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Cloning of a Rat Glia Maturation Factor–γ (rGMFG) cDNA and Expression of Its mRNA and Protein in Rat Organs[1]

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We isolated a rat glia maturation factor–γ (rGMFG) cDNA and examined the tissue distribution of GMFG in rat by Northern and Western blot analyses. Sequence analysis of the entire cDNA revealed an open reading frame of 426 nucleotides with a deduced protein of 142 amino acid residues. The deduced amino acid sequence of the putative product is highly homologous (78.9%) to rat glia maturation factor–β (rGMFB). Northern blot analysis indicated that a 0.9-kb mRNA is predominantly expressed in rat thymus, testis, and spleen. GMFG showed a different tissue distribution from GMFB, being present predominantly in proliferative and differentiative organs.

Key words: gene cloning, gene expression, Northern blot analysis, rat glia maturation factor–γ, Western blot analysis.

Glia maturation factor–β (GMFB) is a 17-kDa protein that was initially identified as a growth and differentiation factor acting on neurons as well as glia in the vertebrate brain (*1–4*). Recently, Zaheer and Lim demonstrated new intracellular functions of GMFB. The protein kinase A (PKA)–phosphorylated GMFB is a potent inhibitor of the extracellular-regulated kinase (ERK1/ERK2), a subfamily of mitogen-activated protein (MAP) kinase (*5*), and it is a strong enhancer of p38 MAP kinase (*6*). They also showed that PKA and protein kinase C (PKC) phosphorylate GMFB, which promotes the activity of PKA itself (*7*).

From Northern blot analysis of the expression of GMFB in astrocytes, we found that the GMFB cDNA probe hybridized to both 0.9- and 4.1-kb fragments with a low-stringency wash. Furthermore, several homologous cDNA sequences to GMFB were submitted to the Expression Sequence Tags database. With these facts in mind, we isolated a new homologue of the human GMFB gene (*8*). Sequence analysis of the entire cDNA revealed an open reading frame of 426 nucleotides coding for a deduced amino acid sequence of 142 residues. The deduced amino acid sequence of the putative product is highly homologous to human GMFB (82% identity) and was named glia maturation factor–γ (hGMFG).

It is supposed that rat, the most commonly used animal for neurochemical study, has similar homologues to those observed in human. In this study, we isolated a rat GMFG cDNA and examined the expression of rGMFG mRNA and protein using antibodies prepared against recombinant rat GMFB and GMFG proteins.

METHODS AND MATERIALS

Isolation of Rat GMFG cDNAs—A rat pulmonary artery cDNA library in Uni-ZAP™ XR vector was purchased from Stratagene. Approximately 1×10^6 phage clones were screened by plaque hybridization (*9*) using a full-length human GMFG cDNA (*8*). The cDNA probe was labeled with [α-^{32}P]dCTP (New England Nuclear) by use of a random primer labeling system (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Phage plaques were transferred to Hybond™-N nylon membranes (Amersham Pharmacia Biotech) and hybridized with probe (4×10^5 cpm/filter) at 42°C for 16 h in hybridization solution (50% formamide, 5 × SSC, 1.5 × Denhardt's solution, 0.1% SDS, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA). The filters were washed twice in 2 × SSC containing 0.1% SDS at 42°C for 15 min and once in 0.5 × SSC containing 0.1% SDS at 42°C for 15 min. Autoradiography of air-dried membranes was performed by overnight exposure to Kodak X-OMAT AR film at –80°C with intensifying screens.

Phage DNAs were purified from plate lysates after quaternary positive-clone enrichment screening and amplification (*10*). Plasmid inserts were rescued in pBluescript SK(–) by the *in vivo* excision technique (*11*) using ExAssist Interference-Resistant Helper Phage and *Escherichia coli* SOLR strain (Stratagene). The plasmid DNAs were prepared essentially as described (*12*).

Various clones were sequenced using a Thermo Seque-

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Abbreviations: GMFB, glia maturation factor, beta; GMFG, glia maturation factor, gamma; PBS(–), Ca^{2+},Mg^{2+}-free phosphate-buffered saline.

nase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech) and analyzed on an automatic A.L.F. red sequencing apparatus (Amersham Pharmacia Biotech).

Cloning of Rat GMFB Partial cDNA—Rat GMFB partial cDNA was obtained by the reverse transcription–polymerase chain reaction. Total RNA was isolated from primary cultured rat astrocytes using Isogen (Wako) according to the manufacturer's instructions. The reagents used for RT-PCR were Deoxyribonuclease I, Amplification Grade (DNase I, Amp Grade, Gibco BRL); 10 × DNase I Reaction Buffer; 25 mM EDTA (Gibco BRL); 25 mM $MgCl_2$ (TaKaRa); 10 × PCR buffer (TaKaRa); dNTP mixture (TaKaRa); 0.5 μg/μl Oligo $(dT)_{12\text{-}18}$ Primer (Gibco BRL), Superscript RNase H⁻ Reverse Transcriptase II (Gibco BRL); and 0.1 M DDT (Gibco BRL).

One microgram of the RNA samples, 1 μl of 10 × DNase I reaction buffer, 1 μl of DNase I, and DEPC-treated water to a final volume of 10 μl were added to a 0.5-ml tube, and the tube was incubated for 15 min at room temperature. DNase I activity was inactivated by the addition of 1 μl of 25 mM EDTA to the reaction mixture and heating for 10 min at 70°C. Reverse transcription of the RNA samples was carried out in 20 μl of reaction buffer containing 1 μl of Oligo $(dT)_{12\text{-}18}$ primer, 2 μl of 10 × PCR buffer, 1 μl of 25 mM $MgCl_2$, 2 μl of dNTP mixture, 2 μl of DTT, and 1 μl of Superscript RNase H⁻ Reverse Transcriptase II. First-strand cDNAs were synthesized at 42°C for 50 min. Then mRNA-cDNA chains were denatured, and the reverse transcriptase activity was arrested by heating 70°C for 10 min. The rat GMFB-specific upstream and downstream primers were 5′-CCG CTG ACG GCC GGA AGG-3′ and 5′-TTA TGT CTG GAT CCA GTA TGG TCA GGT-3′, respectively, which yielded a single band corresponding to a 513 base pair cDNA fragment. The reaction was carried out for 35 cycles, using a denaturing step at 94°C for 45 s, an annealing step at 58°C for 45 s, and an extension step at 72°C for 1 min.

PCR-amplified products were extracted with phenol: chloroform (1:1), precipitated, resuspended in 10 μl of TE buffer, and cloned into pCRII vector (Invitrogen). After several clones had been sequenced, we used the one clone without PCR error for the recombinant protein expression and as the probe for Northern blotting.

Northern Blot Analysis—Rat Multiple Choice™ Northern Blot (RB-1002, RB-1012) membranes were purchased from OriGene. Rat GMFB cDNA, rat GMFG cDNA, and human β-actin cDNA fragments (OriGene) were used for hybridization.

Membranes were prehybridized overnight at 42°C in prehybridization solution (50% formamide, 5 × SSC, 5 × Denhardt's solution, 1% glycine, 0.1% SDS, and 500 μg/ml denatured salmon sperm DNA). The fragments were labeled with [α-^{32}P]dCTP by a random primer labeling system (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Membranes were hybridized with each probe (2 × 10^6 cpm/membrane) overnight at 42°C in hybridization solution (50% formamide, 5 × SSC, 1.5 × Denhardt's solution, 0.1% SDS, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA). After hybridization, the rat GMFB and GMFG membranes were washed once with 2 × SSC containing 0.1% SDS for 15 min and once with 0.5 × SSC containing 0.1% SDS for 15 min at 42°C. The human β-actin membranes were washed once with 2 × SSC containing 0.1% SDS for 15 min and once with 0.2 × SSC containing 0.1% SDS for 15 min at 42°C. Autoradiography of air-dried membranes was performed by exposure to Kodak X-OMAT AR film at –80°C with intensifying screens.

Construction of Expression Vectors for Rat GMFB and GMFG—Oligonucleotide primers for rat GMFB and GMFG were arranged to include the *Hin*dIII, *Nde*I, and *Bam*HI restriction sites followed by sequences corresponding to the rat GMFB and GMFG cDNAs. For rat GMFB, one oligonucleotide primer (5′-CAA GCT TCA TAT GAG TGA GTC CTT GG-3′) was designed to include the *Hin*dIII and *Nde*I restriction sites preceding nucleotides 4 to 19 of the rat GMFB cDNA (accession number Z11558). A second oligonucleotide primer (5′-CGG ATC CAT TAG TGG AAA AAT CCA AGT TTC-3′) was synthesized complementary to nucleotides 411 to 434 of the rat GMFB cDNA and located 3′ to a *Bam*HI site. For rat GMFG, one oligonucleotide primer (5′-CAA GCT TCA TAT GTC CGA CTC CCT GG-3′) was designed to include the *Hin*dIII and *Nde*I restriction sites preceding nucleotides 6 to 21 of the rat GMFG cDNA. A second oligonucleotide primer (5′-CGG ATC CAA ACG TCA ACG AAA GAA AGC-3′) was synthesized complementary to nucleotides 420 to 440 of the rat GMFG cDNA and located 3′ to a *Bam*HI site.

PCR was performed using these primers and templates in a Perkin Elmer Cetus Model 480 thermal cycler. PCR conditions were 30 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 60 s. The amplified products were ligated into *Hin*dIII and *Bam*HI-digested pBluescriptII KS(+) vector (Stratagene). After the entire coding regions of several clones had been sequenced, plasmids without PCR error were prepared and digested with *Nde*I and *Bam*HI. The fragments were subcloned into *Nde*I and *Bam*HI-digested pAED4 (a generous gift from Don S. Doering, Whitehead Institute, Cambridge, MA) and introduced into *E. coli* BL21 (DE3) for expression studies.

Expression of Rat GMFB and GMFG in E. coli—Transformed BL21 (DE3) cells harboring the pAED4 expression plasmid were grown to the desired cell density (A_{600} 1.0) in 1 liter of Luria-Bertani medium containing 100 μg/ml ampicillin. Induction was achieved by isopropyl thio-β-D-galactoside supplementation to a final concentration of 0.4 mM. After incubation overnight at 16°C, cells were harvested by centrifugation at 8,000 rpm for 20 min at 4°C. The collected cells were suspended in 40 ml of 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl. The suspension was centrifuged at 12,000 rpm for 30 min at 4°C, and the pellets were resuspended in a solution containing 50 mM Tris-HCl, pH 7.4, 25% sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM pepstatin A and subjected to freezing and thawing three times. Cell debris was removed by centrifugation at 12,000 rpm for 30 min at 4°C, and the supernatant was used as the starting material for purification.

Purification of Recombinant Rat GMFB and GMFG—Using an FPLC system (Amersham Pharmacia Biotech), the supernatant from above was applied to a 5-ml HiTrap Q column (Amersham Pharmacia Biotech), which was equilibrated with 20 mM Tris-HCl, pH 7.4. The column was washed extensively with 20 mM Tris-HCl, pH 7.4 (5 void volumes), and eluted with a two-step linear gradient from zero to 1,000 mM NaCl. Fractions with elevated absorption

compared to corresponding control fractions (from *E. coli* transformed with pAED4 vector without insert) were pooled, electrophoresed on 12% SDS/PAGE gels, and electroblotted (180 mA, 100 V, 1 h, 4°C) onto polyvinylidene fluoride (PVDF) membranes (ProBlot, ABI). Visualization by Ponceau S staining enabled appropriate bands to be excised and subjected to Edman (*13*) degradation sequencing using a Model 476A protein sequenator (ABI).

Preparation of Antibodies against Recombinant Rat GMFB and GMFG—Purified recombinant rat GMFB and GMFG (500 μg each) were emulsified in mixtures of sterile PBS(–) and TiterMax® Gold adjuvant (CytRx Corporation) according to the manufacturer's protocols, and injected into New Zealand white rabbits. Second and third booster injections (500 μg each) were given at four-week intervals after the primary immunization. The titers of the anti-rat GMFB and GMFG sera were evaluated by the double-immunodiffusion method (*14*). The antibodies were concentrated from the rabbit sera (10 ml) by ammonium sulfate precipitation, and the sedimented material was suspended in 1 ml of distilled water prior to dialysis against 0.05 M Tris-HCl, 0.15 NaCl, pH 7.4. The supernatants obtained by centrifugation at 12,000 ×*g* for 20 min at 4°C were employed in subsequent experiments as anti–rat GMFB Ig and GMFG Ig. The concentration of Ig was estimated by absorbance at 280 nm ($A^{1\%} = 14.6$).

Anti–rat GMFB Ig was applied to a rat GMFG-affinity column to remove cross-reacting antibody to GMFG. The rat GMFG affinity column was prepared by coupling 500 μg of recombinant rat GMFG to a HiTrap NHS-activated column (1 ml, Amersham Pharmacia Biotech) following the manufacturer's instructions. Similarly, anti-GMFG Ig was applied to a rat GMFB-affinity column to remove cross-reacting antibody to GMFB. Adsorbed antibodies were eluted from the affinity columns with ActiSep elution medium (Sterogene Bioseparations) and dialyzed twice against 0.05 M Tris-HCl, pH 7.4, containing 0.15 NaCl.

Preparation of Tissue Extracts—Tissues were extracted at 4°C. Cerebral cortex, spleen, and thymus were harvested from an adult pregnant (18 days) female Wistar rat (Chubu-Kagaku Shizai), and testis was obtained from a young adult (11 weeks) male Wistar rat (Chubu-Kagaku Shizai). The tissues were immediately cooled on ice and homogenized individually in 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM pepstatin A. The homogenates were centrifuged at 1,000 ×*g* for 10 min, and the supernatants were transferred to fresh tubes and centrifuged at 100,000 ×*g* for 60 min. The 100,000 ×*g* supernatants were stored at –80°C.

Western Blot Analysis—Protein content was estimated by the bicinchoninic acid method as recommended by Pierce Chemicals. Samples of 40 μg of crude extracts were separated by electrophoresis. The gels were electroblotted onto PVDF membranes (Immobilon-P, Millipore) at 180 mA for 45 min at 4°C using a Western-blotting apparatus (Bio-Rad). Non-specific binding of antibodies was blocked with 5% dried milk in 0.1% Tween 20, 20 mM Tris-HCl, 137 mM NaCl, pH 7.6. Filters were subsequently incubated for 1 h at room temperature with rabbit antiserum diluted (1:1,000) in 20 mM Tris-HCl, 137 mM NaCl, pH 7.6, and 2 % BSA. Immobilized antibody was detected by ECL Western blotting detection set for rabbit antibodies (Amersham Pharmacia Biotech).

Preparation of Digoxigenin-Labeled cRNA Probe of rGMFG—To prepare digoxigenin-labeled cRNA probe for rat GMFG mRNA, a 285-bp sequence of rat GMFG cDNA corresponding to nucleotides 147–432 of rat GMFG cDNA was inserted into pBluescript II KS(+). For the anti-sense probe, this was digested with *Pst*I and *Eco*RI and then transcribed by T3 RNA polymerase in the presence of DIG-UTP (Boehringer Mannheim) to produce a digoxigenin labeled 323-nt fragment consisting of 285 nt of the rat GMFG sequence and 38 nt of the vector sequence. For the sense probe, the vector was digested with *Xho*I and *Hin*cII and then transcribed by T7 RNA polymerase in the presence of DIG-UTP to produce a digoxigenin labeled 349-nt fragment consisting of 285 nt of the rat GMFG sequence and 64 nt of the vector sequence.

In Situ Hybridization of Rat Brain and Testis—*In situ* hybridization with paraffin tissue sections was performed essentially as described by Panoskaltsis-Mortari and Bucy (*15*). Rat brain was harvested from an adult pregnant (18 days) female Wistar rat (Chubu-Kagaku Shizai), and testis was harvested from a young adult (11 weeks) male Wistar rat (Chubu-Kagaku Shizai). Sections were hybridized with 3 ng of heat-denatured cRNA probe for 16 h at 50°C in 100 μl of hybridization solution (50% formamide, 10 mM Tris-HCl, pH 7.6, 200 μg/ml yeast tRNA, 1 × Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, and 1 mM EDTA, pH 8.0). After hybridization, slides were successively washed in 2 × SSC for 30 min at room temperature, treated at 37°C for 30 min with RNase A (10 μg/ml in 500 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA), washed at 50°C for 15 min in 2 × SSC containing 50% formamide, and at 50°C for 15 min in 1 × and 0.2 × SSC. DIG-labeled RNA probes used for hybridization were immunodetected with anti-digoxigenin-alkaline phosphatase Fab antibody (1:2,000; Boehringer Mannheim), and nitroblue tetrazolium 6-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Boehringer Mannheim) as a substrate for the alkaline phosphatase. Staining was allowed to proceed overnight in the dark at room temperature, and the reaction was stopped in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA.

RESULTS

Isolation of Rat GMFG cDNAs—Screening a rat pulmonary artery cDNA library in Uni-ZAP™ XR vector with full-length human GMFG cDNA (*8*) yielded seven positive clones upon quaternary hybridization. The clones were converted into pBluescript SK(–) plasmid clones by *in vivo* excision and were sequenced. One of the positive clones contained a 553 nucleotide long insert and an open reading frame of 426 bp encoding a deduced protein of 142 amino acids (Fig. 1), predicted to be rat GMFG. The deduced amino acid sequence showed 78.9 and 91.5% identity with rat GMFB and human GMFG, respectively (Fig. 2).

Northern Blot Analysis—We examined the tissue distribution of mRNAs by Northern blotting (Fig. 3). A rat GMFG cDNA fragment was used for hybridization on membranes of Rat Multiple Choice™ Northern Blot (RB-1002, RB-1012). A predominant band indicating a 0.9-kb transcript was detected in rat thymus, testis, and lung and to a lesser extent in spleen, skin, brain, small intestine, and stomach. Reprobing the same membranes with a rat GMFB cDNA fragment showed rat GMFB mRNA as a 4.1-

kb band. In contrast to the distribution of GMFG, the 4.1-kb transcript of rat GMFB was detected predominantly in brain, lung, and heart and only faintly in the other tissues.

Expression of Recombinant Rat GMFB and GMFG—Expression vectors comprising pAED4 containing rat GMFB cDNA (pAED4-rGMFB) and rat GMFG cDNA (pAED4-rGMFG) were introduced into *E. coli* BL21(DE3). Each vector contained a cassette encoding a 5′ segment containing an *Nde*I recognition sequence coincident with the ATG translation-start site. SDS-PAGE analysis of extracts from transformed *E. coli* BL21(DE3) which had been induced with 0.4 mM isopropyl thio-β-D-galactoside revealed a band of approximately 17 kDa (data not shown). This band was absent from extracts of control transformants.

The molecular mass of purified recombinant rat GMFB and GMFG from the supernatant-fraction was estimated to be 17 kDa by SDS-PAGE analysis and Coomassie Brilliant Blue staining (Fig. 4A). The bands were transferred to PVDF membranes, excised, and their amino acid sequences were determined. The N-terminal amino acid sequences of the 17-kDa protein were SESLVVXDVA for rat GMFB and SDSLVVXDVD for rat GMFG, which are identical to the predicted amino acid sequences except for the N-terminal methionines.

Antibodies against Recombinant Rat GMFB and GMFG—Purified recombinant rat GMFB and GMFG were used as immunogens for the production of polyclonal antibodies in New Zealand white rabbits. The titers of anti-rat GMFB and GMFG sera were evaluated by dot-blots as well as by double-immunodiffusion. Anti-rat GMFB serum did not cross-react with rat GMFG, and anti-rat GMFG serum did not cross-react with rat GMFB by double-immunodiffusion. By Western blot analysis, however, both antisera showed weak cross-reactivity (data not shown). To isolate the specific antibodies, anti-rat GMFB antibody was applied to a rat GMFG-affinity column, and anti-rat GMFG antibody to a rat GMFB-affinity column. After removing the cross-reacting antibodies, the antibody to GMFB detected only rat GMFB, and the GMFG antibody only rat GMFG. The cross-reacting antibody obtained from the adsorbed fraction of GMFG and GMFB antibody on the GMFB and GMFG-affinity column reacted with both rat GMFB and GMFG (Fig. 4A).

Western Blot Analyses of Rat Tissue Extracts—Western blot analyses of tissue extracts from rat cerebral cortex, spleen, thymus, and testis were performed using anti-rat GMFB and GMFG antibodies. Crude tissue extracts, each containing 40 μg of protein, were separated by 12% SDS/PAGE, transferred to PVDF membranes and probed with anti-rat GMFB and GMFG antibodies. Antibody specific to rat GMFB reacted predominantly with a 17-kDa band in the tissue extracts from rat cortex. In the extracts from rat spleen and thymus, the bands were weak (Fig. 4B).

On the other hand, antibody to rat GMFG reacted predominantly with a 17-kDa band in the extracts from rat spleen and thymus. In the extracts from rat cortex, the anti-rat GMFG antibody reacted very weakly (Fig. 4B). In the extracts from rat testis, the band was not detected with either antibody (data not shown).

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                                                                  AAAC   -1
ATGTCCGACT CCCTGGTGGT GTGTGACGTG GACCCGGAGC TAAAGGAAAC ACTGAGGAAA    60
M  S  D  S   L  V  V   C  D  V   D  P  E  L   K  E  T   L  R  K
TTCCGTTTCC GAAAAGAGAC CAACAATGCC GCCATCATAA TGAAAGTGGA CAAAGACCGG   120
F  R  F  R   K  E  T   N  N  A   A  I  I  M   K  V  D   K  D  R
CAGATGGTGG TGCTAGAAGA TGAATTCCAG AATGTTTCCC CAGAGGAACT TAAGTTGGAG   180
Q  M  V  V   L  E  D   E  F  Q   N  V  S  P   E  E  L   K  L  E
TTGCCAGAGA GACAGCCCAG GTTCGTGGTC TACAGCTATA AGTACGTGCA TGACGACGGC   240
L  P  E  R   Q  P  R   F  V  V   Y  S  Y  K   Y  V  H   D  D  G
AGGGTGTCCT ACCCTTTGTG TTTCATCTTC TCCAGCCCCG TGGGCTGCAA GCCTGAACAA   300
R  V  S  Y   P  L  C   F  I  F   S  S  P  V   G  C  K   P  E  Q
CAGATGATGT ACCCGGGAAG TAAAAACAGG CTGGTGCAGA TCGCGGAGCT TACAAAGGTG   360
Q  M  M  Y   A  G  S   K  N  R   L  V  Q  I   A  E  L   T  K  V
TTTGAAATCC GCACCACAGA CGACCTCAAC GAGACCTGGC TTAAGGAAAA GTTAGCTTTC   420
F  E  I  R   T  T  D   D  L  N   E  T  W  L   K  E  K   L  A  F
TTTCGTTGAC GTTTGAGCCA GGGATTTGGA ATCTTTACTT CGGAAGACTC AGACTGATAG   480
F  R  *
GATATGAACA TCCAAGAGCT AAATAAAGAT TAAAATTTCA GGAATGTTAA AAAAAAAAAA   540

AAAAAAAAAA AAA                                                     553
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Fig. 1. **Nucleotide and predicted amino acid sequence of the rat GMFG cDNA.** The asterisk indicates the presumed terminal codon. The polyadenylation signal is underlined. The nucleotide sequence shown here has been assigned DDBJ, EMBL, and GenBank accession no. AB007364.

A

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Rat GMFB    MSESLVVCDV AEDLVEKLRK FRFRKETHNA AIIMKIDKDK RLVVLDEELE    50
Rat GMFG    MSDSLVVCDV DPELKETLRK FRFRKETNNA AIIMKVDKDR QMVVLEDEFQ    50

Rat GMFB    GVSPDELKDE LPERQPRFIV YSYKYQHDDG RVSYPLCFIF SSPLGCKPEQ   100
Rat GMFG    NVSPEELKLE LPERQPRFVV YSYKYVHDDG RVSYPLCFIF SSPVGCKPEQ   100

Rat GMFB    QMMYAGSKNK LVQTAELTKV FEIRNTEDLT EEWLREKLGF FH           142
Rat GMFG    QMMYAGSKNR LVQIAELTKV FEIRTTDDLN ETWLKEKLAF FR           142
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B

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Rat   GMFG  MSDSLVVCDV DPELKETLRK FRFRKETNNA AIIMKVDKDR QMVVLEDEFQ    50
Human GMFG  MSDSLVVCEV DPELTEKLRK FRFRKETDNA AIIMKVDKDR QMVVLEEEFQ    50

Rat   GMFG  NVSPEELKLE LPERQPRFVV YSYKYVHDDG RVSYPLCFIF SSPVGCKPEQ   100
Human GMFG  NISPEELKME LPERQPRFVV YSYKYVHDDG RVSYPLCFIF SSPVGCKPEQ   100

Rat   GMFG  QMMYAGSKNR LVQIAELTKV FEIRTTDDLN ETWLKEKLAF FR           142
Human GMFG  QMMYAGSKNR LVQTAELTKV FEIRTTDDLT EAWLQEKLSF FR           142
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Fig. 2. **A: Comparison of amino acid sequences of rat GMFG and rat GMFB.** Non-identical amino acid residues are indicated by shadows. Conserved cysteine residues are boxed. Probable phosphorylation sites are underlined. **B: Comparison of amino acid sequences of rat GMFG and human GMFG.** Non-identical amino acid residues are indicated by shadows. Conserved cysteine residues are boxed. Probable phosphorylation sites are underlined.

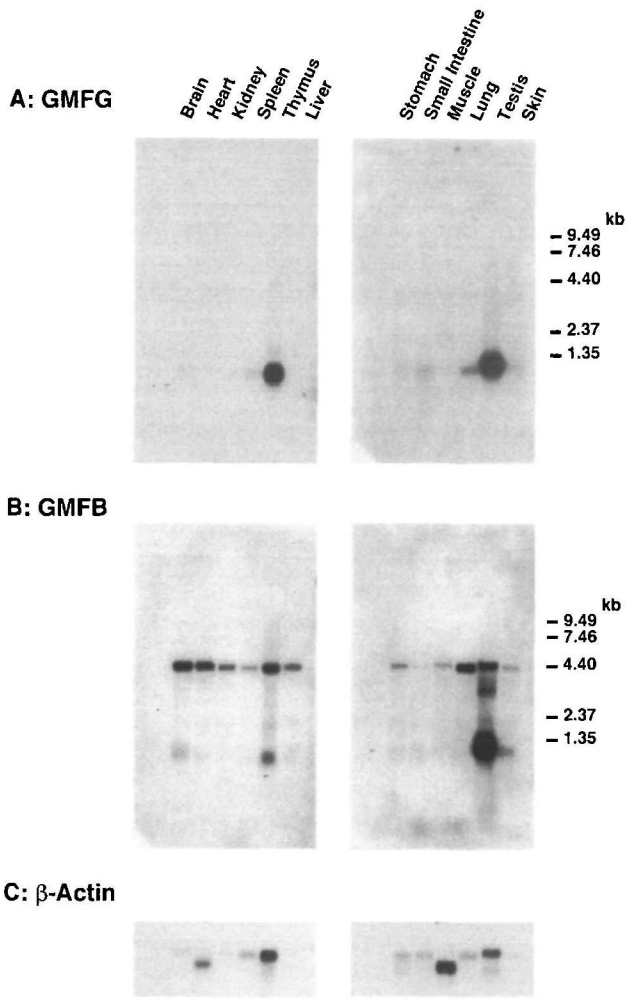

Fig. 3. **Expressions of rat GMFG mRNA in different tissues using a Rat Multiple Choice™ Northern Blot (OriGene).** A: A message of approximately 0.9 kb was detected. The size marker is indicated on the right. Autoradiographic exposure was for 72 h. B: The same filters were reprobed with rat GMFB and a message of approximately 4.1 kb was detected. C: The same filters were reprobed with β-actin as a control.

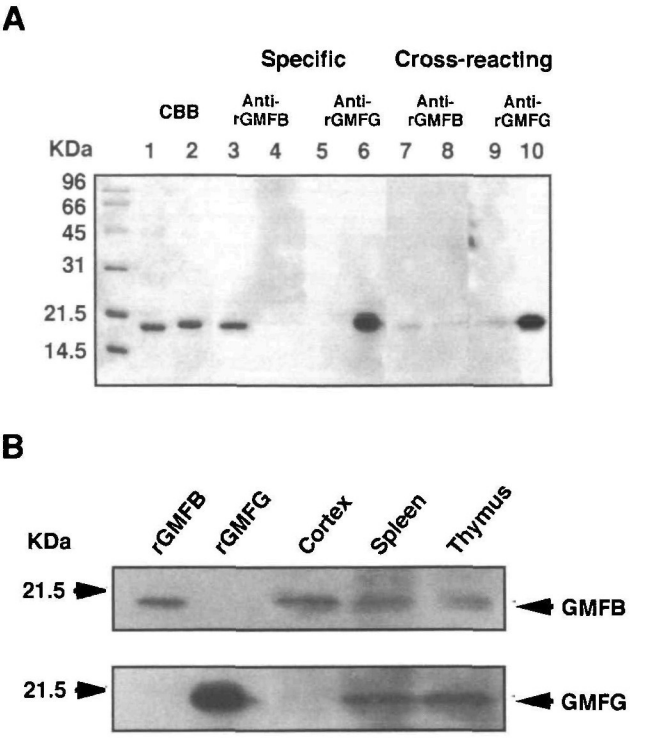

Fig. 4. **A: Western blotting analyses of GMFB and GMFG.** Recombinant preparations of rat GMFB (lanes 1, 3, 5, 7, and 9) and GMFG (lanes 2, 4, 6, 8, and 10) were subjected to SDS-PAGE (left panel; the SDS-PAGE gel stained with Coomassie Brilliant Blue), transferred to Immobilon-P membranes, and probed with specific antibodies (1,000 × dilution), and cross-reacting antibodies (1,000 × dilution) separated from rabbit antiserum to recombinant rat GMFB and GMFG. Immobilized primary antibody was detected using horseradish peroxidase-coupled anti-rabbit IgG. Two specific antibodies recognized their respective 17-kDa proteins without cross-reaction. Numbers at the left indicate molecular masses of protein standards. **B: Expression of GMFB and GMFG proteins in adult rat organs.** Organ extracts were prepared from an adult pregnant rat as described in "MATERIALS AND METHODS." Protein samples (40 μg per lane), and recombinant rat GMFB and GMFG were separated on 12% polyacrylamide gels under reducing conditions and electroblotted on PVDF membranes. Immunodetection was carried out with specific anti–GMFB Ig (upper panel) and anti-GMFG Ig (lower panel). GMFB and GMFG protein bands were detected at approximately 17 kDa.

In Situ Hybridization of Rat Brain and Testis—By Western blotting analysis, GMFG was not detected in rat brain and testis. However, mRNA of rGMFG was detected in both organs, especially testis, by Northern blotting analysis. Therefore, we studied *in situ* hybridization of GMFG in rat brain and testis. In rat brain, mRNA of rGMFG was localized around pyramidal cells within CA3 of hippocampus (Fig. 5C). In rat testis, mRNA of rGMFG was detected in spermatids (Fig. 5A). *In situ* hybridization the using sense probe of rGMFG did not visualize the mRNA in either organ (Fig. 5, B and D).

DISCUSSION

We isolated a rat GMFG cDNA and deduced the sequence of rat GMFG. A homology search using the FASTA program revealed that within the coding region, the cDNA shows 71% identity with the rat GMFB gene and 88% identity with the human GMFG gene. The amino acid sequence of the predicted product, rat GMFG, showed 78.9% identity with rat GMFB, and 91.5% identity with human GMFG (Fig. 2, A and B). The amino acid sequence of rat GMFG contains several noteworthy features. The cysteine residues at position 8, 87, and 96, which are considered to be crucial for the biological activity of GMFB, are completely conserved between human and rat (*16–18*). Zaheer and Lim (*7*) and Lim and Zaheer (*19*) reported that GMFB can be phosphorylated *in vitro* by at least four kinases: protein kinase C (PKC), protein kinase A (PKA), casein kinase II (CKII), and p90 ribosomal S6 kinase (RSK). According to the amino acid sequence of human GMFB, the phosphorylation sites should be serine 72 (SYK) for PKC (consensus: S/T-X-K/R), threonine 27 (KET) and serine 83 (RVS) for PKA (consensus: K/R-X-S/T), serine 53 (SPDEL) for CKII (consensus: S/T-X-X-E/D), and threonine 27 (RFRKET) for RSK (consensus: R-X-R-X-X-S/T). These four phosphorylation

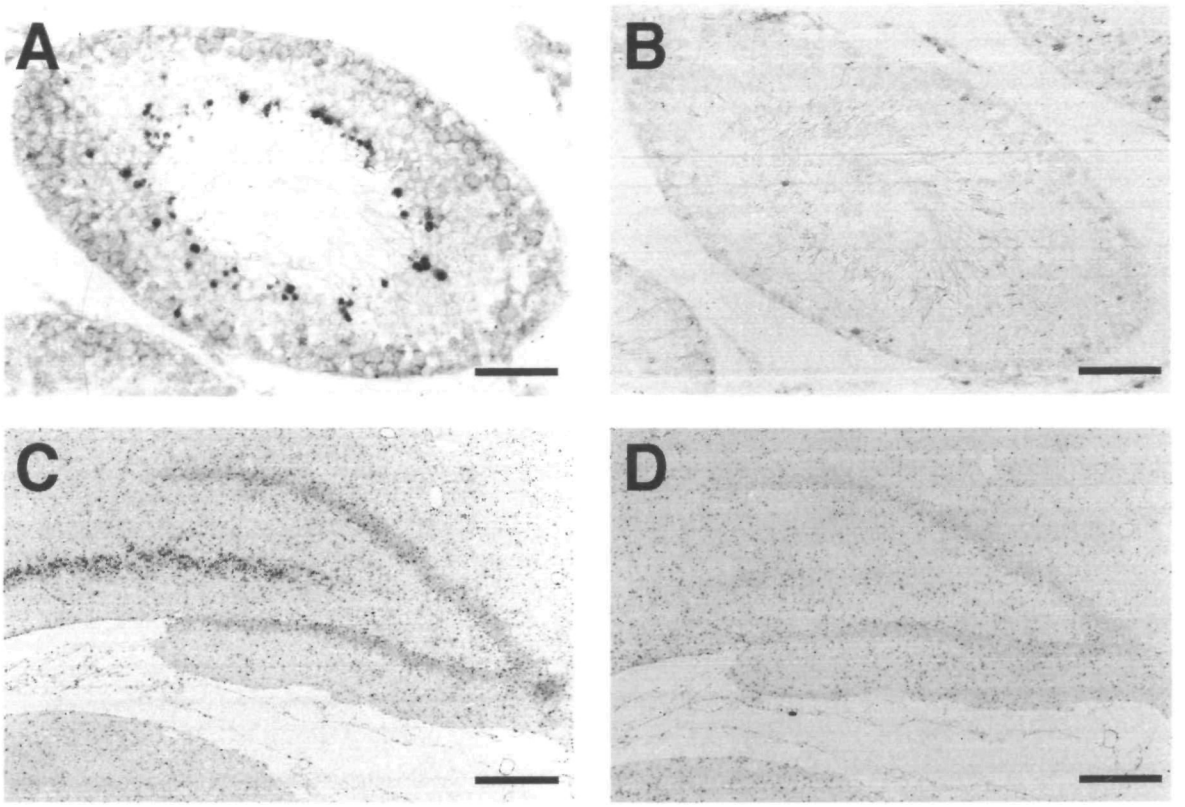

Fig. 5. ***In situ* hybridization of GMFG in rat brain and testis.** Sections of each organ were prepared from an adult pregnant rat and young adult male rat as described in "MATERIALS AND METHODS." Detection of GMFG-mRNA was examined using a digoxigenin-labeled cRNA probe for rat GMFG mRNA. A and B: Sections of rat testis hybridized with anti-sense probe (A) and sense probe (B). C and D: Sections of hippocampus of rat brain hybridized with anti-sense probe (C) and sense probe (D). Scale bars are 50 μm in A and B and 200 μm in C and D.

sites are conserved in rat GMFG, and other putative phosphorylation sites, threonine 17 (TLR) for PKC and threonine 125 (TTDDL) for CKII, are found uniquely in rat GMFG. These phosphorylation sites are consistent with those recently reported by Asai *et al.* (*8*). Zaheer and Lim (*7*) and Lim and Zaheer (*19*) reported that endogenous GMFB in astrocytes was rapidly and transiently phosphorylated at serine and threonine residues after stimulation by phorbol 12-myristate 13-acetate (PMA), and PKA-phosphorylated GMF was an inhibitor of ERK1/ERK2 and an enhancer of p38, both being isoforms of MAP kinase. Furthermore, they recently showed that overexpression of GMF in PC12 cells increased p38, MAPKAP-K2, and tyrosine hydroxylase activity, along with the phosphorylation of Hsp25 and tyrosine hydroxylase (*20*). Overexpression of GMF in C6 rat glioma cells led to restoration of normal cell properties, including morphological differentiation, contact inhibition, and decrease in tumorigenicity and activation of the antioxidant enzyme superoxide dismutase (*21*). These reports suggest that GMF might be a regulator of signal transduction. Considering the four conserved phosphorylation sites and the two additional phosphorylation sites, GMFG may also be involved in a slew of intracellular signal transductions and have functions other than those of GMFB.

By Northern blot analysis (Fig. 3), rat GMFB mRNA was detected predominantly in brain, lung, heart, thymus, and testis and only faintly in other tissues. In contrast to this, a 0.9-kb transcript of rat GMFG was detected predominantly in thymus and testis and faintly in other tissues. Zaheer *et al.* reported that rat GMFB mRNA was predominantly detected in rat brain with trace levels in other organs (*22*). In our experiments, rat GMFB mRNA was predominantly detected not only brain but also in other organs. This discrepancy might be due to developmental variations in mRNAs collected from rats of different ages (newborn, young adult, or adult) (*22*). GMFG mRNAs were expressed more strongly than those of GMFB in undifferentiated cell-rich organs such as thymus. This suggests that GMFG is involved in cell differentiation as well as growth.

To examine the expression of GMFB and GMFG proteins in organs by Western blot analysis we used polyclonal anti-rat GMFB and GMFG antibodies. Figure 4A shows that each specific antibody recognizes the corresponding recombinant protein, and cross-reacting antibody recognizes both GMFB and GMFG. The specific anti-GMFB antibody visualized the bands (17 kDa) predominantly in cortex and weakly in spleen and thymus (Fig. 4B). In contrast, the specific anti-GMFG antibody detected the bands predominantly (17 kDa) in spleen and thymus and very weakly in cortex (Fig. 4B). These results were consistent with the result of Northern blotting except testis (Fig. 3). Although anti-GMFG antibody did not detect the bands in testis, we found that the mRNAs located in spermatids, the cells formed in last stage of spermatogenesis, by *in situ* hybridization. The protein contents of the crude extracts may be

too low for detection by Western blot analysis. Furthermore, anti-GMFG antibody detected the bands very weakly in cortex, but the mRNAs were detected around pyramidal cells within CA3 of hippocampus by *in situ* hybridization (Fig. 5C).

High homology was found between rGMFB and rGMFG (Fig. 2A). However, the two proteins showed clear differences in tissue distribution. GMFG is richer than GMFB in thymus and spleen, which contain cells undergoing proliferation and differentiation. The function of GMFG is not yet fully understood, but these results suggest that it might play important roles in cell differentiation and growth through intracellular signal transduction, in similar ways to GMFB or in other ways.

In this study, we isolated a rat GMFG cDNA and examined expression of rGMFG mRNA. The tissue distribution of GMFG differs from that of GMFB. GMFG is predominantly expressed in organs retaining proliferative and/or differentiative potential.

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