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Molecular Cloning and Characterization of a Serine/Threonine Phosphatase from Rat Brain

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Abstract – A novel serine/threonine protein phosphatase with EF-hand motif, which belongs to PPEF family was partially cloned from rat brain cDNA by employing RT-PCR method. The size of the amplified clone was 1.6kbp. The amplified DNA was subcloned into pGEM-T-Easy vector and the resulting plasmid was named as pGEM-rPPEF2. The nucleotide sequence is shared by 88% with that of mouse PPEF-2 cDNA, and the deduced amino acid sequence reveal 92% homology with that of mouse PPEF-2 cDNA. The N-terminal region of the cloned rat brain PPEF contains a putative phosphatase catalytic domain (PP domain) and the C-terminal region contains multiple Ca^{2+} binding sites (EF region). The putative catalytic domain (PP) and the EF-hand motif (EF) regions were subcloned into pGEX4T-1 and were overexpressed in E. coli DH5 as glutathione-S-transferase (GST) fusion proteins. Expression of the desired fusion protein was identified by SDS-PAGE and also by immunoblot analysis using monoclonal antibody against GST. The recombinant proteins were purified by glutathione-agarose chromatography. This report is first to demonstrate the cloning of PPEF family from rat brain tissues. The clone reported here would be invaluable for the investigation of the role of this new type-phosphatase in rat brain.

Key words □ PPEF, cloning, sequence homology, GST fusion protein, overexpression, purification.

The phosphorylation/dephosphorylation of a protein is a crucial mechanism for the regulation of the cellular functions including cell cycle, metabolism, signal transduction, and gene transcription. The protein phosphatase that catalyze the dephosphorylation of serine and threonine residues have been classified to four types (PP1, PP2A, PP2B, and PP2C) based on their biochemical characteristics, their sensitivity to specific inhibitors and a limited substrate specificity that can be demonstrated *in vitro* (Brautigan, 1994; Cohen, 1989; DePaoli-Roach *et al.*, 1994; Mumby *et al.*, 1993; Shenolikar, 1994). Recently, several additional phosphatases including PP4 (PPX: Brewis *et al.*, 1993), PP5 (Chen *et al.*, 1994; Chinker, 1994), PP6 (Bastians *et al.*, 1996; 1997), and PP7 (Huang *et al.*, 1998) have been identified, but rarely characterized.

It has been reported that Drosophila retinal degeneration C(rdgc) gene encodes a novel serine/threonine phosphatase (Steele *et al.*, 1992) and its gene product dephosphorylates rhodopsin that is the prototype of a protein family known as G-protein coupled receptors (Vinos *et al.*, 1997). Thereafter, several other researchers reported the cloning of rdgc related phosphatases from human and mouse (Steele *et al.*, 1992; Sherman *et al.*, 1997; Montini *et al.*, 1997; Huang *et al.*, 1998). These phosphatases contain characteristic Ca^{++}-binding EF-hand motif (EF-domain) in its sequence in addition to the phosphatase catalytic domain (PP-domain). It is increasingly evident that these phosphatases consist a new type of phosphatase family designated as PPEF family. Interestingly, the first member of this family, rdgc catalyzes the dephospho-

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rylation reaction of rhodopsin. Rhodopsin is a photoreceptor molecule found in retina and is prototype of G protein coupled receptors (GPCR). The dephosphorylation of rhodopsin and GPCR represents a mechanism for resensitization (Kreuger *et al.*, 1997). Therefore, it could be hypothesized that PPEF family of phosphatase performs a role in GPCR signaling in brain.

In this study, we identified and partially cloned a novel serine/threonine phosphatase from rat brain, which is homologous to PPEF family phosphatase. In addition, Ca^{++}-binding and phosphatase motifs were overexpressed in E. Coli as Glutathione S-transferase (GST) fusion protein, which could be used for the biochemical analysis of the function of the clone or antibody production.

MATERIALS AND METHODS

Materials

Trizol, Electrophoresis reagents and reverse transcriptase were obtained from Gibco BRL (Gaithersburg, MD). Oligo(dT)-cellulose was obtained from Qiagen (Hilden, Germany). Restriction enzymes and other enzymes used in molecular cloning were purchased from Promega (Madison, WI). Isopropyl-D-thiogalactopyranoside (IPTG) was purchased from Boehringer Mannheim (Mannheim, Germany). Sepharose CL-4B were obtained from Pharmacia (Uppsala, Sweden). Immunochemical reagents including horseradish peroxidase-conjugated goat anti-mouse IgG or IgM were purchased from Pierce (Rockford, IL). Prestained protein molecular weight marker (myosin, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, α-lactoglobulin, lysozyme) were obtained from Gibco (Gaithersburg, MD). All other reagents were obtained from Sigma Chemicals (St. Louis, MO) and were of the highest grade commercially available.

cDNA preparation and PCR amplification of rat brain cDNA

Total RNA from rat brain was isolated using Trizol reagent according to the manufactor's instruction (GibcoBRL, Gaithersburg, MD). Poly(A)+ mRNA was obtained by affinity chromatography using oligo(dT)-cellulose (Qiagen, Hilden, Germany). First strand cDNA was produced by incubating 1 μg of poly(A)+ mRNA in a solution containing 50 mM KCl, 2m M $MgCl_2$, 0.2 mM dNTP mix, 10 mM Tris-HCl pH 8.3 with 20 pmole of oligo(dT) primer and 200 units of reverse transcriptase (GibcoBRL, Gaithersburg, MD) at 42°C for 50 min.

5 μl aliquots from the first strand cDNA solution was employed as template for PCR reaction. The PCR reaction mixture contained a 5'primer (5'-GGTTTGCATTCATGTTG-GTGTACC-3') matching to the conserved region of catalytic domain of human and mouse PPEF-2 and a 3' oligo(dT) primer. PCR reactions were carried out as follows: 1 min at 95°C, 1 min at 50°C, 1 min at 72°C, 35 cycles, in a solution containing 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTP mix, 10 mM Tris-HCl pH 8.3, and 20 pmole of each primer sets. The size of PCR products were confirmed by agarose gel electrophoresis. The amplified band was extracted and subcloned into pGEM-T vector and the resulting plasmid was named as pGEM-rPPEF2. The sequence of the amplified PCR product was analyzed by dideoxy chain termination methods.

Expression and purification of the GST fusion protein of catalytic and calcium binding domain

Putative catalytic domain (PP domain), and calcium binding EF-hand motif domain (EF domain) of rat brain PPEF-2 were amplified from 10ng aliquots of pGEM-rPPEF2 by PCR. The primer sets used for PCR reaction were as follows:

PP domain,

forward primer: 5'-CTCGAGGGTGAATTCGT-3'

reverse primer: 5'-AATGGCTTACATTCGTG-3'

EF-domain

forward primer: 5'-GGCTGTGAATTCTGCCAC-3'

reverse primer: 5'-CCCGTCCTTGTTGAATTCGATGCT-TCTGGCAAG-3'

The sequence of primer pairs for PP domain and EF domain were slightly modified to contain the restriction site EcoRI-XhoI and EcoRI-EcoRI, respectively. PCR reaction was carried out as follows: 1 min at 95°C, 1 min at 50°C, 1 min at 72°C, 35 cycles, in a solution containing 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTP mix, 10 mM Tris-HCl pH 8.3, and 20 pmol of each primer sets. PCR products were digested with respective restriction enzyme and then ligated into pGEX4T-1, a GST-fusion protein expression vector. The fusion protein expression vector was transformed into E. coli DH5α cells by heat shock method.

E. coli cells transformed with pGEX4T-PP and pGEX4T-EF were grown at 37°C and were induced by 0.1 mM isopropyl-D-thiogalactoside (IPTG) for 4 h at 37°C. Purification of the fusion proteins was performed as previously described (Shin *et al.*, 1998). Briefly, the fusion proteins were solubilized by sonication after the addition of 1.5% N-lauroylsar-

cosine. The supernatant containing solubilized fusion proteins was adjusted to 2% Triton X-100 and incubated with glutathione-Sepharose CL-4B beads for 30 min. After washing, the fusion proteins were eluted with 10 mM reduced glutathione.

SDS-PAGE and Immunoblot analysis

The expression of the fusion protein was identified by SDS-PAGE on 10% polyacrylamide gel followed by staining with Coomassie brilliant blue. In some cases, the resolved protein bands were electrophoretically transferred onto nitrocellulose membrane in methanol/glycine/Tris buffer as described for immunoblot blot analysis (Towbin *et al.*, 1979). After blocking, the nitrocellulose strips were incubated for 2 h at 32°C with monoclonal anti-GST antibody and then were incubated with horseradish peroxidase-conjugated goat anti-mouse IgM diluted 1:3,000 in blocking solution for 1 h at 32°C. Blots were washed and the immunoreactivity was visualized with 3,3'-diaminobenzidine substrate solution (9 mg in 10 ml of 50 mM Tris-HCl, pH 7.5, 0.01% H_2O_2).

RESULTS

PCR amplification and sequence analysis of rat brain PPEF cDNA

A cDNA fragment encoding a serine/threonine phosphatase from rat brain was identified by RT-PCR with a 5' primer matching to the conserved region of catalytic domain of human and mouse PPEF-2 and 3' oligo(dT) primer. The molecular weight of the amplified cDNA fragment was confirmed by agarose gel electrophoresis. The size of the amplified clone was 1.6 kbps. The PPEF clone was subcloned into pGEM-T-Easy vector and the resulting plasmid was named as pGEM-rPPEF2 (Fig. 1).

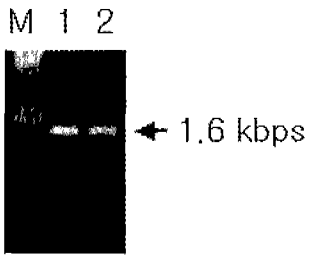

Fig. 1. Agarose gel electrophoretic analysis of the RT-PCR amplification product from rat brain cDNA using a 5' primer matching to the conserved region of catalytic domain of human and mouse PPEF-2 and 3' oligo(dT) primer. Lane M: kb ladder marker, Lane 1, 2: amplified PPEF clone. The size of the amplified DNA fragment was 1.6 kbp.

The nucleotide sequence was shared by 88% with mouse PPEF-2 cDNA (Fig. 2), and the deduced amino acid sequence revealed 92% homology with that of mouse PPEF-2 cDNA (Fig. 3).

Subcloning and overexpression of recombinant proteins

DNA fragments encoding putative catalytic domain and EF-hand motif domain of rat brain PPEF were amplified by PCR. The sequence of primer pairs for the catalytic domain and EF-hand motif were slightly modified to contain restriction sites EcoRI-XhoI and EcoRI-EcoRI, respectively. Both the amplified DNAs are about 600 bp in size (Fig. 4). Each PCR fragment was cloned into respective restriction site of the expression vector pGEX 4T-1, and resulting expression plasmids were transformed into E. Coli DH5. The expression of fusion proteins was confirmed by SDS-PAGE and also by immunoblot analysis using anti-GST antibody (Fig. 5). The fusion proteins purified by GSH affinity chromatography were shown on the gels at apparent molecular weight of 50 kDa, which was predicted from their amino acid sequence.

DISCUSSION

We identified the expression of a rat brain cDNA that encodes a putative serine/threonine protein phosphatase (PPEF-2) that is homologous to mouse brain PPEF-2 (Steele *et al.*, 1992) by RT-PCR. Initially, using more than 15 primer sets, we succeeded to obtain 600 bp fragment which encodes putative Ca^{++}-binding EF-hand motif domain. Hybridization screening of rat cDNA library with the short fragment of PCR-amplified rat brain PPEF-2 homologue was unsuccessful to reveal full length cDNA (data not shown). In order to obtain more larger cDNA fragment, rat brain total RNA was reverse transcribed. PCR was performed with degenerate oligonucleotide primer. As a 5' primer, the region showing high sequence similarity with mouse brain PPEF-2 was selected and oligo(dT) primer was used as a 3' primer. Electrophoretic analysis of PCR product revealed a rather smear band in the first PCR reaction and a band of the expected size (1.6 kbps) in second PCR reaction (Fig. 1). These results and negative hybridization screening results implicate that the PPEF-2 mRNA transcripts are rarely expressed in rat brain tissue.

The PCR amplified cDNA fragment was isolated from the agarose gel and was subcloned into E. coli DH5α cell. The nucleotide and deduced amino acid sequences of the rat

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mouse PPEF-2    1 atgggaagca gctcatccac ccagcaccac tttgcgtttc agaacgctga gaaagccttc
Rat PPEF       61 aaggcagcgg ccctgatcca gagatggtac cggcgctaca tggcccgcct agagatgagg
              121 aggcgatgta cctggaacat cttccagtct atagagtatg ctggacagca agaccaggtc
              181 aagctccatg aattcttcag ctaccttgtg gaccatttca ctcccagcag ccaccatgag
              241 agagatttcc tgaaccgcat gttcaccgaa gagagattcg cccaggacgt ggagacagag
              301 gagggtggag attttgaatc catagaggtg ccagacagct acacggggcc acgcctctcc
              361 ttcccccttc ttcctgacca tgccactgcc ctggtggaag ctttcaggct gcgacaacag
              421 ctccatgctc gatacgtctt gaatcttctg tacgagacca gaaaacacct ggcccagctg
              481 cccaacatca accgagtctc cacctgctac agcgaggagg tcacagtgtg tggagatcta
              541 catggccagt tggatgattt aatatttata ttttataaga acggtctacc atcaccagag
              601 agagcatacg tgttcaatgg tgactttgtg gatcgaggca aggactctgt agaggtcctg
              661 atggttctct ttgccttcat gttggtatac cccaaggagt tccatcttaa cagaggaaac
                        t ttgacttcat gttggtatac cccaaagagt ttctcatcttaa cagaggaaac
              721 catgaggacc atctagtgaa cctacgatat ggcttcacca aggaagtgat gcataaatac
                  catgaggacc atctagtgaa cttacgatac ggcttcacca aggaagtgat gcataaatac
              781 aagatacatg ggaagaaaat cctaaggaca cttcaggatg tcttctgctg gcttccgttg
                  aagatacatg ggaagaaaat cctaaggacg cttcaagatg tcttctgctg gcttccactg
              841 gccactctgg ttgatgagaa agtccttgtt cttcatggtg gagtctcaga caagacagac
                  gccactctgg ttgatgagaa agttcttgtt cttcatggcg gggtctcaga caagacggac
              901 ctggaacttc tggctaaact agacaggcac aagattgttt ctaccatgag gtgcaaaaca
                  ctggaacttc tggctaaact agacaggcac aagattgttt ctaccatgcg gtgaaaaaca
              961 agaaaggaaa gtgagaatcg agaggagcag aagagaaaag ataaccagac aagctctgga
                  agaaaggaaa gtgagaatct ggaggagcag aagagaaaag ataaccagac aagctctgca
             1021 cagaaacctа ctccgtggtt tcttcctcaa agccgctctc tgccctcttc gccttttcac
                  cagaaaccga ctccgtggtt tcttcctcag agccgctctc tccctcttc gccttttcac
             1081 ctgggctctg gctttaaggc ctacaaagcc ggcaggtcct gcagcatccc ctgtggctcc
                  ctgggctctg ggtttaaggc ctacaaagcc tgcaggtcct gcagcatccc ctgtggctcg
             1141 cctaacagca aggagctatc ccggcggggg caggtgcggc gctcagtgga cctggaactg
                  gctagtagca aagagctgtc ccggcagggg caggtgcggc gctccgtgga cctggagctg
             1201 gaacagtgcc ggcagcaagc cggcttcctg gggatcagag agaaggggga gtccttgcct
                  gaactgtgcc ggcagcaagc cggcttcctg gggatccgag agaagagggga gtcttgccc
             1261 ttggccccag atgctgactg tgttgctgat gggggagggg tgctggaacc caccccgag
                  ttggcc---- -----gactg tgacgctgat gcaggagggga tgctgaagcc cactcctgag
             1321 gagtggaagc aggttgtaga tattctgtgg agtgatcccg cggctcagga gggctgcaag
                  gagtggaagc aggttgtaga tattctgtgg agcgatcccа tggctcagga gggctgcaag
             1381 gccaacgctg tccgaggagg aggctgttac tttgggcctg acgtgacaga acggttgatg
                  gccaacactg tccgaggagg aggctgttac tttgggcctg atgtgacaga gcagttgatg
             1441 gaaaaatаca agctgcaact cctgatccgt tcgcatgagt gcaaacccga gggctatgag
                  gagaaatаca agctacaatt cctgatccga tcacatgagt gcaagcccga aggctatgaa
             1501 ttctgtcaca accgcaaggt gttaaccatc ttttctgcct ccaactacta tgaagttggc
                  ttctgtcaca accgcaaggt gttaaccatt ttttctgcct ctaactacta tgaagttggc
             1561 agcaacagag gagcctacgt caagctgggg ccagccctga ctccacatat cgtgcagtat
                  agcaacagga gagcctacgt caaactgggа ccagccctga ctccacatat cgtgcagtac
             1621 caagctaaca aggcgaccca caggctaacc atgaggcaaa ggatcagcag ggtggaggag
                  caagctaaca aggcgaccca caggctgacc atgaggcaaa ggatcagcag ggtggaggag
             1681 tcggccctca gagcccttcg acagaaatta tttgctcatt cgtcggacct gcttgttgag
                  tcggccctca gagccctgcg acaaaaatta tctgctcatt cgtcggacct gcttgttgag
             1741 tttaggaagc gcgacccgga tgagagcggt gtcatcaccc tgagtgattg ggcgactgcc
                  tttaggaagc gcgacccgga tgagagcggt gtaatcaccc tgagtgattg ggcgactgcc
             1801 gtggagtctg tgctgcacct gggactgccc tggcggatgc tgcggccaca actggtgaat
                  gtagaggctg tgctgcacct ggggctgcca tggaggatgc tgcggccaca actggtgaat
             1861 agttcagcgg ataacgtgtt ggaatacagg tcctggttgg acagtttggc caaagaacag
                  agttcagcag acaacgtgtt ggattacaag tcctggttgg acagcttggc caaagagcag
             1921 ctgagccgag agaatattca gtcgagtttg ctggaaaagc tctatcgaaa ccgatccaac
                  ctgagccgag agaatattca gtcgagcttg ctggaaaagc tctatcgaaa ccgatccaac
             1981 ttggagacca tttttaggat catagacagc gatcattcag gattcatctc cctggatgag
                  ttggagacca tttttaggat catagacagc gacccattcag ggttcatctc cctggacgag
             2041 ttcaggcaga cttggaagct cttcagctca catatgagca tcgacatcac agatgacggc
                  ttcagacaga cttggaagct cttcagctct catatgaaca tcaacatcac agatgatggc
             2101 atctgtgacc tcgccagaag catcgacttc aacaaggatg gccacatcga catcaatgag
                  atctgtgacc ttgccagaag catcgacttc aacaaggacg gccacatcga tatcaatgag
             2161 ttcctggagg ccttccgcct cgtggagcag tcctgcttag agggccacgc ctctgcttgc
                  ttcctggagg ccttccgcct cgtccagcag tcctgctcgg agggccacgc ctctgcttgc
             2221 ctgcagtcca cagacactgc tgagagtggc catagcagtc caggcccatg ctga  2274
                  ccgcagtcca cagacacggg ggagagtggc catagcagtc caggcccatg ctga
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Fig. 2. The Comparison of nucleotide sequence of the partially cloned rat brain PPEF-2 with mouse PPEF-2.

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      Mouse PPEF-2                1  MGSSSSTQHH FAFQNAEKAF KAAALIQRWY RRYMARLEMR
      Rat   PPEF-2                   ---------- ---------- ---------- ----------
 41   RRCTWNIFQS IEYAGQQDQV KLHEFFSYLV DHFTPSSHHE RDFLNRMFTE ERFAQDVETE EGGDFESIEV
      ---------- ---------- ---------- ---------- ---------- ---------- ----------
111   PDSYTGPRLS FPLLPDHATA LVEAFRLRQQ LHARYVLNLL YETRKHLAQL PNINRVSTCY SEEVTVCGDL
      ---------- ---------- ---------- ---------- ---------- ---------- ----------
181   HGQLDDLIFI FYKNGLPSPE RAYVFNGDFV DRGKDSVEVL MVLFAFMLVY PKEFHLNRGN HEDHLVNLRY
      ---------- ---------- ---------- ---------- ---------- ---FDFMLVY PKEFHLNRGN HEDHLVNLRY

251   GFTKEVMHKY KIHGKKILRT LQDVFCWLPL ATLVDEKVLV LHGGVSDKTD LELLAKLDRH KIVSTMRCKT
      GFTRKVMHKY KIHGKKILRT LQDAFCWLPL ATLVDEKVLV LHGGVSDKTD LELLAKLDRH KIVLPMRCKT

321   RKESENREEQ KRKDNQTSSG QKPTPWFLPQ SRSLPSSPFH LGSGFKAYKA GRSCSIPC-G SPNSKELSRR
      RKESENLEEQ KRKSNQTSSA QKPTPWFLPQ SRSLPSSPFH LGSGFKAYKA CRSSSIPC-G SASSKELSRQ

391   GQVRRSVDLE LEQCRQQAGF LGIREKGESL PLAPDADCVA DGGGVLEPTP EEWKQVVDIL WSDPAAQEGC
      GQVRPSVDLE LELCRQQAGF LGIRGKRESL PLADCDA---- DAGGELKPTP EEWKQVVDIL WSDPQAQEGC

461   KANAVRGGGC YFGPDVTERL MEKYKLQLLI RSHECKPEGY EFCHNRKVLT IFSASNYYEV GSNRGAYVKL
      KANTVRGGGC YFGPVVTEQL MEKYKLQFLI RSHEVKPEGY EFCHNRKVLT IFSALNYYEV GSNRGAYVKL

531   GPALTPHIVQ YQANKATHRL TMRQRISRVE ESALRALRQK LFAHSSDLLV EFRKRDPDES GVITLSDWAT
      GPALIPHIVQ YQANKATHRL TMRQRISRVE ESALRALRQK LSAHCSDLLV EFRKRDPDES GVITRSDWAT

601   AVESVLHLGL PWRMLRPQLV NSSADNVLEY RSWLDSLAKE QLSRENIQSS LLEKLYRNRS NLETIFRIID
      AVEAVLHLGL PWRMRRPQLV NSSADNVLDY KSWLGSLAKE QLSRENIQSS LLEKAYRNRS NLETIFRIID

671   SDHSGFISLD EFRQTWKLFS SHMSIDITDD GICDLARSID FNKDGHIDIN EFLEAFRLVE QSCLEGHASA
      SDHSGFISLD EFRQTWKLFS SHMEHNITDD GICDLARSID FNKDAHIDIN EFLEAFRLVQ QSCLGGQASA

741   CLQSTDTAES GHSSPGPC    758
      CPQSTDTGES GHSSPGPC
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Fig. 3. The comparison of the deduced amino acid sequence of the partially cloned rat brain PPEF-2 with mouse PPEF-2. Underlines indicate mismatched sequences.

PPEF-2 are compared with those of mouse brain PPEF-2 (Figs. 2. 3). Rat PPEF-2 cDNA exhibits 88% identity to the PPEF found in mouse brain (Steele *et al.*, 1992), and the degree of amino acids sequence conservation is more than 90%. These results suggest that PPEF-2 family is evolutionary well conserved among species and may perform identical biological functions. The deduced amino acid sequence of the rat brain PPEF-2 reveals a putative catalytic domain for protein phosphorylation and multiple direct Ca^{2+}-binding sites. Three residues from amino acid 423 to 425 in mouse are truncated in rat brain PPEF-2. The putative catalytic sequence was more conserved in amino acid sequence compared with multiple Ca^{2+}-binding sites. A domain structure similar to the mouse PPEF-2 protein in which the domain containing the EF hand is appended to catalytic domain, is present in the rdgc protein (Steele *et al.*, 1992) and the human Ca^{2+}-activated neural protease(Aoki *et al.*, 1986). The structural similarity suggests that rat PPEF-2 may be regulated directly by calcium level.

As a next step, we attempted to express the putative catalytic and EF-hand motif domain of rat PPEF-2 cDNA. Each domain was amplified by PCR using the sequence of primer pairs for the catalytic domain and EF-hand motif domain slightly modified to contain restriction sites EcoRI-XhoI and

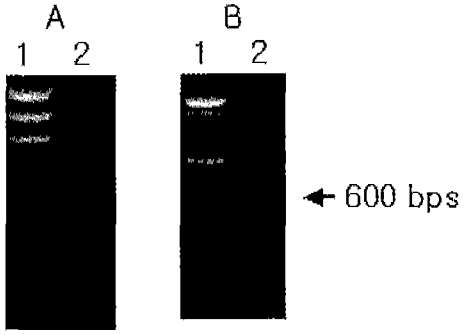

Fig. 4. Agarose gel electrophoretic analysis of PCR products from pGEMT-rPPEF2 using primer sets for putative catalytic domain (PP domain), and EF-hand motif domain (EF domain) of rat brain PPEF-2. A: amplified PP domain region. B: amplified EF domain region. Lane M: molecular size marker, Lane 1: amplified PCR product. Arrows indicate the PCR amplified products.

EcoRI-EcoRI, respectively. Both the amplified DNAs are about 600 bp in size (Fig. 4). Each PCR fragment was cloned into respective restriction site of the expression vector pGEX 4T-1, and transformed into E. coli DH5α. The expression of fusion proteins was confirmed by SDS-PAGE and also by immunoblot analysis using anti-GST antibody (Fig. 5). The fusion protein was expressed in high yield but was found entirely in inclusion bodies. Both the fusion proteins were

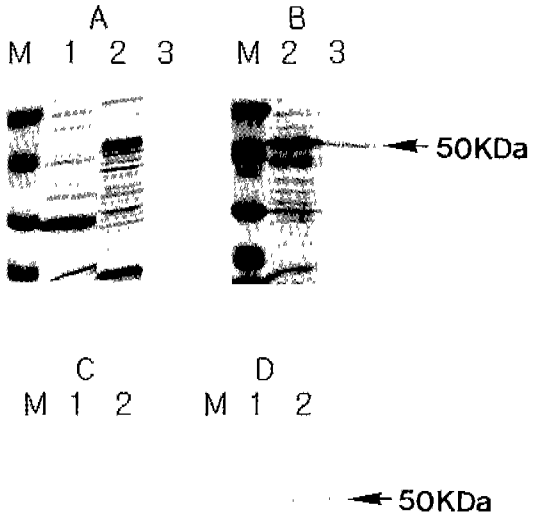

Fig. 5. Identification of the pGEX4T-PP(A, C, PP domain)-, and pGEX4T-EF(B, D, EF domain)-GST fusion protein expression. A, B) SDS-PAGE: E. coli DH5α transformed with pGEX4T-PP or pGEX4T-EF were grown in LB broth until OD_{600} of 0.5 was reached. The cultures were induced with 0.5 mM IPTG for 4 h at 37°C. An aliquot of the culture (100 μl) was electrophoresed on 10% polyacrylamide gel and the protein bands were visualized by staining with Coomassie brilliant blue. B, D) Western blot: After SDS-PAGE, the protein bands were electrically transferred on nitrocellulose membrane. The strips were incubated with monoclonal anti-GST antibody for 2 h at room temperature and probed with HRP-conjugated goat anti-mouse IgG (1:3,000 dilution). Bands were visualized with diaminobenzidine. Lane M: molecular weight marker, Lane 1: purified GST Lane 2: cell lysate, Lane 3: purified fusion protein.

purified from inclusion bodies under denaturing condition by GSH affinity chromatography using N-lauroyl sarcosine as a solubilizer. N-lauroyl sarcosine has been reported to effectively solubilize those proteins found in inclusion body without affecting their biological function (Frangioni and Neel, 1993; Shin *et al.*, 1998). In this study, other detergents including Tween 20, Triton X-100 and SDS did not solubilize the fusion proteins (data not shown) Electrophoretic and Western blot analysis revealed that the apparent molecular weight of the fusion proteins was 50 kDa, which was predicted from their amino acid sequence (Fig. 5). The purified fusion proteins could be used as antigens for monoclonal antibody production and also for the functional analysis of the catalytic domain and EF-hand motif of rat brain PPEF-2.

Further work will be necessary to demonstrate that these recombinant protein is functional Ser/Thr protein phosphatase *in vivo* and to define their biochemical characteristics, their sensitivity to specific inhibitors and their substrate specificity. The cDNA clone of rat brain PPEF-2 would be invaluable for the preparation of DNA probe and monoclonal antibodies, which is indispensible for histochemical and biochemical characterization of rat brain PPEF-2. With the ongoing complete cloning of rat brain PPEF-2, it would also be possible to test whether this new family of phosphatase participate in the regulation of G-protein coupled receptor signaling in brain.

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