Cloning and Characterization of a Mammalian Lithium-sensitive Bisphosphate 3'-Nucleotidase Inhibited by Inositol 1,4-Bisphosphate*

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Discovery of a structurally conserved metal-dependent lithium-inhibited phosphomonoesterase protein family has identified several potential cellular targets of lithium as used to treat manic depression. Here we describe identification of a novel family member using a "computer cloning" strategy. Human and murine cDNA clones encoded proteins sharing 92% identity and were highly expressed in kidney. Native and recombinant protein harbored intrinsic magnesium-dependent bisphosphate nucleotidase activity (BPntase), which removed the 3'-phosphate from 3'-5' bisphosphate nucleosides and 3'-phosphoadenosine 5'-phosphosulfate with $K_v$ and $V_{max}$ values of 0.5 $\mu$M and 40 $\mu$mol/min/mg. Lithium uncompetitively inhibited activity with a $K_i$ of 157 $\mu$M. Interestingly, BPntase was competitively inhibited by inositol 1,4-bisphosphate with a $K_i$ of 15 $\mu$M. Expression of mammalian BPntase complemented defects in hal2/met22 mutant yeast. These data suggest that BPntase's physiologic role in nucleotide metabolism may be regulated by inositol signaling pathways. The presence of high levels of BPntase in the kidney are provocative in light of the roles of bisphosphorylated nucleotides in regulating salt tolerance, sulfur assimilation, detoxification, and lithium toxicity. We propose that inhibition of human BPntase may account for lithium-induced nephrotoxicity, which may be overcome by supplementation of current therapeutic regimes with inhibitors of nucleotide biosynthesis, such as methionine.

Lithium is a major drug used to treat manic depression, yet its molecular mechanism of action has not been conclusively elucidated. Insight into lithium's pharmacological activity has come with the identification of a magnesium-dependent phosphomonoesterase family whose members are inhibited by lithium at subtherapeutic concentrations (1). Despite relatively low overall sequence similarity, in some cases undetectable, the family shows a conserved three-dimensional core structure. Functional and structural studies of three members, fructose 1,6-bisphosphatase (fbptase), inositol monophosphatase (impase), and inositol polyphosphate 1-phosphatase (1ptase), indicate that residues involved in metal binding and catalysis are conserved within a sequence motif D-X$_a$-EE-X$_b$-DP(i/l)D(s/g) a/T-X$_c$-WD-X$_d$-GG (2–7). Of interest, several studies indicate that lithium interacts at one or more of the metal binding sites (6, 8, 9). Together, these data support the idea that the signature motif confers the common enzymatic characteristics of the family, including lithium sensitivity.

The lithium-sensitive family includes proteins with multiple cellular roles. The roles of fbptase in gluconeogenesis, and impase and 1ptase in inositol signaling, are well established. Additionally, this family includes HAL2(MET22), SAL1, and cysQ, gene products implicated in sulfur assimilation and salt tolerance in yeast, plants, and bacteria (10–14). Met22p and isoalactic Hal2p were identified as yeast proteins involved in methionine biosynthesis (10) and sodium tolerance (11, 12). SAL1 was identified in a screen for plant genes that produced salt tolerance in yeast (13). The bacterial gene cysQ was found to be involved in the biosynthesis of cysteine (14). Biochemical analysis of these proteins demonstrated a Mg$^{2+}$-dependent, Li$^+$-sensitive phosphomonoesterase activity on 3' phosphoadenosine 5' phosphate (PAP) and 3' phosphoadenosine 5' phosphosulfate (PAPS) (11–13). Additionally, SAL1 has been reported (13) to possess 1ptase activity which removes the 1-position phosphate from either inositol 1,4-bisphosphate (Ins(1,4)P$_2$) or Ins(1,3,4,5)P$_4$ (15, 16). As SAL1 overexpression confers salt tolerance on yeast and complements the methionine auxotrophy of hal2 mutants (13), roles were ascribed for both hydrolytic activities in the functioning of SAL1.

Direct evidence links physiological effects of lithium to members of this phosphomonoesterase family. Treatment of cells with lithium results in accumulation of inositol mono- and polyphosphates, suggesting that impase and 1ptase are inhibited in vivo. Dichtl et al. (17) recently showed that the mechanism of lithium toxicity in yeast involves inhibition of the cytosolic RNA processing enzyme Xrn1p due to the inhibition of Hal2p and subsequent accumulation of PAP. Concurrent PAP-independent lithium-mediated inhibition of RNase MRP leads to 1-phosphatase; PAP, 3'-phosphoadenosine 5'-phosphate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; Ins(1,4)P$_2$, inositol 1,4-bisphosphate; Ins(1,3,4,5)P$_4$, inositol 1,3,4,5-tetrakisphosphate; Ins(1,3,4,5,6)P$_5$, inositol 1,3,4,5,6-pentakisphosphate; InsP$_6$, inositol hexakisphosphate; BSA, bovine serum albumin; GST, glutathione S-transferase; PCR, polymerase chain reaction; FBS, fetal bovine serum; HPLC, high performance liquid chromatography; AP, ammonium phosphate; PMSF, phenylmethylsulfonyl fluoride; EST, expressed sequence tag; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pair(s); bp, base pair(s); APS, 5' adenosine phosphosulfate.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AF125042 and AF125043.

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The abbreviations used are: fbptase, fructose 1,6 bisphosphatase; impase, inositol monophosphatase; 1ptase, inositol polyphosphate

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to an accumulation of immature ribosomal RNA molecules (17). In addition, the data of Acharya et al., indicate that 1p21 is a target of lithium in Drosophila melanogaster (18). Defects in neuronal function observed in Drosophila 1p21 mutants were phenocopied precisely by the administration of lithium to wild-type flies (18).

The conservation of the sequence motif of this family is important for identifying novel family members from human genome sequencing efforts. To this end, we have used this motif to clone a novel mammalian metal-dependent lithium-inhibited bisphosphate 3'-nucleotidase (BPnTAse) from expressed sequence tag (EST) data bases. Characterization and tissue distribution of a mammalian BPnTase suggest that it is involved in nucleotide metabolism, sodium homeostasis, and the physiological effects of lithium.

MATERIALS AND METHODS

Protein concentrations were determined by the method of Bradford (19), using bovine serum albumin (BSA) as a standard or by measuring A280 (assuming an extinction coefficient of 1 cm/ml/mg). SDS-PAGE was run using the Laemmli method (20). Gels were silver-stained as described within the Silver Stain Plus system (Bio-Rad). DNA-manipulating enzymes were from Life Technologies, Inc. or Roche Molecular Biochemicals. All other materials were reagent grade, typically purchased from Sigma. Polyclonal antibodies were generated by injecting PAP-agarose-purified enzyme as described below into rabbits following a standard protocol (Alpha Diagnostic, San Antonio, TX). The Western blot procedure followed a standard protocol and was developed with a phosphorescent reagent activated by horseradish peroxidase conjugated to donkey-anti-rabbit IgG (Amersham Pharmacia Biotech).

Clone Identification and Sequencing—Mouse and human EST data bases were searched with BLASTP, gapped BLASTP, or TBLASTN (21). Candidate cDNAs were obtained within the Silver Stain Plus system (Bio-Rad). DNA-manipulating procedures were recommended by the manufacturer. The reaction was stopped by the addition of formamide to a concentration of 0.1 M COOH, 0.5 M NH4COO. The membrane was stripped and reprobed with radiolabeled human actin DNA supplied with the blot.

Preparation of 5'-32P/PAP Substrate—Nucleotide 3' -monophosphates (3'-AMP, -dAMP, -CMP, -GMP, -TMP, and -UMP) (Sigma) were 5'-labeled with 32P isotope by incubating an excess of the unlabeled nucleotide with [γ-32P]ATP (NEN Life Science Products) and T4 polynucleotide kinase (Roche Molecular Biochemicals) under conditions recommended by the manufacturer. The mixture was applied to a 25-μl Dowex (AG 1-X8 Resin, 200–400mesh, formate form; Bio-Rad) column equilibrated in formate. Following extensive washing, the product was eluted with 0.03 M COOH, 1.05 M NH4COO.

Preparation of PAPS—PAPS (Sigma) is supplied as a liquid salt. Lithium was removed by desalting over a gravity-flow 5 × 280-mm G10-Sepharose gel filtration column equilibrated in 1 mM Tris-CI, pH 8.7, 50 mM KCl as described (25).

Expression and Purification—Bacterial expressions were performed by growing recombinant bacteria in 1-liter cultures of LB broth (10 g/liter Bacto-tryptone, 5 g/liter yeast extract, and 5 g/liter NaCl) containing 100 μg/ml ampicillin. At mid-log phase (OD600 = 0.6–1.0), cultures were induced with 0.2–0.4 mM isopropyl-1-thio-β-D-galactopyranoside and grown at 37 °C for an additional 4–6 h.

Sf9 cells were maintained in spinner flasks at 28 °C in Grace's medium (Life Technologies, Inc.) at pH 6.1 supplemented with 3.3 g/liter BSA, 1 mg/liter antifoam, 0.3 μg/liter gentamycin, and 10% heat-inactivated fetal bovine serum (FBS) until cell densities reached 1 × 10⁹ cells/ml (log phase). Viral infections were performed by incubating cells (1 × 10⁹) with approximately 15 μl of virus solution for 4 h at 28 °C with periodic gentle agitation. The cells were then diluted to 1 liter with Grace's medium complete with 10% FBS. The infected cell suspension was incubated with stirring for 8 h at 28 °C. Cells were harvested by centrifugation at 6000 × g for 15 min, followed by washing with phosphate-buffered saline. Cells were either frozen immediately at −80 °C for later use or were lysed and purified at once.

Purification of the GST fusion protein was performed using glutathione agarose as described in the manual provided by Amersham Pharmacia Biotech. Purification of the non-fusion recombinant mouse BPnTase initially involved phenyl-Sepharose high performance liquid chromatography (HPLC). Cell pellets were resuspended in lysis buffer (25 mM NaCl, 250 mM KCl, 50 mM Na2 EDTA, 10 mRNA and the Bam-HI-NotI fragment of pCRHYQ were cloned into Bam-HI/NotI-linearized pRS426GAL to create pRSHAL2 and pRcEsYQ. The Ndel-BclI fragment of pCRHAL2 and the Nhel-BamHI fragment of pCRHYQ were isolated and ligated into Ndel/BamHI-linearized pET11c or Nhel/BamHI-linearized pET11c bacterial expression vector (Novagen, Madison, WI) to create pKH12 and pRcEsYQ. These plasmids were transformed into the E. coli strain BL21(DE3) for overexpression.

Recombinant virus was made by co-transfecting Spinodopera frugi-perda (SF9) cells (2 × 10⁶) with 2 μg of sterile pVL439033 and 1 μg of BaculoGold baculovirus DNA (PharMingen) as described in the BaculoGold user manual. Viable, recombinant viruses were harvested 4 days post-transfection and amplified to 300-ml quantities in SF9 cells. Virus stocks had an approximate titer of 1 × 10⁹ and were stored at 4 °C.
protein was eluted with a 15-column volume reverse linear gradient from 30% to 0% ammonium sulfate saturation in buffer A. The protein eluted in 18–22% ammonium sulfate saturation.

In order to improve the yield and reliability of the purification, an affinity purification step was developed based on the enzyme’s activity on PAP-phosphorylated nucleotides. Enzyme was incubated with both 10 mM HEPES, pH 7.5, 10 mM CaCl₂, and 50 mM KCl and poured into a 10-mL disposable polypylene chromatography column (Bio-Rad). A 1-mL bed volume was sufficient to bind up to 6 mg of enzyme. Calcium, a potent inhibitor of homologous phosphomonoesterases, was added to 1-mL bed volume of Dowex equilibrated in formate buffer containing 1 mM PMSF, and the cells were disrupted in a French pressure cell and centrifuged as above. The supernatant was applied to the column by gravity flow, and the column was washed with 15 column volumes of PAP-agrose buffer plus 0.5 M NaCl and then reequilibrated with PAP-agrose buffer containing no additional salt. The enzyme was eluted with PAP-agrose buffer containing 300 μM 2’/3’,5’-PAP (Sigma). The protein was concentrated and the PAP removed by dialysis in Microcon centrifugal concentrators, 30,000 nominal molecular weight limit (Millipore; Bedford, MA). The protein was purified further from small molecules by size exclusion chromatography on Sephadex G50 (fine) QuickSpin columns (Roche Molecular Biochemicals). The protein was stored in 50 mM NaHEPES, pH 7.5, 100 mM NaCl, and 0.02% NaN₃. Aliquots were frozen at −80 °C and were stable for at least 3 months with no detectable loss of activity.

Native protein was also isolated with PAP-agrose affinity purification. Kidneys, lungs, liver, and heart from a freshly dissected mouse were homogenized separately with a Dounce homogenizer in 1 ml of ice-cold PAP-agrose buffer containing 1 mM PMSF. The homogenates were spun at 22,000 × g in a 4 °C microcentrifuge. The soluble fractions were applied to 50-μL PAP-agrose columns, which were washed and eluted as above. The eluants were analyzed by SDS-PAGE and visualized with silver staining and Western blotting with the anti-BPnase antibody. Eluted enzyme from the kidney preparations was pooled, and BSA was added to a final concentration of 0.1 mg/ml. The protein was dialyzed approximately 50,000-fold against a reaction mixture containing 50 mM NaHEPES, pH 7.5, 100 mM KCl, 3 mM MgCl₂, and 0.02% NaN₃. Aliquots were frozen at −80 °C and were stable for at least 3 months with no detectable loss of activity.

**Determination of Substrate Specificity**—Purified GST-439033 fusion protein was utilized to investigate reactions catalyzed by the protein. Sequence homology with the consensus Li⁺-sensitive phosphomonoesterase family active site motif led us to predict that the enzyme hydrolyzed small phosphorylated molecules, possibly including inositols and nucleotides, in a Mg²⁺-dependent manner. Trace molar amounts of [3H]inositol standards (American Radiolabel Corp., St. Louis, MO) were incubated with protein in HEKM. Following incubation at 37 °C, the reaction was stopped with 1 volume of 20 mM ammonium phosphate (AP) and was loaded onto a 4.5 × 250-mm PartiSIL 10 SAX-HPLC strong anion exchange column (Whatman, Clifton, NJ). Pre-reaction inositol standard and reaction potential inositol products typically were separated with linear gradients from 10 mM to 1.7 mM AP over 50 min. Radioactivity of the eluant was measured continuously using a Beta-RAM in-line detector (INUS Systems, Tampa, FL).

Alternatively, GST-439033 was used to treat PAP and PAPS as potential substrates. Enzyme was incubated with both 10 μM unlabeled PAPS (Sigma) and approximately 1000 nM 5’-D[32P]PAP. Following incubation at 37 °C in HEKM, the reaction was stopped as above and loaded onto the PartiSIL 10 SAX column. In this case, the gradient used was 10 mM to 1.02 mM AP over 60 min. One-milliliter fractions were collected and monitored for absorbance at λ = 260 nm and then for 32P radioactivity by scintillation counting. The experimental chromatogram was compared with a standard chromatogram containing commercially obtained 5’-adenosine 5’-phosphosulfate (APS), 5’-AMP, PAP, and PAPS (Sigma).

**Assays of Enzyme Kinetics**—Phosphatase activity was analyzed in the following manner. Unless otherwise noted, reactions were carried out in HEKM buffer containing 0.4 mg/ml BSA. For inhibitor studies, the reaction mixtures contained appropriate concentrations of LiCl, unlabeled PAPS, or unlabeled Ins(1,4,5)P₃. Enzyme was added to reaction mixtures containing appropriate concentrations of transfected 3H-inositols or 5,32P-bisphosphorylated nucleotides. Reactions were performed at 37 °C for specific times and were stopped with 20 volumes of appropriate formate buffer (see below). The stopped reaction mixture was applied to a 200-μL bed volume of Dowex equilibrated in formate buffer. Reaction products were eluted by washing with 20 column volumes of formate buffer whereas higher phosphorylated product re-
using a gapped BLASTP search (version 2.0.6; BLOSUM62 matrix). Probability scores (in brackets) were used to rank a number of similar proteins including: U42833, *Caenorhabditis elegans* CEESN37F [2e-54]; AL032655, *C. elegans* cDNA EST yk255e11.5 [1e-20]; L08488, human 1ptase [3e-14]; U14003, *E. coli* cysQ (amtA) [1e-07]; P29218 -human impase [3e-06]. As clone 645079 was most related to 1ptase and PAP phosphatase, a multiple sequence alignment was performed among 645079 (hBPntase), 1ptase (h1pt), CEESN37F (U42833), and yeast and plant PAP phosphatase (HAL2 and SAL1) using the program MATCHBOX (27) as shown in Fig. 1 (panel A). Despite relatively low overall sequence identities, eleven regions conserved throughout all five proteins were found as indicated by lowercase lettering (determined without human intervention), and the gray boxes (for enhanced visualization).

The core structure of the lithium-sensitive family encompasses 160 residues and is composed of 5 α-helices, 11 β-strands, and at 2 metal binding sites. A stereo image of the 1ptase α-carbon core is shown in Fig. 1B. To characterize the sequence/structure relationship, the secondary structure elements were mapped onto the alignment as shown by the gray arrows (strands) and coils (helices) in Fig. 1A. Remarkably, the MATCHBOX alignment identified 11 out of 16 core structure regions, providing further evidence that clone 645079 is structurally and functionally related to the lithium-sensitive protein family.

In order to determine the expression pattern and size of the 645079 transcript, a multitissue Northern blot analysis was performed. A radiolabeled 0.4-kb region of the human cDNA was used to probe 2 μg of human mRNA from a variety of tissue sources, and the results are shown in Fig. 2. A single 2.5-kb message was visible at various levels in all tissues examined, indicating that the clone 645079 was approximately full-length. As a control for amount of mRNA loaded, the same blot was reprobed with β-actin probe, as shown in the lower panel. Relative expression was determined by comparing the
FIG. 2. Distribution of clone 645079 mRNA in human tissues. A human multiplex Northern blot was probed for the expression of the 645079 message. The blot was probed with a radiolabeled fragment of the human gene and exposed to film for 32 h at −80 °C with two intensifying screens (top). The same filter was then stripped and re-probed with a radiolabeled actin cDNA fragment and exposed to film for 2 h at room temperature (bottom). Radioactivity for each band was quantitated using a PhosphorImager, and expression levels of 645079 are provided relative to actin and normalized to levels observed in lung (assigned a value of 1). RNA molecular weight standards (kb) are displayed.

ratio of 645079 and actin radioactivity (as quantified by phosphorimage analysis). The lowest level of expression was observed in lung and was assigned a value of 1.0 arbitrary units to which the other tissues were normalized. The highest level of expression was observed in kidney, which has 90.5-fold increased level relative to lung. Additionally, Northern analysis of murine kidney mRNA using a radiolabeled fragment of clone 439033 demonstrated a single band of 1.4 kb consistent with size observed from sequencing of the clone (data not shown). Of interest, the relative amount of expression in brain was low.

Substrate Specificity Determination—Computer cloning represents a unique "reverse" biochemistry problem, i.e. determining the substrate specificity for a clone of unknown function.

Sequence homology with proteins of the lithium-sensitive phosphomonoesterase family led us to predict that the enzyme would be metal-dependent and hydrolyze small phosphorylated inositols, possibly including phosphorylated inositols and nucleotides. To investigate this possibility, the coding sequence from the murine clone 439033 was inserted into a pGEX vector and expressed in bacteria generating a GST-439033 fusion protein. Recombinant GST-439033 was readily purified using glutathione affinity resin and incubated in Mg2+ buffer containing radiolabeled Ins(1,4)P2, Ins(1,3,4)P3, and Ins(1,4,5)P3. Reactants from GST control, GST-439033, or pure 1Pase were separated by strong anion HPLC, and representative radioograms of eluted compounds are shown (Fig. 3A, lower, middle, and upper traces, respectively). GST and untreated (not shown) samples show three peaks: B, D, and E. Treatment with 1Pase provides additional standards Ins(4)P (peak A) and Ins(3,4)P2 (peak C), consistent with removal of the 1-position phosphate. Treatment with GST-439033 shows qualitatively similar results in that peaks A and C have appeared at the expense of disappearance of peaks B and D. In both cases, Ins(1,4,5)P3 is not hydrolyzed, as the size of peak E is unchanged. As GST-439033 does not metabolize the Ins(4)P product to inositol nor does it hydrolyze Ins(1)P (data not shown), we conclude it does not function as an impase. Additionally, Ins(3,4)P2 is not converted, indicating that GST-439033 is not acting as a 3- or 4-phosphatase. Furthermore, other inositol polyphosphates that were tested included Ins(1,3,4,5)P4, Ins(1,3,4,5,6)P5, and InsP6, none of which was dephosphorylated. Together, these data demonstrate that GST-439033 functioned qualitatively as an inositol polyphosphate 1-phosphatase.

Given the sequence similarity to HAL2, cysQ, and to a lesser extent SAL1, we tested GST-439033 using PAP and PAPS substrates. GST-439033 or GST protein was incubated in the presence of Mg2+, 5'-[32P]PAP, and unlabeled PAPS, the reagents were separated by HPLC, and fractions were collected and monitored by absorbance at 260 nm (dashed lines) and 32P liquid scintillation counting. Letters and arrows indicate elution times of commercially prepared 5'-AMP (A), 5'-APS (B), PAP (C), and PAPS (D).

FIG. 3. Analysis of reactions catalyzed by 439033. A, various inositol mono- and bisphosphates were treated with: 439033-GST (1), 1Pase (2), and GST control (3). Reaction products were separated on PartiSil 10 SAX-HPLC using a linear gradient from 10 m M to 1.7 m AP over 50 min. Radioactivity was measured continually with an in-line liquid scintillation counter. Elution times of commercially prepared Ins(1,4)P2 (B), Ins(1,3,4)P3 (D), and Ins(1,4,5)P3 (E) are indicated by the letters and corresponding arrows. Products of the 1Pase reaction correspond to peaks A (Ins(4)P) and C (Ins(3,4)P2). B, 5'-[32P]PAP and unlabeled PAPS were treated for 1 h with GST-439033 (1) and GST (2). Reaction products were separated by HPLC using a linear gradient from 10 m M to 1.02 m AP over 60 min. One-milliliter fractions were collected and monitored for substrate via absorbance at λ = 260 nm (dashed lines) and 32P liquid scintillation counting. Letters and arrows indicate elution times of commercially prepared 5'-AMP (A), 5'-APS (B), PAP (C), and PAPS (D).
with GST-439033, indicating that each of the nucleotides tested is a substrate for the enzyme (data not shown).

**Purification of Non-fusion Protein**—In order to determine detailed biochemical parameters of mammalian nucleotide/inositol phosphatase, purification of non-fusion protein was performed. The open reading frame of the mouse clone was inserted into a baculovirus cloning vector. Recombinant virus was amplified and used to infect SF9 cells in medium containing 10% FBS. Typical infections resulted in production of approximately 10 mg of soluble enzyme/liter of cells, representing around 4% of the total soluble protein.

Initial investigations indicated that the protein flowed through both S-Sepharose cation exchange columns and Q-Sepharose anion exchange columns at pH 7.5. Therefore, a negative purification step involving both ion exchange columns in series was employed. Subsequently, ammonium sulfate was added to 30%, and soluble proteins were applied to an HPLC phenyl-Sepharose column. The protein eluted in 18–22% ammonium sulfate. Following concentration and removal of ammonium sulfate, typical yields were on the order of 20% with purification of approximately 25-fold.

In order to increase yield, an improved affinity purification method based on previously reported PAP-agarose chromatography (28) was developed. Calcium exhibits micromolar inhibitory activity of other members of the lithium-sensitive family. Cell lysates therefore were applied to protein was concentrated and dialyzed to remove calcium and free PAP prior to enzymatic assays. Aliquots were stored frozen at −80 °C for up to 3 months with no apparent loss of activity.

In order to confirm the *in vivo* expression, we isolated it from freshly harvested mouse tissues. Following the success of using a PAP-agarose affinity matrix to obtain purified baculovirus-expressed 439033 protein in a single step, we employed this technique to isolate the native enzyme. The soluble fraction from centrifuged mouse kidney homogenate was applied directly to a PAP-agarose column equilibrated in 10 mM CaCl₂. As shown by the silver stain in Fig. 4A, a single band comigrating with recombinant BPnase appears in the column elution fraction. In addition to similar PAP-agarose binding and SDS-PAGE migration, polyclonal antibodies to recombinant 439033 protein react with the 37-kDa band (Fig. 4C), giving further evidence that the native enzyme has been isolated from fresh mouse tissues. Several mouse tissues, including kidney, lung, heart, and liver, were analyzed (Fig. 4A–C). Western blot data corroborated Northern blot analysis (Fig. 2), indicating highest levels of expression in the kidney and little expression in lung.

**Kinetics of Phosphomonoesterase Activity**—Previously reported kinetic parameters for bisphosphate nucleotidase activity were determined using non-radioactive PAP and/or PAPS substrate. Due to relative insensitivity of this assay, Michaelis–Menten constants for all nucleotides were approximated. As a means to accurately determine the affinity constant and catalytic efficiency of mammalian protein, a radiolabeled nucleotide assay was developed. [γ-³²P]PAP labeled in the 5' position was generated simply by incubating 3'-AMP with [γ-³²P]ATP and T4 polynucleotide kinase. In this way, nucleotide concentrations in

**Fig. 4. Overexpression of recombinant BPnase and purification of recombinant and native proteins.** Proteins were separated on 9% SDS-PAGE. Standards (kDa) migrated as indicated. A, Coomassie Brilliant Blue staining of recombinant mouse BPnase produced in a baculovirus/Sf9 system. Lane 1, Sf9 crude supernatant, 60 h after infection (5 μg of total protein); lane 2, PAP-agarose flow-through and 7 column volume wash (5 μg); lane 3, final wash; lanes 4–9, profile of elution with 300 μM free PAP. B, silver staining of native BPnase isolated from mouse kidney. Lane 1, crude mouse kidney supernatant (1 μg); lane 2, mouse liver PAP-agarose flow-through and 7 column volume wash (1 μg); lane 3, final wash; lanes 4–6, free PAP elutions. C, Western blot analysis using antibodies against recombinant BPnase (rabbit host). Lane 1, pure recombinant mBPnase (50 ng); lanes 2–5, crude mouse tissues (30 μg of total protein/lane) (lane 2, kidney; lane 3, lung; lane 4, heart; lane 5, liver); lanes 6–9, third elution from PAP-agarose purifications (lane 6, kidney; lane 7, lung; lane 8, heart; lane 9, liver).
approximate association constant for the sulfated nucleotide. As expected, inhibition was competitive (data not shown), with a $K_i$ of 700 nM (Table I, part B).

Purified native protein was assayed for hydrolysis of PAP and Ins(1,4)P$_2$. Native enzyme hydrolyzes PAP ($K_m$ = 475 nM, $V_{max}$ = 60 $\mu$mol/min/mg) and Ins(1,4)P$_2$ ($K_m$ = 130 $\mu$M, $V_{max}$ = 8 $\mu$mol/min/mg) in a Li$^+$-sensitive manner and with kinetics similar to those determined for the recombinant protein (data not shown).

PAP hydrolysis is dependent on the presence of Mg$^{2+}$. Optimum activity was obtained within a Mg$^{2+}$ concentration range of 2.5–3.5 mM. Similar to other enzymes in the lithium-sensitive family, activity is inhibited at high concentrations of Mg$^{2+}$ (Fig. 6A) (2). A Hill plot of data within a range of Mg$^{2+}$ concentrations of 0.01–0.2 mM gives a Hill coefficient of 1.60, indicating positive cooperativity but an undetermined number of metal binding sites (Fig. 6B). The pH optimum for PAP hydrolysis is approximately 7.5 (Fig. 6C). Similar results were obtained for the hydrolysis of Ins(1,4)P$_2$ and Ins(1,3,4)P$_3$ (data not shown).

Inhibition of Phosphatase Activity—Based on its limited sequence similarities to other Mg$^{2+}$-dependent phosphomonooesterases in the 1ptase family, we predicted that BPntase would also be uncompetitively inhibited by Li$^+$. The velocity of PAP phosphatase activity versus lithium concentration at various substrate concentrations was determined and displayed as a Dixon plot in Fig. 7A. Parallel lines in the Dixon plot indicate that lithium inhibition is uncompetitive with respect to PAP. The $K_i$ for this inhibition is 157 ± 17 $\mu$M LiCl (Table I, part B). Similarly, Li$^+$ inhibition is uncompetitive with respect to Ins(1,4)P$_2$, and the calculated $K_i$ is 540 $\mu$M (data not shown).

As BPntase markedly favors nucleotides, we sought to determine if the inositol substrates compete for identical sites. The data are summarized in Table II. The cellular ranges of inositol polyphosphates are estimated to be near or below micromolar levels in unstimulated cells and 10–50 $\mu$M in stimulated cells. Thus we analyzed inhibition at 50 and 100 $\mu$M concentrations. Remarkably, 50 $\mu$M Ins(1,4)P$_2$ inhibited 84.4% of PAP hydrolytic activity, whereas other inositol polyphosphates even at 100 $\mu$M concentrations had less potent inhibitory activity. This was carefully analyzed in Fig. 7B. Nucleotide hydrolysis in the

![Fig. 5. Kinetic parameters of BPntase phosphomonoesterase activity. Reactions were run as described under “Materials and Methods.”](image-url)

TABLE I

| Biochemical parameters of nucleotide bisphosphatases | A. Substrates | Recombinant mNIP | Native mNIP | HAL2 | CysQ |
|---------------------------------------------------|---------------|-----------------|------------|------|------|
| PAP                                               | $k_{cat}/K_m$ | $4.4 \times 10^7$ | $6.9 \times 10^7$ | $2.8 \times 10^7$ | $2.3 \times 10^7$ |
| PAPS                                              | Active         | ND              | Active     | ND   | ND   |
| Ins(1,4)P$_2$                                     | $4.6 \times 10^4$ | $3.4 \times 10^4$ | $1.0 \times 10^4$ | $0.2 \times 10^4$ |
| Ins(1,3,4)P$_3$                                   | $7.2 \times 10^4$ | ND              | ND         | ND   | ND   |
| InsP                                              | 0              | 0               | ND         | ND   | ND   |
| Ins(1,4,5)P$_3$                                   | 0              | 0               | ND         | ND   | ND   |
| PNP*                                              | Active         | ND              | ND         | ND   |

| B. Inhibitors of PAP hydrolysis | mNIP ($K_i$) |
|--------------------------------|--------------|
| PAPS                            | 0.7          |
| Ins(1,4)P$_2$                   | 15           |
| LiCl                            | 157          |

*Active, detectable activity with undetermined efficiency.

$^a$ND, activity not determined.

$^b$PNP includes 2’dPAP, 4P, PGP, PTP, and PUP.

PAP hydrolysis is dependent on the presence of Mg$^{2+}$. Optimum activity was obtained within a Mg$^{2+}$ concentration range of 2.5–3.5 mM. Similar to other enzymes in the lithium-sensitive family, activity is inhibited at high concentrations of Mg$^{2+}$ (Fig. 6A) (2). A Hill plot of data within a range of Mg$^{2+}$ concentrations of 0.01–0.2 mM gives a Hill coefficient of 1.60, indicating positive cooperativity but an undetermined number of metal binding sites (Fig. 6B). The pH optimum for PAP hydrolysis is approximately 7.5 (Fig. 6C). Similar results were obtained for the hydrolysis of Ins(1,4)P$_2$ and Ins(1,3,4)P$_3$ (data not shown).

Inhibition of Phosphatase Activity—Based on its limited sequence similarities to other Mg$^{2+}$-dependent phosphomonoesterases in the 1ptase family, we predicted that BPntase would also be uncompetitively inhibited by Li$^+$. The velocity of PAP phosphatase activity versus lithium concentration at various substrate concentrations was determined and displayed as a Dixon plot in Fig. 7A. Parallel lines in the Dixon plot indicate that lithium inhibition is uncompetitive with respect to PAP. The $K_i$ for this inhibition is 157 ± 17 $\mu$M LiCl (Table I, part B). Similarly, Li$^+$ inhibition is uncompetitive with respect to Ins(1,4)P$_2$, and the calculated $K_i$ is 540 $\mu$M (data not shown).

As BPntase markedly favors nucleotides, we sought to determine if the inositol substrates compete for identical sites. The data are summarized in Table II. The cellular ranges of inositol polyphosphates are estimated to be near or below micromolar levels in unstimulated cells and 10–50 $\mu$M in stimulated cells. Thus we analyzed inhibition at 50 and 100 $\mu$M concentrations. Remarkably, 50 $\mu$M Ins(1,4)P$_2$ inhibited 84.4% of PAP hydrolytic activity, whereas other inositol polyphosphates even at 100 $\mu$M concentrations had less potent inhibitory activity. This was carefully analyzed in Fig. 7B. Nucleotide hydrolysis in the
Enzyme overexpression was monitored by changes in specific activities. At 1 μM PAP, the cultures had the following specific activities in nanomoles of PAP hydrolyzed/min/mg of total protein: HAL2, 130.4; mBPntase, 746.2; cysQ, 66.1; 1Ptase, 0; vector control, 41.7. These values were obtained from three experiments with standard deviations of 6% or less.

Inositol phosphates were analyzed for their ability to inhibit the PAP hydrolysis activity of NIP. Reactions were performed with 1 μM PAP. Data are displayed as percent inhibition and represent the average of three experiments with standard deviations of 6% or less.

| Inhibitor   | 50 μM inhibitor | 100 μM inhibitor |
|-------------|-----------------|------------------|
| Ins(1)P     | 0.6             | 4.1              |
| Ins(1,4)P_5 | 84.4            | 96.7             |
| Ins(1,3,4)P_3 | 21.7          | 38.6             |
| Ins(1,4,5)P_3 | 30.1           | 50.3             |
| Ins(1,2,6)P_4 | 8.6            | 8.9              |
| Ins(1,4,5,6)P_4 | 14.5        | 22.3             |
| Ins(1,3,4,6)P_4 | 24.5          | 46.6             |
| Ins(3,4,5,6)P_6 | 30.2         | 49.2             |
| Ins(1,3,4,5,6)P_6 | 9.7           | 17.7             |
| InsP_6      | 9.5             | 9.2              |

TABLE II
Inhibition of PAP hydrolysis by inositol phosphates

Ins(1,4)P_2 displays competitive inhibition with a $K_i$ of 15 μM (Fig. 7B; Table I, part B). This provides a basis that Ins(1,4)P_2 may play a regulatory role via inhibition of phosphatase activity as opposed to hydrolysis in the action of BPntase in vivo.

Functional Complementation of Yeast HAL2—The similar substrate selectivity of BPntase, SAL1, and Hal2p prompted experiments to determine if BPntase could functionally complement hal2 yeast mutants. The HAL2 gene therefore was disrupted in diploid W303 S. cerevisiae by single gene replacement with the LEU2 marker gene. The diploid yeast were sporulated, tetrads were dissected, and spores were found to segregate 4:0 on rich media consistent with a nonessential function. Previous studies show that the hal2 disruption confers methionine auxotrophy (10); therefore, spores with the disruption are expected to be methionine auxotrophic and leucine prototrophic. Replica plating tetrads on Leu− and Met− plates showed a 2:2 segregation of both leucine and methionine auxotrophy (data not shown).

Complementation of the hal2 disruption was examined by overexpressing mBPntase, Hal2p, cysQ protein, and human 1P/ase. The genes were expressed from a high copy plasmid (pRS426) containing a galactose-inducible promoter. Following growth in media containing methionine and 2% galactose, cultures were inoculated at 10^4 cells/ml into synthetic media lacking methionine and containing 2% galactose. Fig. 8 displays the functional complementation of the hal2 knockout. Exogenous Hal2p, mBPntase, and cysQ complement at similar levels. As expected, the vector control displays methionine auxotrophy. In addition, 1P/ase, which does not hydrolyze nucleotides, does not complement the defect. This is consistent with the idea that hydrolysis of bisphosphorylated nucleotides and not inositol polyphosphates is required for growth in the absence of methionine.

Enzyme overexpression was monitored by changes in specific activities. At 1 μM PAP, the cultures had the following specific activities in nanomoles of PAP hydrolyzed/min/mg of total protein: HAL2, 23.7; mBPntase, 16.7; cysQ, 3.5; 1P/ase, 0; vector control, 0. At 8 μM Ins(1,4)P_2, the cultures had the following specific activities (pmol/min/mg): HAL2, 130.4; mBPntase, 415.7; cysQ, 66.1; 1P/ase, 746.2; vector control, 41.7. These
Complementation analysis on haploid cells was performed with indicated gene products on a 2 μm plasmid (pRS426), selected by uracil prototrophy, and under control of a GAL promoter. Transformed yeast were inoculated at 10^6 cells/ml in synthetic media lacking uracil and methionine and containing 2% galactose.

Data suggest the following: 1) that the cultures were expressing the recombinant proteins near expected levels; 2) that overexpression of HAL2, as well as cysQ, results in a significant increase in specific activity against Ins(1,4)P_2, suggesting that these proteins have hydrolytic activity against bisphosphorylated inositols. This result contradicts an earlier report that suggested that HAL2 does not hydrolyze Ins(1,4)P_2 (12). To address this, we analyzed the kinetic properties of recombinant Hal2p and cysQ. Hal2p hydrolyzes PAP (K_m = 720 nM) and Ins(1,4)P_2 (K_m = 430 μM). Similarly, cysQ hydrolyzes PAP (K_m = 1.1 μM) and Ins(1,4)P_2 (K_m = 1.2 μM). Catalytic efficiencies are displayed in Table I, part A.

**DISCUSSION**

The Lithium-sensitive Phosphomonoesterase Family—Lithium’s role in biology is of intense interest, yet the mechanisms by which it exerts its effects in human disease and development remain to be firmly established. The identification of a family of structurally conserved lithium-sensitive phosphomonoesterases has provided insight into this mechanism in several ways. First, biochemical and biological characterization of these proteins demonstrated that potent inhibition of enzymatic activity occurs at submillimolar concentrations, indicating that they are relevant targets. In vivo studies have validated this point. Perhaps most compelling are genetic studies of Drosophila 1ptase mutants demonstrating that deletion of the ipp phenocopies treatment of normal flies with lithium (18). Additionally, lithium induces changes in the levels of substrates and products of several family members in vivo. Second, the initial connection of lithium to inositol signaling pathways was made through impasse leading to an “inositol depletion” hypothesis (30). Discovery of other lithium-sensitive signaling and metabolic proteins, such as 1ptase, flptase, Hal2p, SAL1, and now BPntase, has greatly expanded our view of pathways involved. The notion that manic depression is a polygenic disorder is consistent with the involvement of multiple signaling pathways. Third, the conservation of the structures of this family has provided a key demonstration that they have evolved from a common ancestor. The ability to identify members such as flptase required knowledge of the three-dimensional structure, as the overall sequence similarity is undetectable by current local alignment strategies. Importantly, the comparison of the structures has resulted in the identification of the signature motif of this family: D-X_n-EE-X_n-DP(i/b)Dx/g/a/T-X_m-WD-X_11-GG.

The hypothesis that proteins having this motif will also function as metal-dependent lithium inhibited phosphomonoesterases has been validated by this study. This is of significant importance especially in the upcoming “post-genomic” era of modern biology. Repeating the strategy defined in this study on emerging or completed EST and genome data bases will facilitate rapid identification of novel human lithium targets. The demonstration that BPntase was uncompetitively inhibited by Li^+ with the lowest inhibition constant described to date is significant. Additionally, structural information was obtained from multiple sequence alignments with 1ptase and mapping regions of conservation to the core elements. Of note, although the signature motif is able to definitively identify a protein’s membership in the family and lithium sensitivity, it appears unable to predict a protein’s substrate selectivity. This is evident from characterization of the mammalian BPntase that appeared more related to 1ptase than to nucleotidases, yet the substrate preference favored bisphosphate nucleotides.

The Role of Nucleotidase Versus Inositol Phosphatase Activities of BPntase—The ability of BPntase to hydrolyze both nucleotide and inositol polyphosphates raises the question which activity is important biologically? Previous reports characterizing nucleotidase biochemical parameters were limited in interpretation due to the relative insensitivity of measuring phosphate release from unlabeled substrate and/or spectroscopic measurements. This was overcome by developing a simple radiolabeling procedure for making 5^-labeled PAP, and our data show that the Michaelis affinity constant for nucleotides in the nanomolar range, an order of magnitude below previously estimated values (13, 28, 29, 31). Previous biochemical studies of Hal2p nucleotidase showed that it did not possess 1-phosphatase activity (12). In contrast, our data clearly demonstrate that Hal2p does have 1-phosphatase activity, with similar selectivity and catalytic efficiency to mammalian and plant counterparts (13). We demonstrate that the catalytic efficiency of BPntase, Hal2p, and CysQ toward nucleotide substrates is highly favored. Furthermore, 1ptase, which does not possess nucleotidase activity, is unable to complement hal2 mutant yeast providing further biological support favoring the nucleotidase function. Thus, we conclude that the biologically relevant activity is the 3^-nucleotidase.

An unexpected finding of our studies was that inositol 1,4-bisphosphate potently inhibits nucleotidase activity, having an inhibitory constant in the physiological range. This provides a novel connection that, rather than functioning to hydrolyze inositol polyphosphates, BPntase is potently regulated by these potential messengers. Kidney tissue has been shown to possess ample levels of 1ptase, which has a catalytic activity 100 times higher than the 1-phosphatase activity of BPntase (16). Of interest, a previous report of Quintero et al. (13) suggested that the 1-phosphatase activity of SAL1 was important for mediating the sodium/lithium efflux response. This interpretation was based on the observation that both the inhibition of inositol-specific phospholipase C using the inhibitor 48/80, and the overexpression of SAL1 promote lithium efflux in cells. The authors concluded from these experiments that phospholipase-mediated inositol signaling regulates cation efflux through ENA pumps and that SAL1 participates in this process by promoting turnover of messenger molecules. Another interpretation, consistent with our model, is that blocking phospholipase C activity reduces levels of inositol 1,4-bisphosphate, relieving inhibition of the nucleotidase activity, thereby mimicking the effects of overexpression. Further studies are needed to clarify which model is correct.

The ability of this family of nucleotidases to utilize either bisphosphate nucleosides or PAPS has further confirmed determination of the physiologically relevant substrate. Muta-
tions in either HAL2/MET22 or cysQ result in defective sulfur assimilation (10, 14), providing evidence the PAPS nucleotidase activity is biologically relevant. Neuwald et al. suggested that PAPS or a derivative may be cytotoxic when allowed to accumulate (14). As support for this idea, they cite the fact that poor growth due to mutations in the PAPS-utilization pathway can be rescued by inhibiting the formation of PAPS with additional mutations in cysC, a 5′-APS 3′-kinase (14). Furthermore, Peng and Verma have shown that supplementation of media with methionine but not sulfite supports growth of hal2 mutants, indicating that the PAPS nucleotidase activity is most relevant (31).

Alternatively, due to their similar structures, PAP and PAPS may play cooperative roles in the sulfur assimilation pathway. For example, Ozeran et al. showed that a PAPS translocase transports PAPS across mitochondria membranes via an antiport mechanism with PAP as the returning ligand (32, 33). BPnTase may function to deplete the pool of PAP, thereby facilitating the transport of PAPS across membranes and into the vicinity of the sulfotransferase machinery. The PAPS translocase itself was found to be competitively inhibited by PAP with respect to PAPS (32, 33). Therefore, accumulation of PAP via a decrease in BPnTase activity could have drastic effects on sulfur assimilation pathways. In addition to a possible effect on sulfur assimilation, recent evidence points to a role of PAP nucleotidase activity in regulating RNA processing. Dichtl et al. (17) reported deletion of hal2 results in defects in Xrn1p-mediated RNA processing due to direct inhibition by PAP. This enzyme is not essential, but the redundant function is accomplished by RNase MRP, an enzyme that may itself be inhibited directly or indirectly by lithium. Therefore, Dichtl et al. propose that lithium toxicity, at least in yeast, is mediated by inhibition of RNase MRP and by concurrent inhibition of the cytosolic enzyme Xrn1p via inhibition of HAL2 and subsequent PAP accumulation (17). Under growth conditions containing high Na⁺ or Li⁺ concentrations, overexpression of the PAP-metabolizing enzymes HAL2 and SAL1 would rescue growth by an increase in enzyme activity, thus reducing accumulated PAP pools. Methionine supplementation would also rescue growth by down-regulating the production of PAP from PAPS (34). As it appears that BPnTase is a true functional homologue of HAL2 and SAL1, our results augment the findings of Dichtl et al. BPnTase is active on bisphosphorylated nucleotides other than PAP such as PGP and PCP. Thus, other bisphosphorylated nucleosides that may accumulate in the absence of nucleotide phosphatase activity may also inhibit Xrn1p.

The Role of BPnTase in Kidney Function—It is particularly interesting that the levels of nucleotidase are highest in kidney, consistent with the notion increases in nucleotidase activity are associated with resistance to salt. Both SAL1 and HAL2 were identified on screens for proteins that, when overexpressed, conferred tolerance to cations such as sodium and lithium. Furthermore, the up-regulation of SAL1 expression in response to increased tonicity (13) suggests a physiological role for nucleotidase activity in osmoregulation.

In addition, one of the major side effects of lithium treatment is nephrogenic diabetes insipidus, characterized by polyuria and polydipsia. The root of this side effect may lie in the inappropriate inhibition of BPnTase phosphatase activity by Li⁺. Disruption of sodium extrusion machinery on either the luminal or basal side of kidney cells could upset sodium balances. Such a disruption in sodium homeostasis could be manifested as the movement of large amounts of fluid across kidney cells, resulting in the voiding of copious amounts of liquid. Of particular clinical significance is the ability of methionine supplementation to suppress lithium toxicity in yeast, through a mechanism that down-regulates production of PAP and ultimately affects RNA processing, sulfur assimilation, and cation efflux pathways. We suggest that a similar strategy in which lithium is co-administered with methionine in patients treated for bipolar disorder may prove clinically useful in abrogating the harmful effects of nucleotidase inhibition.

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