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Cloning and Expression of a Human Placenta Inositol 1,3,4,5-tetrakisphosphate and Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase

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Distinct inositol and phosphatidylinositol polyphosphate 5-phosphatases have recently been cloned. Primers were designated coding for highly conserved amino acid regions that are shared between sequences of 5-phosphatases. We used degenerate primers to amplify polymerase chain reaction products from rat brain cDNA. A product with a novel sequence was identified and used to clone a 4.9 kb cDNA from human placenta cDNA libraries (hp51CN). COS-7 cells transfected with a C-terminal truncated form of this cDNA showed an increase in Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3 hydrolyzing activity, but not in Ins(1,4,5)P_3 5-phosphatase. Enzymatic activity was inhibited in the presence of 2,3-bisphosphoglycerate and p-hydroxymercuribenzoate. The presence of an SH2 domain and proline-rich sequence motifs within hp51CN suggests that this 5-phosphatase interacts with various proteins in signal transduction.

Hydrolysis of PtdIns(4,5)P_2 by phospholipase C produces the second messengers Ins(1,4,5)P_3 and diacylglycerol, that function in mobilization of intracellular calcium and activation of protein kinase C, respectively [1]. In addition, Ins(1,4,5)P_3 can be phosphorylated by an Ins(1,4,5)P_3 3-kinase to form Ins(1,3,4,5)P_4 [2]. Phosphoinositide 3-kinase, that produces the putative second messenger PtdIns(3,4,5)P_3 from PtdIns(4,5)P_2, plays a central role in the transduction of growth factor signals in the cell [3]. The enzymes that terminate the signal transduction processes and regulate the levels of soluble inositol phosphate and phospholipid messengers are essential for proper cell function. Distinct forms of the inositol and phosphatidylinositol polyphosphate 5-phosphatases selectively remove the phosphate from the 5′-position of the inositol ring of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3. Their substrate specificity, regulatory mechanisms, subcellular localization and tissue specificity indicate that the different 5-phosphatase isoforms may play specific roles in inositol phosphate and phosphatidylinositol metabolism. A 43 kDa in bovine brain and 75 kDa 5-phosphatases in human platelets were identified from enzyme purification [4,5]. The protein encoded by the gene mutated in Lowe's oculocerebrorenal syndrome (OCRL) was demonstrated to be a 5-phosphatase [6,7]. The deduced protein sequences of the 5-phosphatase isoforms that hydrolyze inositol phosphate or phospholipid messengers share a conserved domain of about 100 amino acids. In this paper, we describe the use of degenerate PCR to identify sequences for 5-

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Abbreviations: Ins(1,4,5)P_3: inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4: inositol 1,3,4,5-tetrakisphosphate; PtdIns(4,5)P_2: phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P_3: phosphatidylinositol 3,4,5-trisphosphate; bp: base pairs, MW: molecular weight; PCR: polymerase chain reaction.

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phosphatases: a cDNA encoding a novel type of Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3 5-phosphatase was isolated.

MATERIALS AND METHODS

Materials. [^{3}H]Ins(1,4,5)P_3, [^{3}H]Ins(1,3,4,5)P_4 and Ptd[2-^{3}H]Ins(4,5)P_2 were purchased from DuPont-NEN. Ortho[^{32}P]phosphoric acid, [α-^{32}P]dCTP, [α-^{35}S]dATP were from Amersham. Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, PtdIns(4,5)P_2, Random Priming labeling kit, Expand DNA polymerase and restriction enzymes were obtained from Boehringer Mannheim. Moloney murine leukaemia virus reverse transcriptase and *Taq* polymerase were from Life Technologies Inc. [3-^{32}P]Ins(1,3,4,5)P_4 and [3-^{32}P]PtdIns(3,4,5)P_3 were prepared as in [8,9].

DNA amplification and molecular cloning. Degenerate oligonucleotides, coding for two stretches of conserved amino acids within the 43 kDa, 75 kDa and OCRL 5-phosphatases, were synthesized based on the following sequences: GD(L/F)N(F/Y)R (sense 5′-catcgaagcttggiga(t/c)tiaa(t/c)t(t/a)(t/c)(c/a)g-3′) and PAWCDR (antisense 5′-gatgcggatccc(g/t)(a/g)tc(a/g)caccaigcigg-3′) with added *Hind*III and *Bam*HI restriction sites (underlined) to facilitate subcloning. First strand cDNA was obtained by incubating 2 μg rat brain RNA [10] with random hexamers and moloney murine leukaemia virus reverse transcriptase in a reaction volume of 20 μl for 1 h at 37°C as described [11]. 5 μl of cDNA was used in PCR under the following conditions: 8 cycles of 1 min denaturation at 94°C, 3 min annealing and elongation at 50°C, followed by 25 cycles of 1 min denaturation at 94°C, 3 min annealing and elongation at 60°C. Amplified products were subcloned in pBluescript SK(−) vector (Stratagene) and sequenced. One of the clones obtained by PCR, which encoded a potential novel partial 5-phosphatase, was used to screen a human placenta cDNA library in λEXLOX (provided by Dr P. Chambon, Strasbourg France). This yielded a 2973bp cDNA insert (clone hp21). A 574 bp *Xho*I/*Eco*RI fragment from the 3′-region (position 2399-2973) of this clone was used to screen a human placenta λZAP cDNA library (oligo-dT, Stratagene) to obtain the complete sequence. In the second screen, amongst other clones, an insert of 2021 bp was obtained which contained an additional 1935 bp of 3′-end sequence (clone hp10). The full length clone is referred to as hp51CN.

Northern blot analysis. A blot containing poly(A)-rich RNA from a variety of human tissues was purchased from Clontech. The membrane was probed with a 550 bp *Eco*RI/*Sma*I hp51CN fragment (position 301-866). (Pre)hybridization was performed in 5×SSPE, 10×Denhardt's solution, 100 μl/ml salmon sperm DNA, 2% SDS at 42°C according to manufacturers protocol. The blot was washed twice in 0.2×SSC at 22°C for 10 min, and twice in 2×SSC, 0.1% SDS at 65°C for 30 min.

Generation of antibodies and Western blot analysis. A 685 bp fragment (position 1991-2676) from the conserved 5-phosphatase region of hp51CN was amplified using PCR to incorporate *Eco*RI and *Hind*III sites on the 5′ and 3′ end, respectively. The digested PCR product was cloned into pMAL-cRI vector [12]. Antibodies against the 70 kDa fusion protein were prepared in a New Zealand male rabbit by standard procedures [13]. Antiserum was absorbed with heat-inactivated *E. coli* lysate expressing maltose binding protein prior to use [14]. Western blots were incubated with the antiserum diluted 500-fold.

Expression in COS-7 cells. A construct for expressing amino acids 1-941 of hp51CN was prepared by PCR amplification between a sense oligonucleotide containing nucleotides 141-160 of hp51CN, and an antisense nucleotide containing nucleotides 2944-2963. The PCR product was cloned into the pcDNA3 expression vector (Invitrogen). COS-7 cells were grown and transfected as described [15]. Cells were transfected with pcDNA3 vector alone, with pcDNA3 vector containing hp51CN or with a pcDNA3 construct containing the 43 kDa 5-phosphatase [15]. After 60 h, the transfected cells were harvested, pelleted and resuspended in homogenization buffer A (15 mM Tris-HCl pH 7.5; 2 mM $MgCl_2$; 0.3 mM EDTA; 1 mM EGTA; 2.5 μM leupeptin; 0.4 mM Pefabloc). For PtdIns(3,4,5)P_3 5-phosphatase assays, the cell lysate was centrifuged at 12.000 × g for 10 min. The supernatant was diluted twofold with ethylene glycol and used to determine 5-phosphatase activity.

5-phosphatase assays. Assays using [^{3}H]Ins(1,4,5)P_3, [3-^{32}P] or [^{3}H]Ins(1,3,4,5)P_4 were performed as reported [4]. PtdIns(4,5)P_2 phosphatase activity was determined in the presence of 50 μM [^{3}H]PtdIns(4,5)P_2 (3500 cpm/nmol) in 0.5% cholate, 3 mM $MgCl_2$, 150 mM NaCl, 200 μg/ml bovine serum albumin, 20 mM Tris,pH 8.0. PtdIns(3,4,5)P_3 5-phosphatase assays were performed in the presence of 5 mM $MgCl_2$, 0.5 mM EGTA, 50 mM Tris, pH 7.4, 0.5% cholate and 30 μM [3-^{32}P]PtdIns(3,4,5)P_3 as described [9].

RESULTS

Cloning and sequencing. We have used PCR to identify putative 5-phosphatase sequences by designing degenerate primers coding for highly conserved amino acid sequence regions within the catalytic domain of known inositol and phosphatidylinositol polyphosphate 5-phosphatases. Reverse-transcription PCR on rat brain RNA amplified fragments of approximately 250-360 bp, representing the rat sequences of the 43 kDa 5-phosphatase [11], the 75 kDa 5-phosphatase [16], the OCRL protein [6] and two novel sequences. A Blast search of the DNA

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   1 M V P C W N H G N I T R S K A E E L L S R T G K D G S F L V
  31 R A S E S I S R A Y A L C V L Y R N C V Y T Y R I L P N E D
  61 D K F T V Q A S E G V S M R F F T K L D Q L I E F Y K K E N
  91 M G L V T H L Q Y P V P L E E E D T G D D P E E D T E S V V
 121 S P P E L P P R N I P L T A S S C E A K E V P F S N E N P R
 151 A T E T S R P S L S E T L F Q R L Q S M D T S G L P E E H L
 181 K A I Q D Y L S T Q L A Q D S E F V K T G S S S L P H L K K
 211 L T T L L C K E L Y G E V I R T L P S L E S L Q R L F D Q Q
 241 L S P G L R P R P Q V P G E A N P I N M V S K L S Q L T S L
 271 L S S I E D K V K A L L H E G P E S P H R P S L I P P V T F
 301 E V K A E S L G I P Q K M Q L K V D V E S G K L I I K K S K
 331 D G S E D K F Y S H K K I L Q L I K S Q K F L N K L V I L V
 361 E T E K E K I L R K E Y V F A D S K K R E G F C Q L L Q Q M
 391 K N K H S E Q P E P D M I T I F I G T W N M G N A P P P K K
 421 I T S W F L S K G Q G K T R D D S A D Y I P H D I Y V I G T
 451 Q E D P L S E K E W L E I L K H S L Q E I T S V T F K T V A
 481 I H T L W N I R I V V L A K P E H E N R I S H I C T D N V K
 511 T G I A N T L G N K G A V G V S F M F N G T S L G F V N S H
 541 L T S G S E K K L R R N Q N Y M N I L R F L A L G D K K L S
 571 P F N I T H R F T H L F W F G D L N Y R V D L P T W E A E T
 601 I I Q K I K Q Q Q Y A D L L S H D Q L L T E R R E Q K V F L
 631 H F E E E E I T F A P T Y R F E R L T R D K Y A Y T K Q K A
 661 T G M K Y N L P S W C D R V L W K S Y P L V H V V C Q S Y G
 691 S T S D I M T S D H S P V F A T F E A G V T S Q F V S K N G
 721 P G T V D S Q G Q I E F L R C Y A T L K T K S Q T K F Y L E
 751 F H S S C L E S F V K S Q E G E N E E G S E G E L V V K F G
 781 E T L P K L K P I I S D P E Y L L D Q H I L I S I K S S D S
 811 D E S Y G E G C I A L R L E A T E T Q L P I Y T P L T H H G
 841 E L T G H F Q G E I K L Q T S Q G K T R E K L Y D F V K T E
 871 R D E S S G P K T L K S L T S H D P M K Q W E V T S R A P P
 901 C S G S S I T E I I N P N Y M G V G P F G P P M P L H V K Q
 931 T L S P D Q Q P T A W S Y D Q P P K D S P L G P C R G E S P
 961 P T P P G Q P P I S P K K F L P S T A N R G L P P R T Q E S
 991 R P S D L G K N A G D T L P Q E D L P L T K P E M F E N P L
1021 Y G S L S S F P K P A P R K D Q E S P K M P R K E P P P C P
1051 E P G I L S P S I V L T K A Q E A D R G E G P G K Q V P A P
1081 R L R S F T C S S S A E G R A A G G D K S Q G K P K T P V S
1111 S Q A P V P A K R P I K P S R S E I N Q Q T P P T P T P R P
1141 P L P V K S P A V L H L Q H S K G R D Y R D N T E L P Y H G
1171 K H R P E E G P P G P L G R T A M Q
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FIG. 1. Deduced amino acid sequence of human placenta 51CN 5-phosphatase. Amino acids are numbered beginning at the first ATG, the putative initiation codon. The SH2 domain is *underlined.* Two highly conserved regions between inositol and phosphatidylinositol polyphosphate 5-phosphatases on which the primers for PCR were based, are *boxed.* The DNA and deduced amino acid sequence of hp51CN was deposited with the EMBL sequence database under accession number X98429.

databases identified one as 51C (GenBank Accession No. L36818). The other novel PCR fragment, referred to as 51CN, showed a high degree of similarity to the 51C sequence; 61% nucleotide sequence identity in a 225bp overlap (48% amino acid identity). The 51CN PCR fragment was used as a probe to screen human placenta cDNA libraries. The deduced amino acid sequence of human placenta 51CN (hp51CN) is presented in Figure 1. The 4.9 kb cDNA contained a single large open reading frame encoding a 1188-amino acid protein with a predicted molecular mass of 133 kDa. The putative initiation codon at position 141 is flanked

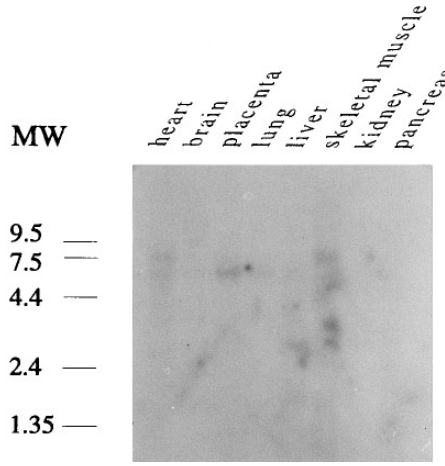

FIG. 2. Tissue distribution of 51CN 5-phosphatase. A Northern blot containing poly-A mRNA from various tissues was hybridized to hp51CN 5-phosphatase cDNA as described in Methods.

by a sequence that fits Kozak's criteria for a translation initiation codon [17]. The domain structure of the predicted hp51CN protein showed several interesting features. The N-terminal region contains an SH2 domain, most similar to the SH2 domains of Ab1 and Grb2. The central region contains the highly conserved amino acid sequence regions present in 5-phosphatases. The C-terminal region shows several proline-rich motifs, with consensus sites for SH3-domain interactions [18].

Northern blot analysis. A Northern blot containing a variety of tissues was analyzed for hp51CN expression (Figure 2). Poly(A) mRNA from human placenta revealed a transcript corresponding to approximately 5 kb, indicating that the composite cDNA sequence of 4.9 kb is likely to represent the full length hp51CN cDNA. A transcript of similar length was found in heart and, very faintly, in brain and lung. In addition, heart and skeletal muscle contained a larger transcript of 8 kb, which may represent alternatively spliced forms of 51CN. No signal was detected in liver, kidney or pancreas.

Expression in COS-7 cells. We examined if the isolated cDNA encoded a functional 5-phosphatase by transfecting COS-7 cells. Figure 3 shows the expression of hp51CN in COS-7 cells assessed by Western blotting. The observed MW (approx. 100 kDa) coincides with the molecular mass predicted for the C-terminal truncated hp51CN protein (106 kDa). Cell homogenates were assayed for their ability to hydrolyze 5-phosphatase substrates (Table 1). Ins(1,3,4,5)P_4 was used as substrate by cells expressing hp51CN, as was demonstrated by the dephosphorylation of [^{3}H]Ins(1,3,4,5)P_4 to [^{3}H]InsP_3 (Table 1), and [3-^{32}P]Ins(1,3,4,5)P_4 to [3-^{32}P]InsP_3 (not shown). In addition, hp51CN hydrolyzed [3-^{32}P]PtdIns(3,4,5)P_3 to [3-^{32}P]-PtdInsP_2 (Table 1). Ins(1,4,5)P_3 and PtdIns(4,5)P_2 were not converted by hp51CN. Thus the hp51CN protein shows a substrate specificity for inositol and phosphatidylinositol phosphates phosphorylated at the 3-position, in contrast to the 43 kDa 5-phosphatase enzyme which hydrolyzed both inositol phosphates, but not the phosphatidylinositol phosphate substrates. The effect of various phosphatase inhibitors on Ins(1,3,4,5)P_4 dephosphorylation were tested in homogenates of transfected COS-7 cells. 2,3-Bisphosphoglycerate and *p*-hydroxymercuribenzoate were previously shown to inhibit 43 kDa Ins(1,4,5)P_3 5-phosphatase activity [11, 12]. As demonstrated in Table 2, the presence of these compounds inhibited hp51CN and 43 kDa 5-phosphatase activity expressed in COS-7 cells. Ins(1,4,5)P_3 had only a slight inhibitory effect on hp51CN activity, whereas the 43 kDa 5-phosphatase was inhibited by 60% at 100

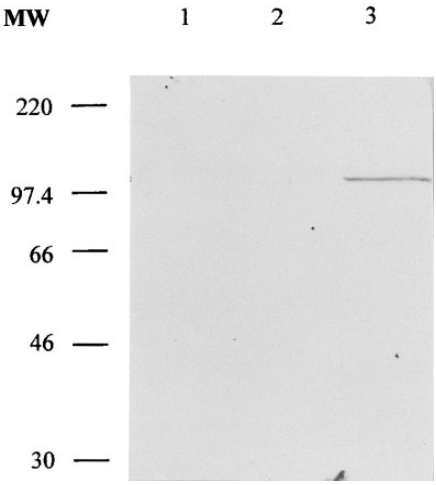

FIG. 3. Western blot analysis of hp51CN protein in COS-7 cells. COS-7 cells transfected with pcDNA3 vector alone (lane 1), COS-7 cells transfected with DNA encoding 43 kDa 5-phosphatase (lane 2), and COS-7 cells transfected with DNA encoding hp51CN (lane 3) were subjected to SDS-polyacrylamide gel electrophoresis (10% gels) and electrophoretically transferred to nitrocellulose. The blot was incubated with antibodies to hp51CN 5-phosphatase and developed by ECL (Amersham).

μM. In the presence of PtdIns(3,4,5)P_3, hp51CN and 43 kDa 5-phosphatase activities were markedly reduced at high concentrations (100 μM). Furthermore, addition of EDTA inhibited Ins(1,3,4,5)P_4 hydrolysis of both isoforms.

DISCUSSION

We report the molecular cloning of a new type of inositol and phosphatidylinositol polyphosphate 5-phosphatase using a PCR based strategy. The hp51CN protein sequence shares a conserved domain with the OCRL protein, synaptojanin, 75 and 43 kDa 5-phosphatases, which is proposed to form the catalytic domain. The central region of hp51CN is most similar to that of 51C [19], which suggests 51C encodes 5-phosphatase activity. Northern blotting showed hp51CN was differentially expressed, with a strong signal in heart, placenta and skeletal muscle, and low expression in brain and lung. The hp51CN 5-phosphatase is unique in that it preferred the 3-phosphorylated substrates Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3, but did not hydrolyze Ins(1,4,5)P_3 and PtdIns(4,5)P_2. In contrast, the 43 kDa 5-phosphatase showed a preference for the soluble inositol phosphate substrates and did not show any phosphatase

TABLE 1
5-Phosphatase Activities of Transfected COS-7 Cells

	Ins(1,4,5)P_3	Ins(1,3,4,5)P_4	PtdIns(4,5)P_2	PtdIns(3,4,5)P_3
Vector	14.9	0.19	nd	0.13
hp51CN	15.7	1.54	nd	2.01
43 kDa	234	60.7	nd	0.19

Note. Cells were transfected with pcDNA3 vector alone (vector), with C-terminal truncated hp51CN (hp51CN), or the 43 kDa 5-phosphatase (43 kDa). Dephosphorylation of Ins(1,4,5)P_3 (10 μM), Ins(1,3,4,5)P_4 (1 μM), PtdIns(4,5)P_2 (50 μM) and PtdIns(3,4,5)P_3 (30 μM) is expressed as nmol/min per ml. PtdIns(4,5)P_2 5-phosphatase isolated from bovine brain was used as a positive control (not shown). nd: not detectable.

TABLE 2
Effect of Various Agents on Ins(1,3,4,5)P_4 Dephosphorylation in hp51CN and 43 kDa Transfected COS-7 Cells

Agent	Concentration	Activity (% of control)	
		hp51CN	43 kDa
None	—	100	100
2,3-bisphosphoglycerate	0.1 mM	95 ± 8	57 ± 6
	1 mM	58 ± 16	29 ± 12
	10 mM	17 ± 9	5 ± 5
p-hydroxymercurybenzoate	5 μM	43 ± 10	11 ± 8
	10 μM	3 ± 2	7 ± 6
	50 μM	10 ± 1	3 ± 3
Ins(1,4,5)P_3	0.1 μM	89 ± 26	93 ± 11
	1 μM	68 ± 25	92 ± 2
	10 μM	76 ± 14	83 ± 5
	100 μM	73 ± 6	40 ± 16
PtdIns(3,4,5)P_3	1 μM	82 ± 17	99 ± 6
	10 μM	71 ± 7	95 ± 1
	100 μM	42 ± 9	52 ± 18
EDTA	10 mM	27 ± 7	10 ± 4

Note. Ins(1,3,4,5)P_4 5-phosphatase was assayed at 1 μM Ins1,3,4,5)P_4 in the presence of various agents. PtdIns(3,4,5)P_3 was prepared as described previously [26]. The data are presented as the mean ± SD of four experiments.

activity when PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3 were used as substrate. The 75 kDa 5-phosphatase showed a wide substrate utilization, at least *in vitro;* it recognized as substrate Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 [16,20]. Synaptojanin and an N-terminal truncated OCRL protein were shown to hydrolyze Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and PtdIns(4,5)P_2 [21, 7]; PtdIns(3,4,5)P_3 as substrate was not tested. We characterized hp51CN enzymatic activity by using 2,3-bisphosphoglycerate and *p*-hydroxymercuribenzoate, previously reported to inhibit specifically certain 5-phosphatase isoforms, e.g. the 43 kDa 5-phosphatase is inhibited by both compounds and by *N*-ethylmaleimide, whereas the PtdIns(3,4,5)P_3 5-phosphatase from rat brain is not inhibited by 2,3-bisphosphoglycerate or *N*-ethylmaleimide [9]. Both compounds inhibited hp51CN. The inhibition by *p*-hydroxymercuribenzoate suggests a cysteine residue is essential for catalytic activity. It is particularly interesting, as it was recently shown that cysteine-348 of 43 kDa 5-phosphatase is the amino acid residue modified by *N*-ethylmaleimide (Communi and Erneux, manuscript submitted). This residue lies within the sequence PAWCDRIL which is conserved within the 75 kDa, OCRL protein and hp51CN 5-phosphatases, but not synaptojanin. This suggests the presence of at least two different types of PtdIns(3,4,5)P_3 5-phosphatases; those containing an active site cysteine residue like hp51CN, and those like rat brain PtdIns(3,4,5)P_3 5-phosphatase, which do not. Another residue, Arginine-350 of 43 kDa 5-phosphatase (also within the sequence PAWCDRIL) was shown to be involved in substrate binding [22]. This conserved domain is therefore proposed to be a signature for substrate binding in inositol phosphate and phosphatidylinositol 5-phosphatases. This may also hold for yeast, where similar motifs have been found [23].

During preparation of this manuscript, sequences were reported in mouse (named SHIP) [24] and human (named SIP) [25] which are similar to our hp51CN sequence. Several differences between hp51CN and human SIP sequences were noted. In the coding sequence hp51CN did not contain a tyrosine residue at amino acid residue 118 due to the absence of 3 nucleotides,

and residue 1168 was found to be a tyrosine instead of a histidine. Furthermore, we extended the sequence by 20bp in the 5′-untranslated region. Expression of an N-terminal truncated SIP protein in COS-7 cells confirmed the substrate specificity of this 5-phosphatase for Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3 [25]. The presence of an SH2 domain and proline-rich sequence motifs within hp51CN suggests this 5-phosphatase interacts with various proteins during signal transduction. It is interesting to note that the SHIP and SIP proteins were shown to form complexes with Shc in hemopoietic cells in response to cytokines [24, 25]. Considering the specificity of hp51CN for substrates phosphorylated at the 3′-position of the inositol ring, this 5-phosphatase may play a modulatory function in the phosphatidylinositol 3-kinase and Ins(1,3,4,5)P_4 signalling pathways.

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