The 75-kDa inositol polyphosphate 5-phosphatase (5-phosphatase II) hydrolyzes various signaling molecules including the following: inositol 1,4,5-trisphosphate, inositol 1,3,4,5-tetraakisphosphate, phosphatidylinositol 4,5-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate. Although studied extensively, a demonstrably full-length cDNA encoding 5-phosphatase II has yet to be isolated. In this study we used a human partial 2.3-kilobase pair (kb) cDNA to screen mouse brain and kidney cDNA libraries, resulting in the isolation of a 3.7-kb cDNA (M5), which by multiple criteria represents a full-length cDNA encoding a 115-kDa 5-phosphatase II. We also isolated a smaller cDNA (M22) with a unique N terminus that encodes a 104-kDa polypeptide. Analysis of these cDNAs suggests a further 87-kDa isoform may arise from differential splicing resulting in translation at methionine 234 in M5. RNA analysis of tissues demonstrates expression of two mRNA species of approximately 4.0 or 3.0 kb, respectively. Probes unique to the 5' end of M5 or M22 hybridized to the 4.0- or 3.0-kb transcripts, respectively. RNA analysis using probes derived from sequence 3' to the potential splice site in M5 and M22 hybridized to both transcripts. Expression of the recombinant 115-kDa protein, or a smaller recombinant protein lacking the N terminus transiently in COS-7 cells, showed localization of enzyme activity to the membrane. Removal of the C-terminal CAAX motif resulted in a significant translocation of the protein lacking the N terminus but not the 115-kDa 5-phosphatase to the cytosol. Western blot analysis of membrane and cytosolic fractions of multiple mouse tissues confirmed the 115-kDa 5-phosphatase II was located in the membrane, whereas the 104- and 87-kDa isoforms were prominent in the cytosol. Collectively these studies demonstrate the widespread expression of at least three isoforms of 5-phosphatase II derived from RNA splicing events. This allows differential distribution of the 5-phosphatase II activity between the membrane and cytosol of the cell and thereby may regulate enzyme access to-phosphoinositide-derived signaling molecules.

The phosphoinositide-derived signaling cascade is initiated following cellular activation by many agonists including growth factors, neurotransmitters, hormones, and cytokines. Phosphatidylinositol (PtdIns)3 can be phosphorylated by a phosphatidylinositol 4-kinase and phosphatidylinositol 4-phosphate 5-kinase generating PtdIns(4)-P and PtdIns(4,5)P2, respectively. Phospholipase C-mediated hydrolysis of PtdIns(4,5)-P2 leads to the generation of the second messengers inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) and diacylglycerol, which mobilize intracellular calcium and activate protein kinase C, respectively (1, 2). An alternative signaling pathway involving phosphoinositides results from phosphoinositide 3-kinase-mediated phosphorylation of PtdIns(3)-P, PtdIns(3,4)P2, and PtdIns(3,4,5)P3, respectively. The 3-position phosphoinositides are not hydrolyzed by phospholipase C but represent signaling molecules in their own right, with proposed roles in activating various protein kinase C isoforms and the serine-threonine kinase Akt (reviewed in Ref. 3).

Many of the signaling molecules derived from the metabolism of phosphoinositides are regulated by removal of the 5-position phosphate of the inositol ring, which is carried out by a family of enzymes known as inositol polyphosphate 5-phosphatases (5-phosphatases). Nine family members have been cloned to date and all contain a common "5-phosphatase domain" (4, 5). This 300-amino acid domain contains two distinct motifs, WXGDXNXR and PXWCDXRXL, located 60 amino acids apart, which may be part of the catalytic or substrate binding site (6–9).

The 5-phosphatase family includes the 43-kDa 5-phosphatase (5-phosphatase I) which hydrolyzes only Ins(1,4,5)P3 and Ins(1,3,4,5)P4 (10–14). Recent studies using an antisense strategy have shown that decreased cellular levels of this enzyme in the unstimulated cell are associated with increased Ins(1,4,5)P3, intracellular calcium levels, and a transformed phenotype (15).

Other 5-phosphatases such as synaptojanin, the 75-kDa 5-phosphatase (5-phosphatase II), and the 5-phosphatase mutated in Lowe’s syndrome have a broader substrate specificity hydrolyzing Ins(1,4,5)P3, Ins(1,3,4,5)P4, PtdIns(4,5)P2, and PtdIns(3,4,5)P3 (16–21). The 75-kDa 5-phosphatase shares substantial sequence homology even outside the 5-phosphatase domain with the 5-phosphatase mutated in Lowe’s syndrome which is a rare X-linked disorder characterized by mental and...
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growth retardation, cataracts, and renal tubular abnormalities leading to metabolic bone disease (22, 23).

The most recently described 5-phosphatase, p150SHP, has a unique substrate specificity as it hydrolyzes Ins(1,4,5,5)P₄ and PtdIns(3,4,5)P₃ (24–27). In addition to this novel 5-phosphatase participates in cytokine and growth factor-mediated tyrosine kinase signaling pathways via a number of structural motifs including an N-terminal SH2 domain and a C-terminal proline-rich domain (reviewed in Refs. 4 and 28).

The 5-phosphatase II was originally purified from human platelet cytosol as a 75-kDa polypeptide (16). A partial human cDNA encoding the enzyme was initially obtained by screening for β-galactosidase fusion proteins that bind to Ins(1,4,5,5)P₄. As RNA analysis demonstrated a 4.4-kb transcript in human megakaryocytes, the 2.3-kb human cDNA did not represent a full-length clone (29).

RNA analysis of 5-phosphatase II expression suggests that more than one transcript is expressed in a variety of different tissues, indicating tissue-specific differential splicing may occur (30). Here we describe cDNA clones encoding several isoforms of mouse 5-phosphatase II, and we show these isoforms have a discrete tissue expression. The larger 115-kDa isoform appears to be associated with the membrane, whereas the 104-kDa isoform is more prominently expressed in the cytosol. These isoforms do not arise from proteolytic cleavage but rather result from RNA splicing events. Finally we show that membrane association of 5-phosphatase II is mediated not only by farnesylation of the CAAX motif but also by the N-terminal region of the 115-kDa 5-phosphatase II.

**EXPERIMENTAL PROCEDURES**

**Materials**—[α-32P]Phosphoric acid, [α-32P]dCTP, and [35S]methionine were from NEN Life Science Products. Restriction and DNA-modifying enzymes were from Promega or New England Biolabs. Sequenase II kit was purchased from Amersham Corp. Oligonucleotides were obtained from Brescat, Australia. The mouse kidney Agt10 and mouse brain AZAP cDNA libraries were from Stratagene and were a kind donation by Ora Bernard, Walter and Eliza Hall Institute, Melbourne, Australia. All reagents unless otherwise stated were from Sigma.

**Cloning of Mouse cDNAs**—A 2384-bp human cDNA clone originally isolated by Ross et al. (29) was used to clone a mouse 5-phosphatase II cDNA. A Agt10 mouse cDNA library (1 × 10⁶ recombinants) was screened using the human 2.3-kb cDNA as a probe, resulting in the isolation of two clones M6 and M22, whose identity was verified as mouse homologues of the human 5-phosphatase II by DNA sequencing. As neither of these mouse cDNAs represented full-length clones, M6 and M22 were used to screen a mouse brain AZAP II cDNA library (Stratagene). Seven positive clones were identified, plaque-purified, and subcloned into the vector Bluescript II KS (pBlueSKII/m-Sptase). The longest cDNA (3.7 kb), designated M5, was sequenced on both strands by the Sanger dideoxy sequencing method using an “oligo walk” strategy (sequencing).

**Expression Vector Construction**—The full-length mouse 5-phosphatase II (M5) was excised from pBlueSKII/m-5-phosphatase by digestion with the restriction enzymes NotI/DraI, which digest in the Bluescript vector immediately prior to the 5’ end of the cDNA and at nucleotide 3464 in the M5 sequence, respectively, and blunt end-ligated into the pSVTF expression vector (construct A) (31). A smaller form of 5-phosphatase II, lacking the N-terminal and insert sequences, was derived by XhoI/DraI restriction digestion, which cuts M5 at nucleotides 913 and 3464, respectively, and blunt end-ligated into pSVTF (construct B). The CAAX motif and additional 3’ sequences were removed from the full-length cDNA (M5) by digestion with XhoI/DraI, which removes 3’ nucleotides (2738–3464), followed by ligation into pSVTF (construct C). Finally, construct D was created by XhoI/DraI digestion of the five 5-phosphatases (nucleotides 913 and 2788 in M5), followed by ligation of the excised 1875-bp fragment into pSVTF. Each of the constructs were expressed in COS-7 cells and also translated in vitro as described below.

**In Vitro Translation of 5-Phosphatase II Constructs**—Each of the four constructs (A–D) in the pSVTF vector was linearized by digestion with SacI and cloned into the TNT-coupled wheat germ extract system (Promega), and was translated in vitro in the presence of [35S]methionine. Translated products were analyzed by 7.5% SDS-PAGE and visualized by fluorography.

**Expression of the Mouse 5-Phosphatase II in COS-7 Cells**—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium, 10% (v/v) fetal calf serum, containing 2 mM glutamine. The cells were transfected with the various constructs using a DEAR-dextran procedure and were allowed to grow for 2 days (32). Cells were harvested 60 h after transfection and assayed for expression of PtdIns(4,5)P₂ and Ins(1,4,5)P₃ 5-phosphatase activity (17). Enzyme assays were performed using at least three protein concentrations, so that linearity of enzyme activity per μg of protein in the assay was achieved, and less than 30% of the substrate PtdIns(4,5)P₂ or Ins(1,4,5)P₃ was consumed. To isolate the enzyme from the plate the cytosol and washed three times, and the pellet was resuspended in buffer A (10 mM imidazole, pH 7.2, containing 3 mM MgCl₂, 1 mM EDTA, 5 mM β-mercaptoethanol, 50 μg/ml phenylmethylsulfonil fluoride) and sonicated 3 × for 15 s on ice (LABSONIC 1510). The lysate was centrifuged briefly at 1,000 × g to remove intact cells, and the supernatant was centrifuged at 100,000 × g at 4 °C for 1 h. The supernatant from this centrifugation represents the cytosolic fraction. The pellet was resuspended in buffer A plus 1% Triton X-100, and detergent-soluble membrane proteins were extracted at 4 °C, with gentle agitation overnight. The solution was centrifuged again for 1 h at 100,000 × g, and the supernatant from this step represents the detergent-soluble membrane fraction.

Ins(1,4,5)P₃ and PtdIns(4,5)P₂ 5-Phosphatase Enzyme Assays—Ins(1,4,5)P₃ and PtdIns(4,5)P₂, 5-phosphatase activity was assayed on cell-free extracts prepared previously (33). Ins(1,4,5)P₃ hydrolysis was measured by extraction of released 32PO₄ as described previously, using as substrate concentration 30 μM and representing the mean of three separate enzyme assays (10). PtdIns(4,5)P₂ 5-phosphatase assay was performed as described previously using 250 μM [3H]PtdIns(4,5)P₂ (3500 cpm/nmol) and representing the mean of three assays, using three linear protein concentrations (17).

**Northern Analysis**—A membrane containing mRNA from various mouse tissues was purchased (Mouse Multi-Tissue Northern blot, CLONTECH). Using standard procedures (described below), the membrane was hybridized to a [α-32P]dCTP-labeled full-length cDNA (M5) probe at 42 °C. All Northern blots were prehybridized in 0.05 M sodium phosphate, 7.4, 0.005 M EDTA, 0.75 mM NaCl, 10°C. Denhardt’s solution, 100 μg/ml salmon sperm DNA, 50% formamide, 2% SDS at 42 °C for 2 h with continuous agitation. The labeled probe was then added and further incubated for 18 h at the same temperature. The membranes were then washed at room temperature three times with 2× standard saline citrate (0.03 M sodium tri-citrate, pH 7.0, 0.3 M NaCl), 0.05% SDS over 40 min. The membranes were subsequently washed twice in 0.1× standard saline citrate, 0.1% SDS at 50 °C for 30 min. The multi-tissue Northern membrane was subsequently probed with an actin probe supplied with the membrane. RNA was isolated from NIH3T3 cells and was transferred to a nylon membrane as described (32), so that each lane contained 15 μg of mRNA. The probes used for analysis were derived as follows. The full-length cDNA encoding the mouse 5-phosphatase II (clone M5, probe A) was excised from pBlueSKII/m-5-phosphatase II by restriction digestion with NotI/DraI. Probe B, representing a 446-bp fragment from the 5’ end of clone M5, was isolated by NotI/HindIII digestion which digests at the 5’ end of the clone and at nucleotide 446. Probe C, a 300-bp fragment encomprising nucleotides 613–913 of M5, was isolated by EcoRI/XhoI digestion of the mouse 5-phosphatase II (clone M5, probe A). Fragment D, a fragment from nucleotides 1913 to 1117 bp, was derived by XhoI/DraI digestion of pBlueSKII/m-5-phosphatase II. Probe E (530 bp) was isolated by EcoRI/NsiI digestion of clone M22. All DNA fragments were isolated by agarose gel electrophoresis and purified (Brescalex, Brescat, Australia). Membranes containing mRNA were incubated with α-32P-labeled probe (A–E) at 42 °C, using standard conditions. After autoradiography the membranes were allowed to decay for 2 months and then reprobed with an
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RESULTS

Isolation and Characterization of a Full-length Mouse 5-Phosphatase Clone (M5)—A 2.3-kb human cDNA was used as a probe to screen a mouse kidney Agt11 cDNA library resulting in the isolation of 1.6-kb (M6) and 1.5-kb (M22) cDNAs, both of which contained sequences highly homologous to the human 3.0-kb cDNA (6). To obtain a full-length mouse clone the 1.6-kb cDNA (M6) was used to screen a mouse brain Agt11 cDNA library. Seven positive clones were obtained following screening 1 × 10⁶ plaques, and the largest of these contained a 3.7-kb (M5) insert, as determined by Southern blot and restriction analysis. Sequence analysis of the 1.6- (M6) and 3.7-kb (M5) clones demonstrated that the 3687-bp cDNA (M5) has 2 kb further 3' sequence than the 1.6-kb (M6) cDNA (Fig. 1a).

The 3.7-kb mouse brain cDNA (M5) comprises a 3054-bp open reading frame, with the initiator methionine occurring at nucleotide 124 and the termination codon at 3175 (Fig. 1b). The nucleotide sequence surrounding the proposed initiator methionine is consistent with the consensus sequence proposed by Kozak (35). The open reading frame between the initiator methionine and the termination codon encodes 1017 amino acids. The 5'-untranslated region contains a number of in-frame stop codons, prior to the initiator methionine. The cDNA contains a 510-bp 3'-untranslated region, with a poly(A) tail commencing at nucleotide 3670. The region prior to the initiator methionine of M5 and after the termination codon bears no homology with the human 5-phosphatase II, consistent with species differences in the 5' and 3'-untranslated regions.

Amino acid sequence alignment of the predicted protein from M5 with the human 5-phosphatase II are as shown (Fig. 1c). M5 shares approximately 83% sequence identity over 915 amino acids with the human protein, excluding two regions of unique sequence (underlined in Fig. 1c) that are not present in the human 5-phosphatase II sequence (6). The first of these regions extends from residues 180 to 258 and bears no sequence identity with any previously described 5-phosphatase, including the 5-phosphatase II, or any other protein in the various data bases. We believe this region represents genuine mouse 5-phosphatase II sequence and not a cloning artifact for the following reasons. First, there is no in-frame stop codon within the insert region, and the reading frame is maintained throughout. Second, the identical cDNA sequence was in two distinct cDNAs isolated from mouse brain and kidney libraries. Finally, RNA analysis using probes derived from the novel insert sequence hybridizes to 5-phosphatase II mRNAs (see below).

The second region of novel sequence encodes 18 amino acid residues (aa 813–831). Because of the extra residues in these two regions the translation of the open reading frame of mouse 5-phosphatase II predicts a polypeptide of 115 kDa compared with translation of the human sequence which predicts a 100-kDa polypeptide. The predicted hydrophobic regions found within the full-length protein are demonstrated in Fig. 1d and are most prominent in the N terminus and C terminus of the protein.

Isolation of Further Mouse 5-Phosphatase 18 Isoforms (M22)—We also isolated a partial cDNA encoding a smaller form of 5-phosphatase II, with a novel N terminus (Fig. 1, a and e). This 1.5-kb (M22) kidney cDNA is identical to the 3.7-kb cDNA in the indicated overlapping areas (Fig. 1a) from nucleotide 824 in M5 and nucleotide 586 in M22; however, it contains unique 5' sequences. The proposed initiator methionine for M22 is at nucleotide 205, followed by an open reading frame comprising a novel 127 amino acids, after which the sequence returns to common 5-phosphatase II sequence (indicated by...
Multiple Isoforms of 5-Phosphatase II

Fig. 1—continued
Multiple Isoforms of 5-Phosphatase II

**MOUSE**

|  |  |
|---|---|
| AIGDGDVSLQIVLST/DYKVEVYESGELILGSVEVTQLYTAELHKLVQPPLFGSHRTT | AIGDGDVSLQIVLST/DYKVEVYESGELILGSVEVTQLYTAELHKLVQPPLFGSHRTT |
|  |  |
| M61 | H61 |
| FKGVRAPCPEGD/FYTRDFREMLRLV/CARKFLSMRPAFPRMN@TRVRQ/SRFEQLSEAI | FKGVRAPCPEGD/FYTRDFREMLRLV/CARKFLSMRPAFPRMN@TRVRQ/SRFEQLSEAI |
| h121 | m121 |
|  |  |
| MN56/S4GKSDFMNCSGCGLELAGTFFCPSGEEFEPLVaAmCGQGGPVGEEQPFQRGRKQRLZAD | MN56/S4GKSDFMNCSGCGLELAGTFFCPSGEEFEPLVaAmCGQGGPVGEEQPFQRGRKQRLZAD |
|  |  |
| m241 | h180 |
| WEMASGSRGPEPDCAG/SV/SRPGGPGGP/GTFSGAC/RKPKBEI/S17-RCNERESNMSK | WEMASGSRGPEPDCAG/SV/SRPGGPGGP/GTFSGAC/RKPKBEI/S17-RCNERESNMSK |
|  |  |
| m301 | h222 |
| VRS/T/YSDH/SHelSMQRPKFLD/FQVRSHEL/QKEEY/TYIQNFVF/GTINNQGSEPKE | VRS/T/YSDH/SHelSMQRPKFLD/FQVRSHEL/QKEEY/TYIQNFVF/GTINNQGSEPKE |
|  |  |
| m361 | h282 |
| C3K1WSLHA/SIAAPV/DGVRFQELDLSSKEAFFHHTIDPKEEWD7KAVS8I/BFD4K4VEF | C3K1WSLHA/SIAAPV/DGVRFQELDLSSKEAFFHHTIDPKEEWD7KAVS8I/BFD4K4VEF |
|  |  |
| m421 | h458 |
| VPRGVI8MLLLLYVQHRAA1TVSVEIATVTQGIMGWS/FQGQGGVAFRQPLHNT31CVV | VPRGVI8MLLLLYVQHRAA1TVSVEIATVTQGIMGWS/FQGQGGVAFRQPLHNT31CVV |
|  |  |
| m481 | h508 |
| NSHLA/H/EYXRNQDY/DICSRMMFQ/GDPSPPLIT/NHRVILLSDGLNRIEELDV | NSHLA/H/EYXRNQDY/DICSRMMFQ/GDPSPPLIT/NHRVILLSDGLNRIEELDV |
|  |  |
| m498 | h528 |
| NSHLA/H/EYXRNQDY/DICSRMMFQ/GDPSPPLIT/NHRVILLSDGLNRIEELDV | NSHLA/H/EYXRNQDY/DICSRMMFQ/GDPSPPLIT/NHRVILLSDGLNRIEELDV |
|  |  |
| m541 | h556 |
| CRKEVLL/EKFRQFQ/YLH/VQADQK/RQVAT/GYFQFQGDITFTQDGYSTQEDDDTSEA | CRKEVLL/EKFRQFQ/YLH/VQADQK/RQVAT/GYFQFQGDITFTQDGYSTQEDDDTSEA |
|  |  |
| m601 | h560 |
| RAPANCDRLMKGHNQTLQ/SQSHM/ATSDEKHSVSTFDGVRVVDONGLRYKREAIVR | RAPANCDRLMKGHNQTLQ/SQSHM/ATSDEKHSVSTFDGVRVVDONGLRYKREAIVR |
|  |  |
| m601 | h573 |
| RAPANCDRLMKGHNQTLQ/SQSHM/ATSDEKHSVSTFDGVRVVDONGLRYKREAIVR | RAPANCDRLMKGHNQTLQ/SQSHM/ATSDEKHSVSTFDGVRVVDONGLRYKREAIVR |
|  |  |
| m661 | h644 |
| SLDDMNANPIDTVSLKSEAYCF/PXQVYQOGQQ/TQESTITNH/QVCFQ/EFSREPEST/CRCQ | SLDDMNANPIDTVSLKSEAYCF/PXQVYQOGQQ/TQESTITNH/QVCFQ/EFSREPEST/CRCQ |
|  |  |
| m681 | h679 |
| WLYAEB8HOFPLPS/SVIELELFLNVRSTAK/NSQKIDTREDIV/LHDEGKDEVLFVS | WLYAEB8HOFPLPS/SVIELELFLNVRSTAK/NSQKIDTREDIV/LHDEGKDEVLFVS |
|  |  |
| m721 | h748 |
| WDJANGHOFPFLPS/UEVILAVNLR/VKAT/NSQKIDTREDIV/LHDEGKDEVLFVS | WDJANGHOFPFLPS/UEVILAVNLR/VKAT/NSQKIDTREDIV/LHDEGKDEVLFVS |
|  |  |
| m781 | h865 |
| QTRLFTSCAPA/FSTSTCSCUGF/PLDLFPL/PQTVLG/LEVSF/S/FDTQ/TAAD | QTRLFTSCAPA/FSTSTCSCUGF/PLDLFPL/PQTVLG/LEVSF/S/FDTQ/TAAD |
|  |  |

**E. coli**

|  |  |
|---|---|
| D86LQMPFHMEJ/EKPNLWMDY/IYNHLMQEDLFQ/GRLEEF/HIRKCDLYGCMDCGCAC | D86LQMPFHMEJ/EKPNLWMDY/IYNHLMQEDLFQ/GRLEEF/HIRKCDLYGCMDCGCAC |
|  |  |
| m901 | h739 |
| DGSQDGSP/FIPKELWMDY/IYNHLMQEDLFQ/GRLEEF/HIRKCDLYGCMDCGCAC | DGSQDGSP/FIPKELWMDY/IYNHLMQEDLFQ/GRLEEF/HIRKCDLYGCMDCGCAC |
|  |  |
| m951 | h799 |
| NHSAVAAKLLFFLESAPAR/EFICTSTTHCRLCCOQG/AASKQIVT/IVAFAEFVENYPP/TPMA | NHSAVAAKLLFFLESAPAR/EFICTSTTHCRLCCOQG/AASKQIVT/IVAFAEFVENYPP/TPMA |
|  |  |
| m961 | h857 |
| FL/GELLHسك/AYLDEHLAS/FUAYCSEMPAV/F-KLM/EERKQGQHFIPQGCLNPL | FL/GELLHسك/AYLDEHLAS/FUAYCSEMPAV/F-KLM/EERKQGQHFIPQGCLNPL |
|  |  |
| h857 |  |
Searches of sequence data bases revealed the N-terminal region in M22 is unique and shows no homology to any known 5-phosphatases, including human 5-phosphatase II.

Assuming the remainder of M22 sequence is identical to M5, and Northern blot analysis as described below suggests this is the case, the predicted protein encoded by this cDNA is 104 kDa.

A potential RNA splice site that conforms to the GT-AG rule occurs in M22 at leucine 128, corresponding to the methionine (aa 234) in M5 (35). Following this potential splice site, the sequence in M22 and M5 is identical. We predict differential splicing events occurring at this methionine could result in a third 5-phosphatase II isoform. Translation from this methionine (aa 234) in the M5 3.7-kb cDNA would generate an 87-kDa polypeptide (Fig. 1f).

RNA Analysis of Mouse 5-Phosphatase II Expression—The
full-length mouse 3.7-kb cDNA (M5) was used to probe a membrane containing mRNA isolated from mouse heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis (Fig. 2). To compare the level of mRNA in each sample, the membrane was subsequently hybridized to an actin probe. Although cardiac and skeletal muscle have two forms of actin mRNA, comparison of the levels of the larger species present in all tissues showed the amount of RNA in each sample is comparable. RNA analysis demonstrated the presence of two distinct 5-phosphatase II transcripts, differentially expressed in discrete tissues. An approximately 4.0-kb transcript was highly expressed in kidney, liver, and brain, whereas a 3.0-kb transcript was observed in lung, liver, kidney, and testis. Low level expression of the 3.0-kb transcript was also seen in several other tissues. The ratio of the two transcripts varied between different tissues.

To investigate which of the two sequences (M5 and M22) were represented by the 4.0- and 3.0-kb transcripts, we designed specific probes from various regions of each cDNA and probed RNA isolated from mouse NIH3T3 cells (Fig. 3). The full-length 3.7-kb cDNA (M5) (probe A) hybridized to both transcripts, as in the multi-tissue analysis (Fig. 3b). The 5’ 446-bp fragment (probe B) derived from the 3.7-kb cDNA (M5) and a probe containing the novel insert sequence (probe C) hybridized only to the larger 4.0-kb transcript, indicating these sequences are unique to this RNA. A probe (D), corresponding to the region which is after a potential RNA splice site present in both M5 and M22, hybridized to both the 4.0- and 3.0-kb transcripts with equal intensity, indicating this region is common to both RNAs. Finally the novel 5’ region of M22 (probe E) only hybridized to the smaller 3.0-kb transcript (Fig. 3b).

These studies indicate alternative splicing results in the generation of at least two RNA transcripts, the larger corresponding to the 3.7-kb cDNA (M5) and smaller to the M22 cDNA. To confirm M22 was represented by the smaller of the two RNA transcripts, the multi-tissue RNA blot was reprobed with probe E. Only the smaller of the two RNA species hybridized to this probe, indicating this clone does not represent a cloning artifact, and represents a genuine form of the 5-phosphatase II (results not shown).

We could not detect the presence of a third RNA transcript corresponding to the proposed 87-kDa 5-phosphatase II, generated by alternative splicing of M5 at residue 234. The size of the 104 kDa and 87 kDa encoding RNAs would be very similar (approximately 3.0-kb), and we have no unique sequence encoding the putative 87-kDa isof orm.

**In Vitro Translation of 5-Phosphatase II Isoforms—**To establish the 3.7-kb cDNA encoded 5-phosphatase II, the full-length M5 cDNA (construct A) was cloned into the expression vector pSVTf and translated in vitro to determine the size of the polypeptide. This construct was also used for transient transfection studies in COS-7 cells. To investigate the properties of the smaller isof orm of 5-phosphatase II lacking the N-terminal insert sequence, a construct commencing at nucleotide 913 in M5, which makes methionine 297 an initiation codon, was generated (Fig. 1, b and c) (construct B). To investigate the role...
of the CAA motif in membrane localization of the 115-kDa 5-phosphatase II, a construct lacking 128 amino acids from the C terminus of full-length 5-phosphatase, including the CAA

![Diagram of constructs A-D](image)

| Construct | Cytosol | Membrane |
|-----------|---------|----------|
|           | PtdIns(4,5)P₂ hydrolyzed | Percentage in cytosol | PtdIns(4,5)P₂ hydrolyzed | Percentage in membrane | Total enzyme activity |
| Vector    | 0.3 ± 0.08 | 0.6 ± 0.1 | 250 | 64 | 7.45 |
| A         | 2.48 ± 0.5 | 4.96 ± 1.1 | 36 | 64 | 6.1 |
| B         | 2.2 ± 0.45 | 3.9 ± 0.9 | 63 | 6.4 |
| C         | 2.24 ± 0.5 | 4.0 ± 1.2 | 20 | 7.3 |
| D         | 5.7 ± 1.5 | 1.6 ± 0.36 | |

Each construct was used to direct mRNA synthesis in vitro and translation in a wheat germ expression system in the presence of [35S]methionine. The resulting labeled proteins were analyzed by SDS-PAGE and fluorography (Fig. 4b). Translation of the full-length 3.7-kb cDNA (M5) gave rise to a prominent polypeptide species of greater than 104 kDa, which is consistent with the molecular mass of the protein predicted by the open reading frame of the 3.7-kb cDNA (115 kDa). Translation of the construct, which represents sequence common to both M5 and M22 but lacking the N terminus of either, resulted in the production of an 83-kDa protein, whereas the M5 construct lacking the C-terminal CAAX motif translated a 93-kDa polypeptide. Finally the M5 construct missing both the N terminus and CAAX motif produced a 65-kDa polypeptide as predicted.

### Table I

**Expression of mouse 5-phosphatase II in COS-7 cells**

Constructs A–D in the pSVTF vector (as shown in Fig. 4a) were transiently transfected into COS-7 cells. Cytosolic and Triton-soluble membrane fractions were isolated as described under “Experimental Procedures.” Top, subcellular fractions were assayed in triplicate using [3H]PtdIns(4,5)P₂, 250 μM (3500 cpm/nmol) as a substrate. Enzyme (nmol/min/mg) activity represents the mean ± S.E. from three separate transfections for each construct (A–D) or the vector alone. Bottom, subcellular fractions were assayed in triplicate using Ins(1,4,5)P₃, 30 μM as a substrate. Enzyme activity (nmol/min/mg) represents the mean ± S.E. of the enzyme activity from three distinct transfections for each transfected construct (A–D) or the vector alone.
tivity was detected in the Triton-insoluble membrane fraction (results not shown).

Transient expression of the smaller 5-phosphatase II construct, lacking the N terminus and insert sequence (construct B), resulted in an approximate 20% decrease in enzyme activity for PtdIns(4,5)P$_2$ and Ins(1,4,5)P$_3$ in both the membrane and the cytosolic fractions, compared with M5. However, the relative distribution of enzyme activity between these two fractions was unchanged, with 64% of the PtdIns(4,5)P$_2$ 5-phosphatase activity located in the membrane.

Previous studies by Jefferson and Majerus (6) have indicated the CAAX motif mediates 5-phosphatase II membrane localization of the human 5-phosphatase II. We removed the whole of the CAAX motif from the C terminus (construct C) of the full-length 115-kDa recombinant protein, leaving the N terminus intact. Surprisingly, expression of the full-length “CAAX-less construct” did not alter the relative distribution of the recombinant enzyme between the membrane and cytosolic fractions, although a decrease in total enzyme activity (15–20%) using either Ins(1,4,5)P$_3$ or PtdIns(4,5)P$_2$ as a substrate was routinely observed. In contrast, expression of the 65-kDa 5-phosphatase II (construct D), which lacks both the N terminus and the CAAX motif, resulted in no significant reduction in total enzyme activity but a significant redistribution of 5-phosphatase II from the membrane to the cytosolic fraction of the cell. This was most marked using PtdIns(4,5)P$_2$ as a substrate, where 80% of the enzyme activity was in the cytosol, when both the N terminus and CAAX motif were removed. These results indicate that membrane localization of 5-phosphatase II is mediated by both discrete N- and C-terminal (CAAX) domains.

We also performed Western blot analysis of the transfection studies demonstrated in Table I, top and bottom, using antibodies to the recombinant 5-phosphatase II, or anti-C-terminal, and or N-terminal antipeptide antibodies (Fig. 5a). This analysis was performed on each of three distinct transfections for each construct, and the relative intensity of the signal between membrane and cytosolic expression was determined by densitometry (Fig. 5b). The results of these analyses support the contention that 5-phosphatase II membrane association is dependent on both N-terminal and C-terminal domains. Transfection of the full-length cDNA (construct A) was almost entirely membrane-associated (>85%), with removal of the N-terminal or C-terminal domains alone resulting in little alteration of membrane expression. Removal of both the N-terminal and C-terminal domains resulted in translocation of 80% of the recombinant enzyme to the cytosol. The results of enzyme assays indicate greater cytosolic expression of 5-phosphatase II than demonstrated by Western analysis, suggesting the cytosolic recombinant 5-phosphatase II is more active than the membrane-associated enzyme. de Smedt and colleagues (36) have reported cytosolic 43-kDa 5-phosphatase has a higher specific activity than the membrane-associated enzyme. However, as we consistently observed proteolysis of the cytosolic recombinant 5-phosphatase II, despite the presence of protease inhibitors, the precise estimation of the specific activity of cytosolic 5-phosphatase II is difficult.

**Tissue Distribution of 5-Phosphatase II Isoforms**—To examine the expression of the 115- and 104-kDa 5-phosphatase II protein isoforms, Western blot analysis of cytosolic and detergent-extracted membrane fractions of a variety of mouse tissues were undertaken. Affinity purified antibodies to the recombinant C-terminal human 5-phosphatase II recognized an approximately 115-kDa polypeptide in the membrane fraction of mouse brain, liver, lung, testis, and, most prominently, in kidney (Fig. 6a). Heart and spleen membrane fractions were non-reactive as predicted by Northern analysis (results not shown). Western blot analysis of the cytosolic fraction of the same mouse tissues demonstrated a 115-kDa isoform in brain, liver, and lung cytosol (Fig. 6b). In addition in all mouse cytosolic fractions the affinity purified antibody recognized two or three other polypeptides of approximately 100, 85, and 55 kDa. We postulate the 100-kDa isoform corresponds to the 104-kDa polypeptide predicted by M22, and the 85-kDa immunoreactive band may correspond to the 87-kDa polypeptide predicted by proposed alternative splicing of M5 (aa 234). The 55-kDa polypeptide appears to result from proteolysis as shown below. Collectively, these studies suggest the 115-kDa isoform is predominantly expressed in the membrane, with the smaller 104-kDa isoform more prominently expressed in the cytosol.

**Expression of 5-Phosphatase II in Human Platelets**—The 5-phosphatase II was originally purified from human platelet cytosol as a 75-kDa polypeptide (16). N-terminal amino acid sequence analysis of the purified platelet protein showed that it starts with a basic amino acid, suggesting that it is proteolysed from a larger precursor (Fig. 1e) (29). We therefore investigated the expression of various isoforms of 5-phosphatase II in human platelets. Isolation, washing, and preparation of cytosolic and detergent-soluble membrane fractions of human platelets was as described previously (17). Western analysis of these fractions gave similar results to those using mouse tissues. The
isolated as described under “Experimental Procedures,” and 100 μg from each subcellular fraction was analyzed by 7.5% reduced SDS-PAGE, followed by immunoblotting with affinity purified polyclonal antibody to recombinant human 5-phosphatase II (antiserum 732–916). Western blots were developed using enhanced chemiluminescence with the migration of molecular weight (MW) markers shown on the left. b, cytosolic fractions from mouse brain (A), heart (B), kidney (C), liver (D), lung (E), testis (F), and spleen (G) (100 μg) were analyzed as described for a. Both a and b are representative of at least three immunoblot analyses.

115-kDa 5-phosphatase II was most highly expressed in the membrane, whereas an 80-kDa immunoreactive species was present equally in the membrane and cytosol (Fig. 7a). In addition, a 100-kDa polypeptide was faintly reactive in the cytosolic and membrane fractions. As shown in mouse tissues a 55-kDa polypeptide was also observed in cytosolic fractions.

We also developed antibodies to N-terminal and C-terminal peptides of the full-length 5-phosphatase II, using sequences common to both mouse and human proteins. Antibodies to the C-terminal peptide should recognize all 5-phosphatase II isoforms, whereas the antibodies to the N-terminal peptide should only react with the 115-kDa isoform. Western blot analysis of pooled cytosolic and detergent-extracted platelet membrane fractions was performed using affinity purified anti-peptide antibodies. Antibodies to the N-terminal peptide recognized only a 115-kDa polypeptide (Fig. 7b). In contrast C-terminal affinity purified anti-peptide antibodies reacted with 115-, 107-, 100-, and faintly with 87-kDa polypeptides and in addition some smaller molecular species of 55 kDa (Fig. 7c). These studies indicated the presence of several platelet isoforms of 5-phosphatase II, with unique N-terminal regions, consistent with the splice variants predicted by the mouse tissue expression studies.

**Proteolysis of the 115-kDa 5-Phosphatase II by Platelet Lysate**—To investigate if the 80- or 55-kDa polypeptides observed in platelets are derived by proteolytic cleavage of the 115-kDa enzyme, the full-length cDNA (M5) encoding the mouse 5-phosphatase II was translated in vitro in the presence of 35S-methionine. Translated products were incubated in the presence or absence of platelet lysate (either control or thrombin-stimulated) or buffer alone for 30 min, and reaction products were analyzed by reduced SDS-PAGE. Incubation of the 35S-labeled 115-kDa polypeptide with platelet lysate resulted in rapid cleavage of the protein to a 55-kDa polypeptide (Fig. 8a, lanes B and C). No difference was observed between control and thrombin-stimulated platelet lysates.

Time course analysis of the proteolysis by platelet lysate was performed in the presence or absence of EDTA. The formation of an intermediary 104- or 87-kDa polypeptide was not observed (Fig. 8b). The 115-kDa enzyme was rapidly sensitive to proteolysis when expressed in the cytosol, and the presence of EDTA only partially inhibited proteolysis. The latter studies suggest the 55-kDa polypeptide immunoblotted in mouse and human platelet cytosol is derived from proteolysis of the 115-kDa 5-phosphatase II.

**DISCUSSION**

We have demonstrated significant diversity in the expression of the 5-phosphatase II mRNA and protein. The studies reported here suggest RNA splicing results in the expression of at least three 5-phosphatase II isoforms as follows: a 115-kDa 5-phosphatase located in the membrane, a second isoform of 104-kDa, expressed in both the membrane and cytosol, and a potential third 87-kDa isoform. Multi-tissue RNA analysis shows the widespread expression of two RNA species, with differential expression occurring in discrete tissues. Western blot analysis of numerous mouse tissues and human platelets demonstrates up to five cross-reacting species and specifically
shows the presence of two major forms of the enzyme, a 115-kDa polypeptide prominently expressed in the membrane fraction and a 104-kDa form in the cytosol.

Other members of the 5-phosphatase family have been shown to have differential membrane localization or cellular expression as a result of RNA splicing events. Ramjaun and McPherson (38) have recently shown that there are two isoforms of synaptotagmin with different intracellular location. The larger 170-kDa isoform is more tightly associated with membrane fractions than the smaller 140-kDa form, resulting from the alternative use of an exon containing a stop codon. In addition, several studies have indicated that there are several isoforms of the SH2 containing 5-phosphatase (SHIP) (145 kDa), with a smaller 110-kDa form designated SIP, for signaling inositol phosphatase. The predicted amino acid sequences of the human SIP and the mouse SHIP suggest they are alternatively spliced products of a single gene (27). Both SIP and SHIP hydrolyze PtdIns(3,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ and form complexes with GRB2. Analysis of peripheral blood leukocytes using antibodies to SHIP has demonstrated the presence of seven distinct immunoreactive species, with the expression of various isoforms altering with both the developmental stage and cell lineage (39).

We believe the mouse 3.7-kb M5 cDNA represents a full-length 5-phosphatase II cDNA for the following reasons. There are a number of “in-frame” stop codons before the proposed initiator methionine, and the untranslated region of the mouse cDNA bears no homology with the human. In addition Western blot analysis of both human and mouse tissues failed to detect any larger protein than the 115-kDa polypeptide predicted by translation of the 3.7-kDa M5 cDNA. Finally RNA analysis identified a 4.0-kb transcript consistent with M5 representing a full-length cDNA.

We propose that both insert sequences found within the full-length mouse 5-phosphatase II derive from RNA splicing events. Analysis of the cDNA at the start and end of each insert shows evidence of consensus splice sites conforming to the “GT-AG” rule (37). Furthermore, only the 4.0-kb transcript hybridized to the cDNA specific to the larger insert sequence. The function of the insert sequence is not clear, as no homology with any known structural or signaling domain was found. However, we have demonstrated that the N-terminal region of the 115-kDa 5-phosphatase II, prior to and including the insert sequence, is just as important as the C-terminal CAA motif.

TABLE II

| GenBank™ accession numbers are as follows: BLR1 (P32310), GUSV (P32326), GCRT (P43657), CRKR, (P1679), GPR2, (P46992), and LCR1, (P30991). |
|---|
| **Sequence alignment of the N-terminal region of mouse 5-phosphatase II with other membrane proteins** |
| **5-phosphatase II** | **VQNLTAELSLSVLPFG** |
| BLR1-human | FHIAVAADLILVFLLPFPA |
| GUSV-bovine | LNLQVADDLILFLTLPFW |
| GCRT-human | INLQVADLILFVTLPFR |
| CRKR-human | LNLQVADLILFVTLPFW |
| GPR2-human | LNLQVADLILFFLPFA |
| LCR1-bovine | LHQLVADLILVPFLPG |

associated, without the CAA motif. It is only when the N terminus, insert sequence, and CAA motif are removed that cytosolic localization predominates. These studies imply the insert sequence, in conjunction with the N terminus, contains a membrane anchoring site. Thus isoprenylation contributes to, but is not necessary for, membrane attachment of the larger 115-kDa 5-phosphatase II isoform. We propose the presence of two membrane anchoring domains at either end of the protein should give stronger membrane attachment than observed with either alone. Such stable membrane association would allow the 5-phosphatase II access to its two principal lipid substrates PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$. Our previous findings have shown that the affinity of 5-phosphatase II for PtdIns(4,5)P$_2$ is considerably enhanced ($k_m$ 30 µM versus 250 µM) in platelet membranes compared with platelet cytosol, respectively (17).

Jefferson and Majerus (6) showed that lack of isoprenylation of the C-terminal CAAX motif was associated with a 30% reduction in PtdIns(4,5)P$_2$ 5-phosphatase activity when the recombinant enzyme was expressed in Sf9 cells. We consistently observed a 15–20% decrease in total PtdIns(4,5)P$_2$ 5-phosphatase and Ins(1,4,5)P$_3$ 5-phosphatase activity in transient transfection studies in COS-7 cells when either the N terminus or the CAAX motif was removed, suggesting such decrease in enzyme activity may correspond to incorrect folding of the 5-phosphatase when it is anchored only by one end of the molecule in the membrane. Restoration of both PtdIns(4,5)P$_2$ and Ins(1,4,5)P$_3$ 5-phosphatase activity occurred when both the N terminus and CAAX motif were either removed or restored. The 43-kDa type I 5-phosphatase is the only other mammalian 5-phosphatase described to date that contains a C-terminal CAAX motif. Deletion or mutation of the CAAX motif results in significant translocation of the majority, but not all of the 43-kDa 5-phosphatase, from the membrane to the cytosol (36). In addition De Smedt and co-workers (36) demonstrated a significant increase in the specific activity of the cytosolic versus membrane-associated 43-kDa 5-phosphatase. Proteolysis of both recombinant and native 5-phosphatase II in cytosolic fraction of cellular extracts precluded precise determination of the enzyme’s specific activity, but a similar inhibition of enzyme activity resulting from membrane attachment cannot be excluded.

We have also identified a smaller form of 5-phosphatase II (M22) with a novel N terminus. Evidence that this is a genuine isoform is as follows: RNA analysis using the unique 5’ end of the M22 cDNA hybridized only to the smaller 3.0-kb RNA transcript. We observed a 104-kDa polypeptide in Western blots of both human and mouse tissues, which is consistent with the size of the protein predicted by M22 sequence analysis.

It is noteworthy that we have consistently immunoblotted an approximate 87-kDa polypeptide in the cytosol of both human platelets and multiple mouse tissues. The 87-kDa polypeptide may represent another spliced variant of 5-phosphatase II.
rather than a proteolytic fragment of the 115-kDa isoform. We were able to generate a 55-kDa polypeptide, by in vitro proteolysis of the 115-kDa 5-phosphatase II by platelet lysates, but production of a 87-kDa isoform was not observed. We cannot exclude that the 104-kDa isoform is proteolyzed to the 87-kDa polypeptide, and we are currently generating recombinant 104-kDa 5-phosphatase II to determine its intracellular location, proteolysis by platelet lysate, and enzyme activity. However, it is equally tenable that the potential RNA splice site identified in both M22 and M5 results in the generation of the smaller 87-kDa isoform. The 87-kDa 5-phosphatase II appears to be more prominently located in the cytosol, perhaps due to the lack of N-terminal anchoring sequences and incomplete farnesylation of the CAAAX motif. Such cytosolic location would enable 5-phosphatase II access to the water-soluble second messengers Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 and thereby regulate intracellular calcium homeostasis.

Finally, the 5-phosphatase II was originally purified as a 75-kDa enzyme from human platelet cytosol (16). Consistent with these results we were able to detect an approximate 80-kDa immunoreactive polypeptide in human platelets, in Western blots using polyclonal antibodies to the recombinant 5-phosphatase II. In addition to mouse cytosolic fractions, two or three immunoreactive polypeptides were observed ranging in molecular mass from 75 to 87 kDa. It is most likely the larger of these species is derived from RNA splicing events, with the smaller species derived from either in vitro or in vivo proteolysis. The purified platelet 75-kDa 5-phosphatase II commences with a basic amino acid, consistent with proteolysis from a larger precursor. The enzyme activity of this smaller 5-phosphatase is comparable to that of the larger recombinant human 5-phosphatase II (6, 16, 27). Our studies extend these earlier observations and suggest that multiple isoforms of 5-phosphatase II reflect differential compartmentalization of activity in the membrane and cytosol, which may provide access to various phosphoinositide-derived second messenger molecules.

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Distinct Membrane and Cytosolic Forms of Inositol Polyphosphate 5-Phosphatase II: EFFICIENT MEMBRANE LOCALIZATION REQUIRES TWO DISCRETE DOMAINS

Maria Matzaris, Cindy J. O'Malley, Anne Badger, Caroline J. Speed, Phillip I. Bird and Christina. A. Mitchell

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