GBPI, a novel gastrointestinal- and brain-specific PP1-inhibitory protein, is activated by PKC and inactivated by PKA

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The activities of PP1 (protein phosphatase 1), a principal cellular phosphatase that reverses serine/threonine protein phosphorylation, can be altered by inhibitors whose activities are themselves regulated by phosphorylation. We now describe a novel PKC (protein kinase C)-dependent PP1 inhibitor, namely GBPI (gut and brain phosphatase inhibitor). The shorter mRNA that encodes this protein, GBPI-1, is expressed in brain, stomach, small intestine, colon and kidney, whereas a longer GBPI-2 splice variant mRNA is found in testis. Human GBPI-1 mRNA encodes a 145-amino-acid, 16.5 kDa protein with pI 7.92. GBPI contains a consensus PP1-binding motif at residues 21–25 and consensus sites for phosphorylation by enzymes, including PKC, PKA (protein kinase A or cAMP-dependent protein kinase) and casein kinase II. Recombinant GBPI-1-fusion protein inhibits PP1 activity with IC50 = 3 nM after phosphorylation by PKC. Phospho-GBPI can even enhance PP2A activity by > 50% at submicromolar concentrations. Non-phosphorylated GBPI-1 is inactive in both assays.

INTRODUCTION

Protein phosphatases 1 and 2A (PP1 and PP2A) are prominent mammalian serine/threonine protein phosphatases. These phosphatases are major signal-transducing enzymes whose activities can be regulated by exogenous and endogenous agents [1–4]. Phosphatase regulation can contribute significantly to the modulation of key cellular processes including transcription, protein synthesis, metabolism, muscle contraction and cell division [5]. PP1 regulation can also alter information processing in neurons by regulating proteins that include neurotransmitter receptors, voltage-gated ion channels and ion pumps [3,6].

PP1 is regulated, and much of PP1 specificity is conferred, by a number of regulatory proteins that change PP1 activities when they are themselves phosphorylated and dephosphorylated by kinases and phosphatases [7], whose activities can be changed by altered cellular signals. For example, the phosphorylation state of the PP1 inhibitor DARPP-32 (dopamine- and cAMP-regulated phosphoprotein; molecular mass, 32 kDa) is increased by cocaine and amphetamine in striatum [8]. PKA (protein kinase A or cAMP-dependent protein kinase) phosphorylates the PP1 inhibitors DARPP-32, Inhibitor-1 and NIPPI (nuclear inhibitor of PP1) [3,9]. PKG (protein kinase G or cGMP-dependent protein kinase) phosphorylates the PP1 inhibitor G-substrate [10]. Glycogen synthase kinase 3 phosphorylates the PP1 inhibitor Inhibitor-2 [11]. PKC phosphorylates CPI (PKC-potentiated PP1 inhibitor) [12], PHI (phosphatase holoenzyme inhibitor) [13] and KEPI (kinase-enhanced phosphatase inhibitor) [4]. Recently, integrin-linked kinase and Rho kinase phosphorylation of CPI have also been documented [14–16]. Phosphatases that terminate the activities of PP1-regulatory phosphoproteins [17] include PP2B (calcineurin), which dephosphorylates DARPP-32 and inactivates its PP1-inhibitory functions [18].

Phosphorylation and dephosphorylation events can dramatically change the ability of PP1 inhibitors to alter PP1 activities. PP1 inhibition increases 1000-fold owing to phosphorylation of DARPP-32. Inhibitor-1, G-substrate, CPI-17, PHI and KEPI [4,5,9,13,19]. In contrast, phosphorylation of NIPPI and Inhibitor-2 decreases their abilities to inhibit PP1 [11,20].

Thus, PP1 inhibitors are active cellular regulators that can alter patterns of cellular phosphorylation on the basis of signals received through broad classes of GPCRs (G-protein-coupled

Abbreviations used: PKA, protein kinase A; PKC, protein kinase C; DARPP, dopamine- and cAMP-regulated phosphoprotein; PP1, protein phosphatase 1; CPI, PKC-potentiated PP1 inhibitor; GBPI, gut and brain phosphatase inhibitor; GPCR, G-protein-coupled receptor; GST, glutathione S-transferase; KEPI, kinase-enhanced phosphatase inhibitor; NIPPI, nuclear inhibitor of PP1; ORF, open reading frame; PHI, phosphatase holoenzyme inhibitor; RT, reverse transcriptase; THOP, testis homologue of PHI; for brevity, the one-letter system for amino acids has been used: e.g. E28 is Glu28.

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The nucleotide sequence data reported have been submitted to the GenBank® Nucleotide Sequence Databases under the following accession numbers: human GBPI-1 cDNA, AY050671; mouse GBPI-1 cDNA, AF408400; human PHI-2, AY179331; rat GBPI-1 cDNA, AY122322; human CPI cDNA, AY050670; mouse CPI cDNA, AY050672; rat CPI cDNA, AY050673; rat PHI-1 cDNA, AY122323; pig PHI-1 cDNA, AY122324; and Drosophila melanogaster KEPI cDNA, AY050669.
receptors) and other cell-surface receptors. Therefore PP1 inhibitors probably influence different functions in different cell types in which they are expressed. Protein phosphatase regulators could change cell-cycle parameters in dividing cells. They play many roles influencing mainly post-nicotic neurons in brain circuits, producing changes in neuronal processes [21].

We reported recently the cloning of a novel PKC-potentiated PP1 inhibitor, KEPI, which we identified by its up-regulation of mRNA in brains of morphine-treated rodents [4]. On the basis of the interesting profile of KEPI expression and regulation, characterizing the nature, roles and expression patterns of each of the protein phosphatase-regulatory phosphoproteins is of interest. We now describe GBPI (gut and brain phosphatase inhibitor), which has been identified by its similarity to KEPI sequences. We demonstrate that GBPI mRNAs are expressed in several brain regions as well as in gut. We identified an alternative transcript, GBPI-2, expressed in testis. We demonstrate that GBPI-1 is a potent and powerful inhibitor of PP1 enzymic activity when phosphorylated by PKC. Phospho-GBPI-1 enhances PP2A activity, although with lower potency. We show that inhibition of PP1 by PKC-phosphorylated GBPI-1 can be reversed by its subsequent phosphorylation by PKA. Each of these results demonstrates that GBPI is a new member of the interesting class of PP1-regulatory phosphoproteins and probably plays important roles at the junctions between PKC and PKA phosphoregulatory pathways in tissues such as brain, gut and testis.

EXPERIMENTAL

Production and purification of GBPI-1-fusion protein

Initial human and mouse cDNAs were identified to be human and mouse ESTs (expressed sequence tags) with sequence homologies to KEPI, including accession numbers AI767585 (GenBank®), 2382912 [IMAGE; A.T.C.C., Manassas, VA, U.S.A.], BG966186 (GenBank®) and 4984667 [IMAGE; Invitrogen, Carlsbad, CA, U.S.A.], cDNA sequences were confirmed by dideoxynucleotide sequencing. The 770 bp human cDNA, cloned in EcoRI and NotI sites of pT7T3D-Pac, contains an ORF (open reading frame) that includes a translation-initiation methionine and a stop codon. A translation-initiation site adapter with the sequence CCAATTTCTCATGgTGTTCTT (where ‘g’ represents a nucleotide replacement that provides a new NcoI restriction site) and a T3 primer were used to amplify a 0.7 kb segment of the cDNA. PCR products were digested with NcoI at the translation-initiation site and with HindIII at the polylinker site and cloned into NcoI–HindIII sites of the bacterial expression vector pET-30 (Novagen, Madison, WI, U.S.A.). The initiation methionine of pET30-GBP1-1 was positioned such that the ORF followed the His-tag peptide in-frame. Purification of the His–GBPI-1-fusion protein was positioned such that the ORF followed the initiation methionine of pET30-GBPI-1 was grown at 37 °C in Lennox broth/ampicillin (50 μg/ml) to reach A660 0.7, induced with 0.2 mM isopropyl β-d-thiogalactoside and grown at 30 °C for 2 h. Expressing bacteria were lysed by sonication, the lysate was centrifuged at 10 000 g for 10 min at 4 °C and GST-tagged GBPI-1 protein was purified from the supernatant by affinity chromatography according to the manufacturer’s instructions (GSTrap FF column; Amersham Biosciences). GST tag (224 amino acids) was removed from GBPI-1 protein by incubation with thrombin protease at 25 °C for 16 h. GBPI-1 protein was eluted from glutathione–Sepharose 4B columns, quantified by Bradford assays (Bio-Rad, Hercules, CA, U.S.A.) and checked for purity by SDS/PAGE (15 % gel). Purified recombinant GBPI-1 protein thus contained a glycine and a serine residue, N-terminal to the initiation methionine.

Full-length rat GBPI-1 cDNAs and human, mouse and rat cDNA clones for CPI and PHI were obtained by low-stringency screening of cDNA libraries, using the mouse GBPI-1 cDNA insert as a probe ([22], but also see [22a]) and by RT (reverse transcriptase)–PCR amplification using primers derived from EST alignments of cDNAs from rodent brain. Drosophila melanogaster KEPI (dKEPI) EST clones RE14405, RE18945, RE20685 and RE35817 were obtained from ResGen (Invitrogen) and inserted into BamHI and Xhol sites of the pFLC-1 vector. Shorter 1243 bp (RE14405 and RE20685) and longer 2344 bp (RE18945 and RE35817) transcripts were observed due to alternative polyadenylation of dKEPI mRNAs.

RT–PCR to identify GBPI-1 transcripts in different tissues

Human cerebral cortical samples were obtained from rapidly frozen tissues obtained post mortem. Mouse brain and peripheral tissues were dissected after killing and immediately frozen in liquid nitrogen. Total RNA was extracted from brain tissues using RNAzol B (Tel-Test, Friendswood, TX, U.S.A.). Single-strand cDNA was synthesized from total RNA using SuperScript™ first-strand synthesis system for RT–PCR ( Gibco BRL, Rockville, MD, U.S.A.). Human MTC (multiple-tissue cDNA) panels MTC-I, including heart, brain, placenta, lung, liver, skeletal muscles, kidney and pancreas, and MTC-II, including spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leucocyte (Clontech, Palo Alto, CA, U.S.A.), served as templates to study human GBPI transcripts. Primer Express software (Applied Biosystems, Foster City, CA, U.S.A.) was used to design RT–PCR primers. hGBP1-5 (AGCTCCAGCGCTGGCTGAGAT- GAG) sense primer and hGBP1-3 (TCTGAGGCCGGCTGAGT- GAG) antisense primer were used to amplify a 239 bp amplicon corresponding to human GBPI-1 mRNA. mGBP1-5 sense (GAGAACAGAAGGGGCGGAGTACG) and mGBP1-3 antisense (TTTTCCAGCATTACGAGCTGCAG) primers were used to amplify a 437 bp amplicon corresponding to mouse GBPI-1 mRNA. mPHI-5 sense primer (CAAGGGAAGTTCAC- CGTCAAGTACG) and mPHI-3 antisense primer (TGCGGT- GTGCTCAGGTCTCGCATGC) were used to amplify a 227 bp amplicon corresponding to mouse PHI mRNA. Two-step PCR programmes were used to amplify GBPI transcripts using brain region- and tissue-specific cDNAs as templates. Thermal cycling used a 95 °C, 8 min ‘hot start’, followed by seven cycles of 30 s at 94 °C and 2 min cycles that began at 72 °C but decreased in temperature by 1 °C for each cycle, followed by 45 cycles of 30 s at 94 °C, 2 min at 65 °C, followed by a final extension for 5 min at 65 °C. The amplified RT–PCR fragments corresponding to GBPI transcripts were analysed using 2 % agarose gels stained with ethidium bromide and 5 % acrylamide gels stained with SYBR® Green I (BioWhittaker Molecular Applications, Rockland, ME,
GBPI: a novel protein kinase C-dependent PP1 inhibitor

U.S.A.), using ΦX174 RF DNA/HaeIII fragments (Invitrogen) as molecular-mass standards. The amplicons were excised from agarose gels, purified using Consert™ PCR purification kits (Gibco BRL) and sequenced to confirm the sequence specificities of GBPI-1 amplification products.

Sequence analyses and homology searches
cDNA sequences were assembled using Sequencher (version 3.0; Gene Codes Corp., Ann Arbor, MI, U.S.A.). BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST7), Ensemble (http://www.ensembl.org) and Celera databases (http://www.celera.com) were used to map human and mouse genes. Relationships between the sequences of GBPI, CPI, PHI and KEPI were assessed by BLAST searches (Genetics Computer Group, Madison, WI, U.S.A.). Potential phosphorylation sites were predicted with ExPaSy Molecular Biology Server (http://us.expasy.org).

Phosphorylation of recombinant GBPI-1 protein
Recombinant GBPI-1 protein was tested as a substrate for purified rat brain PKC (protein kinase C; Calbiochem, La Jolla, CA, U.S.A.) and bovine heart PKA catalytic subunit (Cell Signaling Technology, Beverly, MA, U.S.A.). PKC phosphorylation reactions were performed at 30 °C in 100 μl of 20 mM Hapes (pH 7.4), 0.1 mM CaCl₂, 10 mM MgCl₂, 100 μg/ml phosphatidylserine, 20 μg/ml diacylglycerol, 100 μM g/ml Tris/HCl (pH 7.4), 10 mM MgCl₂, 200 μM [γ-32P]ATP, 10 units of PKA and 3 mM His–GBPI-1 or GST–GBPI-1 protein. PKC-phosphorylated GBPI-1 was purified by PD-10 Sephadex column chromatography (Amersham Biosciences). PKC-phosphorylated GBPI-1 was also used as PKA substrate in experiments in which PKA phosphorylations were performed at 30 °C in 100 μl of 50 mM Tris/HCl (pH 7.4), 10 mM MgCl₂, 200 μM [γ-32P]ATP, 10 units of PKA and 3 μM His–GBPI-1 or GST–GBPI-1 protein. For sequential phosphorylation studies, PKA- or PKC-phosphorylated GBPI-fusion proteins were purified, exchanged into appropriate buffers by PD-10 Sephadex column chromatography and then phosphorylated by PKC and PKA respectively. For time-course studies, reaction aliquots were sampled after 5, 10, 20, 30, 60, 90 and 120 min incubations. For kinetic studies, different concentrations of GBPI-1-fusion proteins were used as PKC and PKA substrates for 60 min. Phosphorylation was stopped by adding 1 ml of 25% (v/v) trichloroacetic acid and 10 μl of 1 mg/ml BSA at 4 °C. Phospho-GBPI-1 was precipitated by centrifugation at 10000 × g for 10 min at 4 °C and washed twice with 7.5% trichloroacetic acid at 4 °C. 32P-labelled proteins were assessed by scintillation counting and by SDS/PAGE followed by autoradiography. Triplicate data were analysed by non-linear regression using GraphPad (San Diego, CA, U.S.A.).

Phosphopeptide mapping
His–GBPI-1, GST–GBPI-1 and GST–GBPI-M3-fusion proteins that were 32P-labelled by incubation with PKC or PKA were separated by SDS/PAGE, stained with Coomassie Blue and detected by autoradiography. A radioactive band migrating with a mass of 30 kDa of His–GBPI or 52 kDa of GST–GBPI was excised from the gel, washed overnight with 10% (v/v) methanol and freeze-dried. Dried gel fragments were digested in 1 ml of trypsin solution (50 μg/ml in 50 mM NH₄HCO₃, pH 8.0) at 37 °C overnight. The peptide solution was separated from the remaining gel fragments, freeze-dried and dissolved in 10 μl of electrophoresis solution (acetic acid/formic acid/water, 3:1:16, by vol.). Tryptic phosphopeptides were analysed by two-dimensional peptide mapping on cellulose TLC plates (Selecto Scientific, Suwanee, GA, U.S.A.) using electrophoresis as the first dimension and ascending chromatography in n-butanol/pyridine/acetic acid/water (13:10:2:8, by vol.) as a second dimension, as described in [23,24].

Site-directed mutagenesis
Mutagenesis was performed using a two-step megaprimer PCR method [25] using the following oligonucleotides: M1, CCAGATGGGGAGAACCCATGTGGAGTCCACTGGCCTTC; M2, CATGTAAGAGGTTCCACGGGCTTCTGGGAGGA; and M3, GACCCAGCGCGCTGAAGTAATGACC (mutated nucleotides are shown in boldface). First-step PCR used the antisense primer GAGAGTCCAGGGGCTTCGCA containing a BamHI site, the megaprimer derived from the first-step PCR and the antisense primer from the first-step PCR to produce GBPI mutants. Second-step PCR products were digested with BamHI at a site located in the vector pGEX-4T and with SstI at a site located in GBPI, and the resulting restriction fragment was shuttled into the wild-type pGEX-GBPI-1 at the same unique restriction sites. Sequences of the resulting mutant constructs pGEX-GBPI-M1 and pGEX-GBPI-M2 were confirmed by DNA sequencing. Mutant GST–GBPI-1-fusion proteins were expressed in E. coli BL21 and confirmed by SDS/PAGE.

PP1 and PP2A assays
A protein phosphatase assay system (Gibco BRL) provided differential measurements of PP1 and PP2A activities in dephosphorylating the glycolytic enzyme phosphorylase a. Phosphorylase a was labelled with [γ-32P]ATP, 10 units of PKA and 3 μM His–GBPI-1 or GST–GBPI-1 protein. For sequential phosphorylation studies, PKA- or PKC-phosphorylated GBPI-fusion proteins were purified, exchanged into appropriate buffers by PD-10 Sephadex column chromatography and then phosphorylated by PKC and PKA respectively. For time-course studies, reaction aliquots were sampled after 5, 10, 20, 30, 60, 90 and 120 min incubations. For kinetic studies, different concentrations of GBPI-1-fusion proteins were used as PKC and PKA substrates for 60 min. Phosphorylation was stopped by adding 1 ml of 25% (v/v) trichloroacetic acid and 10 μl of 1 mg/ml BSA at 4 °C. Phospho-GBPI-1 was precipitated by centrifugation at 10000 × g for 10 min at 4 °C and washed twice with 7.5% trichloroacetic acid at 4 °C. 32P-labelled proteins were assessed by scintillation counting and by SDS/PAGE followed by autoradiography. Triplicate data were analysed by non-linear regression using GraphPad.
A 437 bp amplicon specific for mouse GBP1-1 was observed in mouse intestine, kidney, lung, spleen, stomach and brain. No significant mouse GBP1 expression was noted in heart, liver or muscles. Studies of the regional expression of GBP1-1 expression in brain revealed highest levels of expression in cerebral cortex, hippocampus and midbrain (Figure 2B). In contrast, a 277 bp amplicon specific for mouse PHI was observed ubiquitously in cDNAs amplified from the same RNA extracted from all mouse tissues (results not shown).

Amplification of mRNA from mouse testis produced a 497 bp amplicon (Figure 2B). Sequence alignment of the testis-specific amplicon revealed a differentially spliced variant that contained an additional exon lying between exons 1 and 2 of the genomic sequences that encoded the GBP1-1 isoform. This additional mouse exon was flanked by consensus splice donor and acceptor sites in mouse genomic sequence. Interestingly, this additional exon yields both an additional amino acid sequence and a frameshift (Figure 1B). Thus, the resulting predicted 175-amino-acid translation product shares the 86 N-terminal amino acids with the isoform expressed in other tissues, but contains 89 novel amino acids at its C-terminus. We term the testis-specific protein ‘GBP1-2’. GBP1-2 is larger and more basic (pl 10.94) than GBP1-1. GBP1-2 does retain a PP1 consensus binding site (KKVHW) and a PKC phosphorylation site (RLTVK) and several sites for phosphorylation by PKC and PKA and several sites for phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II. A consensus site for PKC phosphorylation (RLTVK) in human; Figure 1A) is similar phosphorylation by casein kinase II.
GBPI: a novel protein kinase C-dependent PP1 inhibitor

Figure 2  Tissue distribution of PKC-potentiated PP1 inhibitor mRNAs

GBPI: RT–PCR of (A) human cDNA panel, and (B) mouse cDNA panel. Molecular-mass standards (MW) were φX174/HaeIII fragments. Hrt, heart; Kid, kidney; Liv, liver; Lng, lung; Mus, muscles; Pnc, pancreas; Pla, placenta; Col, colon; Int, intestine; Lek, leucocyte; Ova, ovary; Prs, prostate; Spl, spleen; Stm, stomach; Tes, testis; Thy, thymus; Brn, brain; Olf, olfactory bulb; Ctx, cortex; Hip, hippocampus; Str, striatum; Mid, mid brain; Tha, thalamus; Hth, hypothalamus; Brs, brain stem; Cer, cerebellum; Spn, spinal cord.

Figure 3  Purification of GST–GBPI-1-fusion protein

Fusion protein was induced in the absence (−) or presence (+) of 0.2 mM isopropyl β-D-galactoside (IPTG). GBPI-GST, GST–GBPI-1 purified by affinity chromatography using a GST FF column. GBPI, cleavage product of the GBPI-1 fusion protein following the removal of the GST tag.

PKC and PKA phosphorylation of the recombinant GBPI-1

Recombinant GBPI-1 protein can be phosphorylated by purified rat brain PKC preparations that contain mainly the conventional PKC isoforms α, β1, β2 and γ. Under these conditions, recombinant protein is phosphorylated with apparent $K_m = 0.4 ± 0.05 \text{ mM}$, $V_{max} = 30.47 ± 0.96 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ and half-life ($t_{1/2}$) = 21.82 ± 2.73 min (Figure 4A, upper panel). At plateau time points, a near-stoichiometric ratio of phosphorylation, 0.95 ± 0.126 mol of P/mol of GBPI-1, can be achieved. When the mobility of 32P-labelled GBPI-1 was analysed by SDS/PAGE and autoradiography (Figure 4A, middle panel), a dominant 30 kDa 32P-labelled band corresponding to GBPI-1 was observed. Phosphopeptide mapping demonstrates one predominantly tryptic, 32P-labelled peptide phosphorylated by PKC (Figure 4A, lower panel).

GBPI-1 could also be phosphorylated by the catalytic subunit of PKA, with results suggesting multiple potential PKA phosphorylation sites. After initial curve-fitting, we used two-phase exponential association and two-site hyperbolic models to analyse the time course and kinetics of PKA phosphorylation of GBPI-1. $t_{1/2}$ values of 1.71 ± 0.29 and 42.75 ± 4.54 min (Figure 4B, upper panel) provided the best two-phase fit. Phosphorylation of recombinant GBPI-1 displayed $K_{m1} = 4.77\ \text{µM}$, $K_{m2} = 34.36\ \text{µM}$ and $V_{max} = 6.56\ \text{nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$. SDS/PAGE analyses of GBPI-1-fusion protein subjected to PKA phosphorylation displayed a 32P-labelled band with motility (Figure 4B, middle panel) similar to that displayed by GBPI-1-fusion proteins phosphorylated by PKC. Phosphopeptide mapping of PKA-pretreated GBPI-fusion protein revealed two 32P-labelled tryptic GBPI peptide fragments (Figure 4B, lower panel). Thus, both phosphopeptide mapping
and kinetic data point towards the existence of at least two sites for the PKA phosphorylation of GBPI-1, although the sites could differ in their abilities to serve as PKA substrates.

When PKC- and PKA-phosphorylated GBPI-1 proteins were subsequently phosphorylated by PKA and PKC respectively, each displayed phosphorylation kinetics nearly identical with those displayed by GBPI-1 that had not been previously phosphorylated (results not shown). Each of these observations fails to support the existence of any substantial overlap between PKC and PKA phosphoacceptor sites on GBPI-1.

Inhibition of PP1 and activation of PP2A activity by PKC-phosphorylated GBPI-1

Phospho-GBPI-1 was produced by PKC preincubation of His–GBPI-1 or GBPI-1 cleaved from GST. Phospho-GBPI-1 inhibits the ability of recombinant rabbit muscle PP1α to dephosphorylate 32P-labelled phosphorylase a with IC50 = 2.76 ± 0.17 nM (Figure 5A). PKC-phosphorylated GBPI-1 can also activate PP2A activity by more than 50% (Figure 5B) at high concentrations.

Analyses of the activities of GST–GBPI-fusion protein mutants strengthened our confidence in several features conferred by specific features of this protein’s sequence. Mutant M1 substituted two acidic glutamic residues for the two basic lysine residues at N-terminal positions 21 and 22 of GBPI-1. Thus, the potential PP1-binding site KKVHW was converted into EEVHW. Mutant M2 replaced the aromatic amino acid tryptophan with alanine to convert this site into KKVHA (Figure 6). Mutant M3 altered the PKC consensus phosphorylation site found at GBPI residues 56–60 (RLT58VK), replacing the phosphoacceptor threonine with threonine.

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Figure 5  Inhibition of PP1 and activation of PP2A by PKC-phosphorylated GBPI-1
(A) PP1 inhibition and (B) PP2A activation by PKC-phosphorylated GBPI-1. ▲, Unphosphorylated GBPI-1; ■, PKC-phosphorylated GBPI-1; ●, PKC- and PKA-dual-phosphorylated GBPI-1.

Figure 6  Effects of site-directed mutagenesis on PP1 inhibition by wild-type and mutant GST-GBPIs
Mutated nucleotides are shown in boldface. Mutated amino acids are in boldface and marked with asterisks. Black bars, GST–GBPI-fusion proteins treated with PKC; open bars, non-phosphorylated GST–GBPI-fusion proteins.

Figure 7  Major PKC phosphorylation site of GBPI-1 responsible for PP1 inhibition
(A) SDS/PAGE and autoradiography of PKC-phosphorylated wild-type GST–GBPI-1 (WT) and mutant GST–GBPI-M3 (M3). (B) Phosphopeptide mapping of PKC-phosphorylated GST–GBPI-1 and GST–GBPI-M3. 'E' represents the direction of electrophoresis and 'C' the direction of chromatography. Arrowheads point towards $^{32}$P-labelled phosphopeptides.
Phosphorylated phosphopeptides (Figure 7B). The loss of the T5E phosphorylation site (Figure 7B) fits well with the loss of PKC-activated PP1-inhibitory activity of the mutant GST–GBPI-M3.

**PP1 inhibition: competition between PKC-phosphorylated GBPI and potential PP1-binding peptides**

We used 50 μM concentrations of decapeptides, whose core sequences correspond to the PP1 consensus binding sites (R/KVXF/W) of human GBPI (residues 19–28), CPI (residues 5–14), KEPI (residues 17–26) and PHI-1 (residues 24–33) to compete for the PP1-inhibitory activity of PKC-phosphorylated GST–GBPI-1 fusion protein. GBPI (19–28), KEPI (17–26) and PHI-1 (24–33) decapeptides could each almost completely block inhibition of PP1 by PKC-phosphorylated GST–GBPI-1, although the decapeptide corresponding to CPI (5–14) had no effect (Figure 8). The CPI (5–14) core sequence of decapeptide (RVLS) does differ from PP1-binding consensus sequences (R/KVXF/W).

The inhibition displayed by GBPI (19–28), KEPI (17–26) and PHI-1 (24–33) decapeptides displays competitive features. Whereas 50 μM concentrations of these three decapeptides could produce these effects when PKC-phosphorylated GST–GBPI-1 concentrations were less than 30 nM, no decapeptide could effectively compete with 90 nM concentrations of PKC-phosphorylated GBPI-1 (Figure 8). Studies at 30 nM concentrations of PKC-phosphorylated GST–GBPI-1 suggested the rank order of efficacy as follows: KEPI (17–26) > GBPI (19–28) > PHI-1 (24–33) > CPI (5–14). Thus, 50 μM KEPI (17–26) or GBPI (19–28) or PHI-1 (24–33) decapeptide appeared to increase IC50 values for PKC-phosphorylated GST–GBPI-1 2–3-fold.

**DISCUSSION**

The present study functionally identifies a novel member of the PKC-potentiated PP1 inhibitor family that we term GBPI. We discuss this work in relationship to the comparative patterns of GBPI mRNA expression, structure–function relationships of GBPI and examples of the ways in which GBPI expression might alter the properties of expressing cell types and tissues and provide a novel drug target. The present study defines a molecule that can act at important modulatory junctions between PKC and PKA pathways in gut, brain and tests.

GBPI is now one of four PKC-potentiated PP1 inhibitors that have been functionally identified in humans and mice: GBPI, CPI, KEPI and PHI. There is only a single apparent *D. melanogaster* PP1 inhibitor family member, namely dKEPI. Neither *Caenorhabditis elegans* nor *Saccharomyces cerevisiae* has a clear homologue [27–29]. Unique patterns of tissue- and brain-region expressions also distinguish these transcripts. PHI is ubiquitously expressed. GBPI, CPI and KEPI are each differentially expressed in different tissues, different cell types and even different subcellular regions. GBPI-1 is prominently expressed in gastrointestinal tissues and moderately expressed in several brain regions. GBPI and CPI mRNAs are rare in skeletal muscles, but abundant in gastrointestinal or aortic smooth-muscle tissues [19]. KEPI is highly expressed in heart and skeletal muscles, but not in gastrointestinal tissues.

This family may have another member. Mouse EST sequences AV267143, AV280527, AV278668, AV204981, AV268589, AV040646, AV046230, A1429188, AK005938, A159538, AA062392 and AK016819 align closely with the mouse 1A4 locus whose sequences provide the closest fit to PHI. Since each of these ESTs derives from testsis, we tentatively name this new potential family member ‘testis homologue of PHI’ or ‘THOP’.

Genomic THOP sequences found in the contig NW000166 display a single exon whose ORF encodes an apparent THOP protein that retains its PP1-binding motif ‘RYVF’, but lacks the consensus PKC phosphorylation site found in each of the other family members. Taken together, the present results suggest that GBPI is thus a member of a family of perhaps seven related sequences encoded by at least five distinct genes (Figure 9).

The structure–function results from the present mutagenesis studies may provide insights into the molecular basis for these physiological observations. Many proteins that interact with PP1 contain a canonical PP1-binding sequence (R/KVXF/W) [7,26]. The potential PP1-binding site (R7KKIQF11) of GBPI is similar to that found in the PKA-potentiated PP1 inhibitor DARPP-32 (‘KKIQF’), which has also been characterized by phosphopeptide inhibition and mutagenesis studies [30,31]. Double mutations of K21E – K22E in GBPI-1 increased IC50 only 2-fold, suggesting that the basic amino acids in the R/KVXF/W sequence might be less important than aromatic residues (Figure 6).

These observations are similar to those observed in DARPP-32. Phosphopeptides that mimic the PP1 recognition motif of DARPP-32 and PKA phosphorylation site are equipotent with or without a basic amino acid at its KKIQF site [32]. K7E mutation increases IC50 only 4-fold and K8E mutation decreases IC50 1.5-fold [30]. Peptide modelling (GCG PepPlot) predicts that the GBPI R/KVXF/W motif forms an amphipathic β-sheet that might interact with the interface of the two PK1 β-sheets, which form a ‘β-sandwich’ in the PK1 crystal structure [33,34]. Thus, interactions between GBPI β-sheet and PP1 β-sandwich provide a plausible interaction that could well modulate the activities of the PK1 phosphatase active site, which is located opposite to the β-sandwich in PP1 crystal structures [35]. Interestingly, substituting glutamic residues for lysine residues 21
and 22 in the putative PP1-regulatory/binding site of GBPI does not appear to alter the molecule’s PepPlot β-sheet conformation score, whereas the W25A mutant eliminates β-sheet conformation scores for these sequences.

Results from mutagenesis studies and phosphopeptide mapping provide molecular characterization of GBPI phosphoregulation. Substitution of the consensus PKC phosphoacceptor site threonine with glutamate in the mutant T58E inactivates GBPI-1, as it does with mutagenesis of the corresponding residue in CPI (T38E) [36]. Phosphopeptide mapping of His–GBPI-1, GST–GBPI-1 and GST–GBPI-M3 appears to confirm that T58 is the primary PKC phosphorylation site and is thus probably responsible for the PKC actions in activating the ability of GBPI-1 to inhibit PP1. Further, this phosphopeptide is eliminated in the T58E GST–GBPI-M3 mutant, leaving only background phosphorylation of the GST-fusion tag. A weaker phosphopeptide spot representing a highly mobile peptide migrating near the top of the PKC phosphopeptidase maps could relate to an additional PKC phosphorylation site, as noted for CPI [37]. Phosphorylation of this second CPI site required prior phosphorylation of the residue (T38) homologous with T58 of GBPI, and appeared to be not related to PKC activation [15].

The subsequent PKA activity could reverse the physiological effects of prior PKC activation of GBPI. However, several lines of evidence indicate that the two kinases were not acting at the same site. (1) The consensus PKA sites [R/K(2)XS/T] in GBPI-1, namely T32, S33, S55 and S141, are different from the consensus PKC sites (S/TXR/K), S27, S45, S51 and T58. (2) PKA phosphorylation kinetics was virtually identical whether the His–GBPI-1 substrate used had been previously phosphorylated by PKC or not. (3) PKC phosphorylation kinetics was virtually identical whether the His–GBPI-1 substrate used had been previously phosphorylated by PKA or not. (4) PKA conferred similar patterns of phosphorylation on wild-type GST–GBPI-1 and the T58E mutant GST–GBPI-M3 in which the PKC phosphoacceptor site was eliminated (results not shown). (5) Initial mass spectrographic peptide ‘fingerprint’ studies identify one of the PKA sites, consistent with phosphorylation of T32, separate from the T58 site for PKC phosphorylation, indicated by phosphopeptide mapping, to lie on a separate tryptic fragment (A.S. Woods, Q.-R. Liu and G.R. Uhl, unpublished work). In contrast, it is quite plausible that nearby interactions could be responsible for the interactive effects of PKC- and PKA-mediated phosphorylations. PKA phosphorylation of the GBPI residue T32 could influence PKC-activated GBPI-1-inhibitory activity by interference with PP1 binding, owing to the proximity of T32 to the crucial PP1-binding residue W25. PKA phosphorylation of the GBPI residue S55 could even influence PKC-activated GBPI-1-inhibitory activity by interference with the sites for PKC activation, since S55 is close to T58.

Interactions between different phosphatase inhibitors and different phosphatases display complexities as well as similarities. PP1-regulatory binding sites probably differ from inhibitor to inhibitor in ways that involve more than primary amino acid sequences. The consequences of inhibitor–phosphatase interaction could also differ from phosphatase-interacting target protein to phosphatase-interacting inhibitory protein, and from phosphatase to phosphatase. Whereas many proteins that do not bind to PP1 display the R/KVXF/W motif [26], the potent PKC-potentiated PP1 inhibitor CPI does not contain an R/KVXF/W motif. Deletion experiments in which 34 N-terminal amino acids of CPI were deleted and PP1-inhibitory potencies retained do fit [36] with our peptide competition data in which CPI 5–14 sequences including the 8-KRVLS-12 motif are ineffective in competing for PP1 binding, owing to the proximity of T32 to the crucial PP1-binding residue W25. PKA phosphorylation of the GBPI residue S55 could even influence PKC-activated GBPI-1-inhibitory activity by interference with the sites for PKC activation, since S55 is close to T58.

GBPI and its family members are thus well positioned biochemically to play a significant role in integrating PKC and PKA signals and plausibly signals from other cellular signalling pathways.
cascades. In initial experiments, we have identified apparent phosphorylation on one or more of the multiple consensus casein kinase II phosphorylation sites identified in GBP1’s sequence (results not shown). Integrin-linked kinase, calmodulin kinase II, Rho kinase, p21-activated kinase, PKG and PKN (protein kinase N) have also been shown to phosphorylate and possibly regulate GBP1 family members [14–16,37,39,40]. Nevertheless, it is a specific activity of GBP1 to allow simultaneous or even subsequent PKA activation to negate the effects of PKC activation that has drawn our attention, and has not been clearly documented to our knowledge. GBP1 could provide a locus for physiological integration between ligands that differentially activate the GTP-binding protein Gs and those that couple with Gi/oes.

The discovery of GBP1 and KEPI that couple with Gi/o/oes, and has not been clearly documented to our knowledge. GBP1 and KEPI could provide a locus for physiological integration between ligands that differentially activate the GTP-binding protein Gs and those that couple with Gi/oes.

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