# Characterization of an adapter subunit to a phosphatidylinositol (3)P 3-phosphatase: Identification of a myotubularin-related protein lacking catalytic activity

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**The D3-phosphoinositides act as second messengers by recruiting, and thereby activating, diverse signaling proteins. We have previously described the purification of a rat phosphatidylinositol 3-phosphate [PtdIns(3)P] 3-phosphatase, comprising a heterodimer of a 78-kDa adapter subunit in complex with a 65-kDa catalytic subunit. Here, we have cloned and characterized the cDNA encoding the human 3-phosphatase adapter subunit (3-PAP). Sequence alignment showed that 3-PAP shares significant sequence similarity with the protein and lipid 3-phosphatase myotubularin, and with several other members of the myotubularin gene family including SET-binding factor 1. However, unlike myotubularin, 3-PAP does not contain a consensus $HCX_5R$ catalytic motif. The 3-PAP sequence contains several motifs that predict interaction with proteins containing Src homology-2 (SH2) domains, phosphotyrosine-binding (PTB) domains, members of the 14-3-3 family, as well as proteins with SET domains. Northern blot analysis identified two transcripts (5.5 kb and 2.5 kb) with highest abundance in human liver, kidney, lung, and placenta. 3-PAP immunoprecipitates isolated from platelet cytosol hydrolyzed the D3-phosphate from PtdIns(3)P and PtdIns 3,4-bisphosphate [PtdIns(3,4)$P_2$]. However, insect cell-expressed 3-PAP recombinant protein was catalytically inactive, confirming our prior prediction that this polypeptide represents an adapter subunit.**

Phosphoinositide 3-kinase (PI 3-kinase; ref. 1)-derived, membrane-anchored phosphoinositides regulate diverse cellular processes, including proliferation, cell survival, vesicular trafficking, cytoskeletal remodeling, and metabolism (2, 3). Lipid products produced by PI 3-kinase activity include the following: phosphatidylinositol 3-phosphate [PtdIns(3)P], phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)$P_2$], phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)$P_2$], and phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)$P_3$]. Diverse proteins are regulated by binding to the D3-phosphorylated phosphoinositides PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$, including the serine/threonine kinase Akt, a potent suppressor of apoptosis (4). PtdIns(3)P, the most abundant product of PI 3-kinase activity in mammalian cells, specifically interacts with a protein module designated the "FYVE domain" and functions as a regulator of endosomal trafficking (5).

Molecular genetic and biochemical evidence indicates that specific lipid phosphatases terminate the signaling function of phosphoinositides. PTEN dephosphorylates the D3-phosphate of PtdIns(3,4,5)$P_3$ forming PtdIns(4,5)$P_2$ (6). The lipid 3-phosphatase activity of PTEN is mediated by the consensus catalytic motif comprising $HCX_5R$ and is critical for its tumor suppressor function (7).

The locus MTM1, mutated in human X-linked recessive myopathy, encodes the protein myotubularin, which contains an $HCX_5R$ catalytic motif (8) and functions as a PtdIns(3)P 3-phosphatase (9, 10). Numerous human homologues of myotubularin have been identified (11), including the SET-binding factor 1 (Sbf1), which interacts with the SET domain of the oncogene *Mll* (mixed lineage leukemia) (12). Sbf1 lacks a functional consensus catalytic motif and, in overexpression studies, has been shown to function as an anti-phosphatase.

We have previously purified a PtdIns(3)P 3-phosphatase to homogeneity from rat brain lysates, comprising two polypeptides: a homodimer of a 65-kDa polypeptide, and a heterodimer consisting of the 65-kDa polypeptide together with a 78-kDa polypeptide (13). Both complexes exhibited PtdIns(3)P 3-phosphatase activity with a 3-fold greater enzyme activity for the 65-kDa/78-kDa heterodimer, compared with the 65-kDa homodimer. We predicted that the 65-kDa polypeptide represented the phosphatase catalytic subunit and that the 78-kDa polypeptide was a regulatory, or adapter subunit (13).

In this report, we describe the molecular cloning and characterization of a cDNA encoding the human 3-phosphatase adapter protein (3-PAP), which has sequence similarity to myotubularin, but lacks the $HCX_5R$ motif. Recombinant 3-PAP does not dephosphorylate PtdIns(3)P; however, immunoprecipitates of 3-PAP from platelet lysates dephosphorylate D3-phosphate from PtdIns(3)P and PtdIns(3,4)$P_2$, confirming that this polypeptide functions as an adapter molecule.

## Materials and Methods

**Materials.** Restriction and DNA-modifying enzymes were from New England Biolabs, Fermentas (Hanover, MD), or Promega. DNA and protein sequencing and synthesis of oligonucleotide primers were undertaken by appropriate support facilities in Monash University, Clayton, Australia; University of New Mexico, Albuquerque, NM; and Washington University, St. Louis. Cell culture media were obtained from Trace Biosciences (Melbourne, Australia) and GIBCO. All other reagents were from Sigma, unless otherwise stated.

Abbreviations: PI 3-kinase, phosphoinositide 3-kinase; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(3,4)$P_2$, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,5)$P_2$, phosphatidylinositol 3,5-bisphosphate; PtdIns(3,4,5)$P_3$, phosphatidylinositol 3,4,5-trisphosphate; Sbf1, SET-binding factor 1; 3-PAP, 3-phosphatase adapter protein; MHR, myotubularin-homology region; MTR, myotubularin-related.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY028703).

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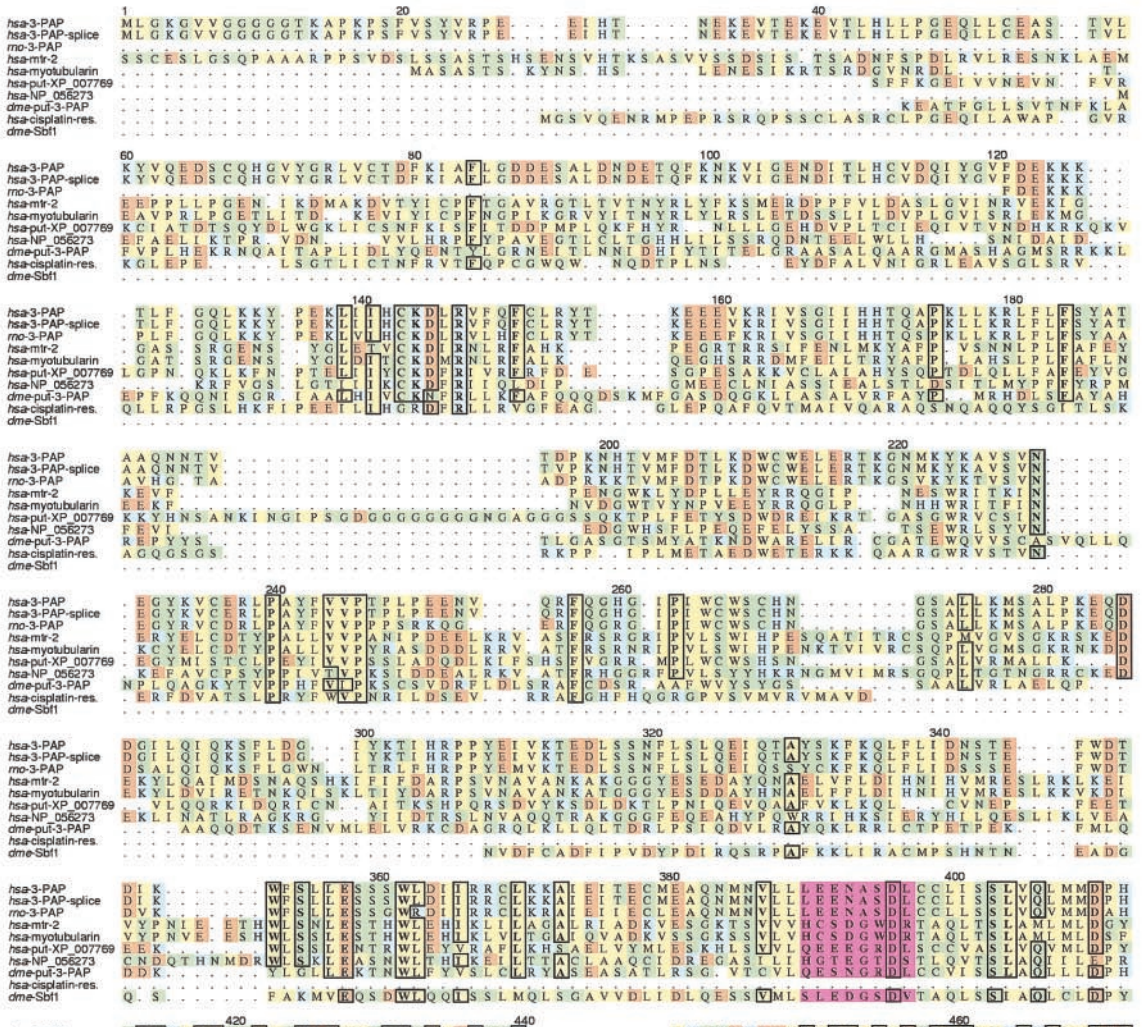


(*Legend appears at the bottom of the opposite page.*)

**Isolation of Rat and Human cDNA Clones.** The 78-kDa polypeptide purified to homogeneity from rat brain lysates (13) was digested with lyslendopeptidase *in situ* (14) or was electroeluted from a 10% SDS/PAGE, followed by digestion with trypsin (15). The digests were separated by reverse-phase chromatography, and peptides were sequenced (15). A total of 15 peptide sequences comprising 160 aa were obtained. Degenerate oligonucleotide primers were designed for use in reverse transcription (RT)-PCR, by using rat brain total RNA as the template, and a unique 286-bp product was generated. Sequence information from this cDNA was utilized to identify a longer rat cDNA, and the full-length human cDNA was isolated by a combination of library screening (rat brain and K562 cell line) and RT-PCR (mRNA from rat brain and K562 cells).

**Northern Blot Analysis.** A membrane containing poly(A)$^+$ mRNA representing various human tissues (CLONTECH) was probed with the expressed sequence tag R86970 labeled with [$\alpha$-$^{32}$P]dCTP (Amersham Pharmacia-Pharmacia Biotech, oligo-labeling kit) and washed according to the manufacturer's instructions. The membrane was allowed to decay and was hybridized with an actin cDNA probe (CLONTECH).

**Production of Antipeptide Antibody.** Rabbits were immunized with synthetic peptides (Chiron-Mimotopes, Melbourne) representing the amino acid sequence N′-(730)-DEDDLAKREDEFVD-(743)-C′, which was unique to the 3-PAP. Antisera (anti-3-PAP antibody) was affinity-purified from the immune sera by binding to the specific peptide couple to thiopropyl-Sepharose resin (Chiron-Mimotopes).

**Preparation of Platelets and Immunoprecipitations.** Platelets were obtained from healthy volunteers and were washed as described (16), and the Triton X-100-soluble lysate was prepared as described (17). Before immunoprecipitations, 500 $\mu$l (9 mg/ml) Triton X-100-soluble platelet lysate was precleared with protein A-Sepharose. To detect PtdIns(3)P 3-phosphatase activity, 500 $\mu$l of the precleared platelet lysate was thoroughly mixed with 0–5 $\mu$g of anti-3-PAP antibody adjusted to a total of 5 $\mu$g by addition of nonimmune IgG antibody, and incubated with rocking at 4°C for 2 h, followed by the addition of 60 $\mu$l of a 50% slurry of protein A-Sepharose in Tris·NaCl and overnight incubation. The protein A-Sepharose was pelleted and washed twice with 1 ml Tris·NaCl containing 1% (vol/vol) Triton X-100, twice with Tris·NaCl, and once with phosphatase assay buffer (see below).

**Production and Immunoprecipitation of Recombinant 3-PAP.** Recombinant 3-PAP with an N′-terminal 6-histidine tag was expressed in the Sf9 insect cell line by using the pBlueBacHis vector and the MaxBac baculovirus expression system (both from Invitrogen, Groningen, The Netherlands) according to the manufacturer's protocol. Either insect cells infected with baculovirus expressing 3-PAP, or parental (uninfected) were centrifuged, and the pellets (12 mg) were resuspended in 500 $\mu$l of lysis buffer (final concentration: 20 mM Tris·HCl, pH 7.4/1% (vol/vol) Triton X-100/5 mM EGTA/2 $\mu$g/ml leupeptin/2 $\mu$g/ml aprotinin/1 mM benzamidine) and incubated at 4°C for 1 h, precleared with protein A-Sepharose, and incubated with the anti-3-PAP antibody (5 $\mu$g). Subsequent immunoprecipitation and washing was conducted as described above.

**Lipid 3-Phosphatase Assays.** PtdIns(3)[3-$^{32}$P]P, PtdIns(3,4)[3-$^{32}$P]$P_2$, and PtdIns(3,4,5)[3-$^{32}$P]$P_3$ were prepared by using PtdIns (140 $\mu$g), PtdIns(4)P (56 $\mu$g), and PtdIns(4,5)$P_2$ (28 $\mu$g), respectively, with 20 $\mu$g of phosphatidylserine (PtdSer), 1 $\mu$g of affinity purified recombinant PI 3-kinase (18), unlabeled ATP (50 $\mu$M), 20 $\mu$l of [$\gamma$-$^{32}$P]ATP (0.2 mCi, 3000 Ci/mM, ICN), 5 $\mu$l of 20× kinase buffer (400 mM Tris·HCl, pH 7.4/100 mM $MgCl_2$/10 mM EGTA) in a reaction volume of 100 $\mu$l. The products of phosphorylation by PI 3-kinase were extracted and suspended in lipid resuspension buffer (20 mM Tris·HCl, pH 7.4/1 mM EGTA) and also analyzed by deacylation and HPLC (19).

Lipid 3-phosphatase assays (100 $\mu$l) were performed by adding $^{32}$P-labeled phosphoinositide to the washed immunoprecipitates in the presence of phosphatase assay buffer (final concentration: 20 mm Tris·HCl, pH 7.4/5 mM $MgCl_2$/0.5 mM EGTA) and incubated 37°C for 30 min (19). In some experiments, PtdIns(3)P 3-phosphatase activity was also analyzed by using the above phosphatase buffer but lacking $MgCl_2$. Extracted lipids were analyzed by TLC in a tank preequilibrated with 130 ml propan-1-ol, 8 ml glacial acetic acid, 0.68 ml orthophosphoric acid, and 62 ml deionized water. The phosphoinositide products were excised from the TLC and counted by using liquid-scintillation counting.

**Phylogenetic Analysis of the Myotubularin-Homology Region (MHR) Domains of the Myotubularin Homologues.** A phylogenetic analysis was performed by using an alignment of the MHR. The 3-PAP sequence was used in a DBCLUSTAL BLASTP search of GENPEPT (20) and selected all sequences with an Expect score $<0.01$. The alignment was edited to remove sequences N- and C-terminal to the MHR domain, all gaps, identical sequences, and short fragments. The MHR domains were realigned by using CLUSTALW (21) running on a Silicon Graphics Octane workstation. Bootstrapping (1,000 trials) was performed by using CLUSTALW, and the phylogenetic tree was constructed by using NJPLOT (22). Clades supported by bootstrapping values $>95\%$ are statistically significant. The active site (or equivalent residues) region from every sequence was removed, and the analysis was repeated with similar results.

**Regression Analysis.** The program GRAPHPAD PRISM v. 3 was used to analyze the co-relation between D3–3-phosphatase activity, and increments in anti-3-PAP antibody used for immunoprecipitation.

## Results

**Cloning of the cDNA Encoding the Human 3-PAP and Sequence Analysis.** We obtained fifteen peptide sequences comprising 160 aa from tryptic digests of the purified rat 78-kDa polypeptide. Degenerate oligonucleotide primers were synthesized based on three peptide sequences, and the primers were utilized to isolate a partial rat and human cDNA by a combination of PCR and

**Fig. 1.** (*On the opposite page.*) Multiple sequence alignment of the human 3-PAP with other putative homologues. Species identifiers are in italics and are as follows: hsa, *Homo sapiens*; rno, *Rattus norvegicus*; dme, *D. melanogaster*. Hydrophobic residues are in yellow, polar non-charged residues in green, acidic residues in brown, and basic residues in blue. Conserved residues ($>70\%$) are boxed and in bold. The following sequences are shown: human 3-PAP, splice variant of human 3-PAP (accession no. NP 061934), rat 3-PAP, human protein similar to myotubularin-related protein-2 (accession no. XP 005992, 23% primary sequence identity); human myotubularin (accession no. XP 010178, 22% identity); human putative protein XP_007769 (38% identity); human unnamed protein NP_056273 (20% identity); *Drosophila* 3-PAP (Putative, accession no. AAF48390, 27% identity), human cisplatin resistance associated alpha protein (accession no. AAB36952, 23% identity), and *Drosophila* Sbf-1 (accession no. AAF54700, 30% identity). All sequences were identified by using PSI-BLAST (19), and returned highly significant Expect scores ($<3 \times 10^{-49}$). The active site motif ($HCX_5R$) of myotubularin and *hsa*-mtr-2, and the corresponding positions in 3-PAP and related proteins are highlighted in magenta.

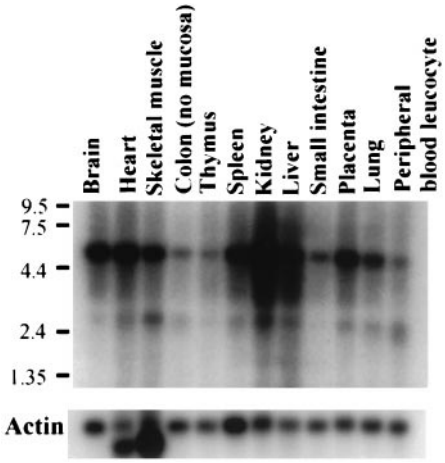


**Fig. 2.** Tissue expression of the 3-PAP mRNA. Human multiple tissue Northern blot probed with the human expressed sequence tag R86970 corresponding to amino acids 601–747 of the composite cDNA. The migration of the RNA standards in kilobases is indicated. The blot was stripped and reexamined with an actin probe as a loading control.

conventional library hybridization. The human sequence predicts for an 86-kDa polypeptide, which we have designated 3-PAP (3-phosphatase-associated protein; Fig. 1). Additional partial clones were also isolated that contained a longer 3′ UTR (3.0 kb). These two species of clones with varying 3′UTR are consistent with the two transcripts of 2.5 kb and 5.5 kb identified by Northern blot analysis (Fig. 2).

Position-specific iterated (PSI)-BLAST (23) search of the GenBank database by using the amino acid sequence of the human 3-PAP protein as a probe identified significant sequence similarity with myotubularin, overall 21% amino acid identity (Expect score = $e^{-173}$). However, from amino acid 310 to 449, there was 40% identity (Expect score = $5e^{-25}$; Fig. 1). We have termed this region the myotubularin-homology region (MHR), because it is common to all myotubularin-related gene family members (11). Myotubularin contains the canonical phosphatase catalytic motif $HCX_5R$ (8), but this motif is absent in 3-PAP (Fig. 1). We have further identified two yet uncharacterized putative human proteins in the database (GenBank accession nos. hsa-XP_007769, has-NP_056273) that, like 3-PAP, contain the MHR domain but also lack the $HCX_5R$ catalytic motif. Myotubularin and Sbf1 contain a C-terminal domain within the MHR that permits interaction with the SET domain of the oncogene *Mll* (12), called the SET-interacting domain (SID), which is also conserved within 3-PAP sequence (amino acid 449–500, Fig. 1; amino acid identity 53%). In addition, we have identified a transcriptional variant of 3-PAP in the GenBank database (GenBank accession no. NP_061934) with a deletion of the SID (missing 110 aa: 449–559), which predicts for a 73-kDa polypeptide. The 3-PAP N′-terminal region (amino acids 65–265) shares significant sequence similarity (amino acid identity 24%, Expect score = $3e^{-56}$) with two cDNAs isolated by differential display analysis of cisplatin-resistant ovarian tumor cells (GenBank accession nos. AAB36952.1 and AAB36953.1, respectively). The 3-PAP amino acid sequence contains 12 tyrosine residues in the context "Y-X-X-hydrophobic," predicting possible interaction with Src homology-2 (SH2) domains (24). There are three

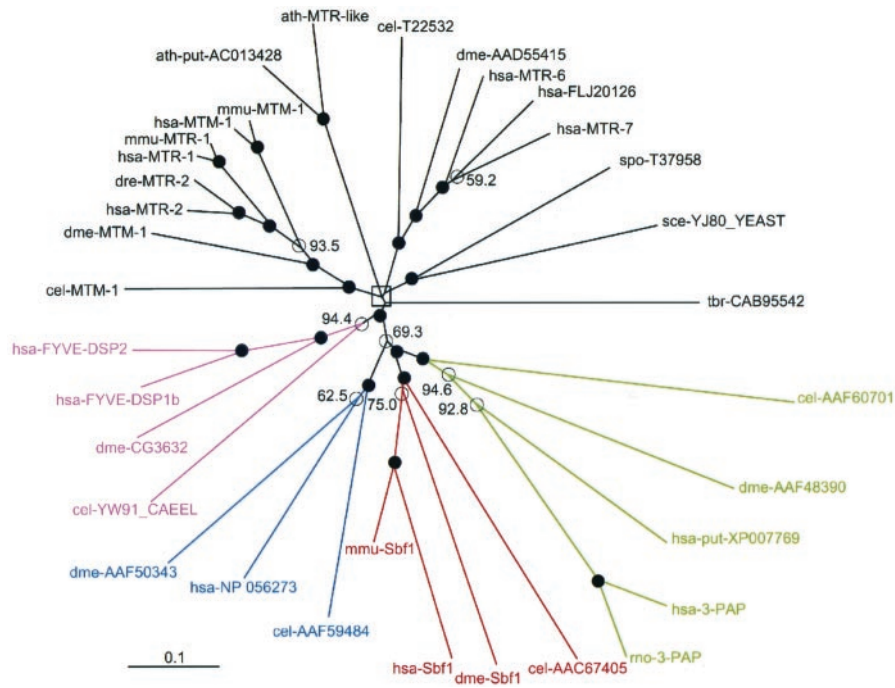


**Fig. 3.** Unrooted distance tree depicting the phylogenetic relationships between members of the myotubularin family. Abbreviations used are: cel, *Caenorhabditis elegans*; mmu, *Mus musculus*; dre, *Danio rerio*; spo, *Schizosaccharomyces pombe*; sce, *Saccharomyces cerevisiae*; tbr, *Trypanosoma brucei*; and ath, *Arabidopsis thaliana*. Protein abbreviations are as follows: MTM, myotubularin; MTR, myotubularin-related protein; FYVE-DSP, FYVE domain-containing dual specificity phosphatase; 3-PAP; 3-phosphatase adapter protein; Sbf1, SET-binding factor 1. Descriptive names are given, where defined in GenBank; otherwise GenBank accession number or SWISSPROT protein ID is shown. Nodes supported by bootstrap values at or above 95% significance threshold are indicated by filled circles; <95% are marked with open circles and the relevant values are shown. An open rectangle marks three nodes at the center of the tree for which bootstrap values were <95%; however, for the sake of clarity these values are not shown. The scale bar indicates the proportion of mutations in a sequence along a branch. The clades containing myotubularin homologues with mutations in the catalytic motif are depicted as follows: green, 3-PAP family; red, Sbf1 family; and blue, which contains the human sequence NP 056273 and related orthologues. Myotubularin homologues that contain a catalytic motif are identified in black and magenta.

"N-X-X-Y" motifs that may mediate interaction with phosphotyrosine-binding (PTB) domains (24), and three motifs for possible complex formation with the 14-3-3 family of adapter proteins (24).

We performed a phylogenetic analysis of the MHR of the myotubularin superfamily, including 3-PAP (Fig. 3). This analysis indicated that the myotubularin-related (MTR) family members form at least five distinct clades. A major clade comprises proteins containing the $HCX_5R$ catalytic motif and includes MTM-1 (myotubularin), MTR-1, MTR-2, MTR-6, and MTR-7 (boot strapping data 98.5%). The FYVE-containing dual specificity phosphatases (FYVE DSP1 and DSP2) are clustered together (shown in pink), based on the presence of the FYVE domain and homology with the MHR domain (bootstrapping data 94.4%). Proteins in which the $HCX_5R$ motif is absent map to three distinct clades (identified by blue, red, and green in Fig. 3) and include Sbf1, a novel clade (shown in blue) that is yet to be characterized, and 3-PAP. The 3-PAP family is extremely well supported (bootstrapping data 98.7%) and includes a *Drosophila melanogaster* orthologue and two human proteins (3-PAP and the putative protein XP007769, shown in green). Collectively, this analysis demonstrates that 3-PAP is a member of the myotubularin family of proteins. Within this family, there are distinct subfamilies of proteins including SbF1 and 3-PAP that lack the catalytic $HCX_5R$ motif.

**3-PAP Tissue Expression Profile.** A human multi-tissue Northern blot was probed with the 525-bp human expressed sequence tag cDNA (GenBank accession no. R86970) encoding human 3-PAP. Two transcripts of 5.5 and 2.5 kb were prominent in brain, heart, kidney, liver, placenta, and lung (Fig. 2). A polyclonal antibody (anti-3-PAP antibody) raised to a unique peptide sequence at the 3-PAP C terminus (amino acid 730–743) recognized the predicted 86-kDa polypeptide in a variety of tissues and cell lines (not shown).

**3-PAP Immunoprecipitates Contain Lipid 3-Phosphatase Activity.** 3-PAP immunoprecipitated from human platelet Triton X-100-soluble lysates dephosphorylated the $^{32}$P-labeled D3-phosphate from PtdIns(3)P. Phosphatase activity in the immunoprecipitates exhibited a linear increase (regression coefficient $r^2 = 0.9$) with increments in the anti-3-PAP antibody (Fig. 4*A*). Activity was 570 cpm of the D3-labeled-$^{32}$P from PtdIns(3)[3-$^{32}$P]P released per microgram of anti-3-PAP antibody in the immunoprecipitates, relative to the control immunoprecipitate by using 5 $\mu$g of nonimmune antibody. We previously reported that the 65/78-kDa heterodimer PtdIns(3)P 3-phosphatase activity did not depend on the presence of added metal ions and was inhibited by divalent cations (25). Here, we confirm that, in the absence of $Mg^{2+}$, activity was 1.5-fold ($\pm$0.15 SD, $n = 3$) that of enzyme activity in the presence of 5.0 mM $Mg^{2+}$. Recombinant 3-PAP immunoprecipitated from Sf9 cytosol did not contain PtdIns(3)P 3-phosphatase activity (Fig. 4 *B* and *C*). There was also no phosphatase activity in immunoprecipitates of COS-7 cells transfected with 3-PAP (data not shown).

We have also examined the ability of the 3-PAP immunoprecipitates from human platelet cytosol to dephosphorylate other D3-phosphorylated phosphoinositides. As shown in Fig. 5 *A* and *B*, dephosphorylation of the $^{32}$P-labeled D3-phosphate from PtdIns(3,4)$P_2$ was observed with similar degree of efficiency as for PtdIns(3)P. However, no significant dephosphorylation of PtdIns(3,4,5)$P_3$ was noted.

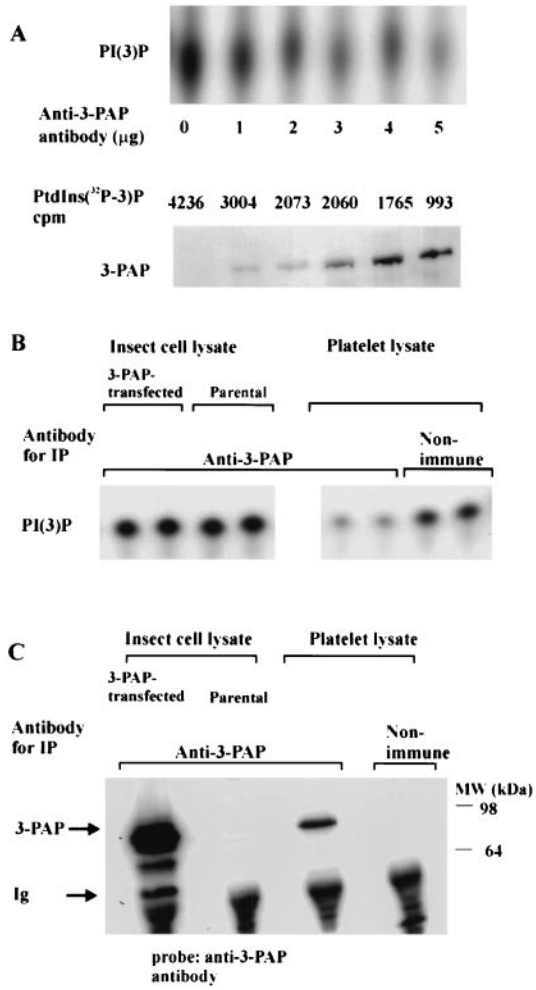


**Fig. 4.** 3-PAP immunoprecipitates from platelets contain lipid 3-phosphatase activity. (*A*) Five hundred microliters of Triton X-100-soluble human platelet lysate was immunoprecipitated with 0–5 $\mu$g of anti-3-PAP antibody. The total antibody used was adjusted to 5 $\mu$g by using reciprocal quantities of nonimmune Ig, captured on protein A-Sepharose and PtdIns(3)[3-$^{32}$P]P 3-phosphatase assays performed on washed immunoprecipitates. Phosphoinositides were extracted, analyzed by TLC, and measured by liquid-scintillation counting. Duplicate immunoprecipitates made with 0–5 $\mu$g of anti-3-PAP antibody were analyzed by immunoblot and probed with the anti-3-PAP antibody. (*B*) Triton X-100-soluble human platelet lysate (500 $\mu$l, 9 mg/ml), or Triton X-100-soluble insect Sf9 cell lysate (prepared from 12 mg of cell pellet) from either parental cells, or cells infected with 3-PAP-expressing baculovirus were incubated with 5 $\mu$g of anti-3-PAP antibody, or 5 $\mu$g nonimmune antibody as indicated. Immunoprecipitates were performed in duplicate and were analyzed for PtdIns(3)P 3-phosphatase activity. (*C*) Lysates prepared from Sf9 insect cells expressing recombinant 3-PAP and from parental Sf9 cells, and lysates prepared from human platelets were incubated with 5 $\mu$g of either anti-3-PAP antibody or nonimmune antibody, as indicated. Immunoblot analysis was performed by using the anti-3-PAP antibody. The migration of 3-PAP, the Ig heavy chain (Ig), and molecular weight markers (MW) (kDa) are indicated.

## Discussion

In this study, we have described the isolation and characterization of a cDNA encoding an adapter subunit to a PtdIns(3)P 3-phosphatase (3-PAP) that coimmunoprecipitates lipid 3-phosphatase activity, with specificity for PtdIns(3)P and PtdIns(3,4)$P_2$. Several lines of evidence support a role for the 86-kDa 3-PAP as an adapter subunit. First, the purification of this protein from rat brain cytosol demonstrated PtdIns(3)P 3-phosphatase activity in the presence of a copurified 65-kDa polypeptide. Second, recombinant 3-PAP showed no enzyme activity when expressed in mammalian or insect cells. Third, 3-PAP immunoprecipitates from human platelet cytosol demonstrated both PtdIns(3)P and PtdIns(3,4)$P_2$ 3-phosphatase activity, suggesting that the polypeptide is in a complex with an

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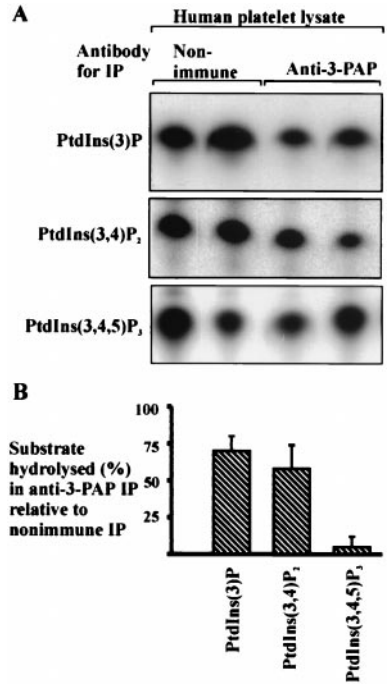


**Fig. 5.** Immunoprecipitates of 3-PAP dephosphorylate multiple D3-phosphorylated substrates. (*A*) Five hundred microliters of Triton X-100-soluble platelet lysate was incubated with either 5 $\mu$g of anti-3-PAP antibody, or 5 $\mu$g nonimmune antibody and washed immunoprecipitates incubated with PtdIns(3)[3-$^{32}$P]P, PtdIns(3,4)[3-$^{32}$P]$P_2$, or PtdIns(3,4,5)[3-$^{32}$P]$P_3$ in lipid phosphatase assays. (*B*) Immunoprecipitates were analyzed for lipid 3-phosphatase activity against the indicated phosphoinositides, as described above. Values represent mean ± SD of three experiments, each containing multiple replicates.

active enzyme *in vivo*. Fourth, the 3-PAP sequence does not predict for any known phosphatase catalytic motif. However, the presence of highly significant sequence identity with myotubularin clearly defines 3-PAP as an evolutionary member of the myotubularin family.

The adapter subunit may influence the function and/or localization of the 65-kDa catalytic subunit. We have previously shown that the rat 65-kDa homodimer has 3-fold lower PtdIns(3)P 3-phosphatase activity and a decreased preference for PtdIns(3)P over the soluble substrate Ins(1,3)$P_2$, compared with the heterodimer consisting of the rat 78-kDa and the 65-kDa polypeptides (13). Thus, by *in vitro* analysis, 3-PAP alters substrate specificity and enhances catalytic activity toward the lipid substrate. The adapter subunit may also target the 65-kDa subunit to specific signaling networks. Sequence analysis of the human 3-PAP has revealed numerous motifs that predict a variety of protein–protein interactions. These motifs include several tyrosine residues that may associate with phosphotyrosine-binding (PTB) domain-containing and Src homology-2 (SH2) domain-containing proteins, and three motifs that conform to the consensus motif for binding with 14-3-3 proteins. Interaction with 14-3-3 proteins may modify the heterodimer of 3-PAP and catalytic subunit, either to enhance or inhibit enzyme activity. The association of 14-3-3 with p85 inhibits PI 3-kinase catalytic activity (17) whereas, in contrast, binding of recombinant 14-3-3 to the platelet 43-kDa 5-phosphatase enhances inositol 1,4,5-trisphosphate 5-phosphatase catalytic activity (26).

Phylogenetic analysis has identified that 3-PAP represents a discrete subfamily, distinct from Sbf1, within the myotubularin superfamily. Sbf1 is proposed to function as a "protective factor" or as an "antiphosphatase" analogous to STYX proteins (27). However, it is unlikely that the 3-PAP acts as an "antiphosphatase" because purification studies have shown that the rat 78-kDa subunit enhances catalytic activity of the 65-kDa catalytic subunit (13), rather than compete for the same substrate.

The identity of the catalytic subunit that forms a complex with the 3-PAP adapter subunit remains to be determined and is the subject of active ongoing laboratory studies. The rat 65-kDa catalytic subunit originally identified in purification studies demonstrated significant PtdIns(3)P and Ins(1,3)$P_2$ 3-phosphatase activity, and like myotubularin shows a preference for PtdIns(3)P (13). However, database searches have identified many mammalian myotubularin homologues that contain an $HCX_5R$ motif (see Fig. 3), any of which, including myotubularin, are potential candidates for the catalytic subunit.

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