The Major Acidic Fibroblast Growth Factor (aFGF)-stimulated Phosphoprotein from Bovine Liver Plasma Membranes Has aFGF-stimulated Kinase, Autoadenylylation, and Alkaline Nucleotide Phosphodiesterase Activities*

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The major acidic fibroblast growth factor (aFGF)-stimulated phosphoprotein (MAFP) purified from bovine liver exhibits kinase, autoadenylylation, and alkaline nucleotide phosphodiesterase activities depending upon reaction conditions. In the presence of divalent ions, MAFP showed intrinsic and aFGF-stimulated kinase activities (autophosphorylation) using either [γ-32P]ATP or [γ-32P]GTP as a substrate. The autophosphorylation activity of MAFP was stimulated at low concentrations of Ca2+, Mg2+, or Mn2+ (0.2–2 μM). Depletion of the divalent ions by EDTA abolished the autophosphorylation activity but enhanced the autoadenylylation activity of MAFP. [α-32P]ATP as well as [α-32P]NAD could serve as substrates for autoadenylylation activity of MAFP. aFGF appeared to enhance the autoadenylylation activity of MAFP with an optimal concentration (0.6–1.2 mM). P1P3-di(adenosine-5')-triphosphate (AP3A) was found to be a potent inhibitor for the autophosphorylation and autoadenylylation activities of MAFP. Analyses by automated Edman degradation of the adenylated and phosphorylated peptides derived from autoadenylated and autophosphorylated MAFP revealed that both autoadenylation and autophosphorylation occurred at residue Thr204.

The kinase and autoadenylylation activities of MAFP had an optimal pH of 6.9–7.4. However, at pH 8.9, MAFP showed intrinsic and aFGF-stimulated phosphodiesterase activities. aFGF appeared to stimulate the phosphodiesterase activity of MAFP without altering the Km (−0.2 mM) of its substrate.

Bovine liver major acidic fibroblast growth factor (aFGF)1-stimulated phosphoprotein (MAFP) is a newly identified ectoprotein Ser/Thr kinase whose activity is augmented by aFGF (1, 2). MAFP appeared to cochromatograph with the aFGF receptor/protein tyrosine kinase on wheat germ lectin-Sepharose 4B column chromatography and DEAE-cellulose ion exchange chromatography during purification of the latter from bovine liver plasma membrane extracts (2). The kinase activity of MAFP was modulated by divalent ions (Mg2+, Mn2+, and Ca2+) and pyrophosphate (PPi). At an equal molar ratio of Mn2+ and PPi, MAFP exhibited maximum activity with respect to intrinsic and aFGF-stimulated kinase activities (1, 2).

The glycoprotein MAFP is a 260-kDa homodimer linked together by disulfide bonds (1). The amino acid sequence analysis of proteolytic peptides of MAFP and a computer search of sequence homology with other known proteins revealed ~80–100% amino acid sequence homology of MAFP peptides to human and mouse plasma cell membrane glycoproteins (PC-1) whose cDNA were recently cloned and sequenced (3). Together with their identical subunit structures, the close amino acid sequence homology of bovine MAFP and human and mouse PC-1 suggested that MAFP is a bovine version of PC-1. PC-1 has been used as a cell surface differentiation marker of plasma cells for 2 decades (4, 5). The function of PC-1 is unknown. The identification of MAFP, an ectoprotein kinase, as PC-1 provides a new insight into the functional role of MAFP (PC-1) in the cell biology of plasma cells.

MAFP (PC-1) is an ectoprotein kinase possessing a novel alkaline nucleotide phosphodiesterase activity (1, 2, 6). Although the MAFP (PC-1) molecule possesses kinase-specific motifs, the amino acid sequence of MAFP (PC-1) does not appear to be very homologous to those of other known cytoplasmic protein Ser/Thr and Tyr kinases (1–3), suggesting that MAFP (PC-1) is an unique protein kinase. For these reasons, we wished to characterize further the enzymic activities of MAFP. In this paper we demonstrate that MAFP purified from bovine liver has autoadenylylation activity in addition to kinase and alkaline nucleotide phosphodiesterase activities. We also show that the autoadenylylation and autophosphorylation occur at the same threonine residue in the MAFP molecule.

EXPERIMENTAL PROCEDURES

Materials

[γ-32P]ATP (4,500 Ci/mmol), [α-32P]ATP (3,000 Ci/mmol), [γ-32P]GTP (1,500 Ci/mmol), and [α-32P]NAD (250 Ci/mmol) were purchased from ICN Radiochemicals and diluted with unlabeled nucleotides to have a specific radioactivity of 10–25 Ci/mmol unless otherwise noted. Molecular weight standards, ATP, GTP, UTP, CTP, dATP, dGTP, UDP-glucose, UDP-galactose, thymidine 5'-monophosphate p-nitrophenyl ester, P1P3-di(adenosine-5')-triphosphate (AP3A), poly-L-lysine (molecular weight 16,000–30,000), poly-L-argi-
nine (molecular weight 18,000–70,000), myelin basic protein, histone, and protamine sulfate were obtained from Sigma. Biochimnica acid was obtained from Pierce. Endoproteinase Lys-C was purchased from Boehringer Mannheim. Wheat germ lectin-Sepharose 4B, monoclonal antibody to MAFP from bovine liver (D6.5), and monoclonal antibody D6.5-Sepharose 4B were prepared as described previously (1, 2). aFGF was purified from bovine brain according to the procedure described (1, 2). Thin-layer polyethyleneimine-cellulose plates and thin-layer cellulose plates were obtained from Macheney-Nagel. Thin-layer Silica Gel 60 plates were purchased from EM Separations.

**Purification of MAFP from Bovine Liver Plasma Membranes with Monoclonal Antibody Affinity Column Chromatography**

MAFP was purified from bovine liver plasma membranes as described previously (1, 2) with some modifications. Plasma membranes were prepared from fresh bovine liver by differential centrifugation (1, 2). The Triton X-100 extracts from crude plasma membranes were subjected to sequential column chromatography on wheat germ lectin-Sepharose 4B and monoclonal antibody D6.5-Sepharose 4B. MAFP was eluted from monoclonal antibody D6.5-Sepharose 4B with 3 M KCl in 20 mM HEPES, pH 7.4, containing 10% glycerol and 0.2% Triton X-100 (HEPES/Triton X-100 buffer), dialyzed against HEPES/Triton X-100 buffer and concentrated by ultrafiltration. The Triton X-100 extracts from crude plasma membranes were subjected to sequential column chromatography on wheat germ lectin-Sepharose 4B and monoclonal antibody D6.5-Sepharose 4B. MAFP was eluted from monoclonal antibody D6.5-Sepharose 4B with 3 M KCI in 20 mM HEPES, pH 7.4, containing 10% glycerol and 0.2% Triton X-100 (HEPES/Triton X-100 buffer), dialyzed against HEPES/Triton X-100 buffer and concentrated by ultrafiltration.

**Assay for the Kinase Activity (Autophosphorylation) of MAFP**

The reaction mixture (50 μl) contained ~3 μg of MAFP, 0.5 mM MnCl₂ (Mn²⁺), 0.5 mM PPi, 2.5 μM [γ32P]ATP or [γ32P]GTP, and various concentrations of aFGF in HEPES/Triton X-100 buffer. The reaction mixture was preincubated at 0 °C for 30 min prior to the addition of [γ32P]ATP or [γ32P]GTP. The phosphorylation reaction was carried out at 0 °C for 10 min. The [32P]-labeled MAFP was prepared with 10% trichloroacetic acid, washed with ethanol/ether (2:1, v/v), and then analyzed by 6% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The relative autophosphorylation of MAFP was quantitated by direct counting or by densitometry of the autoradiograms.

**Assay for Autoadenylation Activity of MAFP**

The reaction mixture (50 μl) contained ~3 μg of MAFP, 5 mM EDTA, 2.5 μM [α-32P]ATP or [α-32P]NAD, and various concentrations of aFGF in HEPES/Triton X-100 buffer. The reaction mixture was preincubated at 0 °C for 30 min prior to the addition of [α-32P]ATP or [α-32P]NAD. After incubation at 37 °C for 30 min, the reaction mixture was analyzed by 6% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The relative autoadenylation of MAFP was estimated by densitometry of the autoradiograms.

**Phosphoamino Acid Analysis**

Phosphorylated or autoadenylated MAFP in the reaction mixture was precipitated by 10% trichloroacetic acid, washed with ethanol/ether (2:1, v/v), and subjected to acid hydrolysis as described previously (7). The acid hydrolysates were analyzed by thin-layer cellulose electrophoresis at pH 3.5 followed by autoradiography (7).

**Piperidine Treatment of Adenylylated MAFP**

After 10% trichloroacetic acid precipitation and washing with ethanol/ether (2:1, v/v), adenylylated MAFP was suspended in 0.5 M piperidine and incubated at 37 °C for 2 h. After 10% trichloroacetic acid precipitation and washing with ethanol/ether (2:1, v/v), the precipitates containing adenylylated MAFP were suspended in 0.5 M piperidine and incubated at 37 °C for 2 h. Piperidine was then removed under vacuum by Speed-Vac. After 10% trichloroacetic acid precipitation and washing with ethanol/ether (2:1, v/v), the precipitates containing adenylylated MAFP were suspended in 0.5 M piperidine and incubated at 37 °C for 2 h. Piperidine was then removed under vacuum by Speed-Vac.

**Identification of Phosphorylation or Adenylation Site(s) in the MAFP Molecule**

*Preparation of MAFP*—About 50 μg of MAFP was incubated with 100 μCi of [γ32P]ATP (4,500 Ci/mmol) and 1.2 mM aFGF in HEPES/Triton X-100 buffer containing 0.5 mM MnCl₂ and 0.5 mM sodium pyrophosphate. After incubation at 0 °C for 2 h, 0.5 mM ATP was added into the reaction mixture. The reaction mixture was further incubated at 0 °C for 4 h. The [32P]-labeled MAFP was then precipitated with 10% trichloroacetic acid (three times) and washed with ethanol/ether (2:1, v/v) (twice).

*Preparation of Adenylylated MAFP*—About 50 μg of MAFP was incubated with 100 μCi of [α-32P]ATP (3,000 Ci/mmol) and 1.2 mM aFGF in HEPES/Triton X-100 buffer containing 0.5 mM MnCl₂ and 0.5 mM sodium pyrophosphate. After incubation at 0 °C for 2 h, 0.5 mM ATP was added into the reaction mixture. The reaction mixture was then incubated at 0 °C for 4 h. The [32P]-labeled MAFP was precipitated with 10% trichloroacetic acid (three times) and washed with ethanol/ether (2:1, v/v) (twice). The [32P]-labeled (phosphorylated or adenylylated) MAFP was dissolved in 50 μl of 8 M urea, 0.4 M ammonium bicarbonate, and 5 μl of 45 mM dithiothreitol and incubated at 50 °C for 15 min. After reduction and alkylation, the reaction mixture was diluted with 140 μl of water and then treated with 0.6 μg of endoproteinase Lys-C at 37 °C for 24 h. The endoproteinase Lys-C digests were subjected to reverse phase HPLC with an Applied Biosystems 130A separation system using a linear gradient from 0 to 70% acetonitrile in 0.1% trifluoroacetic acid. The peptide peak was detected by the absorbance at 220 nm. The radioactivity of the peptide peak was counted with a scintillation counter. The amino acid sequences of the peptide peaks containing radioactivity were determined on an Applied Biosystems model 477A gas/liquid phase protein sequenator equipped with online Applied Biosystems model 120A PTH-amino acid analyzer. For detection of phosphorylated or adenylylated amino acid residues(s), the radioactivity of the PTH-derivative from each cycle of automated Edman degradation was determined by a scintillation counter and by autoradiography. In the autoradiography detection, the PTH-derivatives were spotted on Silica Gel 60 plates. The radioactivity of the PTH-derivative on silica gel plates was visualized by autoradiography.

**Assay for Alkaline Nucleotide Phosphodiesterase Activity**

The assay for nucleotide phosphodiesterase activity was carried out according to the procedure described by Razzell (9). Briefly, the reaction mixture (200 μl) containing ~0.1 μg of MAFP, 0.5 mM thymidine 5′-monophosphate p-nitrophenyl ester and aFGF (0 and 0.6 mM) in 0.1 M Tris-HCl, pH 8.9, and 3.01% Triton X-100 was incubated at 37 °C for 10 min prior to the addition of thymidine 5′-monophosphate p-nitrophenyl ester. The reaction was terminated by the addition of 1 ml of 1.0 M NaOH. The reaction product, p-nitrophenol, was determined by the absorbance at a wavelength of 405 nm (the molar absorption coefficient = 1.5 × 10⁵). Protein concentration was estimated by the bicinchoninic acid method using bovine serum albumin as a standard. The experiments were done in triplicate.

**RESULTS**

**MAFP Utilizes Either ATP or GTP as a Substrate for Its Autophosphorylation Activity**—As reported previously (1, 2), MAFP exhibited a kinase activity when [γ32P]ATP was used as a substrate. Since both [γ32P]ATP and [γ32P]GTP can serve as substrates for some kinases (10, 11), the substrate activity of [γ32P]GTP for MAFP was examined. As shown in Fig. 1, MAFP exerted an autophosphorylation activity with an optimal pH of ~7.4 using either [γ32P]ATP (Fig. 1A) or [γ32P]GTP (Fig. 1B) as a substrate. The autophosphorylation of MAFP with [γ32P]GTP as a substrate was stimulated by aFGF in a dose-dependent manner (Fig. 2A). At 0.6–1.2 mM, aFGF showed a maximal stimulation (~2-fold) of autophosphorylation of MAFP. This optimal concentration of aFGF was essentially identical with that previously reported to stimulate the autophosphorylation of MAFP with [γ32P]ATP as a substrate (1, 2). Phosphoamino acid analysis of MAFP that was labeled with [γ32P]GTP revealed that the autophosphorylation occurred at threonine residues (Fig. 2B). The autophosphorylation of MAFP with [γ32P]ATP as a substrate was also previously shown to be threonine-specific (1).
Fig. 1. Effect of pH on the autophosphorylation of MAFP using [γ-32P]ATP (panel A) or [γ-32P]GTP (panel B) as a substrate.
The reaction mixture contained MAFP, 0.5 mM MnCl₂, 0.5 mM sodium pyrophosphate, and 2.5 μM [γ-32P]ATP or [γ-32P]GTP with (+) or without (−) 1.2 mM aFGF in 20 mM Tris acetate buffer, pH 5.8-8.9 or pH 6.0-9.2, 0.2% Triton X-100, and 10% glycerol. The phosphorylation reaction was carried out at 0 ºC for 10 min. The 32P-labeled MAFP was analyzed by 6% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The arrow indicates the location of 32P-labeled MAFP. The relative autophosphorylation of MAFP was quantitated by densitometry of the autoradiogram.

In addition to autophosphorylation activity, MAFP elicited kinase activity toward protein substrates such as histone and myelin basic protein when [γ-32P]ATP was used as a cosubstrate (1, 2). It was of interest to see if MAFP could utilize [γ-32P]GTP for its kinase activity toward protein substrates. Under similar conditions for protein phosphorylation using [γ-32P]ATP as a cosubstrate (1, 2), MAFP was found not to catalyze the phosphorylation of myelin basic protein or histone in the presence of [γ-32P]GTP (data not shown). The reason for the inability of [γ-32P]GTP to serve as cosubstrate for protein phosphorylation is not known. [γ-32P]GTP differed from [γ-32P]ATP with respect to its affinity to MAFP. As shown in Fig. 3, the maximal autophosphorylation of MAFP was observed at 25–30 μM [γ-32P]GTP. The apparent Kₘ (~12 μM) of [γ-32P]GTP estimated by the double-reciprocal plot analysis of the [γ-32P]GTP concentration against the autophosphorylation of MAFP appeared to be two times lower than the apparent Kₘ (~25 μM) observed for [γ-32P]ATP (1, 2).

In contrast to other known kinases (12, 13), the kinase activity of MAFP was inhibited by nucleotide pyrophosphate compounds such as UDP-monomosaccharides (1). To explore the potential physiological significance of the inhibition by nucleotide pyrophosphate compounds, we investigated the effect of AP₃A on the autophosphorylation of MAFP. AP₃A has been found to be released with ATP from platelets (14, 15). As shown in Fig. 4, AP₃A inhibited the autophosphorylation of MAFP using either [γ-32P]ATP (Fig. 4A) or [γ-32P]GTP (Fig. 4B) as a substrate. At 50 μM, AP₃A almost abolished autophosphorylation of MAFP with [γ-32P]ATP as substrate, whereas about 85% inhibition of MAFP autophosphorylation with [γ-32P]GTP as substrate was observed at 50 μM AP₃A. These results suggest that like other nucleotide pyrophosphate compounds (1), AP₃A showed a potent inhibitor activity for MAFP.

MAFP Shows an aFGF-stimulated Autoadenylylation Activity in the Presence of EDTA—We reported previously that the kinase activity of MAFP could be modulated by divalent ions (Mg²⁺, Ca²⁺, and Mn²⁺), and without the addition of exogenous divalent ions, MAFP showed a significant kinase activity (1, 2). To see if divalent ions were required for its kinase activity, we investigated the effect of EDTA on the autophosphorylation activity using [γ-32P]ATP, [γ-32P]GTP, or [α-32P]ATP as a substrate. As shown in Fig. 5, EDTA completely abolished the 32P labeling of MAFP by either [γ-32P]ATP (Fig. 5A) or [γ-32P]GTP (Fig. 5B), suggesting that divalent ions were required for the kinase activity of MAFP. This suggestion was supported by the observation that the addition of exogenous divalent ions further increased the autophosphorylation activity of MAFP.

![Fig. 2. Effect of aFGF concentration on the autophosphorylation of MAFP using [γ-32P]GTP as a substrate (panel A) and phosphoamino acid analysis of 32P-labeled MAFP (panel B). Panel A: the reaction mixture contained MAFP, 0.5 mM MnCl₂, 0.5 mM sodium pyrophosphate, and 2.5 μM [γ-32P]GTP, and various concentrations of aFGF as indicated in HEPES/Triton X-100 buffer. The phosphorylation reaction was carried out at 0 ºC for 10 min. The 32P-labeled MAFP was analyzed by 6% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The arrow indicates the location of 32P-labeled MAFP. The relative autophosphorylation of MAFP was quantitated by densitometry of the autoradiogram. Panel B: MAFP was labeled with [γ-32P]GTP in the presence (+) and absence (−) of aFGF (1.2 mM) as described above and subjected to 6% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The arrow indicates the location of 32P-labeled MAFP. The relative autophosphorylation of MAFP was quantitated by densitometry of the autoradiogram.

![Fig. 3. Effect of [γ-32P]GTP concentration on the autophosphorylation of MAFP. The reaction mixture contained MAFP, 0.5 mM MnCl₂, 0.5 mM sodium pyrophosphate, and various concentrations of [γ-32P]GTP (5, 10, 15, 20, 25, and 30 μM) in HEPES/Triton X-100 buffer. After 10 min at 0 ºC, the reaction mixtures were analyzed by 6% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The arrow indicates the location of 32P-labeled MAFP. The relative autophosphorylation of MAFP was quantitated by densitometry of the autoradiogram.]
A

\[ \text{APsA (mM)} \]

\[ 0 ~ 0.05 ~ 0.5 ~ 5 \]

\[ \text{aFGF} \]

\[ 205 \text{kDa} \]  
\[ 116 \text{kDa} \]  
\[ 97 \text{kDa} \]  
\[ 66 \text{kDa} \]

B

\[ \text{[\(\gamma\)-32P]GTP} \]

\[ \text{[\(\gamma\)-32P]ATP} \]

FIG. 4. Inhibition of the autophosphorylation of MAFP by APsA. The reaction mixture contained MAFP, 0.5 mM MnCl\(_2\), 0.5 mM sodium pyrophosphate, and 5 \(\mu\)M [\(\gamma\)-32P]ATP (panel A) or [\(\gamma\)-32P]GTP (panel B) in the presence of various concentrations of APsA with (+) or without (−) aFGF (1.2 nM). After 10 min at 0 °C, the reaction mixtures were analyzed by 6% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The arrow indicates the location of 32P-labeled MAFP.

the autophosphorylation activity of MAFP (Fig. 5C). In the presence of [\(\gamma\)-32P]GTP, Mg\(^{2+}\) and Ca\(^{2+}\) stimulated the kinase activity (≈1.5-fold) of MAFP with an optimal concentration of 0.2–2 \(\mu\)M (Fig. 5C). At higher concentrations, the stimulating activity of these divalent ions diminished (Fig. 5C). Mg\(^{2+}\) (MnCl\(_2\)) also showed a dose-dependent effect on the MAFP autophosphorylation activity similar to those of Mg\(^{2+}\) and Ca\(^{2+}\) (data not shown). However, when [\(\alpha\)-32P]ATP was used as a substrate, EDTA did not inhibit but stimulated the 32P labeling of MAFP. In the presence of EDTA, aFGF stimulated the 32P labeling of MAFP in a dose-dependent manner (Fig. 6A). About 1.5-fold stimulation was observed at the concentrations of 0.6–1.2 nM aFGF. At 1.2 nM aFGF, the stoichiometry of the 32P labeling of MAFP was estimated to be 0.9 mol of 32P incorporated/mol of MAFP. The optimal pH of the intrinsic and aFGF-stimulated 32P labeling of MAFP was determined to be 7.4 (data not shown). This 32P labeling of MAFP appeared to result from the cleavage of the pyrophosphate bond between the \(\alpha\)- and \(\beta\)-phosphate groups of [\(\alpha\)-32P]ATP and subsequent incorporation of the [32P]AMP moiety into the MAFP molecule. As shown in Fig. 6B, [32P]AMP could be released from the 32P-labeled MAFP following treatment with piperidine and identified on thin-layer polyethyleneimine-cellulose chromatography. These results suggest that the depletion of divalent ions by EDTA enhanced the autoadenylation activity of MAFP. To define which amino acid residues were adenylated, the 32P-labeled MAFP was subjected to phosphoamino acid analysis following acid hydrolysis. As shown in Fig. 6C, phosphothreonine was detected in the acid hydrolysates of 32P-labeled MAFP by thin-layer chromatography on polyethyleneimine cellulose using two solvent systems of 0.5% formic acid and 0.15 M lithium formate, pH 3.0, followed by autoradiography. Standard NAD, AMP, and ADP were cochromatographed with piperidine hydrolysates and were identified under a UV light. Panel C, MAFP labeled with [\(\alpha\)-32P]ATP in the presence (+) and absence (−) of aFGF (1.2 nm) was hydrolyzed in 6 N HCl at 100 °C for 1 h. The acid hydrolysates of 32P-labeled MAFP were then analyzed by thin-layer cellulose electrophoresis at pH 3.5. The 32P-labeled amino acid was visualized by autoradiography. Standard phosphoamino acids were coelectrophoresed and identified by ninhydrin reaction.
cellulose electrophoresis at pH 3.5. This result suggests that the autoadenylylation of MAFP occurred at threonine residues.

The kinase and adenylylation activities of MAFP involved the cleavages of pyrophosphate bonds between the β- and γ-phosphate groups and between the α- and β-phosphate groups, respectively. We hypothesized that the chelating of divalent ions (Mg²⁺ or Mn²⁺) with phosphate groups, possibly β- and γ-phosphate groups, facilitated the cleavage by MAFP of the pyrophosphate bond between the β- and γ-phosphate groups and that the depletion of divalent ions by EDTA allowed MAFP to attack preferentially the pyrophosphate bond between the α- and β-phosphate groups. This hypothesis has been supported by the observation that the divalent ions inhibited the autoadenylylation of MAFP, but the presence of EDTA enhanced the autoadenylylation activity of MAFP.

To test the above hypothesis further, we investigated the autoadenylylation of MAFP using [α-³²P]NAD (β-nicotinamide adenine dinucleotide) as a substrate. [α-³²P]NAD contains an adenosine pyrophosphate moiety. If the above hypothesis is correct, [α-³²P]NAD should be able to serve as a substrate for the autoadenylylation of MAFP. As shown in Fig. 7A, MAFP was indeed ³²P-labeled in the presence of [α-³²P]NAD with an optimal pH of 6.9. In addition, aFGF stimulated the ³²P labeling (−1.4-fold) of MAFP in a dose-dependent manner with an optimal concentration of 0.6–1.2 nM (Fig. 7B). This ³²P labeling or adenylylation was confirmed by the identification of [³²P]AMP on thin-layer silica gel chromatography following treatment of the ³²P-labeled MAFP with piperidine (Fig. 7C). Phosphoamino acid analysis of ³²P-labeled MAFP indicated that the autoadenylylation of MAFP with [α-³²P]NAD as a substrate occurred at threonine residues (data not shown). This Thr-specific autoadenylylation of MAFP could also be strongly inhibited by AP₃A, a potent inhibitor for the kinase activity of MAFP (Fig. 8).

Autophosphorylation and Autoadenylylation of MAFP Occur at the Same Thr²⁰⁴ Residue—As described above, MAFP exhibited both kinase and autoadenylylation activities. Since MAFP possesses a single ATP binding site (1), it is possible that the kinase and autoadenylylation activities are catalyzed by the same active site in the MAFP molecule. To test this possibility, we investigated the identity of the sites of autophosphorylation and autoadenylylation in the MAFP molecule. Both adenylylated and phosphorylated MAFP, which were labeled with [α-³²P]ATP and [γ-³²P]ATP, respectively, were subjected to digestion with endoprotease Lys-C following reduction and alklylation. The endoprotease Lys-C-digested peptides were separated on reverse phase HPLC. Fig. 9 shows the HPLC chromatograms of these peptides from adenylylated and phosphorylated MAFP (Fig. 9, A and B, respectively). A major ³²P-labeled peak, namely adenylylated peptide peak 30 (APP30) or phosphorylated peptide peak 28 (PPP28), appeared on each chromatogram. The analysis by automated Edman degradation indicated that APP30 contained an amino acid sequence of EnvNMRPVYPTKXPFPNHYSIVTGYLEPSHGIIDNK²²⁶, and PPP28 contained 2 amino acid sequences of EnvNMRPVYPTKXPFPNHYSIVTGYLEPSHGIIDNK²²⁶ (25%) and EnvXPFPNHYSIVTGYLEPSHGIIDNK²²⁶ (75%) (Table I). These results suggest that the adenylylation and phosphorylation sites are located in the same peptide region. To identify the adenylylated threonine residue or phosphorylated threonine residue in these peptides, we determined the radioactivity of each PTH-derivative obtained from automated Edman degradation of adenylylated and phosphorylated peptides. As shown in Fig. 10A, the ³²P radioactivity started to appear at the 10th PTH-derivative from the amino terminus of APP30, which corresponded to the 10th residue Thr²⁰⁴ of EnvNMRPVYPTKXPFPNHYSIVTGYLEPSHGIIDNK²²⁶. In the phosphorylated peptide (PPP28) (Fig. 10B), the radioactivity started to appear at the 1st and 10th PTH-derivatives, which corresponded to the amino-terminal threonine residue of EnvTFPNHYSIVTGYLEPSHGIIDNK²²⁶ and the 10th residue Thr²⁰⁴ of EnvNMRPVYPTKXPFPNHYSIVTGYLEPSHGIIDNK²²⁶. These results suggest that autoadenylylation and autophosphorylation occurred at Thr²⁰⁴. The continuous appearance of the ³²P radioactivity in

**Fig. 7.** aFGF-stimulated adenylylation of MAFP using [α-³²P]NAD as a substrate. Panel A, the reaction mixture contained MAFP, 2.5 μM [α-³²P]NAD, and 5 mM EDTA with (+) or without (−) 1.2 nM aFGF in 20 mM Tris acetate buffer, pH 6.0–8.2, containing 0.2% Triton X-100 and 10% glycerol. After 10 min at 0 °C, the ³²P-labeled MAFP was analyzed by 6% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The arrow indicates the location of ³²P-labeled MAFP. Panel B, the reaction mixture contained MAFP, 2.5 μM [α-³²P]NAD, 5 mM EDTA, and various concentrations of aFGF (1.2 nM) in HEPES/Triton X-100 buffer. After 10 min at 0 °C, the ³²P-labeled MAFP was analyzed by 6% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The arrow indicates the location of ³²P-labeled MAFP. Panel C, MAFP, labeled with [α-³²P]NAD in the presence of 5 mM EDTA with (+) or without (−) aFGF (1.2 nM), was treated with 0.5 M piperidine at 37 °C for 2 h. The piperidine hydrolysates of ³²P-labeled MAFP were analyzed by thin-layer silica gel chromatography using a solvent system (butanol:acetic acid:water, 5:2:3, by volume). The ³²P-labeled AMP was then visualized by autoradiography.

**Fig. 8.** Inhibition of the adenylylation of MAFP by various concentrations of AP₃A. The reaction mixture contained MAFP, 5 mM EDTA, 5 μM [α-³²P]ATP or [α-³²P]NAD, and various concentrations of AP₃A as indicated with or without aFGF (1.2 nM) in HEPES/Triton X-100 buffer. After reaction at 0 °C for 10 min, the ³²P-labeled MAFP was analyzed by 6% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The arrow indicates the location of ³²P-labeled MAFP.
FIG. 9. Reverse phase HPLC of peptide fragments derived from endoproteinase Lys-C digestion of adenylylated (panel A) and phosphorylated (panel B) MAFP. MAFP was adenylylated and phosphorylated with 100 μCi of [α-32P]ATP and [γ-32P]ATP, respectively, as described under "Experimental Procedures." The 32P-labeled MAFP was subjected to reduction and alkylation followed by endoproteinase Lys-C digestion. The endoproteinase Lys-C digests were then chromatographed on reverse phase HPLC using a linear gradient of acetonitrile concentration from 0 to 70% in 0.1% trifluoroacetic acid. The peptide peaks were monitored by the absorbance at 220 nm. The radioactivity of peptide peaks was measured with a scintillation counter. The amino acid sequences of peptide peaks APP30 and PPP28 containing radioactivity were then determined by automated Edman degradation.

TABLE I
Amino acid sequence analysis of the peptide peaks APP30 and PPP28 from the reverse phase HPLC of endoproteinase Lys-C digests of adenylylated and phosphorylated MAFP

The peptide peaks APP30 and PPP28 were obtained from the reverse phase HPLC of endoproteinase Lys-C digests of adenylylated and phosphorylated MAFP, respectively. APP30 and PPP28 were subjected to amino and sequence analyses by automated Edman degradation. The amino acid residues at the amino terminus and carboxyl terminus are numbered based on the amino acid sequence of human MAFP (PC-1) (3). The amino acid residues are represented by one-letter symbols. X indicates the unidentified residues due to phosphorylation or adenylation.

| Peptide Peak | Amino Acid Sequence                  |
|--------------|--------------------------------------|
| APP30        | 166N M R P V Y P T K X F P N H S I V T G L Y P E S H G I D N K276 (75%) |
| PPP28        | 166N M R P V Y P T K X F P N H S I V T G L Y P E S H G I D N K276 (25%) |

the PTH-derivatives after [32P]Thr204 was because of the tight association of [32P]Thr (released from the [32P]Thr residue at the step of cleavage reaction of automated Edman degradation) or [32P]AMP (released from the adenylylated Thr residue) with positively charged Polybrene-treated cartridge filters (16). The [32P] or [32P]-AMP was gradually released cycle after cycle from the cartridge filters. It is of importance to note that endoproteinase Lys-C was unable to hydrolyze the Lys205-Thr204 (adenylated) peptide bond because of the bulky adenylylate group of the adenylylated threonine residue. Endoproteinase Lys-C appeared to hydrolyze the Lys205-Thr204 (phosphorylated) peptide bond partially.

MAFP Shows an aFGF-stimulated Alkaline Nucleotide Phosphodiesterase Activity—MAFP exhibited an alkaline nucleotide phosphodiesterase activity. The optimal pH of the alkaline phosphodiesterase activity of MAFP was determined to be 9.8 (Fig. 11). To see if aFGF stimulated the alkaline nucleotide phosphodiesterase activity, the phosphodiesterase
been found to modulate the kinase activity of MAFP. The PTH-derivatives was then visualized by autoradiography. APP30 and PPP28. respectively) was spotted on the silica gel plate. The radioactivity of the PTH-derivatives was then visualized by autoradiography.

After 10 min at 37 °C, the reaction product, p-nitrophenol, was measured. The double-reciprocal plot analysis of the thymidine 5'-monophosphate p-nitrophenyl ester concentration against the enzyme activity (μmol of p-nitrophenol/h/mg of protein) of MAFP was performed to estimate the Km and maximal velocity.

different pH buffer solutions (100 mM Tris acetate, 100 mM Tris-HCl, 100 mM diethylamine (DEA), and 100 mM sodium bicarbonate). After 10 min at 37 °C, the reaction product, p-nitrophenol, was determined.

During automated Edman degradation of MAFP, 0.5 mM thymidine 5'-monophosphate p-nitrophenyl ester, 0.01% Triton X-100, 0.5% glycerol, and aFGF (0 and 1.2 nM) in different pH buffer solutions (100 mM Tris acetate, 100 mM Tris-HCl, 100 mM diethylamine (DEA), and 100 mM sodium bicarbonate). After 10 min at 37 °C, the reaction product, p-nitrophenol, was determined.

Fig. 10. Identification of 32P-labeled amino acid residues in APP30 and PPP28. During automated Edman degradation of APP30 (panel A) and PPP28 (panel B) a portion (50 μl out of 140 μl) of the PTH-derivative from each cycle was analyzed by an on-line PTH-amino acid analyzer. The remainder (90 μl) of the PTH-derivatives (1st to 16th cycle and 1st to 23rd cycle for APP30 and PPP28, respectively) was spotted on the silica gel plate. The radioactivity of the PTH-derivatives was then visualized by autoradiography.

FIG. 11. Effect of pH on the alkaline nucleotide phosphodiesterase activity of MAFP. The reaction mixture contained MAFP, 0.5 mM thymidine 5'-monophosphate p-nitrophenyl ester, 0.01% Triton X-100, 0.5% glycerol, and aFGF (0 and 1.2 nM) in different pH buffer solutions (100 mM Tris acetate, 100 mM Tris-HCl, 100 mM diethylamine (DEA), and 100 mM sodium bicarbonate). After 10 min at 37 °C, the reaction product, p-nitrophenol, was determined.

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activity of MAFP was carried out at pH 8.9 in the presence of various concentrations of the substrate, thymidine 5'-monophosphate p-nitrophenyl ester ± aFGF (1.2 nM). Fig. 12 shows the double-reciprocal plot of the substrate concentration and the enzyme activity of MAFP. aFGF appeared to stimulate the enzymic activity of MAFP ~1.2–1.5-fold without altering the Km (~0.2 mM) of the substrate.

Divalent ions and basic proteins or basic polypeptides have been found to modulate the kinase activity of MAFP (1, 2). It was of interest to examine the effect of the compounds on the alkaline nucleotide phosphodiesterase activity of MAFP. As shown in Fig. 13A, Ca2+ and Mg2+ increase the alkaline nucleotide phosphodiesterase activity of MAFP at concentrations of > 0.5 mM. In contrast, the alkaline nucleo-

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Effect of pH on the alkaline nucleotide phosphodiesterase activity of MAFP. The reaction mixture contained MAFP, 0.5 mM thymidine 5'-monophosphate p-nitrophenyl ester, 0.01% Triton X-100, 0.5% glycerol, and aFGF (0 and 1.2 nM) in different pH buffer solutions (100 mM Tris acetate, 100 mM Tris-HCl, 100 mM diethylamine (DEA), and 100 mM sodium bicarbonate). After 10 min at 37 °C, the reaction product, p-nitrophenol, was determined.

Fig. 12. Effect of aFGF on the alkaline nucleotide phosphodiesterase activity of MAFP. The reaction mixture contained MAFP, aFGF (0 and 1.2 nM), and various concentrations of thymidine 5'-monophosphate p-nitrophenyl ester in 0.1 M Tris-HCl, pH 8.9, containing 0.01% Triton X-100, and 0.5% glycerol. After 20 min at 0 °C, the reaction product, p-nitrophenol, was measured. The double-reciprocal plot analysis of the thymidine 5'-monophosphate p-nitrophenyl ester concentration against the enzyme activity (μmol of p-nitrophenol/h/mg of protein) of MAFP was performed to estimate the Km and maximal velocity.

determine the phosphodiesterase activity of MAFP was inhibited by a 0.5 mM equal molar ratio of Mn2+ and PPi (Fig. 13B). Basic proteins or polypeptides (protamine, histone, polylsine, and polyarginine) were also found to enhance the phosphodiesterase activity of MAFP (Table I). These results suggest that like kinase and autoadenylation activities, the alkaline nucleotide phosphodiesterase activity of MAFP could be activated by aFGF and modulated by divalent ions and basic polypeptides (1, 2).

DISCUSSION

MAFP is a novel type II membrane protein that exhibits multiple enzymic activities depending upon the reaction conditions. In the presence of divalent ions and [γ-32P]ATP or [γ-32P]GTP, MAFP showed an intrinsic and aFGF-stimulated autophosphorylation activity. At an equal molar ratio of Mn2+ and PPi (0.5 mM), maximal kinase activity of MAFP was observed (1, 2). The depletion of divalent ions by EDTA shifted the autophosphorylation activity to autoadenylation activity. [α-32P]ATP or [α-32P]NAD could serve as substrate for the autoadenylation activity of MAFP. Both kinase and autoadenylation activities of MAFP showed an optimal pH of 6.9–7.4. At higher pH, the kinase and autoadenylation activities diminished, whereas the alkaline nucleotide phosphodiesterase activity increased. At pH 8.9, MAFP showed intrinsic and aFGF-stimulated nucleotide phosphodiesterase activities. The kinase, autoadenylation, and alkaline nucleotide phosphodiesterase activities of MAFP appear to be catalyzed by the same active site based on the following evidence. (i) MAFP possesses a single known nucleotide binding sequence per polypeptide chain (1). (ii) The substrates of one enzyme activity of MAFP can function as inhibitors for two other enzyme activities. For examples, ATP and GTP are potent inhibitors for phosphodiesterase activity; thymidine 5'-monophosphate p-nitrophenyl ester, a substrate for phosphodiesterase activity of MAFP, is an inhibitor for the kinase and autoadenylation activities of MAFP.2 (iii) All three activities were found to be augmented by aFGF with similar

2. Y. Oda, M.-D. Kuo, S. S. Huang, and J. S. Huang, unpublished results.
optimal concentrations. (iv) The autoadenylylation and autophosphorylation occurred at the same threonine residue.

The exact molecular mechanism by which aFGF stimulates the enzymic activities of MAFP is not known. However, aFGF appears to stimulate the enzymic activities of MAFP without altering the $K_m$ of ATP (2) or thymidine 5'-monophosphate p-nitrophenyl ester. MAFP is a homodimeric protein. We have found no evidence to suggest that aFGF stimulates the activities of MAFP by inducing oligomerization of MAFP. aFGF may simply stimulate the activities of MAFP by inducing conformational change of MAFP molecule. The optimal concentration of aFGF to stimulate the activities of MAFP has been found to be 0.6–1.2 nM. This optimal concentration is comparable to those observed for epidermal growth factor and platelet-derived growth factor to stimulate the activities of their respective receptors but ~10–20-fold higher than those for fibroblast growth factors to stimulate their receptor/protein tyrosine kinase activities (17–20).

MAFP is distinct from other known Ser/Thr- and Tyr-specific protein kinases with respect to its multiple functions. No known protein Ser/Thr or Tyr kinase has been reported to exert autoadenylylation or phosphodiesterase activity. We have tested the autoadenylylation activity of the catalytic subunit of cAMP-dependent protein kinase, epidermal growth factor receptor/protein kinase, and hexokinase. None of these enzymes shows the autoadenylylation activity. The physiological significance of the multiple functions of MAFP is not known. The high optimal pH (pH 9.8) of the nucleotide phosphodiesterase activity and the inhibition of the autoadenylylation activity by low concentrations of divalent ions (Mn$^{2+}$ and Mg$^{2+}$) suggest that the kinase activity of MAFP may be more favorable than its adenylylation and phosphodiesterase activities under physiological conditions.

Recently, the ecto-kinase, adenylylation, and alkaline nucleotide phosphodiesterase activities have been demonstrated in cultured cells and membrane preparations (21–28). The proteins responsible for these activities have not been identified. It is very possible that MAFP or MAFP-like enzymes mediate these activities. It is of importance to note that the major adenylylated protein found in rat liver plasma membrane showed a molecular weight similar to that of MAFP (21).

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REFERENCES

1. Oda, Y., Kuo, M.-D., Huang, S. S., and Huang, J. S. (1991) J. Biol. Chem. 266, 16791–16795
2. Kuo, M.-D., Huang, S. S., and Huang, J. S. (1990) J. Biol. Chem. 265, 16455–16463
3. Buckley, M. F., Loveland, K. A., McKinstrv, W. F., Tong, B. D., Finley, E. M., and Hickman, S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5132–5136
4. van Driel, I. R., and Goding, J. W. (1987) J. Biol. Chem. 262, 4862–4867
5. Takahashi, T., Old, L. J., and Boyse, E. A. (1970) J. Exp. Med. 131, 1325–1341
6. Rebbe, N. F., Tong, B. D., Finley, E. M., and Hickman, S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5132–5136
7. Huang, S. S., and Huang, J. S. (1988) J. Biol. Chem. 261, 9568–9571
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8. Bradley, M. K., Hudson, J., Villanueva, M. S., and Livingston, D. M. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6574-6578
9. Razzell, W. E. (1963) Methods Enzymol. 6, 228-258
10. Marshak, D. R., and Carroll, D. (1981) Methods Enzymol. 200, 154-156
11. Meggio, F., Boldyreff, B., Martin, O., Marchiori, F., Perich, J. W., Issinger, O.-G., and Finna, L. A. (1997) Eur. J. Biochem. 255, 929-945
12. Edelman, A. M., Blumenthal, D. K., and Krebs, E. G. (1987) Annu. Rev. Biochem. 56, 567-613
13. Hunter, T. (1991) Methods Enzymol. 200, 2-37
14. Flodgaard, H., and Kienow, H. (1982) Biochem. J. 206, 737-742
15. Lüthje, J., and Oligie, A. (1983) Biochem. Biophys. Res. Commun. 115, 293-299
16. Wang, Y., Fiol, C. J., DePaoli-Roach, A. A., Bell, A. W., Hermodson, M. A., and Roach, P. J. (1988) Anal. Biochem. 174, 537-547
17. Huang, J. S., Huang, S. S., and Kuo, M.-D. (1986) J. Biol. Chem. 261, 11600-11607
18. Maciag, T., and Burgess, W. H. (1989) Annu. Rev. Biochem. 58, 575-606
19. Carpenter, G. (1987) Annu. Rev. Biochem. 56, 881-914
20. Huang, J. S., Huang, S. S., Kennedy, B., and Deuel, T. F. (1982) J. Biol. Chem. 257, 8130-8136
21. José, E. S., Benguria, A., and Villalobo, A. (1990) J. Biol. Chem. 265, 20653-20661
22. Evans, W. H., Hood, D. O., and Gurd, J. W. (1973) Biochem. J. 135, 819-826
23. Skubitz, K. M., and Goueli, S. A. (1991) Biochem. Biophys. Res. Commun. 171, 49-55
24. Culp, J. S., Blytt, H. J., Hermodson, M., and Butler, L. G. (1985) J. Biol. Chem. 260, 8320-8324
25. Piroton, S., Boutrit-Falson, O., Robaye, R., and Boeynaems, J.-M. (1982) Biochem. J. 205, 323-329
26. Maruyama, E., Iwanatsu, A., and Takashima, S. (1993) Biochem. Mol. Biol. Intern. 20, 579-586
27. Hartmann, M., and Schrader, J. (1992) Biochim. Biophys. Acta 1136, 189-196
28. Codini, M., Fini, C., Paolotti, P., and Floridi, A. (1992) Biochem. Intera. 28, 988-997