Identification of Amino Acids in the Tetratricopeptide Repeat and C-Terminal Domains of Protein Phosphatase 5 Involved in Autoinhibition and Lipid Activation†

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Received May 16, 2001; Revised Manuscript Received June 29, 2001

ABSTRACT: Protein phosphatase 5 (PP5) exhibits low basal activity due to the autoinhibitory properties of its N-terminal and C-terminal domains but can be activated approximately 40-fold in vitro by polyunsaturated fatty acids. To identify residues involved in regulating PP5 activity, we performed scanning mutagenesis of its N-terminal tetratricopeptide repeat (TPR) domain and deletion mutagenesis of its C-terminal domain. Mutating residues in a groove of the TPR domain that binds to heat shock protein 90 had no effect on basal phosphatase activity. Mutation of Glu-76, however, whose side chain projects away from this groove, resulted in a 10-fold elevation of basal activity without affecting arachidonic acid-stimulated activity. Thus, the interface of the TPR domain involved in PP5 autoinhibition appears to be different from that involved in heat shock protein 90 binding. We also observed a 10-fold elevation of basal phosphatase activity upon removing the C-terminal 13 amino acids of PP5, with a concomitant 50% decrease in arachidonic acid-stimulated activity. These two effects were accounted for by two distinct amino acid deletions: deleting the four C-terminal residues (496−499) of PP5 had no effect on its activity, but removing Gln-495 elevated basal activity 10-fold. Removal of a further three amino acids had no additional effect, but deleting Asn-491 resulted in a 50% reduction in arachidonic acid-stimulated activity. Thus, Glu-76 in the TPR domain and Gln-495 at the C-terminus were implicated in maintaining the low basal activity of PP5. While the TPR domain alone has been thought to mediate fatty acid activation of PP5, our data suggest that Asn-491, near its C-terminus, may also be involved in this process.

Several years ago we and others identified the protein serine/threonine phosphatase that has been designated PP5[1] (*1*−*3*). The catalytic domain of PP5 is closely related to the catalytic subunits of PP1, PP2A, and PP2B (*4*). Unlike those enzymes, however, PP5 contains regulatory and targeting domains within a single polypeptide chain rather than in separate subunits. PP5 has been implicated in several signaling pathways (reviewed in ref 5). Through its association with heat shock protein 90 (hsp90), PP5 appears to be involved in signaling through the glucocorticoid receptor and to have a potential role as a cochaperone (*6*, *7*). A recent study also demonstrates that overexpression of PP5 can stimulate the growth of cultured cells (*8*). In biochemical studies, PP5 was purified in a search for an arachidonic acid-activated phosphatase that regulates potassium channel activity (*9*). A clear demonstration of the normal biological function of PP5 has yet to be achieved, however, and our understanding of its biochemical regulation remains limited.

A distinctive feature of PP5 is its amino-terminal tetratricopeptide repeat (TPR) domain. TPR domains mediate specific protein−protein interactions (*10*), and the TPR domain of PP5 mediates its binding to hsp90 (*6*) and to other signaling proteins (*1*, *11*, *12*). Using the three-dimensional structure of this domain (*13*) as a guide, we have performed site-directed mutagenesis to identify which portion of the TPR domain was involved in binding to hsp90 (*14*). In this way, we identified a binding groove that contains three basic residues essential for hsp90 binding (*14*). These residues are conserved in all known hsp90-binding TPR proteins and have subsequently been shown, in FKBP52, Hop, and AIP, to interact with the C-terminus of hsp90 (*15*−*17*). TPR domains consist of a series of antiparallel α-helices bundled together to form a cradle-shaped groove (*13*, *17*−*20*). On the basis of our mutagenesis studies, PP5 was suggested to represent a paradigm for TPR proteins, in which consensus TPR sequences form the backbone structure, while variable residues lining the binding groove form the basis for targeting specificity (*10*). Consistent with this postulate, not only do TPR proteins bind to hsp90 via this groove but the binding of Tup1 to Ssn6, which is predicted to have a hydrophobic groove in its TPR domain, is mediated by hydrophobic interactions (*21*). More recently, however, it has been shown that binding of target proteins to some TPR domains can be mediated by loops connecting the TPR helices, in addition to (*19*), or instead of (*20*), the binding groove.

In addition to binding other proteins, the TPR domain of PP5 plays a key role in regulating its phosphatase activity. Partial proteolysis removing the TPR domain results in a concomitant increase in phosphatase activity of up to 50-fold (*9*, *22*). Arachidonic acid, which activates PP5 to the same extent as proteolysis, binds to the TPR domain (*9*, *22*), presumably displacing it from the autoinhibitory position it

† This work was supported by National Institutes of Health Grants HL 47063 and DK 55877 to M.C.

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[1] Abbreviations: PP, protein phosphatase; TPR, tetratricopeptide repeat; pNPP, *p*-nitrophenyl phosphate; BSA, bovine serum albumin.

10.1021/bi010999i CCC: $20.00
Published on Web 08/02/2001

holds in the basal state. Activation of PP5 by arachidonic acid and other long-chain unsaturated fatty acids currently represents the best model for its regulation. High micromolar concentrations are required for full activation in vitro, but native PP5 may gradually lose its ability to be activated during purification, and in the cell the enzyme may be responsive to physiological concentrations of arachidonic acid (*9*). A short C-terminal sequence in PP5 has also been shown to be autoinhibitory (*23*). We have now used site-directed mutagenesis to test whether the groove in the TPR domain that binds to hsp90 also mediates the autoinhibitory function of this domain. We report here that the autoinhibitory face of the TPR domain appears to be distinct from the hsp90-binding groove, corresponding instead to a loop connecting the TPR helices. We also report that Gln-495, four residues from the C-terminus of PP5, is an important autoinhibitory residue. Unexpectedly, Asn-491, near the C-terminus, is required for full activation by arachidonic acid. This is the first evidence for a positive role for the C-terminal sequences, previously described as purely autoinhibitory.

EXPERIMENTAL PROCEDURES

Preparation of Bacterial Expression Constructs. For studies to be described elsewhere, sequences encoding the TPR domain of rat PP5 were amplified by PCR using primers that introduced *Nde*I and *Bam*HI sites. The PCR product was cloned into the corresponding sites of pET15b (Novagen), a vector that introduced an amino-terminal His tag. The absence of undesired mutations was confirmed by sequencing. Full-length PP5 was introduced into this vector by replacing the *Xho*I/*Eco*RI fragment containing sequences encoding the TPR domain (and additional vector sequences) with the 1.9 kb *Xho*I/*Eco*RI fragment of full-length rat PP5 (*3*, *6*).

To introduce mutations into the TPR domain, the PP5 sequences were excised from pET15b-PP5 and subcloned into pGEM-3Zf(−) as an *Xba*I/*Eco*RI fragment. Sequences encoding TPR sequences with single amino acid mutations were excised from previously described constructs (*14*) as *Xho*I/*Nhe*I fragments and used to replace wild-type sequences in the pGEM-3Zf(−)-PP5 construct. After the replacements were confirmed by sequencing, the mutant full-length PP5 constructs were cloned into the *Xba*I/*Eco*RI sites of pET15b.

For C-terminal deletions, PCR was used to generate 3′ sequences with deletions of the indicated lengths. The forward primer (GGCAAAGTGCTGATCATGCATGGAGGCC) encompassed the unique *Nsi*I site in rat PP5, while the reverse primers incorporated an *Eco*RI site, a termination codon, and appropriate sequences to delete from 1 to 13 amino acids from the wild-type C-terminus of PP5. The oligonucleotides used were as follows: 1 amino acid deletion, CCCGAATTCTCACATTCCTAGCTGCAG; 2 amino acid deletion, CCCGAATTCTCATCCTAGCTGCAGCAG; 3 amino acid deletion, CCCGAATTCTCATAGCTGCAGCAGCGT; 4 amino acid deletion, CCCGAATTCTCACTGCAGCAGCGTGTT; 5 amino acid deletion, CCCGAATTCTCACAGCAGCGTGTTGGC; 6 amino acid deletion, CCCGAATTCTCACAGCGTGTTGGCGTA; 7 amino acid deletion, CCCGAATTCTCACGTGTTGGCGTATGC; 8 amino acid deletion, CCCGAATTCTCAGTTGGCGTATGCCAT; 9 amino acid deletion, CCCGAATTCTCAGGCGTATGCCATGGG; 10 amino acid deletion, CCCGAATTCTCAGTATGCCATGGGCTT; 11 amino acid deletion, CCCGAATTCTCATGCCATGGGCTTGAC; 12 amino acid deletion, CCCGAATTCTCACATGGGCTTGACGTT; 13 amino acid deletion, CCCGAATTCTCAGGGCTTGACGTTGGG.

PCR products were cloned into pCR2.1 (Invitrogen) and sequenced to confirm the presence of the desired mutations and the lack of additional mutations. The 0.5 kb *Nsi*I/*Eco*RI fragments were then used to replace the corresponding wild-type fragment in pET15b-PP5, and the replacement was confirmed by sequencing.

Expression and Purification of Recombinant PP5. The pET15b constructs were transformed into *Escherichia coli* BL21(DE3) cells, which were grown at 18 °C, in LB medium containing 50 μg/mL ampicillin, until the OD_{600} reached 0.7. Cells were then induced for 7 h with 1 mM IPTG. Pelleted cells were lysed by sonication in buffer A (20 mM Tris-HCl, pH 8.0, 500 mM NaCl) containing 5 mM imidazole, 1 mM PMSF, and 20 mM benzamidine. All subsequent purification procedures were performed at 4 °C. After centrifugation for 30 min at 20000*g*, the supernatant was applied to a 5 mL nickel-saturated chelating HiTrap column (Amersham Pharmacia Biotech) equilibrated with buffer A containing 5 mM imidazole. The column was washed with equilibration buffer and then with buffer A containing 65 mM imidazole. Elution was then conducted using a linear gradient of 65−250 mM imidazole in buffer A. Active fractions, assayed as described below, were pooled, concentrated by ultrafiltration in an Amicon stirred cell (YM-30), and dialyzed against 20 mM Hepes, pH 7.5, and 50 mM NaCl. The purity of the samples was >95% as assessed by SDS−PAGE and Coomassie Blue staining. Proteins were stored at −80 °C before use.

Preparation of Phosphatase Domains by Partial Proteolysis. After binding to a chelating HiTrap column, with washes and equilibration as described above, the column was filled with 20 mM Tris, pH 8.0, containing 50 μg/mL trypsin, and incubated for 20 min at 30 °C. Trypsin cleaves the N-terminal TPR domain from PP5 but does not cleave the C-terminus (*23*). The catalytic domains were eluted by washing with buffer A containing 10 mM imidazole, while the His-tagged TPR domains remained bound to the column. After addition of 1 mg of soybean trypsin inhibitor, the eluant was dialyzed against buffer B (20 mM Hepes, pH 7.5, 1 mM PMSF, 20 mM benzamidine). The dialyzate was then passed through a 1 mL Mono S column (Amersham Pharmacia Biotech) equilibrated with buffer B, and the wash fractions were pooled and loaded onto a 1 mL Mono Q column (Amersham Pharmacia Biotech). After being washed with buffer B, the catalytic domains were eluted using a linear gradient of 0−300 mM NaCl in buffer B. Fractions containing catalytic activity were pooled and concentrated by ultrafiltration in an Amicon YM-10 cell. Samples were then fractionated on a 24 mL Superose 12 column (Amersham Pharmacia Biotech) equilibrated with buffer B. Active fractions were pooled, dialyzed against 20 mM Hepes, pH 7.5, and 50 mM NaCl, concentrated by ultrafiltration as describe above, and stored at −80 °C.

Phosphatase Assays. Phosphatase activity was measured using either *p*-nitrophenyl phosphate (pNPP) or ^{32}P-labeled casein as substrate. The latter was prepared as described (*24*)

and contained approximately 2.5 nmol of phosphate/mg of protein. To measure activity toward pNPP, except where indicated otherwise, 200 μL reactions contained 50 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 30 mM pNPP, 0.1% β-mercaptoethanol, and 50 nM enzyme in the presence of the indicated concentrations of arachidonic acid. Reactions were allowed to proceed for 10 min at 37 °C and terminated by addition of 0.25 M NaOH. After clarification by centrifugation, absorbance was determined at 410 nm. To measure activity toward casein, 20 μL reactions were performed as above except that 2.5 μM ^{32}P-labeled casein was substituted for pNPP. Reactions were stopped by addition of 170 μL of cold 20% trichloroacetic acid and 10 μL of 3% BSA. After the mixtures were vortexed and incubated at −20 °C for 20 min, samples were centrifuged for 20 min at 19000*g*. The amount of radioactivity released into 100 μL of the supernatant was determined by liquid scintillation counting. Results from representative experiments are presented as the mean ($n = 4$) ± standard error. All results were substantiated using at least three different sets of enzyme preparations (wild-type PP5 and the TPR mutants or wild-type PP5 and the C-terminal mutants).

RESULTS

Effects of Mutations within the TPR Domain on PP5 Activity. We have previously performed site-directed mutagenesis of the isolated TPR domain of PP5 to determine which residues are important for binding to hsp90 (*14*). Mutations were designed to minimize effects on protein folding (most of the mutations were made in polar residues at the protein's surface), and the mutations were distributed throughout the various faces of the TPR domain (*14*). Using this approach, we found that three amino acids lining a groove in the TPR domain (Lys-32, Lys-97, and Arg-101) were required for hsp90 binding. We hypothesized that this same binding groove might mediate the autoinhibitory interactions between the TPR domain and the catalytic domain of PP5 (*14*). To test this hypothesis, we have now introduced the previously described TPR mutations into full-length PP5, and the recombinant proteins were expressed in bacteria and purified by affinity chromatography. We examined the basal and arachidonic acid-stimulated phosphatase activity of wild-type PP5 and each of the mutant proteins. As shown in Figure 1, most of the mutations had no significant effect on basal or stimulated activity. Only one mutation, that of Glu-76, resulted in a substantial elevation of the normally low basal activity of PP5 (Figure 1). Although this was only a partial activation relative to that observed with arachidonic acid, it represented a 10-fold increase over the basal activity of the wild-type enzyme, and this increase was highly reproducible. We have made the same observation using six different enzyme preparations, i.e., that there is activation of the Glu-76 mutant but not of other PP5 mutants. This suggests that Glu-76 is important for the autoinhibitory function of the TPR domain. Mutation of Lys-40 reproducibly led to a 30% reduction in arachidonic acid-stimulated activity, but it is not clear how to interpret this modest effect. It may be due to altered protein folding, or Lys-40 may have a small role in the binding of arachidonic acid.

In the experiments shown in Figure 1A, the activity of PP5 was measured using pNPP as a substrate. Similar

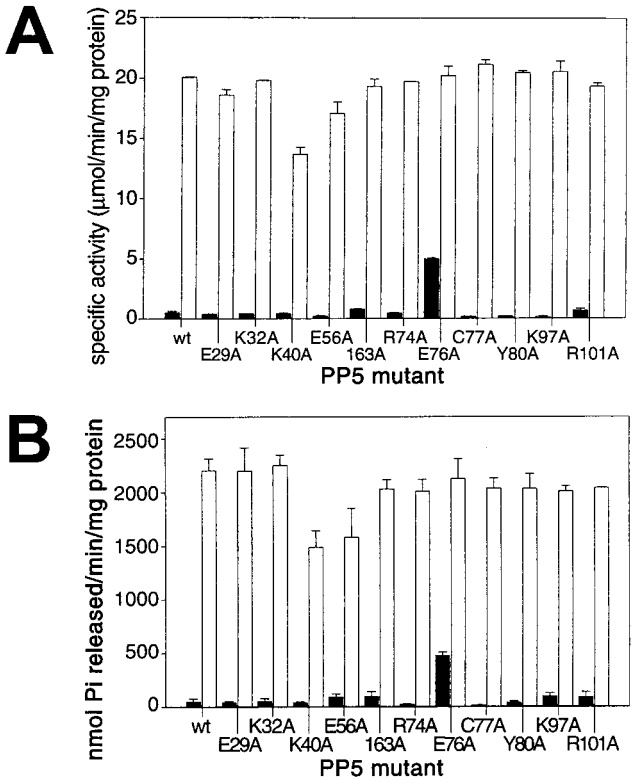

FIGURE 1: Effects of mutations in the TPR domain on arachidonic acid-stimulated phosphatase activity of PP5. Wild-type PP5 or the indicated mutants were assayed for phosphatase activity in the absence (solid bars) or presence (open bars) of 200 μM arachidonic acid, using pNPP (A) or ^{32}P-labeled casein (B) as a substrate. Results from a representative experiment are presented as the mean ($n = 4$) ± standard error. Similar results were obtained using six different preparations of each enzyme.

observations were made when the substrate used was ^{32}P-labeled casein (Figure 1B). These results indicated that the three basic residues (Lys-32, Lys-97, and Arg-101) that lie in the binding groove of the TPR domain, and are essential for hsp90 binding (*14*), are not required for the autoinhibitory function of this domain. Glu-76, whose side chain projects from the edge of the hsp90-binding groove (Figure 2), was the only residue tested whose mutation significantly elevated the basal activity of PP5. Thus, different faces of the TPR domain appear to be important for binding to hsp90 and inhibition of PP5 activity (Figure 2).

Identification of C-Terminal Residues Regulating PP5 Activity. Sinclair et al. (*23*) have reported that deletion of the 13 C-terminal amino acids of PP5 by mutagenesis resulted in activation of bacterially expressed PP5. Full activation was observed, relative to that obtained with arachidonic acid, when casein was used as a substrate, but only about a 50% activation was observed when myelin basic protein was used as a substrate (*23*). In our hands, deletion of the 13 C-terminal amino acids of PP5 resulted in slightly less dramatic elevations of basal activity. Similar to our observations with the Glu-76 mutant, we found that deleting the C-terminal 13 amino acids led to a 10-fold elevation of basal activity, representing approximately 25% of the activation obtained with arachidonic acid. Surprisingly, arachidonic acid-stimulated activity was decreased by approximately 50% in the deletion mutant (Figure 3). This observation was repeated using three different enzyme preparations. Full activation of PP5 by deletion of its C-terminus was never

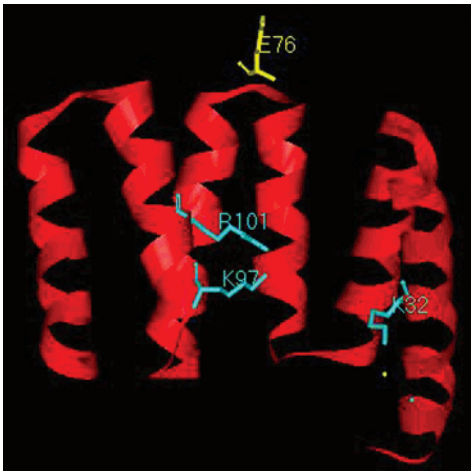

FIGURE 2: Ribbon diagram showing separate interfaces for hsp90 binding and autoinhibition by the TPR domain of PP5. The side chains of Lys-32, Lys-97, and Lys-101 (cyan) are required for binding of hsp90 (*14*) and line a groove formed by the TPR helices (*13*). The side chain of Glu-76 (yellow) is required for maintaining the low basal activity of PP5 and projects away from this groove in a connecting loop between helices A and B of TPR2. This image of the TPR domain of PP5 [Protein Data Bank accession code 1A17 (*13*)] was prepared using WebLab ViewerLite 4.0.

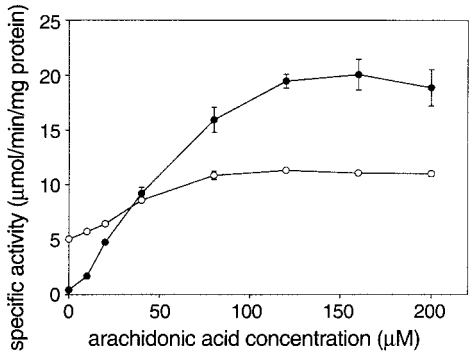

FIGURE 3: Arachidonic acid stimulation of wild-type and C-terminally truncated PP5. Enzyme activity was determined as described in Experimental Procedures, using pNPP as a substrate. Symbols: filled circles, wild-type PP5; open circles, PP5 lacking residues 487−499. Results from a representative experiment are presented as the mean ($n = 4$) ± standard error. Similar concentration−response curves were obtained using three different preparations of each enzyme.

observed, and arachidonic acid-stimulated activity was always dramatically decreased after this deletion.

Because the reduction in arachidonic acid-stimulated activity was unexpected on the basis of the results of Sinclair et al. (*23*), we performed an experiment to test whether it might be due to improper folding of the mutant enzyme in our hands. In this experiment, we purified the wild-type and mutant enzymes, subjected them to partial proteolysis with trypsin to remove their TPR domains, and then purified their catalytic domains. Partial proteolysis with trypsin does not cleave the C-terminus of PP5 (*23*). Enzymatic activity was then measured. The activities of the wild-type and C-terminally truncated catalytic domains, measured using pNPP as substrate, were essentially identical [12.3 ± 0.2 vs 14.6 ± 0.2 μmol min^{-1} (mg of protein)$^{-1}$ for the catalytic domains from the wild-type and truncated enzymes, respectively]. The ability of the deletion mutant to be activated to the same extent as the wild-type protein by partial proteolysis indicated that its catalytic domain was folded properly, even though

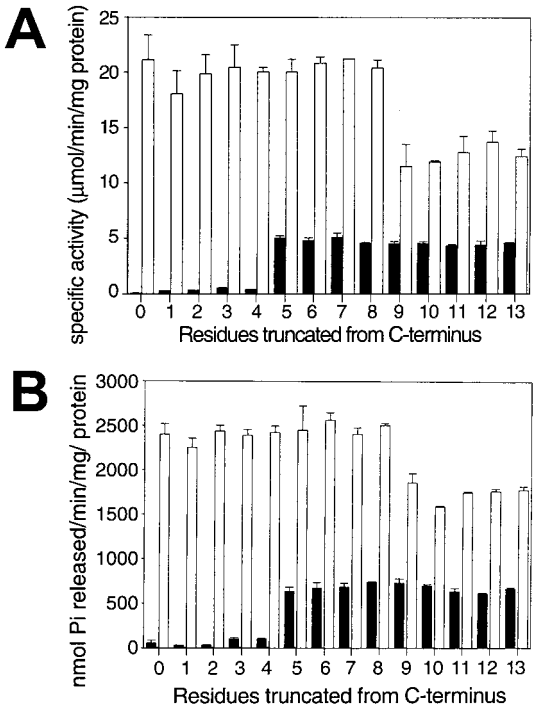

FIGURE 4: Effect of C-terminal truncations on the activity of PP5. Activity was measured in the absence (filled bars) or presence (open bars) of 150 μM arachidonic acid, using pNPP (A) or ^{32}P-labeled casein (B) as a substrate. Results from a representative experiment are presented as the mean ($n = 4$) ± standard error. Similar results were obtained using three different preparations of each enzyme.

the ability of the enzyme to be activated by arachidonic acid was significantly impaired.

We next examined which of the 13 deleted C-terminal residues were involved in elevating basal activity and which were involved in diminishing arachidonic acid-stimulated activity. We performed a systematic series of deletions in which the C-terminal amino acids were removed one by one. As shown in Figure 4, deletion of the four C-terminal residues had no effect on basal or arachidonic acid-stimulated PP5 activity. Deletion of five C-terminal residues, however, resulted in as dramatic an elevation in basal activity as deletion of all 13 residues (Figure 4). Deletion of the next three C-terminal residues had no additional effect on phosphatase activity, but deletion of nine C-terminal residues resulted in a 50% decrease in arachidonic acid-stimulated activity. No further effects were seen upon deletion of up to 13 C-terminal residues (Figure 4). Thus, Gln-495, the fifth residue from the C-terminus, appears to play a role in maintaining the low basal activity of PP5. In addition, and unexpectedly based on previous work, removal of Asn-491 impaired the ability of PP5 to be activated by arachidonic acid. This suggests an active role for C-terminal sequences of PP5 in conformational changes that occur upon activation of the enzyme by arachidonic acid rather than these sequences simply being important for repressing activity in the basal state. Similar results were obtained using ^{32}P-labeled casein as a substrate (Figure 4B), though the effects of deleting Asn-491 and Gln-495 were somewhat less dramatic than those observed using pNPP (Figure 4A).

Kinetic Parameters of the PP5 Mutants. Because activation of PP5 by site-directed mutagenesis was only on the order of 10-fold, as opposed to the 40-fold observed with arachidonic acid, we examined the activity of wild-type PP5 and

Table 1: Michaelis−Menten Kinetic Parameters for *p*-Nitrophenol Production ($n = 4$)[a]

enzyme	arachidonic acid (200 μM)	K_m (mM)	V_{max} [μmol min^{-1} (mg of protein)$^{-1}$]
wild type	−	26.53 ± 0.35	1.47 ± 0.01
	+	9.63 ± 0.37	19.82 ± 0.46
E76A	−	17.95 ± 3.65	5.82 ± 0.83
	+	9.08 ± 0.14	21.88 ± 3.98
C-5	−	27.99 ± 0.64	4.74 ± 0.05
	+	12.98 ± 2.57	22.59 ± 0.85
C-9	−	28.35 ± 2.43	4.82 ± 0.37
	+	15.24 ± 0.67	12.11 ± 0.55

[a] Reaction mixtures and conditions used for kinetic studies were identical to the standard assay using varying concentrations of pNPP in the absence or presence of 200 μM arachidonic acid. Data from these experiments were fitted to double reciprocal plots to calculate K_m and V_{max} values.

of three key mutants in more detail. Activity was characterized in the presence of various concentrations of pNPP and in the absence or presence of arachidonic acid. Kinetic parameters were then calculated and are presented in Table 1. The elevations of basal activity in the E76A mutant and in the C-terminal truncation mutants were due to a severalfold increase in V_{max}. In the absence of arachidonic acid, there was no significant effect on K_m produced by the C-terminal truncations and only a borderline effect on K_m for the E76A mutant. In the presence of arachidonic acid, the E76A mutation had no significant effect on V_{max} or K_m. The V_{max} was also unchanged, in the presence of arachidonic acid, for the 5 amino acid truncation mutant. The V_{max} of the 9 amino acid truncation mutant was, however, reduced by approximately 50% relative to the wild-type enzyme in the presence of arachidonic acid. Small increases in K_m were also observed for the C-terminal truncation mutants, but their significance is uncertain. In summary, the effects of the three mutants on basal and arachidonic acid-stimulated phosphatase activity are primarily due to effects on V_{max}.

DISCUSSION

We previously used a series of mutations in the TPR domain of PP5 to define an interface for its interaction with hsp90 (*14*). The hsp90-binding groove in the TPR domain defined in this way and the importance of conserved basic residues in the binding of hsp90 have been confirmed in both mutagenesis and crystallographic studies of other TPR proteins (*15*−*17*). We hypothesized that the groove involved in hsp90 binding might interact with the catalytic domain of PP5 in a similar manner, accounting for its autoinhibitory properties. We have now tested this hypothesis by introducing the same mutations into full-length PP5 and studying their effects on its enzymatic activity. In doing so, we have confirmed and extended previous observations that the TPR domain is autoinhibitory and that it binds to hsp90 via a groove lined with basic residues. In particular, we have defined a specific autoinhibitory residue within the TPR domain that, in turn, suggests which face of the TPR domain is responsible for autoinhibition (Figure 2). It is clear that mutations of basic residues that interact with hsp90, or other residues in the binding groove, do not disrupt the autoinhibitory function of the TPR domain. We show here, however, that Glu-76, a residue outside the hsp90-binding groove, in a connecting loop between the A and B helices of the second TPR repeat (Figure 2), is required for full autoinhibitory function. This suggests that the previously identified hsp90-binding groove is not the region that interacts with the catalytic domain of PP5. Rather, an edge of the TPR domain containing connecting loops between helices seems to be involved in autoinhibition. This sort of interaction with a TPR domain has recently been observed in crystallographic studies of the binding of PEX5 to a peroxisomal targeting signal as well as of the binding of $p67^{phox}$ to the small GTPase Rac (*19*, *20*). Others have previously shown that different TPR domains within a single protein can serve different functions (*25*, *26*). Our results go farther, suggesting that different faces of the single TPR domain of PP5 are responsible for targeting vs autoinhibitory functions. These mutagenesis studies, however, cannot definitively rule out a role for the hsp90-binding groove in autoinhibition. It is conceivable that residues within this groove that were not mutated in the current study are required for interactions with the catalytic domain.

We have also confirmed and extended work defining a short C-terminal sequence of PP5 as autoinhibitory (*23*). As previously described by other workers, we observed partial activation of PP5 by deletion of these sequences. In contrast to the previous study, however, we found that residues near the C-terminus of PP5 were important not only for autoinhibition but also for full activation by arachidonic acid (Figure 3). We have gone on to define Gln-495 as a key residue for the autoinhibitory properties of the C-terminal domain and to define Asn-491 as important for PP5 activation by arachidonic acid. One interpretation of these results is that the hydrophobic C-terminus of PP5, as well as the TPR domain, may be involved in the binding of arachidonic acid, as well as in inhibition of basal enzyme activity. Whatever the mechanism, Asn-491 appears to be involved in the conformational changes leading to increased activity after treatment with arachidonic acid. The specifics of the interactions between catalytic and autoinhibitory domains may be complex; activity of the isolated catalytic domain was not inhibited by the isolated TPR domain or C-terminal peptide (*23*). It will be important, in future work, to define the residues in the catalytic domain that interact with the autoinhibitory domains and in particular with the autoinhibitory residues defined in the studies presented here.

The results presented here should be interpreted with the usual caveats regarding the possible global effects of site-directed mutagenesis on protein folding. The regulation of PP5 will be best understood upon solution of its three-dimensional structure in both its basal and activated states. The partial proteolysis experiment shown in Figure 3, however, indicates that the loss of arachidonic acid sensitivity observed after deletion of C-terminal residues is not due to misfolding of the catalytic domain of PP5. We believe, therefore, that it is likely that Asn-491 does play a role in the activation of PP5 by arachidonic acid. It should be noted that none of the TPR mutations, which in general involved charged residues, led to full activation of PP5, if full activation is defined as the activity observed in the presence of arachidonic acid. Kinetic analyses of the mutated proteins, however, were consistent in showing severalfold effects on V_{max}. Thus, while our results suggest roles for Glu-76 and Gln-495 in repressing basal activity, other residues are also

likely to be important. It may be that nonpolar TPR residues, which we avoided mutating, act cooperatively with Glu-76 to repress the basal activity of PP5. Alternatively, C-terminal residues such as Gln-495 may act cooperatively with Glu-76 in this regard. It should not be surprising that point mutations do not fully activate PP5, since at least two distinct autoinhibitory domains are present in the enzyme.

Our data suggest multifaceted roles for TPR domains in the regulation of protein function. The evolution of the PP5 TPR domain to contain a hsp90-binding groove, an apparently separate site that performs an autoinhibitory function, and an as yet undefined arachidonic acid binding site suggests that the basic backbone of the TPR domain can accommodate variations that perform multiple functions at multiple sites. It is not yet known whether the lipid binding site overlaps the hsp90-binding or autoinhibitory sites. An immediate question raised by the activation of PP5 by arachidonic acid is whether other proteins containing TPR domains may be regulated in a similar fashion, that is, whether, in addition to their targeting functions, TPR domains may function in other proteins as autoinhibitory domains whose binding of activators results in a relief of their inhibitory function.

While the importance of identifying autoinhibitory residues in PP5 is clear, the importance of residues such as Asn-491, which may be involved in arachidonic activation, is debatable. One could argue that the activation of PP5 by supraphysiological concentrations of unsaturated fatty acids is unlikely to relate to its regulation in vivo and represents a mere detergent effect. On the other hand, the very dramatic effects of these compounds suggest that similar compounds may indeed activate PP5 in the cell or that arachidonic acid may activate PP5 at physiological concentrations in the presence of as yet unidentified coactivators. Further, we have found that activation by long-chain fatty acid compounds is not due to a detergent effect and that changes in assay conditions can lead to PP5 activation at arachidonic acid concentrations that may occur in vivo. Also, we have recently identified lipid compounds capable of activating PP5 in vitro at physiological concentrations (A. J. Ramsey and M. Chinkers, unpublished data). Finally, PP5 may gradually lose its responsiveness to fatty acids during purification (*9*) and thus may eventually prove to respond to physiological concentrations of arachidonic acid in the cell. The question of whether arachidonic acid or similar compounds regulate PP5 in vivo thus remains unanswered—it has neither been ruled out nor shown to occur.

In summary, the data presented here advance our understanding of PP5 regulation in several respects. First, the results suggest that the autoinhibitory function of the TPR domain is unlikely to result from a direct interaction between its hsp90-binding groove and the catalytic domain. Second, in the course of defining what appear to be separate faces of the TPR domain for autoinhibition and hsp90 binding, we have identified a specific TPR residue, Glu-76, that is important for maintaining low basal activity. Finally, we have confirmed and extended previous observations about the C-terminal regulatory domain, identifying a specific residue (Gln-495) that accounts for its autoinhibitory properties and another specific residue (Asn-491) important for lipid activation—previously not known to be mediated by the C-terminal domain.

ACKNOWLEDGMENT

We thank Sherry Whitt for technical assistance, Frank Vogtner for assistance in preparing artwork, and Dr. Andrew Ramsey for careful reading of the manuscript.

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BI010999I