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Activation of protein phosphatase 5 by limited proteolysis or the binding of polyunsaturated fatty acids to the TPR domain

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Abstract Protein phosphatase 5 (PP5) exhibits very low phosphatase activity, which can be stimulated >25-fold by proteolysis. Since proteolysis cleaves the N-terminal tetratricopeptide repeat (TPR) domain from the catalytic domain, these results indicate that the TPR domain shields the active site. Polyunsaturated fatty acids, such as arachidonic acid, and lipids containing polyunsaturated fatty acids, such as phosphatidylinositol, stimulate both bacterially expressed human and native rabbit PP5 activity >25-fold towards casein and myelin basic protein. Phosphatidylinositol binds to the TPR domain, and not to the catalytic domain, indicating that activation by polyunsaturated fatty acids is allosteric and that it may occur by movement of the TPR domain to allow substrate access.

Key words: Protein phosphatase; Protein phosphorylation; Tetratricopeptide repeat (TPR); Arachidonic acid; Phosphatidylinositol

1. Introduction

The dephosphorylation of serine and threonine residues is an essential process in the regulation of many cellular functions. The major (PPP) family of serine/threonine phosphatases can be classified according to the structures of their catalytic subunits into four subfamilies, which are exemplified by PP1, PP2A, PP2B (calcineurin, Ca^{2+}-calmodulin regulated protein phosphatase) and PP5 [1]. Unlike PP1, PP2A and PP2B, PP5 was identified from its cDNA rather than its catalytic activity. Nevertheless PP5 is present throughout eukaryotes from man to yeast (where it is designated PPT), and in mammals PP5 is found in all tissues examined. It is predominantly located in the nucleus but is present at lower levels in the cytoplasm [2].

Although the function(s) of PP5 is (are) not known, identification of higher levels of PP5 in interphase cells in logarithmic growth than in dividing cells and serum deprived cells, together with increased levels of PP5 in the nucleolus subsequent to α-amanitin treatment of cells, suggests a role for PP5 in the regulation of transcription [3]. The identification of PP5 as a protein that interacts with the protein kinase domain of the guanylate cyclase receptor using the yeast two hybrid system [4] suggests a role in cell signalling from this receptor to the nucleus. However, the presence of PP5 in cell types that do not possess the guanylate cyclase receptor and in nuclei indicates a wider role. PP5 is potently inhibited by the tumour promoters okadaic acid and microcystin with IC_{50} values in the nanomolar range [2] and has recently been reported to be more sensitive than PP1, PP2A and PP2B to the fungal toxin fumonisin B_1 with an IC_{50} value of 80 μM [5].

PP5 comprises a catalytic subunit which shows approximately 40% identity to PP1, PP2A and PP2B but, distinct from these enzymes, the catalytic domain of PP5 is preceded by a long N-terminal domain which contains four 34 amino acid repeats, termed tetratricopeptide repeats (TPR) [2,4,6]. Since these motifs are also found in many other proteins which participate in cell cycle events, RNA biosynthesis, protein import into mitochondria and peroxisomes and other processes, it is believed that TPR domains provide interfaces for protein-protein interactions (reviewed in [7]). Therefore the TPR domain of PP5 may target the phosphatase domain close to its substrate.

Since human PP5 expressed in *E. coli* exhibited only low levels of phosphatase activity against a variety of conventional substrates [2], we sought to identify mechanisms that might trigger the activation of this enzyme. Here we report that both bacterially expressed human PP5 and native rabbit PP5 can be activated by proteolysis and by the binding of polyunsaturated fatty acids to the TPR domain. The data indicate that the TPR domain shields the active site from entry by protein substrates and that lipids activate PP5 by movement of the TPR domain away from the active site.

2. Materials and methods

2.1. Materials

All compounds were from Sigma unless otherwise stated. Arachidonic acid, arachidonic ethyl ester, arachidonyl acetate, linoleic acid, oleic acid, linolenic acid, sphingosine, were dissolved at concentrations of 10–60 mM in dimethylsulphoxide. Arachidic acid, stearic acid, nonadecanoic acid, cholesterol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and diacylglycerol were dissolved in chloroform at 10–60 mM. Prostaglandin A_2 was supplied in 10 mg/ml methyl acetate solution. Phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, purified from Folch fractions (Sigma) and dissolved at 3–10 mM in chloroform, were gifts from P. Downes and S.J. James (University of Dundee).

2.2. Bacterial expression and purification of PP5, its TPR and phosphatase domains

Construction of plasmids expressing the virtually entire human PP5 from R16 to M499 (HTW2), the TPR domain from R16 to L181 (HTT1), and the catalytic domain from L181 to M499 (HTP1) in the pT7.7 vector and expression of these proteins in *E. coli* has been described previously [2] (Fig. 1). All proteins were insoluble when expressed at 37°C for 3 h. The insoluble proteins HTT1 and HTP1 were isolated from inclusion bodies, purified to homogeneity on SDS polyacrylamide gels as described previously [2], and used to raise antibodies in sheep. Anti-HTT1 and anti-HTP1 antibodies were affinity purified, and detected 1 ng of HTT1 and HTP1, respectively, on an immunoblot. In cell extracts they detected a single PP5 band of 58 kDa. Soluble forms of HTW2 and HTT1 were obtained by expression of the same constructs in *E. coli* at a lower temperature (23°C) for 7 h.

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Cells were lysed as described in [2]. Soluble HTW2 was purified to near homogeneity (Fig. 2) by 50–67% ammonium sulphate fractionation of the cell cytosol, followed by chromatography on S-Sepharose in buffer A (50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 0.03% Brij-35, 0.1% 2-mercaptoethanol, 5% glycerol, 1 mM benzamidine and 0.1 mM phenylmethylsulphonyl fluoride) plus 1 mM $MnCl_2$, with a 0–500 mM NaCl gradient. HTW2 eluted at 310–340 mM NaCl. It was dialysed against buffer B (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1 mM EGTA, 0.03% Brij-35, 0.1% 2-mercaptoethanol, 50% glycerol) plus 1 mM $MnCl_2$ at 4°C overnight, and stored at −20°C. HTT1 was purified by a 17–33% ammonium sulphate fractionation, followed by chromatography on Mono Q in buffer A. HTT1 eluted at 190 mM NaCl. After heating to 95°C for 5 min and centrifugation at 10000×*g*, the supernatant, which contained homogeneous soluble HTT1 (Fig. 2), was dialysed against buffer B at 4°C overnight and stored at −20°C.

2.3. Preparation of catalytically active phosphatase domain, HTP3

180 μl of a 1 mg/ml trypsin solution was added to 2 mg of purified HTW2 in 20 ml in buffer A (without benzamidine and phenylmethylsulphonyl fluoride). After incubation at 30°C for 10 min, 200 μl of 5 mg/ml lima bean trypsin inhibitor was added to stop the reaction. The sample was applied to a Mono Q column in buffer A. A major casein phosphatase activity eluted at 180–200 mM NaCl. SDS polyacrylamide gel electrophoresis of this fraction revealed a protein of 39 kDa, termed HTP3 (Fig. 2), which was dialysed against buffer B at 4°C overnight and stored at −20°C. N-terminal sequence analysis of this protein was performed by Dr. Nick Morrice on an Applied Biosystems 476A sequencer.

2.4. Purification of native PP5 from rabbit liver

18 g rabbit liver was homogenised in 50 ml buffer C (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 1 mM benzamidine, 0.5 mM phenylmethylsulphonyl fluoride) and centrifuged at 15000×*g* for 10 min at 4°C. PP5 was purified 10-fold from the supernatant by a 45–60% ammonium sulphate fractionation. The precipitated protein was dissolved in 60 ml buffer C, and PP5 was further purified 2.5-fold by a 10–15% polyethylene glycol 6000 fractionation. The pellet was dissolved in 4 ml buffer A. 200 μl of this sample was loaded onto a Mono Q column in buffer A, which was developed with a linear gradient of 0–500 mM NaCl. PP5 eluted at 110–125 mM NaCl with a further 20-fold increase in purity. The overall purification of PP5 through these three steps was 500-fold.

2.5. Protein phosphatase assays

Cyclic AMP-dependent protein kinase (PKA, prepared by L.K. MacDougall) was used to phosphorylate partially hydrolysed bovine casein to 3.5 nmol phosphate per mg [8]. Myelin basic protein was phosphorylated by PKA to 1 mol phosphate/mol protein by H.A Snaith as described in [9]. The specific activity of the [γ-^{32}P]ATP used for all phosphorylations was 106 cpm/nmol. Protein phosphatase assays were performed [10] in the absence of added divalent cations and most were in the presence of 0.33 mg/ml bovine serum albumin and 0.01% Brij-35. However, both albumin and detergent significantly lowered the sensitivity of PP5 to fatty acids. Therefore assays in the presence of fatty acids were conducted in the absence of these components, which did not significantly affect the basal activity of the enzyme. The substrate concentrations in the assays were 6 μM for casein and 5 μM for myelin basic protein. Protein concentrations were determined by Coomassie Protein Assay reagent (Pierce, Rockford, IL, USA). One unit of activity is the amount of enzyme which catalyses the release of 1 μmol [^{32}P]phosphate per min.

2.6. Lipid vesicle binding to PP5 and its separate domains

Large sucrose loaded unilamellar vesicles of phosphatidylinositol were prepared by extrusion through polycarbonate membranes, with pores of 100 nm diameter, using a phospholipid extruder (Lipex Biomembranes) by X. Tang (University of Dundee) according to [11]. 30 μl lipid vesicles were mixed with 30 ng protein in 30 μl buffer D comprising 10 mM HEPES pH 7.4, 3.4 mM EDTA, 150 mM NaCl, and incubated on ice for 10 min. Following centrifugation for 30 min at 4°C at 60000 rpm in a TLA100 rotor, 50 μl supernatant was recovered. The pellet was gently washed with ice-cold buffer D, and resuspended in 60 μl water. 7.5 μl samples of the supernatant and pellet fractions were denatured in SDS sample buffer, electrophoresed on a SDS polyacrylamide gel, transferred to nitrocellulose membranes in order to determine the proportion of PP5 in each fraction. Immunoblotting was performed by incubation of the membranes with 1 μg/ml affinity purified sheep anti-HTP1 or anti-HTT1 antibodies in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% (w/v) Tween-20, 10% (w/v) skimmed milk powder (Marvel) for 2 h at room temperature. Antibody binding was detected using 0.2 μg/ml horseradish peroxidase-conjugated anti-sheep antibodies (Pierce), followed by Enhanced Chemiluminescence (Amersham International, UK).

3. Results

3.1. PP5 is activated by proteolytic cleavage of the TPR domain

Human PP5 from amino acid R16 to the C-terminus (M499) was expressed as a soluble protein in *E. coli*, termed HTW2, with the leader sequence MARIAQ (Figs. 1 and 2), and purified to near homogeneity (Section 2.2). HTW2 had only very low phosphatase activity towards casein (14.7 mU/mg) and myelin basic protein (1.2 mU/mg). The phosphatase activity was found to increase substantially after brief incubation of HTW2 with trypsin. This treatment released a proteolytic fragment of approximately 39 kDa, termed HTP3, which was purified on Mono Q (Fig. 2) and shown to possess the phosphatase activity. The N-terminus of the HTP3 had the amino acid sequence SVVD, which is found at position 160–164 in PP5. Since S160 to the C-terminus of PP5 contains the entire catalytic domain and has a calculated molecular size of 38.6 kDa, HTP3 is very likely to comprise amino acids S160–M499. The specific activities of HTP3 towards casein (382 mU/mg) and myelin basic protein (328 mU/mg) were 26- and 260-fold higher respectively than those of HTW2. This suggests that the TPR domain may suppress the phosphatase activity of PP5.

3.2. The activity of bacterially expressed and native PP5 is stimulated by fatty acids

Various compounds were tested for their ability to stimulate the low phosphatase activity of HTW2. These included molecules reported to influence protein phosphatase activity, such as protamine and spermine (reviewed in [12]), ceramide [9], and arachidonic acid [13]. The last mentioned was also recently shown to activate a bovine brain phosphatase that had peptide sequences with similarities to those of human and rat PP5 [14]. Our results show that the polyunsaturated fatty acids, arachidonic acid, linoleic acid, oleic acid (Fig. 3), and linolenic acid stimulated the casein phosphatase activity of HTW2. The half-maximal activating concentrations (AC_{50}) for these molecules were 125, 194, 213, and 270 μM respectively. Arachidonic acid, the most effective activator, stimulated activity 26-fold, similar to the increase in the casein phosphatase activity produced by proteolytic cleavage of the TPR domain from HTW2. Other compounds related to arachidonic acid, including arachidonic ethyl ester and arachidonyl acetate, also stimulated PP5. In contrast, polysaturated lipids including arachidic acid, nonadecanoic acid and stearic acid failed to stimulate the enzyme. Structurally distinct lipids, including ceramide, sphingosine, cholesterol and prostaglandin A_2, and other compounds including spermine, protamine, and histone H1 also failed to cause stimulation. These results suggest a polyunsaturated fatty acid chain is essential for the stimulation of HTW2. The activity of HTW2 towards myelin basic protein was stimulated 57-fold by arachidonic acid.

The casein phosphatase activity of HTW2 was stimulated by several phospholipids, including phosphatidylinositol, phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate, and phosphatidylserine. Eight- and seven-fold stimulation of HTW2 activity was observed in the presence of 330 μM phosphatidylinositol and 220 μM phosphatidylinositol 4-phosphate, the highest concentrations which could be tested. These compounds appear to stimulate activity because they contain polyunsaturated fatty acid chains and an overall negative charge, since HTW2 was insensitive to the related compounds, phosphatidylcholine, phosphatidylethanolamine and diacylglycerol, which possess polyunsaturated fatty acid components, but are uncharged. However, since arachidonic ethyl ester and arachidonyl acetate stimulated HTW2, a net negative charge is not essential.

We next investigated whether the casein phosphatase activity of native PP5 could also be stimulated by fatty acids. PP5 was partially purified from rabbit liver (Section 2.4). Two peaks of casein phosphatase activity that were stimulated by arachidonic acid were detected in the final chromatography on Mono Q (Fig. 4A). The first peak, eluting at 110–125 mM NaCl, contained a 58 kDa protein that cross-reacted with anti-HTT1 and anti-HTP1 antibodies (Fig. 4B) and was therefore PP5. The basal phosphatase activity in this peak was inhibited by okadaic acid with IC_{50} value of approximately 3 nM, similar to that measured for HTW2 [2]. Other properties of native PP5 were also very similar to the expressed enzyme. For example, the casein phosphatase activity was stimulated when the enzyme was briefly digested with trypsin (data not shown). The nature of the second peak of arachidonic acid stimulated phosphatase activity eluting from Mono Q (fractions 11–13) is not known. Although it was inhibited by 2 nM okadaic acid, it does not appear to be a closely related isoform of PP5 since it showed no cross reaction with anti-HTT1 or anti-HTP1 antibodies.

3.3. The TPR domain of PP5 binds to lipids

Unlike the full length PP5, the catalytic domain, HTP3, was unaffected by arachidonic acid, linoleic acid or oleic acid (data not shown). This suggests that fatty acids may stimulate PP5 by interacting with the TPR domain. Since fatty acids form micelles in solution, whereas phosphatidylinositol will form bilayer lipid vesicles, we used the latter to examine which region of PP5 bound to lipids. We tested whether HTW2, HTP3, and/or HTT1 could bind to vesicles prepared from phosphatidylinositol [11,15] by incubation of these proteins with the lipid vesicles, followed by centrifugation to pellet the vesicles, and examination of supernatant and pellet fractions by gel electrophoresis as described in Section 2.6. The experiments showed that both HTW2 and HTT1, but not HTP3, pelleted reproducibly with phosphatidylinositol vesicles (Fig. 5), demonstrating that the TPR domain is the region of PP5 that interacts with the fatty acid moiety of the lipid.

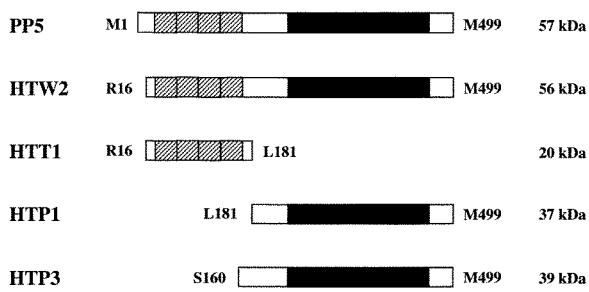

Fig. 1. Human protein phosphatase 5 (PP5) and fragments derived from it by expression in *E. coli* or by proteolysis of the expressed enzyme. The human PP5 sequence (amino acids E7–M499) is described in GenEMBL data base accession number X89416 and [2]. Numbering of the amino acids is by comparison with the rat PP5 [6] which is 98% identical to the human PP5 and has the sequence MAMAEG prior to E7. Bacterially expressed HTW2 is amino acids R16–M499 of human PP5 with the sequence MARIRA preceding R16. HTT1 is the N-terminal TPR domain from R16–L181 with the same leader sequence and terminating with amino acids LGMM after L181. HTP1 is the phosphatase domain from L181–M499 with the leader sequence MARIRAQ. HTP3 is the catalytically active phosphatase domain starting at S160, which was produced by cleavage of HTW2 with trypsin. Hatched boxes denote the tetratricopeptide repeats and filled bars represent the phosphatase domain.

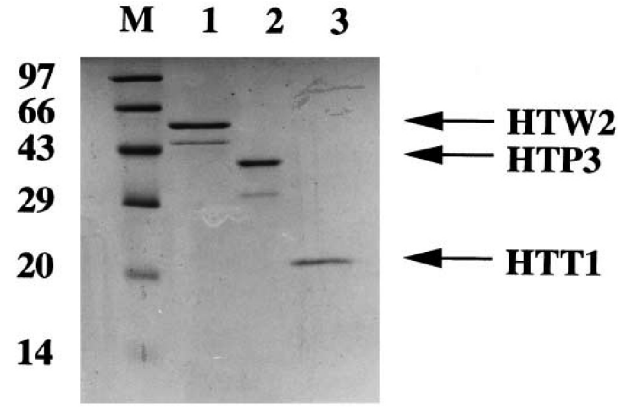

Fig. 2. Purified bacterially expressed human PP5, phosphatase and TPR domain fragments. Approximately 1 μg of each protein was electrophoresed in a 15% SDS polyacrylamide gel and stained with Coomassie Blue. Lane (M) contains molecular mass markers glycogen phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa), α-lactalbumin (14 kDa). Lanes (1) HTW2, (2) HTP3, and (3) HTT1.

4. Discussion

4.1. The TPR domain prevents access of substrates to the active site of PP5

Bacterially expressed (HTW2) and partially purified rabbit liver PP5 had very low phosphatase activity towards all substrates tested, including phosphorylase [2], casein and myelin basic protein. Proteolytic cleavage of the TPR domain stimulated both the expressed and native PP5 towards casein and myelin basic protein by 26- and 260-fold, respectively. It is therefore likely that the TPR domain acts as a shield to prevent access of large substrates to the active site of the phosphatase domain, while permitting entry of small molecules. The very low basal phosphatase activity may indicate that the active site is not completely shielded, but a more likely explanation is that slight proteolytic cleavage gives rise to trace amounts of the fully active phosphatase domain in the HTW2 and PP5 preparations.

4.2. Binding of polyunsaturated fatty acids to the TPR domain allosterically activates PP5

Out of a wide range of substances tested, only polyunsaturated fatty acids (arachidonic acid, linoleic acid, oleic acid) and their esters and negatively charged lipids containing ara-

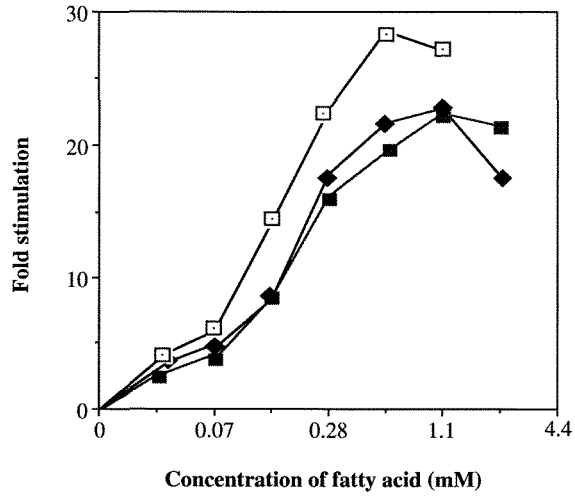

Fig. 3. Activation of bacterially expressed human PP5 (HTW2) by fatty acids. The abscissa is the concentration of the fatty acids and the ordinate is the fold stimulation by fatty acids of the basal casein phosphatase activity, which was measured in the absence of fatty acids. Open squares: arachidonic acid. Filled diamonds: linoleic acid. Filled squares: oleic acid.

chidonic acid, such as phosphatidylinositol, activated bacterially expressed and native PP5. The finding that the interaction of phosphatidylinositol was with the TPR domain, and not the phosphatase domain, demonstrated that the fatty acid component does not directly influence the active site, but does so through binding to the TPR domain. Since arachidonic acid stimulated the casein phosphatase activity of the expressed PP5 by a similar magnitude to that observed on proteolytic cleavage of the TPR domain (26-fold), fatty acid stimulation and proteolytic activation may be occurring by similar mechanisms. Binding of fatty acid to the TPR domain may lead to exposure of the active site by movement of the TPR domain to such a degree as to allow access for protein substrates. This is supported by the fact that inclusion of a 10-fold molar excess of the TPR domain did not affect the casein phosphatase activity of HTW2 or the phosphatase domain, HTP3, in the presence or absence of arachidonic acid (data not shown), demonstrating that lipid-bound TPR domain is not directly stimulating the enzyme. The lower stimulation of HTW2 by arachidonic acid (57-fold) compared to proteolysis (260-fold) when myelin basic protein was the substrate may be due to the binding of the acidic lipids to the basic protein substrate.

Although we have shown here that allosteric activation of native and expressed PP5 by lipids containing polyunsaturated fatty acids occurs in vitro, it is not known whether the activation is of physiological significance. The AC_{50} for stimulation for any compound tested is >100 μM, which is higher than would be expected for PP5 to be regulated by changes in lipid concentrations in vivo. However, it is possible that other molecules or particular subcellular locations may lower the AC_{50} and/or make the response specific to particular lipids. Arachidonic acid has been shown to inhibit the myosin light chain phosphatase activity of PP1 in smooth muscles, with an IC_{50} of 60 μM. The effect of arachidonic acid on PP1 might be somewhat analogous to its effect on PP5, in that it causes dissociation of the M-complex from the PP1 catalytic subunit, which results in activation of the phosphorylase phosphatase activity as well as inhibition of the myosin light chain phosphatase activity [13]. Arachidonic acid is also known to increase in response to signals that activate phospholipase A_2.

TPR domains are thought to provide sites for protein-protein interactions. If PP5 contacts other protein(s) via the TPR domain and is also stimulated by lipids, a membrane location would be an ideal situation for PP5. In this respect it is interesting that PP5 was found to interact with the guanylate cyclase receptor [4]. In addition we have obtained preliminary evidence that PP5 binds to proteins in a subcellular membrane fraction examined by gel electrophoresis (data not shown).

Other protein domains are known which have been found to interact with lipids, for example some PH domains interact with phosphatidylinositol 4,5-bisphosphate [16]. The C_1 domain found in protein kinase C and other proteins binds diacylglycerol and some forms of protein kinase C are also reported to be activated by arachidonic acid [17]. It will be interesting to know if lipid binding is a property of all or a subset of TPR domains.

4.3. *The TPR domain may be required for folding of the phosphatase domain*

Although HTW2 (R16–M499) and the proteolytic fragment HTP3 (S160–M499) possess phosphatase activity indicating

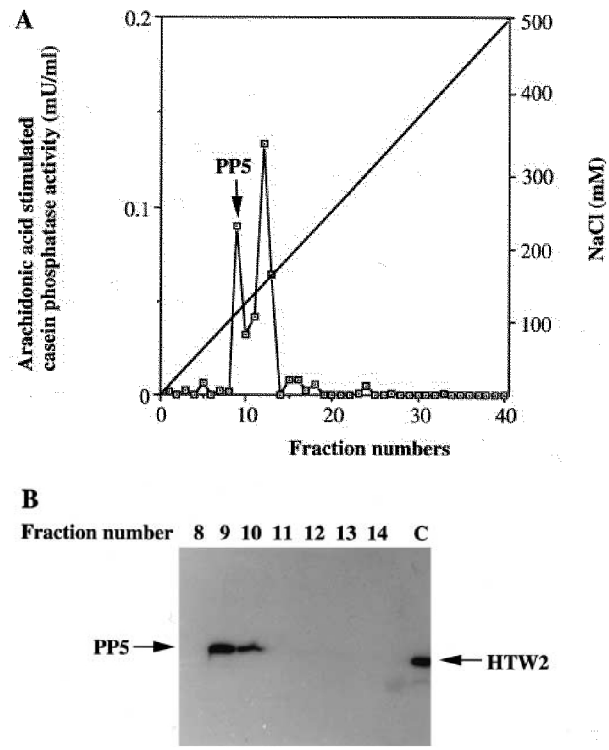

Fig. 4. Arachidonic acid stimulation of native rabbit liver PP5 casein phosphatase activity. Partially purified PP5 was applied to a Mono Q column and a linear 0–500 mM NaCl gradient was used to elute 40 fractions which were assayed for casein phosphatase activity in the presence and absence of 550 μM arachidonic acid. The ordinate shows the difference between casein phosphatase activity in the presence and absence of arachidonic acid and the abscissa the fraction number. Two peaks of arachidonic acid stimulated casein phosphatase activity are detected with maxima at fractions 9 and 12. B: Immunoblot of fractions 8–14 with anti-HTP1 antibodies. PP5 (57 kDa) was present in fractions 9 and 10. Lane C, HTW2 control (56 kDa).

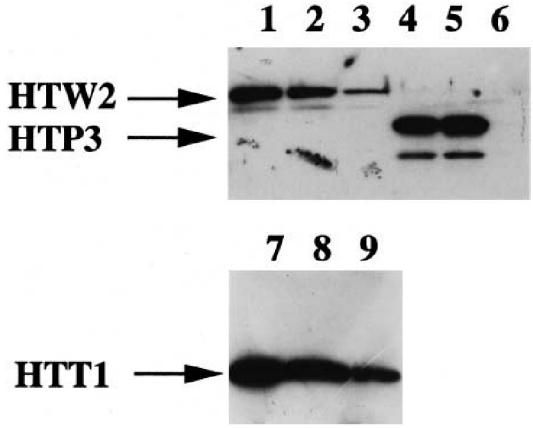

Fig. 5. The TPR domain of PP5 binds to phosphatidylinositol vesicles. HTW2 (comprising both the TPR and phosphatase domains of PP5), HTP3 (the phosphatase domain) and HTT1 (the TPR domain) were incubated with unilamellar vesicles of phosphatidylinositol. Lipid-bound and free protein were separated as described in Section 2. Samples were electrophoresed on a SDS polyacrylamide gel, transferred to nitrocellulose and probed with affinity purified anti-HTP1 antibodies (lanes 1–6) and affinity purified anti-HTT1 antibodies (lanes 7–9). Lanes 1, 2 and 3, HTW2; 4, 5 and 6, HTP3; 7, 8 and 9: HTT1. Lanes 1, 4 and 7, total protein; 2, 5 and 8, 60 000×*g* supernatant; 3, 6 and 9, 60 000×*g* pellet of phosphatidylinositol vesicles.

that they are folded correctly, the bacterially expressed fragment, HTP1 (see Section 2.2), comprising L181–M499 exhibited no measurable phosphatase activity and, as one might expect, could not be activated by proteolysis or arachidonic acid (data not shown). HTP1 is only 21 residues shorter at the N-terminus than HTP3 and therefore, either these 21 residues or the TPR domain must be important for the folding of the protein into the correct conformation. Since the number of amino acids preceding the catalytic domain in HTP1 is the same as that in PP1 and longer than that in some other phosphatases such as PP2A, it appears more likely that the TPR domain will play a role in folding PP5. Fukuda et al. [5] have recently shown that bacterially expressed PP5 comprising M107–M499 possesses phosphorylase phosphatase and histone H1 phosphatase activity although the specific activities were not reported. Since M107–M499 contains most of the third repeat and all of the fourth repeat in the TPR domain, it appears that this section of the TPR domain will suffice for sufficiently correct folding of PP5 to yield activity.

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