J. Biochem. **133**, 607–614 (2003)
DOI: 10.1093/jb/mvg078

Molecular Cloning of a cDNA for the Human Phospholysine Phosphohistidine Inorganic Pyrophosphate Phosphatase

Fumiaki Yokoi*, Hiroyuki Hiraishi† and Kenji Izuhara
Department of Biochemistry, Saga Medical School, Nabeshima 5-1-1, Saga-shi, Saga 849-8501

Received December 8, 2002; accepted February 16, 2003

We previously reported the isolation from bovine liver of a novel 56-kDa inorganic pyrophosphatase named phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPPase). It is a unique enzyme that hydrolyzes not only oxygen-phosphorus bonds in inorganic pyrophosphate but also nitrogen-phosphorus bonds in phospholysine, phosphohistidine and imidodiphosphate *in vitro*. In this study, we determined the partial amino acid sequence of the purified bovine LHPPase. To investigate whether humans have the same enzyme, we isolated a cDNA clone from a HeLa cell cDNA library that encodes for the human homologue of LHPPase. Although its sequence does not include the consensus sequence of a typical inorganic pyrophosphatase, it does contain a similar sequence of the active site in other phosphatases such as protein-tyrosine phosphatase, dual-specific phosphatase and low molecular weight acid phosphatase. Human LHPPase was highly expressed in the liver and kidney, and moderately in the brain. The recombinant protein was produced in *E. coli*. Its ability to hydrolyze oxygen-phosphorus bonds and nitrogen-phosphorus bonds was confirmed. The enzymatic characteristics of this human protein were similar to those of purified bovine LHPPase. Thus, we concluded that the cDNA encoded the human counterpart of bovine LHPPase.

Key words: LHPP, lhpp, LHPPase, phosphatase, pyrophosphatase.

Abbreviations: LHPPase, phospholysine phosphohistidine inorganic pyrophosphate phosphatase; His-hLHPPase, His·Tag human LHPPase fusion protein; *p*-CMPS, *p*-chloromercuriphenyl sulfonic acid; PNP, imidodiphosphate; O-P, oxygen-phosphorus; N-P, nitrogen-phosphorus; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; PPase, inorganic pyrophosphatase; PNPase, imidodiphosphatase.

Inorganic pyrophosphate is produced as a byproduct in many biosynthetic reactions using nucleotide triphosphates in cells. Inorganic pyrophosphatase [EC 3.6.1.1] is an enzyme that hydrolyzes inorganic pyrophosphate (PP_i) to produce two molecules of orthophosphate (P_i) (*1*, *2*). Although the reaction is bidirectional, the equilibrium tends to produce orthophosphate. It is believed that this enzyme is coupled with the reactions producing inorganic pyrophosphate. Consequently, it facilitates reactions that produce orthophosphate and prevents the reactions from reversing (*3*). Inorganic pyrophosphatase exists in many organisms including animals, plants and bacteria. It is an essential enzyme for controlling the cellular level of inorganic pyrophosphate. Human inorganic pyrophosphatase is 94% identical to the bovine one (*4*, *5*). Inorganic pyrophosphatases are classified by their enzymatic characteristics. A mammalian cytosolic enzyme is a homodimer of 30- to 36-kDa subunits and requires Mg^{2+} for its full activity (*6–8*). Its hydrolytic activity on PPi is competitively inhibited by imidodiphosphate (PNP), an analog of PPi (*7–9*). On the other hand, a yeast enzyme hydrolyzes PNP into P_i and amidophosphate in a Mg^{2+}-dependent manner, although the hydrolytic rate of PNP is very low compared with that of PP_i (*10*).

We surveyed phosphatases that hydrolyze PNP in mammals and found a novel 56-kDa enzyme that is a homodimer in bovine liver (*11*). This enzyme hydrolyzes PNP into two molecules of Pi and ammonia. It also hydrolyzes PPi in the presence of Mg^{2+}. The main difference between this enzyme and the yeast enzyme is that the former works as a phosphoamidase (EC 3.9.1.1), while the latter hydrolyzes substrates having diphosphate bonds. We also reported that this enzyme hydrolyzes 3-phosphohistidine and 6-phospholysine in addition to PNP and PPi (*12*). We designated this novel inorganic pyrophosphatase as phospholysine phosphohistidine inorganic pyrophosphate phosphatase and abbreviated it as LHPPase (phospho-Lysine phospho-Histidine inorganic Pyrophosphate Phosphatase). In this study, we determined partial amino acid sequences of the purified bovine LHPPase.

To investigate if humans have the same enzyme, we isolated a human cDNA encoding the corresponding protein. To confirm that the cDNA clone codes for a functional enzyme, we measured the enzymatic activity of the protein to hydrolyze oxygen-phosphorus (O-P) bonds and nitrogen-phosphorus (N-P) bonds by the methods used for bovine LHPPase (*11*).

*To whom correspondence should be addressed at the present address: Department of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, 3614 Beckman Institute, MC-251, 405 North, Mathews Avenue, Urbana, IL 61801 USA. Tel: +1-217-244-3218, Fax: +1-217-244-1726, E-mail: yokoi@uiuc.edu
†Present address: Research Organization of Science and Engineering, Ritsumeikan University, Kusatsu, Shiga 525-8577

MATERIALS AND METHODS

Protein Sequencing of Bovine LHPPase—Bovine LHPPase was purified from the liver as previously described (*11*). The purified enzyme was applied to an Applied Biosystems model 477A/120A protein sequencer. It was also digested with V8 proteinase (Takara, Shiga) in 50 mM ammonium bicarbonate (pH 8.0). The digests were separated by 13% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (*13*). The partial amino acid sequence of a peptide fragment (peptide 1) was determined using the protein sequencer. The purified enzyme was also *S*-carboxymethylated and digested with lysylendopeptidase (Wako Pure Chemical, Osaka). The peptide fragments were separated by reverse phase chromatography with TSK gel ODS-80Ts (4.6 × 150 mm) in a linear gradient of acetonitrile/1% TFA (9:1). A partial amino acid sequence of a second peptide fragment (peptide 2) was determined by using a Shimadzu PSQ-1 Systems protein sequencer in APRO science (Tokushima).

Cloning of a cDNA Encoding the Human Homologue—Based on the partial amino acid sequence of the bovine LHPPase, a nucleotide sequence (accession No. H75601) reported in the EST project (*14*) was selected as a probe to isolate a cDNA encoding a full-length human homologue. Two oligonucleotides, 5′-GGCCGAGCGCCATGGCACCGTGGG-3′ and 5′-CGTTAATCATGTTTTIAATAAGAA-3′, were designed as PCR primers. The DNA fragment was amplified by a polymerase chain reaction using a heat-stable DNA polymerase KOD dash (Toyobo, Osaka) and a human HeLa S3 5′-stretch plus cDNA library (Clontech, Tokyo) as a template. The amplified DNA fragment was ligated into a pT-Adv vector (Clontech). The DNA fragment of 425 bp was labeled with ^{32}P using random primers and used as a screening probe. A cDNA clone was isolated from the HeLa cells S3 cDNA library and subcloned into a plasmid Bluescript II SK M13+ (Stratagene, La Jolla, CA). The nucleotide sequence was determined by the dideoxy chain termination method with an Applied Biosystems model 373A stretch DNA sequencer (*15*). An online homology search of the DDBJ/EMBL/GeneBank database was made with the FASTA software program (*16*, *17*). The gene location was searched for in the EBI human genome database (*18*).

Northern Blot Analysis—The coding region was subcloned into a pT-Adv vector. The DNA fragment was obtained by digesting with *Eco*RI and labeled with ^{32}P by random primer labeling. Two kinds of commercial membranes (Human 12-lane MTN blot and human fetal MTN blot II, Clontech) were hybridized with the labeled probe in ExpressHyb Hybridization Solution (Clontech). They were washed according to the manufacturer's procedure. The signals were detected on Fuji RXU X-ray films (Fuji Film, Tokyo). Human 12-lane MTN blot and human fetal MTN blot II contained approximately 1 μg and 2 μg of polyA+ RNA per lane, respectively.

Preparation of His·Tag Human LHPPase Fusion Protein—The coding region was amplified with *Eco*RI sites at both ends by PCR and subcloned into pET30a plasmid (Novagen, Madison, WI). The His·Tag human LHPPase fusion protein (His-hLHPPase) was expressed in *Escherichia coli* BL21(DE3)pLysS and isolated by use of a Ni^{2+} chelating affinity column (His·Bind, Novagen) according to the manufacturer's instructions. The protein concentration was measured by the Bradford assay (Bio-Rad, Tokyo) (*19*). The fusion protein was dialyzed in 50 mM Tris-Cl (pH 6.5) containing 1 mM $MgCl_2$. The protein was mixed with an equal volume of glycerol and stored at –20°C for the enzyme assay.

Molecular Mass Estimation of Human LHPPase—The molecular mass of human LHPPase was estimated by SDS-PAGE. Five micrograms of the His-hLHPPase was dissolved in 50 mM Tris-Cl (pH6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol in the presence or absence of 100 mM dithiothreitol. Each sample was boiled for five minutes and subjected to electrophoresis on a 15% SDS–polyacrylamide gel (*13*). Proteins were stained with Coomassie Brilliant Blue R250. The molecular mass of human LHPPase was also estimated by gel filtration. Two hundred micrograms of the His-hLHPPase or standard proteins were applied on a Sephadex G-100 superfine column (0.9 × 54 cm, Amersham Pharmacia Biotech, Tokyo) equilibrated with 20 mM Tris-Cl (pH 7.5) and 0.15 M NaCl and fractionated to estimate the molecular mass. The fractionated fusion protein was detected by the Bradford assay (*19*). The molecular mass was estimated from the standard curves calculated by the method of least squares.

Measurement of Phosphatase Activity—Malachite green reagent was used to detect phosphate (*11*). Briefly, solution M was prepared as 1.5 mM Malachite green oxalate (Certistain, Merck, Tokyo) containing 2% polyvinyl alcohol 1500 (Wako Pure Chemical). Solution A was prepared as 40 mM hexaammonium heptamolybdate (Nacalai tesque, Kyoto) in 6 N HCl. Aliquots of solutions M and A were mixed to make the Malachite green reagent containing 0.2 mM Malachite green oxalate, 0.27% polyvinyl alcohol, 5.3 mM hexaammonium heptamolybdate in 0.8 N HCl. The solution was incubated at room temperature for more than 30 min after mixing. One or two ml of Malachite green reagent was added to 100 μl of the reaction mixture described as follows. Absorbance at 630 nm was measured 30 min after the addition of the reagent.

A 100 mM GTA buffer consisting of 33.3 mM 3,3-demethylglutaric acid, 33.3 mM Tris, and 33.3 mM 2-amino-2-methyl-1,3-propanediol was used as reaction buffer. Optimum pH was obtained in 100 μl of 100 mM GTA buffer (pH 4.0–10.0) containing 1 mM $MgCl_2$, 1 mM PP_i or 0.125 mM PNP, and 0.5 μg of the His-hLHPPase. The reaction was performed at 30°C for 30 min in duplicate. One milliliter of Malachite green reagent was added to each sample. The standard curve of phosphate was obtained with 100 μl of 0–300 μM sodium phosphate.

Inorganic pyrophosphatase (PPase) and imidodiphosphatase (PNPase) activities were measured as previously described (*11*). PPase activity was measured in 100 μl of reaction mixture containing 100 mM GTA buffer (pH 5.5), 1 mM $MgCl_2$, 0–4 mM PP_i and 1 μg of the fusion protein at 30°C for 30 min. One milliliter of Malachite green reagent was added. PNPase activity was measured in 100 μl of reaction mixture containing 100 mM GTA buffer (pH 7.0), 1 mM $MgCl_2$, 0–4 mM PNP, and 1 μg of the fusion protein. The reaction was performed in triplicate. Standard deviations were calculated by use of Excel (Microsoft, Tokyo).

```
AAGAACTTTAAAAATCACCTAGGTGTGGGCCGGGCACGGTGGCTAACGCCTGTAATCCCA     60
GCACTTTGAGATGCTGAGGCAGGTGGATCACGAGGTCAGGAGATCGAGACCATCCTGGAT    120
AACACGGAGAAACCCCGGCGGAGCTGAGGAGCAGGGCCGGGCGCCATGGCACCGTGGGGC    180
                        (*)                 M  A  P  W  G      (5)
AAGCGGCTGGCTGGCGTGCGCGGGGTGCTGCTTGACATCTCGGGCGTGCTGTACGACAGC    240
 K  R  L  A  G  V  R  G  V  L  L  D  I  S  G  V  L  Y  D  S   (25)
GGCGCGGGCGGCGGCACGGCCATCGCCGGCTCGGTGGAGGCGGTGGCCAGACTGAAGCGT    300
 G  A  G  G  G  T  A  I  A  G  S  V  E  A  V  A  R  L  K  R   (45)
TCCCGGCTGAAGGTGAGGTTCTGCACCAACGAGTCGCAGAAGTCCCGGGCAGAGCTGGTG    360
 S  R  L  K  V  R  F  C  T  N  E  S  Q  K  S  R  A  E  L  V   (65)
GGGCAGCTTCAGAGGCTGGGATTTGACATCTCTGAGCAGGAGGTGACCGCCCCGGCACCA    420
 G  Q  L  Q  R  L  G  F  D  I  S  E  Q  E  V  T  A  P  A  P   (85)
GCTGCCTGCCAGATCCTGAAGGAGCGAGGCCTGCGACCATACCTGCTCATCCATGACGGA    480
 A  A  C  Q  I  L  K  E  R  G  L  R  P  Y  L  L  I  H  D  G  (105)
GTCCGCTCAGAATTTGATCAGATCGACACATCCAACCCAAACTGTGTGGTAATTGCAGAC    540
 V  R  S  E  F  D  Q  I  D  T  S  N  P  N  C  V  V  I  A  D  (125)
GCAGGAGAAAGCTTTTCTTATCAAAACATGAATAACGCCTTCCAGGTGCTCATGGAGCTG    600
 A  G  E  S  F  S  Y  Q  N  M  N  N  A  F  Q  V  L  M  E  L  (145)
GAAAAACCTGTGCTCATATCACTGGGAAAAGGGCGTTACTACAAGGAGACCTCTGGCCTG    660
 E  K  P  V  L  I  S  L  G  K  G  R  Y  Y  K  E  T  S  G  L  (165)
ATGCTGGACGTTGGTCCCTACATGAAGGCGCTTGAGTATGCCTGTGGCATCAAAGCCGAG    720
 M  L  D  V  G  P  Y  M  K  A  L  E  Y  A  C  G  I  K  A  E  (185)
GTGGTGGGGAAGCCTTCTCCTGAGTTTTTCAAGTCTGCCCTGCAAGCGATAGGAGTGGAA    780
 V  V  G  K  P  S  P  E  F  F  K  S  A  L  Q  A  I  G  V  E  (205)
GCCCACCAGGCCGTCATGATTGGGGACGATATCGTGGGCGACGTCGGCGGTGCCCAGCGG    840
 A  H  Q  A  V  M  I  G  D  D  I  V  G  D  V  G  G  A  Q  R  (225)
TGTGGAATGAGAGCGCTGCAGGTGCGCACCGGGAAGTTCAGGCCCAGTGACGAGCACCAT    900
 C  G  M  R  A  L  Q  V  R  T  G  K  F  R  P  S  D  E  H  H  (245)
CCGGAAGTGAAGGCTGATGGGTACGTGGACAACCTCGCAGAGGCAGTGGACCTGCTGCTG    960
 P  E  V  K  A  D  G  Y  V  D  N  L  A  E  A  V  D  L  L  L  (265)
CAGCACGCCGACAAGTGATGGCCTCCTGGGAGAGCCCCGCCTCCTCCACCCCTGCCTCTC   1020
 Q  H  A  D  K  *                                             (270)
CTCCACCCCTGCCTCCCCTCCACCCCTGCCTCTCCTCCACCCGCCCAGGAGAGCCCCACC   1080
TCCTCCACCCCTGCCTCTCCTCCACCCCTGCCTCCCCTCCACCTGCCCCAGTGCCCAGAC   1140
CAACCAAGGCCCTGACAGCCCTGCCTTCTGCCCTCTGCCCTGCATGGGCAGGCATTTGTT   1200
CCCTACCTGGGTGGCCTGCTCCCCTGCCTGGGCCCTGACTTCAGCTCCCTGTAGTGAAGT   1260
CCAGGAGGGTGGGACAGGCCTGTCAGGCCTCTGGGAATCTCCCAAATCCCAGAACTCACC   1320
ACTCACCATGGGCCTTTAAATGCAGTAAACTCCACCTAACCAGATTCAGGGGCACTATGC   1380
CCACTGCCTCCTCTTCAGACTCTTTGCATTTCAGTGAAGAGCCTGGAAGAAACCCAGGGG   1440
CCTCCTATGCACAGATCTTGCAGCCCAGAACCAAGTCAGCCTCCCTGCGACTGCCCAGGC   1500
ACACTGCCCACCACCCCACCCCCGAAACAATGCCAGCCCGCTGCTTTTTCTATCCTCCCA   1560
GTCACCTTTGCAGACAAAGACCAGGGGCAGCTCCCGAGGGCACTGTGAAGGCTCCCATGC   1620
CACACAGTGAGAACTGTAGCCTCTGCGTCCAAGGCACACAGGGTACTTTCTGGACCCACT   1680
GCTGGACAGACTTGAAGGTGTCATGCCCGGTGTGTGCAGGAGGAAACTAACAGTTCAGTA   1740
AACTCTGCCTTGACCAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA   1800
AAAAAAAAAAAAA                                                  1814
```

Fig. 1. **Nucleotide and derived amino acid sequence of a cDNA coding human LHPPase.** The stop codon is marked by the asterisk. The Kozak sequence in vertebrates is underlined. The amino acids in two putative phosphatase active site sequences are shown in circles. The three leucines in a leucine-zipper–like sequence are shown in squares.

```
hLHPPase    MAPWGKRLAGVRGVLLDISGVLYDSGAGGGTAIAGSVEAVARLKRSRLKV  (50)

hLHPPase    RFCTNESQKSRAELVGQLQRLGFDISEQEVTAPAPAACQILKERGLRPYL (100)
                              ***** ** *********  **
Bp2                           RLGFDVSEGEVTAPAPAAXLIL

hLHPPase    LIHDGVRSEFDQIDTSNPNCVVIADAGESFSYQNMNNAFQVLMELEKPVL (150)
                     ********** ** *
Bp1                  FDQIDTSNPNXVVDA

hLHPPase    ISLGKGRYYKETSGLMLDVGPYMKALEYACGIKAEVVGKPSPEFFKSALQ (200)
hLHPPase    AIGVEAHQAVMIGDDIVGDVGGAQRCGMRALQVRTGKFRPSDEHHPEVKA (250)
hLHPPase    DGYVDNLAEAVDLLLQHADK                                (270)
```

Fig. 2. **Comparison of the amino acid sequences of human and bovine LHPPase.** The determined partial amino acid sequences of bovine LHPPase (Bp1: peptide 1 and Bp2: peptide 2) are indicated under the corresponding human LHPPase sequence (hLHPPase). The amino acid number of human LHPPase is indicated in parentheses. Asterisks indicate identical residues.

PPase activity was also measured in 100 μl of reaction mixture containing 100 mM GTA buffer (pH 5.5), 1 mM $MgCl_2$, 0–10 μM *p*-chloromercuriphenyl sulfonic acid (*p*-CMPS), 1 mM PP_i, and 1 μg of the fusion protein, which was incubated at 30°C for 30 min before the addition of 1 ml of Malachite green reagent. PNPase activity was measured in 100 μl of reaction mixture containing 100 mM GTA buffer (pH 7.0), 1 mM $MgCl_2$, 0–10 μM *p*-CMPS, 0.1 mM PNP, and 0.5 μg of the fusion protein. The reaction mixtures in triplicate were incubated at 30°C for 30 min. Then 2 ml of Malachite green reagent was added.

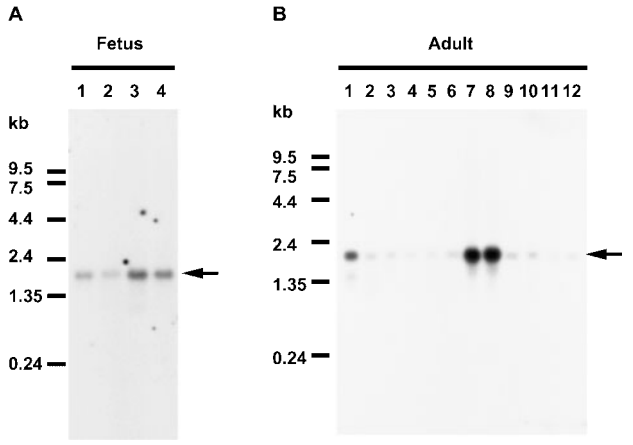

Fig. 3. **Expression of LHPPase in human tissues.** The expression of LHPPase was analyzed by Northern blotting using human fetal MTN blot II and human 12-lane MTN blot (Clontech). Arrows indicate human LHPPase mRNA. (A) Samples from fetal tissues. From left to right: (1) brain, (2) lung, (3) liver, and (4) kidney. (B) Samples from adult tissues. From left to right: (1) brain, (2) heart, (3) skeletal muscle, (4) colon (no mucosa), (5) thymus, (6) spleen, (7) kidney, (8) liver, (9) small intestine, (10) placenta, (11) lung, and (12) peripheral blood leukocyte.

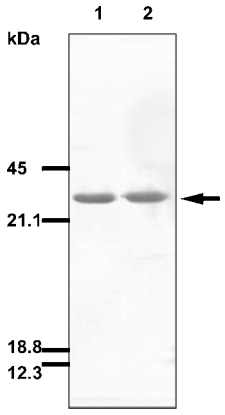

Fig. 4. **SDS-PAGE of the His-hLHPPase.** The migration of His-hLHPPase on SDS-PAGE in the absence (1) or presence (2) of the reducing reagent. The molecular mass was estimated from comparison with the migration of the standard proteins (45-kDa ovalbumin, 21.1-kDa trypsin inhibitor, 18.8-kDa myoglobin, 12.3-kDa cytochrome *c*). The arrow indicates His-hLHPPase.

RESULTS

Partial Amino Acid Sequence of Bovine LHPPase—To investigate whether humans have LHPPase, we analyzed the primary structure of the bovine LHPPase and used the data to isolate a cDNA encoding the human counterpart. Bovine LHPPase was purified from the liver as previously described (*11*) and subjected to amino acid sequencing. However, no substantial sequence was obtained, suggesting that the bovine LHPPase N-terminus was blocked. To obtain its internal sequence, the protein was digested with V8 protease and lysylendopeptidase. Two peptide fragments were isolated and their partial amino acid sequences were determined. A homology search indicated that the two peptides were similar to those encoded in cDNA clone H75601 analyzed in the human EST project (*14*).

A cDNA Encoding the Human Homologue—Although the homology search suggested that the cDNA encoded a human counterpart of the bovine enzyme, the human EST cDNA sequence was incomplete and too short to encode the full length of a corresponding human enzyme. Thus, we tried to isolate a cDNA clone that encodes a full-length human homologue. A cDNA clone encoding an insert of 1,814 bp was isolated from a cDNA library of HeLa cells (Fig. 1). The translation initiation site was predicted from the Kozak's sequence in vertebrates (*20*, *21*). The cDNA contained an in-frame stop codon sequence between 976 bp and 978 bp. There was a stop codon (145–147 bp) upstream of the predicted in-frame initiation codon and no other intervening initiation codon. This indicated that the sequence upstream of the predicted initiation codon did not encode any amino acids. Thus, the open reading frame encoded 270 amino acids, and the molecular mass of the protein was predicted to be 29,192 Da. Because the sequence was similar to the partial amino acid sequences of the bovine LHPPase as shown in Fig. 2, we designated the gene as lhpp.

The human LHPPase sequence had no similarity to the consensus sequences of known inorganic pyrophosphatases (*22*, *23*). Thus, we compared the human LHPPase sequence with that of other phosphatases. Protein-tyrosine phosphatase, dual-specific phosphatase and low molecular weight acid phosphatase are known to have a consensus sequence of $XCX_5RS(T)$ at their active sites (*24*). We compared the sequences around the five cysteine residues of human LHPPase with the consensus sequence and found two homologous regions, as shown in Fig. 1. There is a candidate sequence of XCX_5KS at residues 52–60, although lysine is substituted for arginine, another basic amino acid. Another candidate is XCX_7RT at residues 225–235, although there are seven amino acids between cysteine and threonine instead of five. Motif analysis also indicated that human LHPPase had a leucine-zipper–like sequence (*i.e.* three leucines accounted for every seventh residue at positions 8, 15, and 22).

Expression of Human LHPPase—LHPPase was originally purified from bovine liver, but its presence in other tissues has not been reported. We analyzed the expression of human LHPPase by Northern blotting. Although the mRNA was expressed at low level in other tested tissues, it was highly expressed in the liver and kidney, and moderately in the brain (Fig. 3). The expression patterns were the same in both fetal and adult tissues. The results indicated that LHPPase plays an important role in these tissues. The RNA detected in the Northern blot was 1.8 kb in length.

Subunit Interaction of Human LHPPase—Because the purified bovine enzyme is a homodimer, we analyzed the molecular mass of human LHPPase. We used the His-hLHPPase because it was possible to purify the proteins containing His·Tag sequence in one step by immobilized metal affinity chromatography. The His-hLHPPase was expressed in *E. coli* and purified on a Ni^{2+} chelating affinity column as a single band protein on SDS-PAGE (Fig. 4). The fusion protein was composed of 322 amino acids, and the molecular mass was predicted to be 34,900 Da.

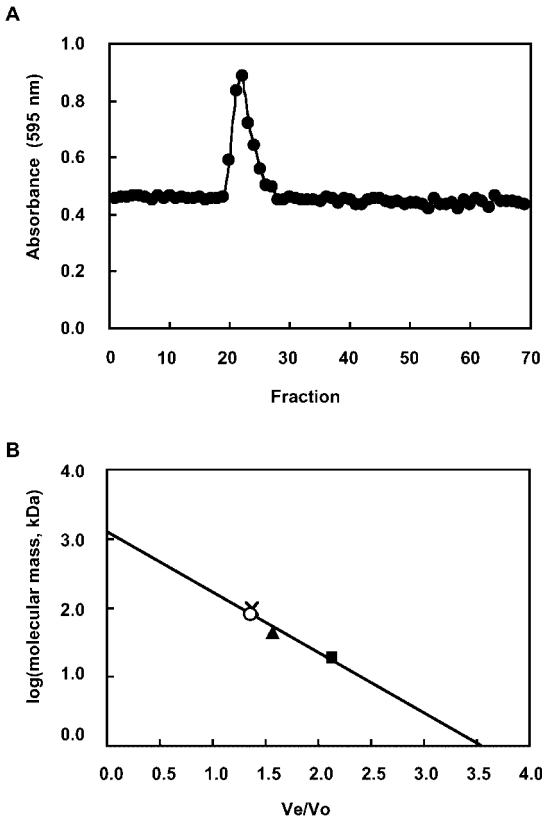

Fig. 5. **Gel filtration of the His-hLHPPase.** (A) Elution profile of the His-hLHPPase from a Sephadex G-100 superfine column. The fusion protein was detected by the Bradford assay at 595 nm. (B) Estimation of molecular mass. The molecular mass was estimated from the standard curves calculated by the method of least squares. Symbols are: open circles, His-hLHPPase; ×, 96-kDa hexokinase; solid triangles, 45-kDa ovalbumin; solid squares, 18.8-kDa myoglobin.

The migration of the fusion protein on SDS-PAGE was the same in the presence or absence of reducing reagents. The fusion protein was fractionated from Sephadex G-100 superfine gel and the molecular mass was estimated with standard proteins (Fig. 5). The fusion protein was eluted in the 80-kDa fraction, and its molecular mass was almost double the 35-kDa molecular mass of the fusion protein estimated from the sequence and migration on SDS-PAGE. These results suggest that the human LHPPase is also a homodimer, at least in this buffer condition, and the disulfide bonds are not used for LHPPase dimerization.

Optimal pH for the Phosphatase Activities—To confirm that the cDNA encodes an active enzyme, the phosphatase activities of the His-hLHPPase were assayed as previously described (*11*). The hydrolysis of nitrogen-phosphorus bonds in PNP and oxygen-phosphorus bonds in PPi by human LHPPase was tested through assays in various pH ranges of GTA buffers (Fig. 6). The optimal pH for the reaction was 7.0 and 5.5, respectively. Accordingly, the activity hydrolyzing PPi appeared to function in a lower pH range than the activity hydrolyzing PNP. These enzymatic characteristics were similar to those of the purified bovine enzyme. The enzyme activity

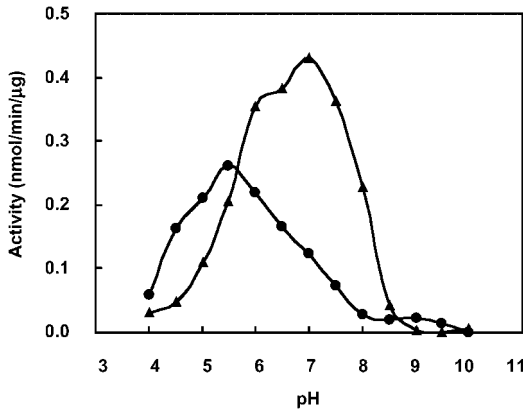

Fig. 6. **The pH curves of His-hLHPPase hydrolyzing PP_i and PNP.** Phosphatase activities of the His-hLHPPase to hydrolyze PP_i (solid circles) and PNP (solid triangles) were assayed under the indicated pH conditions (pH 4.0–10.0). The activities are shown as the released Pi (nmol)/reaction time (min) / enzyme (μg). The average values of duplicate experiments are shown.

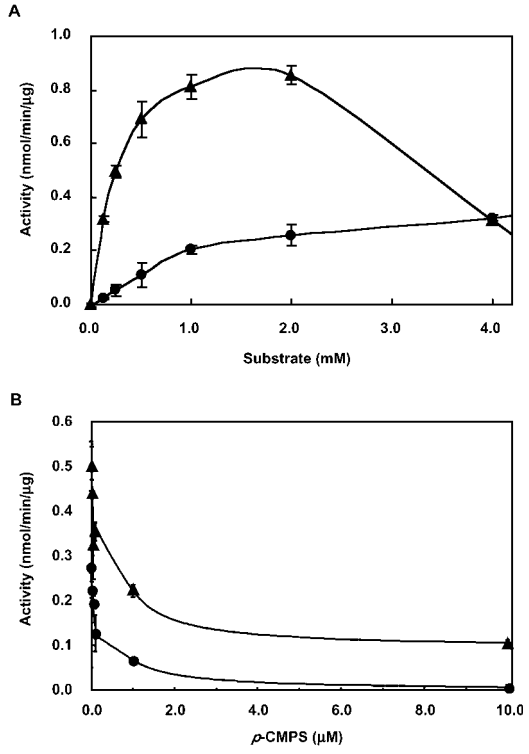

Fig. 7. **Phosphatase activities and the effect of *p*-CMPS.** (A) PPase (solid circles) and PNPase (solid triangles) activities of the His-hLHPPase were measured with PP_i and PNP as substrates, respectively. The activities are shown as the released P_i (nmol)/reaction time (min)/enzyme (μg). The standard deviations of triplicate experiments are shown in bars. (B) PPase (solid circles) and PNPase (solid triangles) activities of the His-hLHPPase were measured in 100 μl of reaction mixture with 0–10 μM *p*-CMPS. The standard deviations of triplicate experiments are shown in bars.

to hydrolyze nitrogen-phosphorus bonds was higher than that for oxygen-phosphorus bonds at the optimal pH.

Effect of a Thiol Reagent—The PPase and PNPase activities of the His-hLHPPase were each measured at their optimal pH (Fig. 7A). High concentration of PNP

inhibited the PNPase activity of human LHPPase. Similar substrate inhibition has been reported for the bovine enzyme (*12*). Because *p*-CMPS inhibited both PPase and PNPase activities of the purified bovine LHPPase, we analyzed the effect of this strong thiol reagent on the human LHPPase. Both PPase and PNPase activities were inhibited by *p*-CMPS as predicted (Fig. 7B). More than half of the full activity of PPase and PNPase were inhibited by 0.1 μM and 1 μM *p*-CMPS respectively. The results indicate that the hydrolytic activity on PPi was slightly more sensitive to *p*-CMPS than that on PNP. These enzymatic characteristics were similar to those of the purified bovine enzyme.

DISCUSSION

LHPPase was found as a novel inorganic pyrophosphatase in bovine liver in 1997 (*11*). Because the enzymatic characteristics of the purified bovine enzyme were unique, it was expected that LHPPase sequence would be quite different from those of typical inorganic pyrophosphatases. We confirmed this hypothesis by sequencing. To investigate whether humans have the same enzyme, we isolated a cDNA clone encoding the human homologue. We registered the human cDNA sequence as lhpp to DDBJ/EMBL/GeneBank database in 2000 (AB049629). After our registration, the human genome projects reported the draft sequence of the whole human genome in 2001 (*25*, *26*). In this paper, we detail the isolation of the human LHPPase cDNA and the enzymatic activities of its translated product. A homology search against the genome sequence database indicated that LHPPase is encoded by seven exons on chromosome 10 q26.13. The official gene name of lhpp is now identified as LHPP in the human genome database.

We suggested that human LHPPase is a dimer, but the disulfide bonds are not used for dimerization. There is a leucine-zipper–like sequence (three leucines at intervals of seven residues at positions 8, 15, and 22). This suggests a possible dimerization domain of LHPPase to form a homodimer. Mutation analysis of these residues will determine the dimerization domain.

The purified bovine enzyme has two different catalytic sites for hydrolysis of imidodiphosphate and *N*-phosphorylated amino acids, respectively (*12*). Two candidates for the active site may function to promote hydrolysis of the different substrates. The actual active site may be determined by point mutation analysis in further studies. A similar sequence was also found in phosphoarginine phosphatase purified from rat liver (*27*–*30*). Although human LHPPase hydrolyzes O-P bonds in inorganic pyrophosphate, it hydrolyzes N-P bonds more effectively. This suggests that another, more suitable substrate than inorganic pyrophosphate exists *in vivo*. One possibility is that LHPPase works as a protein phosphatase which hydrolyzes phospho-proteins containing phospholysine and/or phosphohistidine. The similarity of the putative active site to the consensus sequence of other phosphatases functioning as signal transduction components suggests that LHPPase may transduce signals in a novel signal transduction pathway.

It was suggested that the N-terminal amino acid of the bovine enzyme was blocked. In the case of a phosphoarginine phosphatase, the first methionine was removed and the second alanine was acetylated (*30*). LHPPase may also have such a modification. Tandem mass analysis of the modified N-terminal peptide fragment will determine the type of modification. Although the meaning of the N-terminal amino acid modification is not known at present, the N-terminus of LHPPase does not seem important for the enzymatic activity itself. In fact, the fusion protein containing a large extension at its N-terminal region of this protein has an activity to hydrolyze N-P and O-P bonds at the same level as that reported for the purified bovine enzyme.

The nucleotide sequence reported in this paper has been submitted to the DDBJ/EMBL/GenBank databases with an accession number of AB049629. We would like to express our special thanks to Professor emeritus Akira Kumon for his excellent teaching. We thank Drs. Minoru Tanaka, Eiji Majima, Tatsuya Wakasugi, Tsunehiro Mukai, Shuji Toda, Hajime Sugihara, Yuqing Li and Mr. Toshihiro Kondo for their technical advice, assistance and encouragement. We also thank Ms. Shizuko Furukawa for her secretarial assistance, and Dr. Shinichi Mitsui, Mr. Steve Lamos, Ms. Mai Tu Dang, Mr. Victor Campos, Ms. Mary Lindsey and Dr. Kazuhiko Arima for their critical reading of the manuscript.

REFERENCES

1. Josse, J. and Wong, S.C.K. (1971) Inorganic pyrophosphatase of *Escherichia coli* in *The Enzymes* (Boyer, P.B., eds.) Vol. IV, pp. 499–527, Academic Press, New York
2. Butler, L.G. (1971) Yeast and other Inorganic pyrophosphatase in *The Enzymes* (Boyer, P.B., eds.) Vol. IV, pp. 529–541, Academic Press, New York
3. Stadtman, E.R. (1973) Adenylyl transfer reactions in *The Enzymes* (Boyer, P.B., eds.) Vol. VIII, pp. 1–49, Academic Press, New York
4. Fairchild, T.A. and Patejunas, G. (1999) Cloning and expression profile of human inorganic pyrophosphatase. *Biochim. Biophys Acta* **1447**, 133–136
5. Yang, Z. and Wensel, T.G. (1992) Molecular cloning and functional expression of cDNA encoding a mammalian inorganic pyrophosphatase. *J. Biol. Chem.* **267**, 24641–24647
6. Yoshida, C., Shah, H., and Weinhouse, S. (1982) Purification and properties of inorganic pyrophosphatase of rat liver and hepatoma 3924A. *Cancer Res.* **42**, 3526–3531
7. Yang, Z. and Wensel, T.G. (1992) Inorganic pyrophosphatase from bovine retinal rod outer segments. *J. Biol. Chem.* **267**, 24634–24640
8. Felix, R. and Fleisch, H. (1975) Properties of inorganic pyrophosphatase of pig scapula cartilage. *Biochem. J.* **147**, 111–118
9. Smirnova, I.N., Kudryavtseva, N.A., Komissarenko, S.V., Tarusova, N.B., and Baykov, A.A. (1988) Diphosphonates are potent inhibitors of mammalian inorganic pyrophosphatase. *Arch. Biochem. Biophys.* **267**, 280–284
10. Smirnova, I.N., Baykov, A.A., and Avaeva, S.M. (1986) Studies on inorganic pyrophosphatase using imidodiphosphate as a substrate. *FEBS Lett.* **206**, 121–124
11. Hiraishi, H., Ohmagari, T., Otsuka, Y., Yokoi, F., and Kumon, A. (1997) Purification and characterization of hepatic inorganic pyrophosphatase hydrolyzing imidodiphosphate. *Arch. Biochem. Biophys.* **341**, 153–159
12. Hiraishi, H., Yokoi, F., and Kumon, A. (1998) 3-phosphohistidine and 6-phospholysine are substrates of a 56-kDa inorganic pyrophosphatase from bovine liver. *Arch. Biochem. Biophys.* **349**, 381–387
13. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685

14. Hillier, L.D., Lennon, G., Becker, M., Bonaldo, M.F., Chiapelli, B., Chissoe, S., Dietrich, N., DuBuque, T., Favello, A., Gish, W., Hawkins, M., Hultman, M., Kucaba, T., Lacy, M., Le, M., Le, N., Mardis, E., Moore, B., Morris, M., Parsons, J., Prange, C., Rifkin, L., Rohlfing, T., Schellenberg, K., Soares, M.B., Tan, F., Thierry-Meg, J., Trevaskis, E., Underwood, K., Wohldmann, P., Waterston, R., Wilson, R., and Marra, M. (1996) Generation and analysis of 280,000 human expressed sequence tags. *Genome Res.* **6**, 807–828
15. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA* **74**, 5463–5467
16. Bairoch, A., Bucher, P., and Hofmann, K. (1997) The PROSITE database, its status in 1997. *Nucleic Acids Res.* **25**, 217–221
17. Pearson, W.R. and Lipman, D.J. (1988) Improved tools for biological sequence comparison. *Proc. Natl Acad. Sci. USA* **85**, 2444–2448
18. Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402
19. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
20. Kozak, M. (1991) Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.* **266**, 19867–19870
21. Cavener, D.R. and Ray, S.C. (1991) Eukaryotic start and stop translation sites. *Nucleic Acids Res.* **19**, 3185–3192
22. Lahti, R., Kolakowski, L.F. Jr., Heinonen J., Vihinen, M., Pohjanoksa, K., and Cooperman, B.S. (1990) Conservation of functional residues between yeast and *E. coli* inorganic pyrophosphatases. *Biochim. Biophys Acta* **1038**, 338–345
23. Cooperman, B.S., Baykov, A.A., and Lahti, R. (1992) Evolutionary conservation of the active site of soluble inorganic pyrophosphatase. *Trends Biochem. Sci.* **17**, 262–266
24. Denu, J.M. and Dixon, J.E. (1995) A catalytic mechanism for the dual-specific phosphatases. *Proc. Natl Acad. Sci. USA* **92**, 5910–5914
25. Lander, E. S, Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J.P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J.C., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R.H., Wilson, R.K., Hillier, L.W., McPherson, J.D., Marra, M.A., Mardis, E.R., Fulton, L.A., Chinwalla, A.T., Pepin, K.H., Gish, W.R., Chissoe, S.L., Wendl, M.C., Delehaunty, K.D., Miner, T.L., Delehaunty, A., Kramer, J.B., Cook, L.L., Fulton, R.S., Johnson, D.L., Minx, P.J., Clifton, S.W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J.F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., Gibbs, R.A., Muzny, D.M., Scherer, S.E., Bouck, J.B., Sodergren, E.J., Worley, K.C., Rives, C.M., Gorrell, J.H., Metzker, M.L., Naylor, S.L., Kucherlapati, R.S., Nelson, D.L., Weinstock, G.M., Sakaki, Y., Fujiyama, A., Hattori, M., Yada, T., Toyoda, A., Itoh, T., Kawagoe, C., Watanabe, H., Totoki, Y., Taylor, T., Weissenbach, J., Heilig, R., Saurin, W., Artiguenave, F., Brottier, P., Bruls, T., Pelletier, E., Robert, C., Wincker, P., Smith, D.R., Doucette-Stamm, L., Rubenfield, M., Weinstock, K., Lee, H.M., Dubois, J., Rosenthal, A., Platzer, M., Nyakatura, G., Taudien, S., Rump, A., Yang, H., Yu, J., Wang, J., Huang, G., Gu, J., Hood, L., Rowen, L., Madan, A., Qin, S., Davis, R.W., Federspiel, N.A., Abola, A.P., Proctor, M.J., Myers, R.M., Schmutz, J., Dickson, M., Grimwood, J., Cox, D.R., Olson, M.V., Kaul, R., Raymond, C., Shimizu, N., Kawasaki, K., Minoshima, S., Evans, G.A., Athanasiou, M., Schultz, R., Roe, B.A., Chen, F., Pan, H., Ramser, J., Lehrach, H., Reinhardt, R., McCombie, W.R., de la Bastide, M., Dedhia, N., Blocker, H., Hornischer, K., Nordsiek, G., Agarwala, R., Aravind, L., Bailey, J.A., Bateman, A., Batzoglou, S., Birney, E., Bork, P., Brown, D.G., Burge, C.B., Cerutti, L., Chen, H.C., Church, D., Clamp, M., Copley, R.R., Doerks, T., Eddy, S.R., Eichler, E.E., Furey, T.S., Galagan, J., Gilbert, J.G., Harmon, C., Hayashizaki, Y., Haussler, D., Hermjakob, H., Hokamp, K., Jang, W., Johnson, L.S., Jones, T.A., Kasif, S., Kaspryzk, A., Kennedy, S., Kent, W.J., Kitts, P., Koonin, E.V., Korf, I., Kulp, D., *Lancet*, D., Lowe, T.M., McLysaght, A., Mikkelsen, T., Moran, J.V., Mulder, N., Pollara, V.J., Ponting, C.P., Schuler, G., Schultz, J., Slater, G., Smit, A.F., Stupka, E., Szustakowski, J., Thierry-Mieg, D., Thierry-Mieg, J., Wagner, L., Wallis , J., Wheeler, R., Williams, A., Wolf, Y.I., Wolfe, K.H., Yang, S.P., Yeh, R.F., Collins, F., Guyer, M.S., Peterson, J., Felsenfeld, A., Wetterstrand, K.A., Patrinos, A., Morgan, M.J., Szustakowki, J., de Jong, P., Catanese, J.J., Osoegawa, K., Shizuya, H., Choi, S., and Chen, Y.J. (2001) Initial sequencing and analysis of the human genome. *Nature* **409**, 860–921
26. Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., Smith, H.O., Yandell, M., Evans, C.A., Holt, R.A., Gocayne, J.D., Amanatides, P., Ballew, R.M., Huson, D.H., Wortman, J.R., Zhang, Q., Kodira, C.D., Zheng, X.H., Chen, L., Skupski, M., Subramanian, G., Thomas, P.D., Zhang, J., Gabor Miklos, G.L., Nelson, C., Broder, S., Clark, A.G., Nadeau, J., McKusick, V.A., Zinder, N., Levine, A.J., Roberts, R.J., Simon, M., Slayman, C., Hunkapiller, M., Bolanos, R., Delcher, A., Dew, I., Fasulo, D., Flanigan, M., Florea, L., Halpern, A., Hannenhalli, S., Kravitz, S., Levy, S., Mobarry, C., Reinert, K., Remington, K., Abu-Threideh, J., Beasley, E., Biddick, K., Bonazzi, V., Brandon, R., Cargill, M., Chandramouliswaran, I., Charlab, R., Chaturvedi, K., Deng, Z., Di Francesco, V., Dunn, P., Eilbeck, K., Evangelista, C., Gabrielian, A.E., Gan, W., Ge, W., Gong, F., Gu, Z., Guan, P., Heiman, T.J., Higgins, M.E., Ji, R.R., Ke, Z., Ketchum, K.A., Lai, Z., Lei, Y., Li, Z., Li, J., Liang, Y., Lin, X., Lu, F., Merkulov, G.V., Milshina, N., Moore, H.M., Naik, A.K., Narayan, V.A., Neelam. B., Nusskern. D., Rusch, D.B., Salzberg, S., Shao, W., Shue, B., Sun, J., Wang, Z., Wang, A., Wang, X., Wang, J., Wei, M., Wides, R., Xiao, C., Yan, C., Yao, A., Ye, J., Zhan, M., Zhang, W., Zhang, H., Zhao, Q., Zheng, L., Zhong, F., Zhong, W., Zhu, S., Zhao, S., Gilbert, D., Baumhueter, S., Spier, G., Carter, C., Cravchik, A., Woodage, T., Ali, F., An, H., Awe, A., Baldwin, D., Baden, H., Barnstead, M., Barrow, I., Beeson, K., Busam, D., Carver, A., Center, A., Cheng, M.L., Curry, L., Danaher, S., Davenport, L., Desilets, R., Dietz, S., Dodson, K., Doup, L., Ferriera, S., Garg, N., Gluecksmann, A., Hart, B., Haynes, J., Haynes, C., Heiner, C., Hladun, S., Hostin, D., Houck, J., Howland, T., Ibegwam, C., Johnson, J., Kalush, F., Kline, L., Koduru, S., Love, A., Mann, F., May, D., McCawley, S., McIntosh, T., McMullen, I., Moy, M., Moy, L., Murphy, B., Nelson, K., Pfannkoch, C., Pratts, E., Puri, V., Qureshi, H., Reardon, M., Rodriguez, R., Rogers, Y.H., Romblad, D., Ruhfel, B., Scott, R., Sitter, C., Smallwood, M., Stewart, E., Strong, R., Suh, E., Thomas, R., Tint, N.N., Tse, S., Vech, C., Wang, G., Wetter, J., Williams, S., Williams, M., Windsor, S., Winn-Deen, E., Wolfe, K., Zaveri, J., Zaveri, K., Abril, J.F., Guigo, R., Campbell, M.J., Sjolander, K.V., Karlak, B., Kejariwal, A., Mi, H., Lazareva, B., Hatton, T., Narechania, A., Diemer, K., Muruganujan, A., Guo, N., Sato, S., Bafna, V., Istrail, S., Lippert, R., Schwartz, R., Walenz, B., Yooseph, S., Allen, D., Basu, A., Baxendale, J., Blick, L., Caminha, M., Carnes-Stine, J., Caulk, P., Chiang, Y.H., Coyne, M., Dahlke, C., Mays, A., Dombroski, M., Donnelly, M., Ely, D., Esparham, S., Fosler, C., Gire, H., Glanowski, S., Glasser, K., Glodek, A., Gorokhov, M., Graham, K., Gropman, B., Harris, M., Heil, J., Henderson, S., Hoover, J., Jennings, D., Jordan, C., Jordan, J., Kasha, J., Kagan, L., Kraft, C., Levitsky, A., Lewis, M., Liu, X., Lopez, J., Ma, D., Majoros, W., McDaniel, J., Murphy, S., New-

man, M., Nguyen, T., Nguyen, N., Nodell, M., Pan, S., Peck, J., Peterson, M., Rowe, W., Sanders, R., Scott, J., Simpson, M., Smith, T., Sprague, A., Stockwell, T., Turner, R., Venter, E., Wang, M., Wen, M., Wu, D., Wu, M., Xia, A., Zandieh, A., and Zhu, X. (2001) The sequence of the human genome. *Science* **291**, 1304–1351

27. Kuba, M., Ohmori, H., and Kumon, A. (1992) Characterization of N^{ω}-phosphoarginine hydrolase from rat liver. *Eur. J. Biochem.* **208**, 747–752
28. Yokoyama, K., Ohmori, H., and Kumon, A. (1993) Isolation of N^{ω}-phosphoarginine hydrolase from rat liver and its physical properties. *J. Biochem.* **113**, 236–240
29. Kumon, A., Kodama, H., Kondo, M., Yokoi, F., and Hiraishi, H. (1996) N^{ω}-phosphoarginine phosphatase (17 kDa) and alkaline phosphatase as protein arginine phosphatases. *J. Biochem.* **119**, 719–724
30. Yokoi, F., Hamato, N., Kamei, K., Hara, S., Miyagi, M., Tsunasawa, S., Hiraishi, H., and Kumon, A. (1998) N^{ω}-phosphoarginine phosphatase activity of ubiquitin-conjugating enzymes, type UBC4A/10A and UBC2E. *Res. Commun. Biochem. Cell Mol. Biol.* **2**, 331–346