Identification of Potential Physiological Activators of Protein Phosphatase 5†

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ABSTRACT: The protein serine/threonine phosphatase designated PP5 has little basal activity, and physiological activators of the enzyme have never been identified. Purified PP5 can, however, be activated by partial proteolysis or by the binding of supraphysiological concentrations of polyunsaturated long-chain fatty acids to its tetratricopeptide repeat (TPR) domain. To test whether activation of PP5 by polyunsaturated but not saturated fatty acids was an artifact of the lower solubility of saturated fatty acids, the effects of fatty acyl-CoA esters were examined. Saturated and unsaturated long-chain fatty acids are both freely water-soluble when esterified to CoA. Long-chain fatty acyl-CoA esters activated PP5 at physiological concentrations, with the saturated compounds being more effective. We investigated the effects of chain length and of the CoA moiety on PP5 activation. Chains of 16 carbons or more were required for optimal activation, with no activation observed below 10 carbons. On the basis of competition studies using acetyl-CoA, the function of the CoA moiety appeared to be to increase solubility of the fatty acyl moiety rather than to interact with a specific binding site. These data suggested that long-chain fatty acid-CoA esters might be physiological activators of PP5 and point to a potential link between fatty acid metabolism and signal transduction via this enzyme. Because heat shock protein 90 is also known to bind to the TPR domain of PP5 via its C-terminal domain (C90), we investigated its effect on PP5 activity. C90 activated the enzyme approximately 10-fold. Thus, we have identified two potential physiological activators of PP5.

PP5[1] is a protein serine/threonine phosphatase possessing distinctive structural and enzymatic properties. Related phosphatases typically contain both a catalytic subunit and regulatory subunits responsible for controlling their enzymatic activity, substrate specificity, and subcellular targeting (*1*). PP5 consists of a single polypeptide chain containing a phosphatase catalytic domain near its C-terminus and a regulatory tetratricopeptide repeat (TPR) domain near its N-terminus (*2*−*4*). TPR domains mediate protein−protein interactions (*5*), and there is abundant evidence that the TPR domain of PP5 targets it to other proteins, including heat shock protein 90 (hsp90) (*6*), ASK1 (*7*), the atrial natriuretic peptide receptor (*2*), the anaphase-promoting complex (*8*), the cryptochromes (*9*), and PP2A (*10*). PP5 has unusually low phosphatase activity in vitro (*11*−*13*), and two central problems in the study of this enzyme are to determine under what circumstances it may be activated and toward what substrates.

On the basis of its interactions with other proteins and of studies in which PP5 activity was inhibited using recombinant DNA approaches, potential biological roles for PP5 have begun to emerge (reviewed in ref *14*). PP5 has been suggested to modulate glucocorticoid receptor signaling (*6*, *15*), to promote cell growth (*16*, *17*), and to terminate responses to oxidative stress (*7*). Initially described as a nuclear enzyme (*3*), most subsequent reports describe PP5 as being present in the cytoplasm (*7*, *8*, *18*−*22*). Despite progress in identifying putative biological functions of PP5, our understanding of its biochemical regulation has remained limited during the 8 years since the discovery of this enzyme.

Two observations have yielded preliminary insight into the mechanisms of PP5 regulation. First, PP5 can be activated by partial proteolysis (*12*, *13*). Removal of the TPR domain by treatment with trypsin or other proteases led to activation of the catalytic domain of PP5 (*12*, *13*). Thus, the TPR domain of PP5 has an autoinhibitory function. An additional autoinhibitory sequence was identified at the extreme C-terminus of the enzyme (*13*, *23*). Activation of PP5 in vivo, however, presumably does not occur through proteolysis. PP5 can also be activated in vitro by high micromolar concentrations of polyunsaturated fatty acids (*11*, *12*). Arachidonic acid was one of the most effective of these compounds, while saturated fatty acids were unable to activate PP5 (*11*, *12*). Fatty acids bound to the TPR domain (*12*, *13*) and presumably regulate PP5 by inducing a conformational change in the enzyme. Unfortunately, the concentrations of arachidonic acid reported to activate PP5 in vitro are much higher than those found in the cell, so that this compound is not thought to be a physiological activator. The published experiments, however, raised the possibility that compounds related to polyunsaturated fatty acids might be physiological activators of PP5.

We have now pursued this question further. One class of related compounds already known to play a role in signal

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[1] Abbreviations: PP, protein phosphatase; TPR, tetratricopeptide repeat; hsp, heat shock protein; ASK1, apoptosis signal-regulating kinase 1; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pNPP, *p*-nitrophenyl phosphate; pNP, *p*-nitrophenol; C90, hsp90 C-terminal domain; Hop, hsp organizing protein.

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transduction is the long-chain fatty acyl-CoA esters (*24*−*26*). In the studies described here, we show that physiological concentrations of these compounds fully activate PP5, suggesting that they may be in vivo activators of this enzyme. We also demonstrate that binding of the C-terminus of hsp90, which interacts with the TPR domain of PP5, results in activation of the enzyme.

EXPERIMENTAL PROCEDURES

Purification of Recombinant PP5. A 3.6 L LB culture of BL21(DE3) cells containing pET15-PP5 (*23*) was incubated for 5 h at 22 °C with 60 μg/mL ampicillin and 1 mM isopropyl β-D-thiogalactopyranoside. Cells were then harvested by centrifugation and stored at −20 °C. The following procedures were performed at 4 °C or on ice. Cells were incubated for 30 min, with occasional stirring, in 35 mL of 20 mM Tris, pH 8.0, 0.5 M NaCl, and 5 mM imidazole (buffer A) containing 0.5 mg/mL lysozyme, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 20 mM benzamidine, and 0.1 mM phenylmethanesulfonyl fluoride. The sample was then sonicated and centrifuged for 1 h at 18000*g*. Streptomycin sulfate was added to the supernatant to a final concentration of 2%, and the sample was left on ice for 20 min with occasional stirring before centrifugation at 18000*g* for 15 min. The supernatant was loaded onto a 5 mL HiTrap chelating column (Amersham Pharmacia Biotech) preequilibrated with buffer A. After extensive washing with buffer A, His-tagged PP5 was eluted using a 30−220 mM gradient of imidazole in buffer A. In initial experiments, fractions containing PP5 were combined and dialyzed against 50 mM Hepes, pH 6.8, and 20 mM NaCl (buffer B) and loaded onto a Mono S column (Amersham Pharmacia Biotech) equilibrated with buffer B. PP5 was eluted using a 20−500 mM NaCl gradient in buffer B. Fractions containing essentially pure PP5 were combined and dialyzed against 50 mM Hepes, pH 7.4, and 100 mM NaCl (buffer C). The purified enzyme was then stored at −80 °C and used in the experiments shown in Figures 1 and 2. The gain in purity during the Mono S step was minimal, however, and this step was omitted in subsequent purifications. In those purifications, fractions eluted from the HiTrap chelating column that contained PP5 were combined, dialyzed against buffer C, and stored at −80 °C until needed.

Purification of Native hsp90. A pig liver was homogenized in 40 mM Tris, pH 7.5, 40 mM KCl, 4 mM EDTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 0.1 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 2000*g* for 20 min and then at 22000*g* for 1 h. hsp90 was then purified from the supernatant using the method of Sullivan et al. (*27*). However, the isolated hsp90 contained trace amounts of phosphatase activity at this point and was further purified. The sample was adjusted to contain 1 M ammonium sulfate and loaded onto a high-performance phenyl-Sepharose column (Amersham Pharmacia Biotech) preequilibrated with 1 M ammonium sulfate in 50 mM Hepes, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.5. Elution was then performed using a 1.0−0 M ammonium sulfate gradient in 50 mM Hepes, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.5. The purified hsp90 was then concentrated by ultrafiltration, dialyzed against 50 mM Hepes and 100 mM NaCl, pH 7.4, and stored at −80 °C. Approximately 14 mg of purified hsp90 was recovered.

Purification of the C-Terminus of hsp90 (C90). Bacterially expressed C90 was purified as previously described (*28*). The purified protein was dialyzed against 50 mM Hepes and 100 mM NaCl, pH 7.4, prior to use in phosphatase assays.

Determination of the pH Optimum. A modified version of the end point method of Skinner et al. (*11*) was used. One microgram of PP5 was added to 200 mM pNPP, 50 mM Tris, pH 7.1−9.0, 1 mM EDTA, 1 mM EGTA, 0.1% β-mercaptoethanol, and 0.1% ethanol in a final volume of 100 μL. The reaction was allowed to proceed at 30 °C for 15 min and terminated by addition of 900 μL of 0.25 M NaOH. Absorption at 410 nm was used to monitor pNP production. Control reactions lacking PP5 were used to correct for nonenzymatic pNPP hydrolysis. A molecular absorption coefficient of 17.8×10^3 mol of pNP L^{-1} cm^{-1} at 410 nm was used for samples containing NaOH.

Determination of Optimal Divalent Metal Ion Concentrations. Cuvettes containing 100 mM pNPP in 100 mM Tris, pH 8.0, and the indicated concentrations of $MnCl_2$ or $MgSO_4$ were allowed to equilibrate for 5 min at 30 °C. PP5 (2.7 μg) was then added in a final volume of 1 mL, and absorbance at 410 nm was monitored for 10 min. The initial, linear portion of the slope was used to determine the rate of pNP production. Control reactions lacking PP5 were used to correct for nonenzymatic pNPP hydrolysis. A molecular absorption coefficient of 15.1×10^3 mol of pNP L^{-1} cm^{-1} was used to calculate pNP concentration.

Effects of Lipids on PP5 Activity. Cuvettes containing 50 mM pNPP, divalent metal ions, and the indicated compounds in 100 mM Tris, pH 8.0, were allowed to equilibrate at 30 °C. Except where otherwise indicated, 0.5 mM $MnCl_2$ was present as the divalent cation. PP5 (0.6 μg) was then added in a final volume of 1 mL, and the production of pNP was followed by monitoring the increase in absorbance at 410 nm for 10 min. When present, the concentration of $MgSO_4$ was 25 mM. Activity was expressed as moles of pNP produced per mole of PP5 per second, calculating pNP concentration as described above using a molecular absorption coefficient of 15.1×10^3 mol of pNP L^{-1} cm^{-1}.

Kinetic Parameters of PP5 in the Absence and Presence of Arachidonoyl-CoA. Cuvettes containing 10−200 mM pNPP, 0.5 mM $MnCl_2$, and, where appropriate, 20 μM arachidonoyl-CoA in 100 mM Tris, pH 8.0, were allowed to equilibrate for 5 min at 30 °C. PP5 was added in a final volume of 1 mL, and absorbance at 420 nm was monitored for 10 min. A molecular absorption coefficient at 420 nM of 12.3×10^3 mol of pNP L^{-1} cm^{-1} was used to calculate pNP concentration.

RESULTS

Optimization of PP5 Assay Conditions. For reasons that are unclear, previous studies of the activation of PP5 by fatty acids have employed somewhat unusual assay conditions. Although related phosphatases have optimal activity in the presence of added divalent cations, published studies of PP5 activation by fatty acids have been performed in the absence of added divalent cations (*11*−*13*). We wondered whether this might be due to interference of divalent cations with PP5 activation by fatty acids. The effects of Mg^{2+} and Mn^{2+} on PP5 activity were tested to optimize assay conditions for examining this question. We first varied the pH for the assay

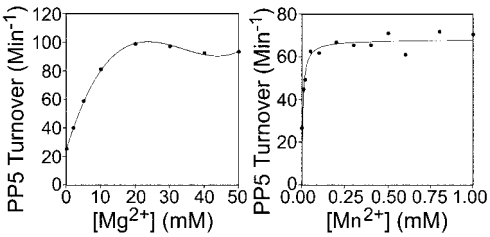

FIGURE 1: Effects of Mg^{2+} and Mn^{2+} on PP5 activity. Hydrolysis of pNPP by PP5 was measured in a continuous spectrophotometric assay at pH 8.0 in the presence of the indicated concentrations of $MnCl_2$ or $MgSO_4$, as described under Experimental Procedures. Each point on the curves shown represents the reaction rate calculated from the linear portion of a reaction in which approximately 40 data points were collected.

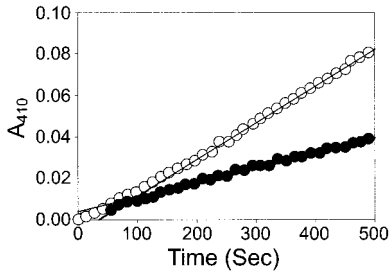

FIGURE 2: Time course of PP5 activity in the absence or presence of Mn^{2+}. Hydrolysis of pNPP by PP5 was measured in a continuous spectrophotometric assay at pH 8.0 in the absence (filled circles) or presence (open circles) of 0.5 mM $MnCl_2$, as described under Experimental Procedures. The data indicate that the reaction in the presence of Mn^{2+} becomes linear only after a 200 s lag.

with an optimum observed at approximately pH 8.0 (data not shown). All subsequent assays were performed at this pH. As shown in Figure 1, Mn^{2+} activated the enzyme at much lower concentrations than Mg^{2+}, but the maximal activity observed with Mg^{2+} was greater than that observed with Mn^{2+}. As expected, however, PP5 activity was elevated in the presence of either divalent cation.

The use of pNPP as a substrate allowed the continuous spectrophotometric assay of PP5 activity. In the analysis of subsequent PP5 assays (below), it was necessary to take into account that, in the presence of metal ions, a significant lag was observed before the maximal rate of pNPP hydrolysis was achieved (Figure 2). In the presence of Mn^{2+}, the onset of the linear phase of pNPP hydrolysis occurred approximately 200 s after the addition of PP5 to the assay. It has been suggested that, in its basal state, the active site of PP5 is relatively inaccessible to solvent (*12*, *13*). The observed lag is consistent with this hypothesis, probably being due to the gradual diffusion of metal ion into the active site.

Effect of Divalent Cations on the Activation of PP5 by Arachidonic Acid. PP5 has been shown to be activated by a variety of unsaturated long-chain fatty acids, and lipids containing such fatty acids, with arachidonic acid being the most potent activator (*11*, *12*). We examined the activation of PP5 by arachidonic acid in the presence of Mn^{2+} (Figure 3). Addition of arachidonic acid to the assay removed the initial lag of pNP production observed in the presence of divalent cations. This observation is consistent with the hypothesis that lipid activators induce a conformational change that renders the active site more solvent-accessible. However, arachidonic acid addition resulted in the rate of

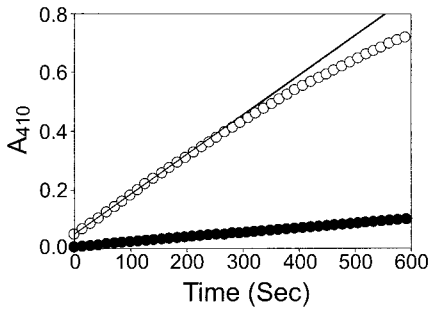

FIGURE 3: Nonlinearity of PP5 assays containing Mn^{2+} and arachidonic acid. Hydrolysis of pNPP by PP5 was measured in a continuous spectrophotometric assay at pH 8.0 containing 0.5 mM $MnCl_2$ in the absence (filled circles) or presence (open circles) of 25 μM arachidonic acid, as described under Experimental Procedures.

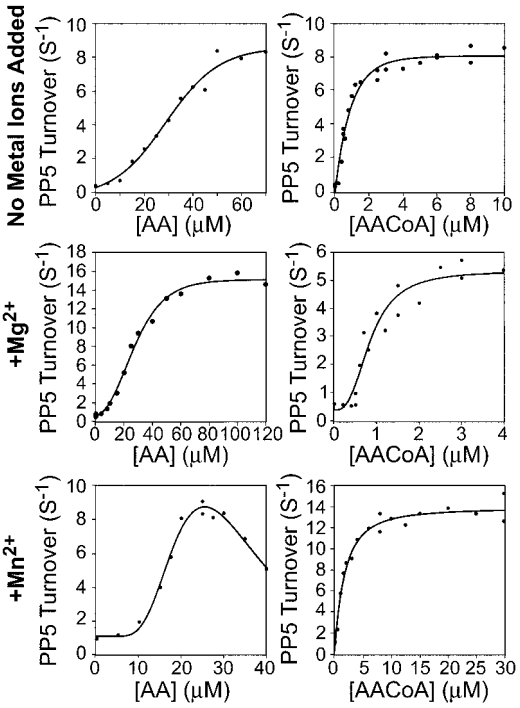

FIGURE 4: Effects of Mg^{2+} and Mn^{2+} on PP5 activation by arachidonic acid (AA) or arachidonoyl-CoA (AACoA). As described under Experimental Procedures, hydrolysis of pNPP was measured in a continuous spectrophotometric assay at pH 8.0 in the presence of the indicated concentrations of activators and in the absence or presence of 25 mM $MgSO_4$ or 0.5 mM $MnCl_2$. Each point on the curves shown represents the reaction rate calculated from the linear portion of a reaction in which approximately 40 data points were collected.

pNP production becoming nonlinear shortly after PP5 addition (Figure 3). The length of the initial linear phase decreased as the arachidonic acid concentration increased.

While arachidonic acid activated PP5 in the absence or presence of Mg^{2+} or Mn^{2+} (Figure 4, left panel), differences were observed. In the absence of added metal ions, PP5 was stimulated half-maximally by approximately 30 μM arachidonic acid, a somewhat lower concentration than previously reported (*11*, *12*) but still higher than would normally be encountered in living cells. In the presence of Mn^{2+}, the interaction between arachidonic acid and PP5 was complex, with phosphatase activity decreasing as arachidonic acid levels rose above 25 μM. This may be partly due to the limited solubility of arachidonic acid under these conditions.

Table 1: PP5 Activation by Long-Chain Fatty Acid and Long-Chain Fatty Acyl-CoA Esters in the Absence or Presence of Divalent Cations[a]

activator	metal added	maximum rate ($M\ M^{-1}\ s^{-1}$)	AC_{50} (μM)
arachidonic acid	none	8.4	29.8
arachidonic acid	Mg^{2+}	15.1	26.8
arachidonic acid	Mn^{2+}	8.8	15.7
arachidonoyl-CoA	none	8.1	0.8
arachidonoyl-CoA	Mg^{2+}	5.3	0.7
arachidonoyl-CoA	Mn^{2+}	13.6	1.6
linoleic acid	Mn^{2+}	10.4	17.6
linoleoyl-CoA	Mn^{2+}	13.7	2.5

[a] One milliliter reaction mixtures containing 50 mM pNPP in 100 mM Tris, pH 8.0, in the absence or presence of 25 mM $MgSO_4$ or 0.5 mM $MnCl_2$ and the indicated lipids were allowed to equilibrate for 5 min at 30 °C before the addition of 0.6 μg of PP5 to initiate the reaction. pNP production was determined by measuring the sample's absorption at 410 nm. A control experiment lacking enzyme was performed for each test sample and used to correct for nonenzymatic hydrolysis of pNPP.

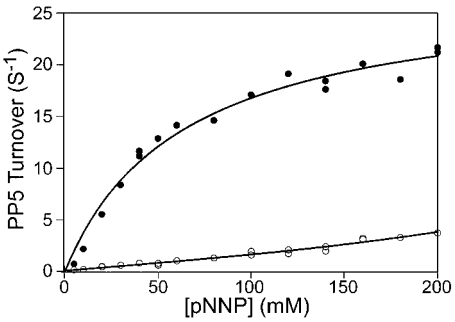

FIGURE 5: Saturation curve of PP5 activation by arachidonoyl-CoA. Hydrolysis of pNPP by PP5 was measured in a continuous spectrophotometric assay at pH 8.0 in the presence of 0.5 mM $MnCl_2$ and in the absence or presence of 20 μM arachidonoyl-CoA, as described under Experimental Procedures. Reactions were initiated by the addition of 1.4 μg of PP5 to the sample lacking arachidonoyl-CoA (open circles) or by the addition of 0.28 μg of PP5 to the sample containing arachidonoyl-CoA (filled circles). Each point on the curves represents the reaction rate calculated from the linear portion of a reaction in which approximately 40 data points were collected.

A simple saturation curve was observed when arachidonic acid was added to PP5 assays containing Mg^{2+} (Figure 4, left panel). Table 1 indicates that the addition of Mg^{2+} to assays containing saturating levels of arachidonic acid resulted in an increase in the maximum rate of reaction.

Activation of PP5 by Fatty Acyl-CoA Esters. Difficulty was encountered in accurately measuring the rate of pNPP hydrolysis in the presence of high concentrations of arachidonic acid. We hypothesized that the observed lack of linearity might reflect the limited aqueous solubility of long-chain fatty acids. We tested this hypothesis by examining the activation of PP5 by fatty acyl-CoA esters. The addition of the CoA moiety to the fatty acyl group results in a large increase in aqueous solubility. To determine optimal assay conditions, we examined the effects of arachidonoyl-CoA on PP5 activity in the absence or presence of Mg^{2+} or Mn^{2+} (Figure 4, right panel). To our surprise, arachidonoyl-CoA activated PP5 much more efficiently than free arachidonic acid. The AC_{50} for arachidonoyl-CoA was less than 1 μM, well within the physiological range for long-chain fatty acyl-CoA esters (*29*). This was much lower than the AC_{50} of approximately 30 μM observed with arachidonic acid. This observation was both unexpected and exciting: we initiated studies of CoA esters to overcome problems associated with the limited solubility of long-chain fatty acids. In doing so, we inadvertently identified a potential physiological regulator of PP5. However, arachidonoyl-CoA comprises only a small component of the cellular fatty acyl-CoA pool. We therefore examined the abilities of linoleoyl-CoA and linoleic acid, compounds that play major roles in long-chain fatty acid metabolism, to activate PP5. Again, the CoA ester activated PP5 at significantly lower concentrations than the corresponding fatty acid (Table 1).

In the absence of added divalent cations, arachidonic acid and arachidonoyl-CoA produced similar values for maximal activation, suggesting that the two compounds produced similar conformational changes in PP5. This is not surprising, given their related structures. The presence of Mn^{2+} or Mg^{2+} was again a complicating factor in the development of assay conditions. In the presence of Mg^{2+}, a saturation curve for arachidonoyl-CoA activation could not be obtained, due to reaction profiles becoming nonlinear at relatively low arachidonoyl-CoA concentrations (Figure 4, right panel, center). However, a simple hyperbolic saturation curve for PP5 activation by arachidonoyl-CoA was obtained in the presence of Mn^{2+} (Figure 4, right panel, bottom). Consequently, subsequent assays were performed in the presence of 0.5 mM Mn^{2+}. These results are summarized in Table 1.

Kinetic Parameters of Activated PP5. Figure 5 shows that, in the presence of arachidonoyl-CoA, PP5 demonstrates the standard hyperbolic relationship between substrate concentration and enzyme activity. A K_m value of 62.8 mM was obtained from this plot, together with a maximum rate of enzyme turnover of 27.3 mol of pNP produced per mole of PP5 per second. A hyperbolic plot could not be obtained for the PP5-catalyzed reaction in the absence of arachidonoyl-CoA (Figure 5). This is consistent with diffusion of the substrate into the enzyme's active site being the rate-limiting step of pNPP hydrolysis in the absence of a lipid activator.

Effects of Chain Length and Saturation on Activation of PP5 by Long-Chain CoA Compounds. Previous reports indicated that polyunsaturated but not saturated fatty acids were able to activate PP5 (*11*, *12*). As shown in Table 2, PP5 was activated by both saturated and unsaturated long-chain acyl-CoA esters. Surprisingly, CoA esters possessing saturated acyl groups were better activators than their unsaturated counterparts. These data support the hypothesis that the previous observations were artifacts of the low aqueous solubility of saturated fatty acids.

A previous investigation had also demonstrated that the length of the acyl group was an important factor in the ability of fatty acids to activate PP5 (*11*). Consequently, we systematically examined the effect of acyl chain length on the ability of CoA esters to activate PP5 (Table 2). The data indicated that CoA esters with acyl chain lengths of 16−20 carbons have broadly similar saturation profiles. Reducing the acyl chain length to below 16 carbons resulted in a severalfold increase in the concentration required to produce 50% activation. CoA esters with acyl groups of below 10 carbons were unable to activate PP5. It is notable that CoA esters with acyl chains of between C12 and C20 produced

Table 2: Effects of Chain Length and Saturation on the Activation of PP5 by Acyl CoA Esters[a]

activator	acyl chain	maximum rate ($M\ M^{-1}\ s^{-1}$)	AC_{50} (μM)
acetyl-CoA	C2:0	no activation	
n-butyryl-CoA	C4:0	no activation	
n-hexanoyl-CoA	C6:0	no activation	
n-octanoyl-CoA	C8:0	no activation	
n-decanoyl-CoA	C10:0	saturation not achieved	
lauroyl-CoA	C12:0	16.04 ± 0.59	18.2 ± 2.4
myristoyl-CoA	C14:0	17.02 ± 0.37	7.17 ± 0.64
myristoleoyl-CoA	C14:1	15.33 ± 0.44	18.5 ± 1.2
palmitoyl-CoA	C16:0	15.91 ± 0.99	2.35 ± 0.23
palmitoleoyl-CoA	C16:1	14.66 ± 1.02	3.37 ± 0.12
stearoyl-CoA	C18:0	15.54 ± 0.29	1.89 ± 0.11
linoleoyl-CoA	C18:2	17.88 ± 1.04	3.36 ± 0.17
arachidoyl-CoA	C20:0	14.32 ± 0.80	0.76 ± 0.04
arachidonoyl-CoA	C20:4	18.63 ± 0.26	2.61 ± 0.16

[a] One milliliter reaction mixtures containing 0.5 mM $MnCl_2$ were analyzed as described in the legend to Table 1. Standard error values were determined using duplicate assays performed on each of two enzyme samples. PP5 turnover in the presence of 100 μM *n*-decanoyl-CoA was 4.72 $M\ M^{-1}\ s^{-1}$.

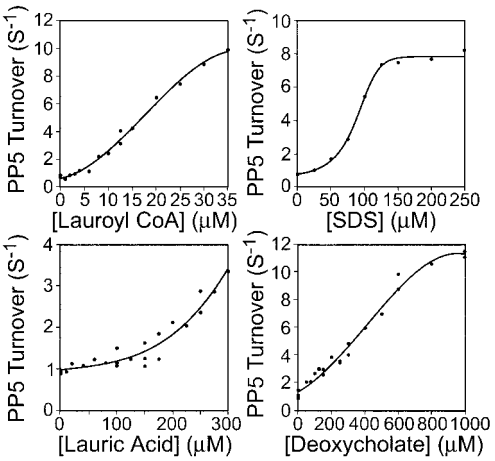

FIGURE 6: Activation of PP5 by lauric acid derivatives. Activation of PP5 by the indicated compounds was measured as described in the Figure 4 legend and under Experimental Procedures.

similar maximum activation values despite large differences in the concentrations required to activate (Table 2).

Investigation of Other Factors Underlying Acyl-CoA Activation of PP5. We used C12:0 compounds to examine other factors affecting activation of PP5. As previously reported by Skinner et al. (*11*), lauric acid had only a limited ability to stimulate PP5 (Figure 6). For this reason, a saturation profile of lauric acid activation could not be obtained. The activation profile of lauric acid was similar to that of deoxycholic acid (Figure 6), suggesting that lauric acid may behave as a detergent, activating PP5 in a nonspecific manner. Several other C12 compounds were tested to determine whether structural modifications resulted in greater activity. The plant hormone traumatic acid (12:1; two carboxy groups), 1,12-dodecanedioic acid (12:0; two carboxy groups), and 5-dodecenoic acid (C12:1) all failed to affect PP5 activity (data not shown). The CoA moiety alone was not sufficient for PP5 activation, as acetyl-CoA did not stimulate PP5 activity (data not shown). SDS was the only lauric acid derivative tested, other than lauroyl-CoA, that significantly activated PP5. The concentration of SDS

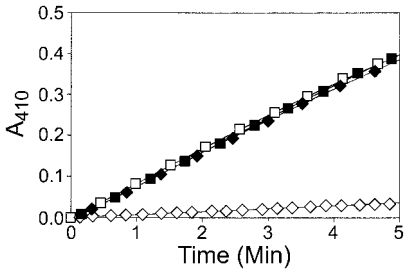

FIGURE 7: Effect of excess acetyl- or malonyl-CoA on PP5 activation by palmitoleoyl-CoA. PP5 (0.5 μg/mL) was added to assays containing 4 μM palmitoleoyl-CoA (open squares), 4 μM palmitoleoyl-CoA plus 400 μM acetyl-CoA (filled diamonds), and 4 μM palmitoleoyl-CoA plus 400 μM malonyl-CoA (filled squares) or to assays containing no CoA esters (open diamonds). Reaction mixtures containing 100 mM Tris, pH 8.0, 0.5 mM $MnCl_2$, and 50 mM pNPP were incubated for the indicated times at 30 °C.

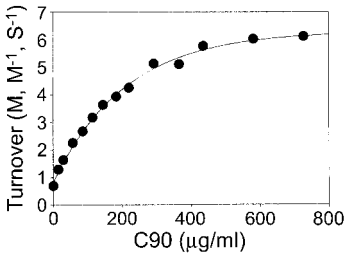

FIGURE 8: PP5 activation by C90. Hydrolysis of pNPP was measured in a continuous spectrophotometric assay at pH 8.0 in the presence of the indicated concentrations of C90 as described under Experimental Procedures. Reactions containing 50 mM pNPP and 0.5 mM $MnCl_2$ were initiated by the addition of 0.5 μg/mL PP5. Each point shown represents the rate calculated from the linear portion of a reaction in which approximately 130 data points were collected. The maximal turnover in the presence of C90 was 6.46 ± 0.23 per second, and half-maximal activation occurred at 97.0 ± 4.8 μg/mL C90. Standard error values were obtained from duplicate assays performed using each of two enzyme samples.

required to produce 50% activation, however, was 89 μM, significantly higher than the 17.6 μM required for 50% activation by lauroyl-CoA (Figure 6). It may be significant that SDS, like lauroyl-CoA, consists of a hydrophobic carbon chain attached to a strong acidic group.

Lack of a Specific CoA Binding Site. Acetyl-CoA and malonyl-CoA are present in the cell at much higher concentrations than the long-chain CoA esters. This raised the possibility that, although the short-chained esters did not activate the enzyme (Table 2 and data not shown), they might interact with a CoA binding site on PP5 and act as competitive inhibitors of its activation by long-chain CoA esters. This would produce a mechanism whereby carbohydrate metabolism could regulate PP5 activity. We tested this hypothesis by measuring PP5 activity in the presence of 4 μM palmitoleoyl-CoA in the absence or presence of a 100-fold excess of acetyl-CoA or malonyl-CoA (Figure 7). The rates of pNP production were indistinguishable in the absence or presence of the short-chain CoA esters (Figure 7). This strongly suggests that the CoA moiety does not interact directly with PP5; its primary role may be to enhance the solubility of long acyl chains in aqueous environments.

PP5 Activation by the C-Terminal Domain of hsp90. PP5 binds via its TPR domain to the C-terminal 12 kDa domain of hsp90 (*18*, *28*), designated C90 (*30*). Figure 8 shows that the addition of the isolated C90 domain to PP5 results in its activation. However, the maximal activation was only

approximately one-third that obtained with acyl-CoA esters. It is of note that half-maximum activation was observed at 97 μg/mL C90, or 5.7 μM. This is similar to the affinity of C90 for the related TPR domain of Hop (*31*). Surprisingly, the addition of 240 μg/mL native full-length hsp90 had no effect on PP5 activity (data not shown). This could be due to steric hindrance by the amino-terminal region of hsp90. It is known that the binding of ATP produces conformational changes in hsp90 (*27*, *32*−*34*), and we hypothesized that ATP binding might produce a conformation of hsp90 that activated PP5. However, even in the presence of 2.5 mM Mg-ATP, native hsp90 at a concentration of 240 μg/mL did not activate PP5 (data not shown). Still, the ability of the C90 domain to activate PP5 suggests the possibility that, under as yet unidentified conditions, hsp90 may also be an activator.

DISCUSSION

Since its discovery 8 years ago (*2*−*4*), the mechanisms by which PP5 is activated in vivo have remained a mystery. The enzyme has extremely low activity in vitro, and until now, PP5 activation had only been achieved by partial proteolysis or by treatment with supraphysiological concentrations of long-chain fatty acids (*11*−*13*). PP5 could be activated by polyunsaturated, but not saturated, fatty acids (*11*, *12*). We examined the effects of long-chain fatty acyl-CoA esters on PP5 activity because we suspected that previous studies of PP5 activation by fatty acids were flawed due to the limited solubility of these compounds. In particular, it seemed possible that the apparent specificity for polyunsaturated fatty acids might be due to their being much more soluble than saturated fatty acids. We eliminated this variable by examining the effects of long-chain fatty acyl-CoA esters on PP5 activity. The CoA esters are freely water soluble, and we report here that they efficiently activate PP5 whether or not their acyl chains are saturated. Indeed, saturated long-chain CoA esters activated PP5 more efficiently than their unsaturated counterparts. Thus, previous observations suggesting a preference for unsaturated acyl chains appear to have been artifacts of the low aqueous solubility of saturated fatty acids.

An unanticipated observation of potential biological importance was that long-chain fatty acyl-CoA esters were dramatically better activators of PP5 than the free fatty acids. Unlike fatty acids, fatty acyl-CoA esters activated PP5 at physiological concentrations. Arachidonic acid, the fatty acid most effective in activating PP5, has previously been reported to produce half-maximal activation at concentrations between 50 and 125 μM, with maximal activation occurring at 100 and 500 μM (*11*, *12*). Such high concentrations of arachidonic acid would not normally occur in vivo. In contrast, we report here that half-maximal activation of PP5 by long-chain fatty acyl-CoA esters occurs at concentrations between 0.8 and 3.4 μM, with maximal activation occurring at concentrations between 2.5 and 10 μM (Figure 4, Table 2). The concentration of cytosolic long-chain fatty acyl-CoA esters has been estimated at 1−4 μM (*29*). Thus, PP5 activity seems likely to be regulated by long-chain CoA esters in vivo. There is ample precedent for regulation of signal transduction by long-chain fatty acyl-CoA esters. These compounds are known to play a role in insulin secretion by β cells (*25*) and in the regulation of gene expression in bacteria, yeast, and mammals (*26*). The best characterized example of the latter may be the finding that long-chain fatty acyl-CoA esters are endogenous ligands for the nuclear receptor, hepatic nuclear factor 4α (*24*).

Unlike its predecessors, this study measured PP5 activity using a continuous assay rather than an end point assay. In the absence of lipid activators, the addition of PP5 to assays containing Mn^{2+} resulted in a lag of approximately 200 s. This was probably due to the Mn^{2+} slowly diffusing into the active site. The lag was not observed in the presence of arachidonic acid, presumably due to arachidonic acid-induced conformational changes making the enzyme's active site solvent-accessible. However, the addition of arachidonic acid to assays containing Mn^{2+} produced traces with increasing levels of curvature. Thus, care had to be taken to use only the linear portion of the plot in determining the reaction rate. It would have been impossible to obtain accurate rates using an end point assay. The use of continuous assays, however, produced its own problems; at saturating or near-saturating activator concentrations, the linear phase of many of the PP5 assays became extremely short. In assays containing arachidonic acid and Mn^{2+}, nonlinear rates were observed at subsaturating arachidonic acid concentrations. Similar nonlinear plots were observed in assays containing arachidonoyl-CoA and Mg^{2+}. The cause of the nonlinearity under these conditions is unclear. However, one possibility is that, in addition to their role as activators, the compounds were partially denaturing the protein by acting as detergents. If this were the case, then nonlinearity might be a function of the substrate used. Tightly bound physiological substrates, unlike pNPP, might be expected to stabilize the PP5−substrate complex and protect the enzyme from putative denaturation due to detergent properties of its activators.

The CoA moiety in the long-chain acyl esters could potentially perform several roles. One would be to bestow enhanced solubility on the fatty acid derivative. This is clearly important in the case of the saturated C18 and C20 compounds, where stearic and arachidic acids are essentially insoluble in water. However, since the CoA group strongly potentiates the ability of lauric acid, which is water-soluble, to activate PP5, it is likely to also function via other mechanisms. One possibility is that the strong negative charge possessed by the CoA moiety enables binding to a positively charged region of PP5. This would be reminiscent of the interactions between the basic residues in the TPR domain of PP5 and acidic residues in hsp90 (*18*, *28*). The replacement of the carboxylic group in lauric acid by the much more electronegative sulfate group in SDS produced a large increase in activating ability. This is consistent with the notion that a negative charge plays a role in PP5 activation. Enthusiasm for this idea, however, is tempered by our failure to observe a specific binding site for the CoA moiety (Figure 7). The hydrophobic acyl moieties and the hydrophilic CoA moieties of the esters presumably interact with their environment via very different mechanisms. The inability of lauric acid analogues with two carboxylic groups to activate PP5 suggests that the acyl moiety interacts with PP5 exclusively via hydrophobic interactions and that this interaction can be disrupted by negative charges. It might be argued that the relatively efficient activation by SDS is due to a detergent effect rather than to its strong negative

charge, but maximal activation was observed at 150 μM, or 0.004% SDS. This is well below levels normally used to denature proteins. Further confusing the issue of the role of negative charges, Skinner et al. have previously reported that the carboxylic acid group in arachidonic acid was required for PP5 activation (*11*), while Chen and Cohen reported opposite findings (*12*). Finally, the enhanced activation brought about by the CoA moiety could be a function of the increased size of the CoA esters compared to the corresponding fatty acids. The mechanism of fatty acid activation of PP5 is not understood in great detail, but both the N- and C-terminal domains are autoinhibitory and potentially important in lipid activation (*13*, *23*). We speculate that the larger CoA esters may be able to interact with both domains better than the corresponding fatty acid.

We found that CoA esters with acyl groups of 16 or more carbons activated PP5 at physiological concentrations, with CoA esters of saturated acyl groups being better activators. It may be significant that the levels of both palmitoyl (C16:0) CoA and the C18 CoA esters in rat liver increase more dramatically than those of other CoA esters during fasting (*35*). Thus, one might predict that the onset of fasting would result in enhanced PP5 activity. Activation of PP5 by long-chain CoA esters may thus provide a novel mechanism for protein phosphorylation to be regulated by the cell's metabolic status.

The current work calls into question some aspects of data obtained in previous investigations of PP5 activation. In our hands, PP5 activation by divalent metal ions, long-chain fatty acids, or long-chain acyl-CoA esters resulted either in initial lag phases or in relatively short linear phases. In either case, the use of a continuous rather than an end point assay is essential in determining accurate reaction rates. The assay's linear phase can be readily identified in continuous assays, but the periods of nonlinearity would be impossible to detect using the end point assays previously presented by others and us (*11*−*13*, *23*). The tailing off of PP5 activity we observed after 4 min in assays containing fatty acids would have resulted in serious underestimations of PP5 activity in 10 or 15 min end point assays. This would explain the AC_{50} values reported here for arachidonic acid being lower than values obtained in previous studies.

PP5 binds to both hsp90 and CoA esters via its TPR domain. We report here that the isolated C-terminal 12 kDa domain of hsp90 (C90) activates PP5. The CoA esters for which saturation profiles were obtained produced similar maximum PP5 activities despite large variations in PP5 binding affinities (Table 2). This suggested that different CoA esters produced similar conformational changes. However, the maximal PP5 activation by C90 was only one-third that observed with CoA esters. This suggests that C90 and the CoA esters activate PP5 by the induction of different conformational changes. This is not surprising, as the interactions of the long-chain acyl groups with PP5 are presumably hydrophobic, while at least some critical interactions of the enzyme with C90 are hydrophilic (*28*). The induction of different conformational changes by the binding of hsp90 and by the binding of lipids would also be consistent with our mutagenesis data, suggesting different sites of PP5 binding by hsp90 and lipid activators (*23*).

The observation that the C-terminal domain of hsp90 activates PP5 while the full-length molecule has no effect on the enzyme's activity was unexpected and remains unexplained. However, if the binding of PP5 to hsp90, one of the most abundant cellular proteins, significantly activated the enzyme, it would render acyl-CoA activation less important and would hinder the modulation of PP5 activation in signal transduction. Still, we speculate that, under some conditions, bound hsp90 may be present in a conformation that can activate PP5. For example, glucocorticoid receptor heterocomplexes contain not only hsp90 and PP5 but also the receptors and the p23 cochaperone (*36*). It is possible that within such a heterocomplex hsp90 may assume a conformation that activates PP5. Nucleotide-mediated conformational switching of hsp90 has been shown to modulate the ability of $p50^{cdc37}$ to bind to protein kinase−hsp90 complexes (*37*, *38*). Alternatively, hsp90 binding to PP5 may activate the enzyme, but the bound hsp90 may sterically hinder the PP5 active site. In this case, PP5 could still have a potential function in dephosphorylating hsp90 itself; it has been suggested that hsp90 activity is regulated by its phosphorylation state (*39*, *40*). At this point, however, we cannot categorically rule out the activation of PP5 by C90 being an artifact of truncation.

In summary, we have used a continuous assay to characterize the activation of PP5 in the presence of added divalent cations. This has allowed a more rigorous analysis of enzymatic activity than has previously been reported. Contrary to previous studies, our investigation suggests that both saturated and unsaturated fatty acyl chains activate PP5. More importantly, we present the first evidence for the activation of PP5 by physiological concentrations of fatty acid derivatives. In addition, our data indicate that although PP5 binding to native hsp90 did not activate the enzyme under the conditions tested, the isolated C-terminal domain of hsp90 can activate PP5. Further studies will be required to understand the structural basis for activation of PP5 by long-chain fatty acyl-CoA esters and C90, to test whether conditions exist under which native hsp90 can activate PP5, and to test whether PP5 mediates some of the biological effects of long-chain CoA esters.

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