in the cell are potentially capable of phosphorylating GMF, we performed an “in-gel” kinase assay. In this procedure, C6 rat glioma cells were stimulated with PMA, and the cell lysate was subjected to SDS-polyacrylamide gel electrophoresis using a piece of gel embedded with recombinant GMF. After electrophoretic separation, the protein bands capable of phosphorylating GMF were revealed by incubation with 32P-labeled ATP and autoradiography. Fig. 4 shows that there were at least three defined protein bands capable of phosphorylating GMF, two of which were identical in electrophoretic mobility with PKA and PKC, whereas a third band was unidentified. Although the results may not necessarily reflect the in vivo situation, when taken together with our earlier findings that phorbol ester (activator of PKC) and forskolin (activator of PKA) stimulate the phosphorylation of endogenous GMF in live cells (4, 6), a strong argument can be made for the activation of endogenous GMF by endogenous PKC and PKA.

To gain an insight into the nature of the phospho-isofoms of GMF created by the action of PKA, PKC, RSK, and CKII, an experiment was conducted where synthetic GMF peptide fragments carrying single putative phosphorylation sites were tested as substrates for each of the four kinases (Table I). Peptide I contains threonine 26, which fits the R/R/K/X(S/T) consensus for PKA (10) and the RXRX(S/T) consensus for RSK; peptide II contains serine 52, which fits the S/TXTEX consensus for CKII (11); peptide III contains serine 71, which fits the S/TX/K/R consensus for PKC (11); peptide IV contains serine 82, which fits the RX(S/T) consensus for PKA (11). The results of the experiment confirmed that threonine 26 is a target for both PKA and RSK, that serine 52 is for CKII, that serine 71 is for PKC, and that serine 82 is for PKA. In other words, PKA appears to phosphorylate at two sites, whereas the other three kinases phosphorylate at single sites, assuming that the four peptides cover all the targets for the four kinases. Except for an overlap between PKA and RSK at threonine 26, there are no overlaps among other kinases.

The results of peptide phosphorylation are in conformity with our previous phosphoamino acid analysis, demonstrating the formation of phosphoserine as a result of PKC and CKII action on intact GMF, and the formation of phosphothreonine as a consequence of RSK action (4). However, a discrepancy exists with PKA in that we formerly detected phosphoserine but not phosphothreonine (4). We believe this was due to incomplete hydrolysis of the PKA-phosphorylated GMF, resulting in the release of phosphoserine but not phosphothreonine. A search of literature (12) revealed that the release of phosphoserine is virtually complete (94%) with a 1-h acid hydrolysis, whereas the release of phosphothreonine peaks at 4 h, and even so, the release is only partial (14%). In fact, we have now verified the time-dependent release of the two phosphoamino acids from PKA-phosphorylated GMF (results not shown).

By correlating the data on peptide phosphorylation with the in vitro effects of the GMF-P isoforms, one may speculate which of the putative sites is essential for which function. For example, PKA and RSK share the Thr26 site, but the Ser82 site is unique to PKA. Because only PKA but not RSK-phosphorylated GMF can enhance the activity PKA, it appears that Ser82, either alone or in combination with Thr26 (to be determined), is responsible for PKA activation. On the other hand, because the PKC target site does not overlap the PKA sites, yet PKC-phosphorylated GMF also activates PKA, it appears that the Ser71 site alone is also sufficient for activating PKA. That both Ser92 and Ser71 sites are independently involved in PKA activation is consistent with the fact that double phosphorylation of GMF by PKA and PKC showed synergistic effect (Fig. 3). Likewise, among the isoforms of GMF-P, only the PKA-phosphorylated form can enhance the activity of p38. Therefore, it appears that the S82 site (unique to PKA) is necessary for p38

FIG. 4. Detection of protein kinase activity in C6 cell lysate by “in-gel” kinase assay. C6 cells were stimulated with PMA (100 ng/ml) followed by cell extraction as described under “Experimental Procedures.” The cell lysates were electrophoresed on 12% SDS-polyacrylamide gel embedded with 0.1 mg/ml recombinant GMF (A) or without GMF (B). The gels were denatured-renatured in situ, and phosphorylation reaction was carried out in the presence of 50 μM [γ-32P]ATP. Kinase activities in the protein bands were revealed by autoradiography for the presence 32P-labeled GMF. C and D are immunoblots of the same cell extract probed with antibodies against PKA catalytic subunit (C) or pan-PKC (D). Molecular size standards (in kDa) are indicated on the sides. The three lanes in each panel represent the time after PMA stimulation of the cells. Note that after subtraction of the radioactive bands in B (which represents autoprophosphorylation of the kinase bands in the absence of added GMF), three bands stand out in A that are capable of phosphorylating GMF: band a corresponds to PKC (~80 kDa); band b is unidentified (~60 kDa); and band c corresponds to the catalytic subunit of PKA (~40 kDa). Also note that the activities of the three bands increased 15 min after PMA stimulation.

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Phosphorylation of GMF peptides containing putative targets of protein kinases

Four peptides were synthesized according to the human sequence of GMF. Each peptide contained a single serine or threonine residue (in parentheses) that by consensus was a probable phosphorylation site of one or more of the kinases known to phosphorylate GMF. Each peptide was tested as a substrate for the kinases according to the method described under “Experimental Procedures,” and the results of phosphorylation are expressed in terms of $^{32}$P radioactivity.

| GMF peptide testeda | PKA | PKC | RSK | CKII |
|---------------------|-----|-----|-----|------|
| I. $^{21}$RFRKET/NNAA$^{52}$ | 34,140 ± 671 | 0 | 5,470 ± 705 | 0 |
| II. $^{47}$ELEGVS/PEDEL$^{56}$ | 0 | 0 | 0 | 0 |
| III. $^{66}$RFIVY/SYKYQ$^{75}$ | 0 | 25,380 ± 929 | 0 | 0 |
| IV. $^{77}$DDGRVS/YPLC$^{86}$ | 7,630 ± 374 | 0 | 0 | 0 |

a Synthetic peptides were N-acetyl blocked and C-amido blocked. Numbers correspond to locations in the intact GMF protein.
b Values are mean ± SD of triplicate reaction tubes from one set of experiment. A second experiment yielded similar results.
c A 15-mer version of peptide IV stretching from amino acid position 75 (Gln) to 89 (Phe) gave the same phosphorylation value.

discussion

activation. Whether it requires the participation of T26 remains to be seen. The inhibition of ERK activity is shared by PKA- and RSK-phosphorylated GMF. We, therefore, speculate that perhaps the T26 site is both necessary and sufficient for ERK inhibition.

Although the information is far from complete, our study on synthetic peptides does reveal important clues to the structure-function relationship of GMF-P isoforms. Future work with phosphopeptide analysis (from intact GMF protein) and site-directed mutagenesis (where putative phosphorylation sites are substituted) should provide a more definitive conclusion.

In many cell types, PKA suppresses the Ras/ERK transduction pathway by phosphorylating and inactivating Raf (two steps upstream of ERK/14, 16). We found previously that PKA can potentially suppress the Ras/ERK pathway in another manner, i.e., by phosphorylating GMF which then inhibits ERK (5). In this regard, the positive feedback of PKA-phosphorylated GMF on the activity of PKA as reported in this paper takes on additional meaning. Because the major role of endogenous GMF appears to be a bifunctional regulator of the MAP kinase cascades (inhibition of ERK and promotion of p38), one can speculate that the mutual augmentation between GMF and PKA serves to maximize the influence of PKA on the regulation of MAP kinase in GMF. In addition, the phosphorylation of GMF by PKC provides yet another trigger for the activation of GMF by PKA. The fact that the IC$_{50}$ of GMF-P on ERK and the EC$_{50}$ of GMF-P on p38 and PKA all fall within the same lower nanomolar range implies that the multiple interplays can take place simultaneously (barring compartmentation).

In the nervous system, the PKA pathway conveys messages imparted by the neurotransmitters and neuuropeptides, whereas the ERK MAP kinase cascade channels information from the neurotransphins and other growth factors. More recently, the Janus kinase/signal transducer and activator of transcription (Jak/STAT) pathway emerges as another signaling traffic important in the nervous system, relaying messages from the neuropoietic cytokines, such as ciliary neurotrophic factor and leukemia inhibitory factor (15, 16). Furthermore, the Jak/STAT pathway has been found to elicit exploratory behavior initiated by leptin (17, 18). Because ERK is essential for the activation of STAT (19, 20), we expect endogenous GMF to be able to regulate the Jak/STAT pathway as well. Thus, by modulating and integrating the functions of several major signal transduction cascades, GMF and its various phospho-isoforms inside the cell could have far-reaching significance both in general cell biology and in neuroscience.

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