

Modulation of type-1 protein phosphatase by synthetic peptides corresponding to the carboxyl terminus

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Protein phosphatase type-1 (PP-1) has a protease resistant catalytic core $M_r = 35000$ (PP-35K) and a carboxyl terminal segment which affects activity with various substrates. We found that micromolar concentration of a synthetic peptide, corresponding to residues 312–326 of the PP-1 carboxyl terminus (P312-326) that is missing from PP-35K, increased the phosphatase activity of PP-35K with phosphorylase and myosin light chains as substrates by decreasing the apparent K_m without a change in V_m . Purified PP-1 and PP-35K were inhibited identically by okadaic acid, but peptide P312-326 only stimulated the activity of PP-35K, not full-length PP-1. Other peptides corresponding to the carboxyl terminus of phosphatase-2A or to the amino terminus of PP-1 did not affect the activity of PP-35K. A sequence conserved in PP-1 from different species, Pro-Ile-Thr-Pro-Pro was implicated as the active region because a derivative peptide, Ala-Pro-Ile-Thr-Pro-Pro-Ala, stimulated the activity of PP-35K to the same extent as peptide P312-326 although at higher concentrations. These results indicate that the carboxyl terminus of PP-1 interacts with the catalytic core to modulate its activity, and suggest that the physiological regulation of PP-1 may involve this segment.

Phosphatase specificity; Okadaic acid; Peptide antibody; Myosin light chain

1. INTRODUCTION

Of the four major cytosolic protein phosphatases involved in cellular regulation, types 1 and 2A are the primary regulators of metabolism [1]. In the past few years, cDNA clones for type-1 and type-2A phosphatases have been isolated and sequenced [2–9]. The sequence for the catalytic subunit of protein phosphatase type-1 is comprised of 330 amino acids and predicts an M_r of 37.4 K [7,9]. Comparison of the type-1 and type-2A sequences reveals that the two phosphatases have about 50% amino acid sequence identity. The available sequences reveal consensus segments that are likely to be functional regions of the phosphatase [6]. However, the carboxyl terminal segments, unlike the rest of the structure, show no identity between type-1 and type-2A phosphatases.

The catalytic subunit of PP-1¹ (M_r 38 000) can be converted to a catalytic fragment, PP-35K (M_r 35 000) by trypsin digestion in the presence of divalent metal [10–12] with an increase in phosphorylase phosphatase activity. It has been established that metal ion is responsible for the activation [13]. Digestion by chymotrypsin [14] has the same effect. Using anti-peptide antibodies,

the apocryphal belief that digestion removes a segment at the carboxyl terminus was confirmed. Synthetic peptides corresponding to only part of the carboxyl terminus of PP-1 interact with PP-35K and cause an increase in activity. Based on these results, we propose that the 'tail' of PP-1 binds to a specific site on the PP-35K fragment and modulates substrate interactions.

2. EXPERIMENTAL

2.1. Materials

Phosphorylase *b* was purified according to Fischer and Krebs [15] and converted to phosphorylase *a* [16]. Glycogen synthase was isolated from rabbit liver [17]. Myosin light chains were isolated from turkey gizzard [18] and phosphorylated by the cAMP-dependent protein kinase, which phosphorylates the same site as myosin light chain kinase [19]. MOPS, PMSF, trypsin, and lima bean trypsin inhibitor were purchased from Sigma. Okadaic acid was from Moana Bioproducts. ¹²⁵I-Protein A and [γ -³²P]ATP were purchased from Dupont-New England Nuclear. Prestained molecular weight standards for electrophoresis were obtained from Diversified Biotech. Other chemicals were purchased from Fisher Scientific.

2.2. Methods

2.2.1. Preparation of type-1 protein phosphatase

The catalytic subunit of type-1 phosphatase was isolated essentially as described previously [20] for preparation of the catalytic fragment, but with modifications to be described elsewhere². The preparations were determined to be type-1 phosphatase by their sensitivity to phosphatase inhibitor-2 and reactivity with anti-(type-1 phosphatase) antibodies detected on immunoblots [21]. The purified proteins had specific activities of 8000–12 000 U/mg for the catalytic subunit and 20 000–30 000 U/mg for the catalytic fragment.

2.2.2. Phosphatase assays

Typically, phosphorylase phosphatase activity was assayed in

Abbreviations: PP-1, catalytic subunit of type-1 protein phosphatase; PP-35K, protease resistant catalytic fragment of PP-1; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride

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duplicate at 30°C as described [22]. Assays were also done with [³²P]-phosphorylase α as substrate. Briefly, at specified time points, 10 μ l of the reaction were spotted on Whatman 3MM filter papers and the papers were washed 3 times in cold 10% trichloroacetic acid for 10 min and then twice with 95% ethanol for 5 min. The papers were dried and the amount of unreacted substrate remaining was quantified by liquid scintillation spectrometry. Glycogen synthase phosphatase activity was assayed according to a published procedure [23] using rabbit liver glycogen synthase D as the substrate. The myosin light chain phosphatase activities of isolated PP-1 and PP-35K were measured with [³²P]MLC (1 μ M; 500–700 cpm/pmol) in standard assay buffer. After incubation at 30°C for 10 min, the reaction was stopped by spotting onto Whatman 3MM filter paper and processed as described with phosphorylase as substrate. Direct measurement of the effect of trypsin digestion on myosin light chain phosphatase activity of PP-1 was done by the lab of Dr. David Hartshorne (Dept. of Muscle Biology, Univ. of Arizona, Tucson, AZ) as described [24]. With both glycogen synthase and myosin light chains, trypsin was included at 10 μ g/ml.

2.2.3. Peptide synthesis.

The synthetic peptide, P312-326, with sequence, Leu-Asn-Pro-Gly-Gly-Arg-Pro-Ile-Thr-Pro-Pro-Arg-Asn-Ser-Ala, was synthesized on the ABI 430A peptide synthesizer (Applied Biosystems, Inc.). Purification of the peptide was done on a Beckman 334 chromatography system with a Hitachi model 100-40 detector at 220 nm. The separation used VYDAC C₁₈ (250 \times 4.6 mm) and C₄ (250 \times 4.6 mm) reverse-phase columns with gradients of acetonitrile in 0.1% trifluoroacetic acid. The amino acid composition of the purified peptide was confirmed with amino acid analysis using a Beckman model 7300 amino acid analyzer. Peptides corresponding to the amino terminus of PP-1, Ser-Asp-Ser-Glu-Lys-Leu-Asn-Leu-Asp-Ser; the carboxyl terminus of PP-2A, Arg-Gly-Glu-Pro-His-Val-Thr-Arg-Arg-Thr-Pro-Asp-Tyr-Phe-Leu; and a derivative of P312-326, Ala-Pro-Ile-Thr-Pro-Pro-Ala, were similarly synthesized, purified and analyzed.

2.2.4. Stimulation by peptide

The effect of peptides on PP-35K was determined by preincubating phosphatase (1–4 nM) with various concentrations of peptides in 20 μ l for 10 min at 30°C followed by the addition of 20 μ l phosphorylase α to initiate the phosphatase reaction. Phosphatase activity was determined as described. The kinetic parameters of PP-35K plus or minus peptide P312-326 were determined with [³²P]phosphorylase α (15–20 cpm/pmol) as substrate, over the range of 6.4 to 32.0 μ M. The amount of substrate present at time zero was quantified. A portion (10 μ l) of the reaction mixture was removed at 4, 8 and 12 min and the ³²P-substrate remaining was quantified by liquid scintillation spectrometry as described. Initial velocities were determined, in duplicate, from linear plots of substrate remaining vs time. The kinetic constants were evaluated with the Michaelis-Menten equation using a BASIC program written for the IBM PC obtained from Dr. D. Quinn (Dept. of Chemistry, Univ. of Iowa, Iowa City, Iowa).

2.2.5. Electrophoresis and immunoblotting

Polyacrylamide gel electrophoresis was performed according to Laemmli [25]. Immunoblotting was done with the procedure of Burnette [26] using affinity purified antibodies directed against the catalytic fragment of type-1 phosphatase (2.5 μ g/ml) and rabbit anti-sheep IgG as the secondary antibody [20]. Detection was accomplished with [¹²⁵I]-Protein A exposed on Kodak XAR-5 film at –70°C with Dupont Cronex Hi-plus screens.

2.2.6. Protein determination

Protein concentrations were determined by the Lowry procedure [27] or by the method of Bradford [28] using lysozyme as the standard.

3. RESULTS AND DISCUSSION

3.1. Comparison of PP-1 and PP-35K

Intact PP-1 catalytic subunit and the catalytic fragment, PP-35K, can be distinguished by functional properties. PP-35K has increased phosphorylase phosphatase activity relative to PP-1 [11,12] (see Fig. 1A). In contrast, purified PP-35K had much lower activity with myosin light chains than PP-1; 107 vs 319 U/mg phosphatase, respectively (Fig. 1B). Protease digestion of PP-1 in situ decreased the myosin light chain phosphatase activity and resulted in an almost complete loss of glycogen synthase phosphatase activity in the absence of Mn²⁺ (33 vs 5 U/ml). The presence of Mn²⁺ mitigated the apparent loss of glycogen synthase phosphatase activity, but did not prevent it. PP-35K has been presumed to be PP-1 lacking its carboxyl terminus because both proteins are resistant to Edman degradation [29]. Here we demonstrate that the difference between PP-1 and PP-35K is the loss of a carboxyl-terminal segment. Polyclonal antibodies to PP-35K [20] reacted with both PP-35K and intact PP-1 as expected (Fig. 2, lanes a and b). In contrast, antibodies to peptides corresponding to PP-1 residues 312–326 (Martin et al., manuscript in preparation) or to residues 317–330 (Olivier et al., unpublished observations) specifically reacted with PP-1 (38K) on immunoblots, but were not reactive with the trypsin fragment, PP-35K (Fig. 2, lanes c and d). These results show that PP-35K is truncated at the carboxyl terminus in the region including residues 312–330 of PP-1. Together, these results show that the carboxyl terminal region is not simply an autoinhibitory segment, like those found in several protein kinases [30], as was suggested for PP-1 by Tung et al. [12]. The removal of an autoinhibitory domain can not account for the reciprocal changes in the activity of PP1 with different substrates.

Although these enzymes were differentially active with different substrates, they did not demonstrate any

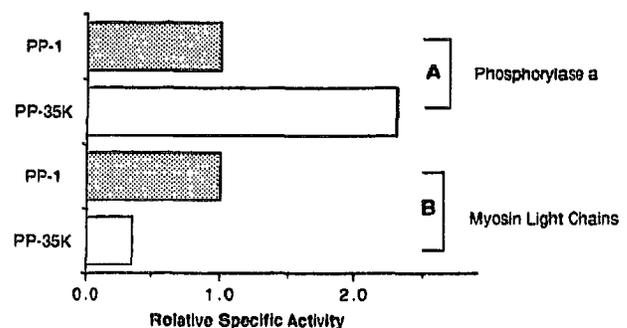


Fig. 1. Activity of PP-1 and PP-35K with different substrates. The specific activities of purified PP-1 (shaded bars) and PP-35K (open bars) with phosphorylase α and myosin light chains were compared using assays described in section 2. The values have been normalized to a value of 1.0 for PP-1 that were 11 800 U/mg with phosphorylase α and 319 U/mg with myosin light chains.

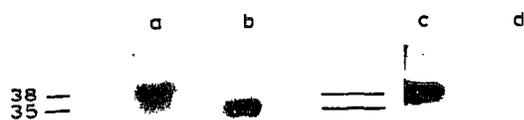


Fig. 2. Western blotting of PP-1 and PP-35K with polyclonal anti-PP-35K and site specific anti-carboxyl terminus peptide antibodies. Samples (2 μ g) of PP-1 (lanes a and c) and PP-35K (lanes b and d) were electrophoresed on a 12% dodecyl sulfate acrylamide gel and subsequently electrotransferred to a nitrocellulose filter. The filter was probed either with antibody to PP-35K (lanes a and b) or with antibody to synthetic peptide P312-326 corresponding to the carboxyl terminus of PP-1 (lanes c and d). Subsequent detection was done with [125 I]-Protein A and autoradiography. Identical results were obtained with antibodies against another carboxyl terminal peptide composed of residues 317-330 (Olivier et al., unpublished observations).

difference in response to phosphatase inhibitors. Both PP-1 and PP-35K are inactivated in an identical time dependent reaction by the protein inhibitor-2 (not shown) and inhibited identically by the marine toxin, okadaic acid (Fig. 3) demonstrating that the carboxyl terminus is not involved in the binding of these inhibitors. Furthermore, these results indicate that the carboxyl terminal segment must not be part of, nor occupy, the catalytic site of PP-1.

3.2. Effects of PP-1 carboxyl-terminal peptides on PP-35K activity

Addition of peptides corresponding to the missing carboxyl terminus selectively enhanced the activity of PP-35K, but not PP-1. The synthetic peptide corresponding to residues 312-326 of PP-1 (P312-326, Table I), stimulated the activity of PP-35K (tested with 3 different preparations) up to 30% (Fig. 4). No increase in activity was observed with 0.01 μ M P312-326 ($n=3$ independent trials). However, with 0.10 μ M P312-326, there was a 25% stimulation of activity ($n=6$; $P<0.01$). At higher concentrations of 1.0 and 10

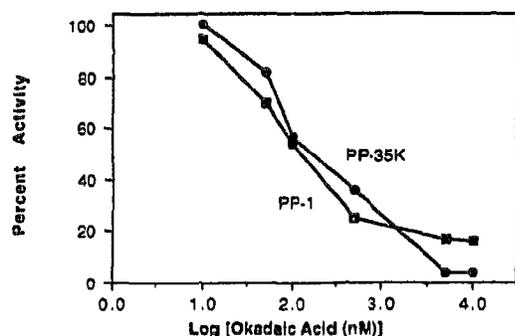


Fig. 3. Inhibition of PP-1 and PP-35K by okadaic acid. The phosphorylase phosphatase activities of PP-1 (closed squares) and PP-35K (closed circles) were assayed in the presence of 10, 50, 100 and 500 nM, and 5 and 10 μ M okadaic acid by measuring 32 P_i release from [32 P]phosphorylase α . Activities are presented relative to a control reaction in the absence of okadaic acid.

Table I

Sequences of synthetic peptides

Peptide	Peptide sequence
P312-326	L-N-P-G-G-R-P-I-T-P-P-R-N-S-A
A318-322A	A-P-I-T-P-P-A
P2-10	S-D-S-E-K-L-N-L-D-S
P295-309	R-G-E-P-H-V-T-R-R-T-P-D-Y-F-L

The sequences of peptides tested as activators of PP-35K are presented in the single letter code: A, alanine; D, aspartic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; N, asparagine; P, proline; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine.

μ M P312-326, the same level of stimulation was observed ($n=8$; $P<0.01$) showing saturation by the peptide. Peptide P312-326, at both 1.0 ($n=3$) and 10 ($n=3$) μ M (see Fig. 4), did not influence the activity of PP-1, with an intact carboxyl terminus. The lack of an effect with PP-1 under identical assay conditions also demonstrated that the effect of peptide was enzyme-directed, not a result of interaction with substrate. This conclusion was corroborated by the observation that activity of PP-35K, but not PP-1, also was stimulated 30% with myosin light chains as substrate (Fig. 4; $n=3$; $P<0.05$).

There was specificity not only for the phosphatase, but also for the peptide. Peptides corresponding to the carboxyl terminus of phosphatase-2A (P295-309, Table I) and the amino terminus of PP-1 (P2-10, Table I) did not substitute for P312-326 (Fig. 4; $n=2$). Therefore, the catalytic core of PP-1, with over 50% overall identity to PP-2A and several regions of perfectly conserved

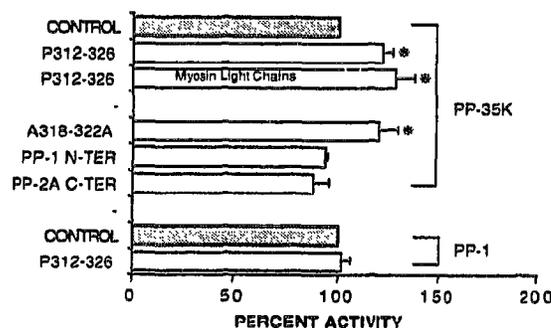


Fig. 4. Effects of various peptides on the phosphorylase phosphatase activity. From top to bottom: PP-35K alone (control; shaded) or with peptide P312-326 (10.0 μ M) was incubated for 10 min at 30°C and phosphorylase activity determined with phosphorylase α or myosin light chains as described. The effect of peptides A318-322A (A318-322A), P2-10 (PP-1 N TER), and P295-309 (PP2A C TER) on PP-35K activity was tested at 10.0 μ M peptide. PP-1 was incubated alone (control; shaded) or with peptide P312-326 (10.0 μ M) for 10 min at 30°C. The activities are expressed as percent of control to normalize over multiple experiments with 3 different preparations of PP-35K; for the experiments shown here the control activity was 0.80 ± 0.09 U/ml. An asterisk (*) denotes a statistically significant difference from control (Student's t -test).

sequences, was able to discriminate between the carboxyl termini of PP-1 and PP-2A and between the amino and carboxyl termini of PP-1.

Within P312-326, the sequence, Arg-Pro-Ile-Thr-Pro-Pro-Arg, is conserved between rabbit and mouse PP-1 with a valine for isoleucine substitution as the only difference [31]. The sequence Thr-Pro-Pro is also present in the *dis2⁺*, the PP-1 gene in *S. pombe* [31]. A derivative of this sequence, Ala-Pro-Ile-Thr-Pro-Pro-Ala (A318-322A, Table I), also stimulated the phosphorylase phosphatase activity of PP-35K (Fig. 4). At 1.0 μ M A318-322A, there was no significant change in the activity of PP-35K ($n = 3$), compared to the same concentration of P312-326 which caused maximal stimulation. However, 10 μ M A318-322A produced significant stimulation ($n = 3$; $P < 0.05$) of PP-35K up to the same level (Fig. 4) as P312-326 showing that both peptides saturated the response. Therefore, specific peptides stimulated PP-35K, but not intact PP-1, and the effect was selective for a sequence with multiple and vicinal proline residues that is conserved between species.

Kinetic analysis showed that the stimulatory effect of P312-P326 (10 μ M) involved a decrease in the K_m for phosphorylase without a change in V_m (Fig. 5). In the absence of peptide P312-326, the value of K_m was 19 ± 1.7 mM and in the presence of 10 μ M peptide, the K_m was 15 ± 1.7 mM. Activity increased in the presence of peptide because of a decrease in K_m for phosphorylase. Based on the K_m in the presence of peptide P312-326, estimation of the predicted initial velocities predicts a 20% increase at 10 μ M phosphorylase, consistent with the stimulation (20–30%) depicted in Fig. 4. The reciprocal changes in PP-1 activity with different substrates found by Johansen and Ingebritsen after digestion with chymotrypsin predominantly involved increases in K_m [14]. These results implicate the carboxyl terminus in regulating activity of the phosphatase.

3.3. Role of the carboxyl-terminal domain

How can both removal of the carboxyl terminus by digestion and its replacement with a synthetic peptide activate the phosphatase? The results can be explained by a model in which the carboxyl terminus interferes with metal ion activation of PP-1. Studies over the past 20 years have demonstrated enhancement of phosphorylase activity by Mn^{2+} [13,32–37] implicating a role for divalent metal in PP-1 activity. Villa-Moruzzi et al. [13] have reported that isolated PP-1 is inactive unless divalent metal ion is added. Although we have isolated an active preparation of PP-1 without the addition of exogenous metal, we find that in the presence of Mg^{2+} , the activity of PP-1 and PP-35K was stabilized. McNall and Fischer [38] demonstrated that PP-1 activity with phosphopeptide substrates required exposure of the enzyme to Mn^{2+} which increased in the order: heterodimer of PP-1 and inhibitor-2 < catalytic

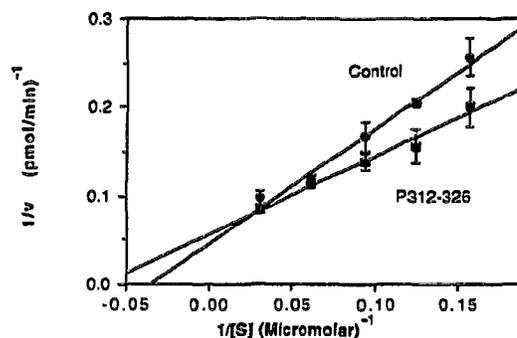


Fig. 5. Effect of peptide P312-326 on PP-35K kinetics. As described in section 2, initial velocities were determined from time courses for the release of $^{32}P_i$ from phosphorylase in the absence (closed circles) and presence (closed squares) of 10 μ M peptide P312-326. Other details of the data handling are in the text. Kinetic parameters were determined using a program written for the IBM PC by Dr. D. Quinn (Dept. of Chemistry, Univ. of Iowa, Iowa City, IA). The line shown was generated using the Graph program of Cricket software.

subunit < trypsin-digested catalytic subunit. This is consistent with previous observations that trypsin plus Mn^{2+} (or Co^{2+}) affords the highest levels of PP-1 activity [10]. Removal of the carboxyl-terminal segment would expose a site on the catalytic core of PP-1 for interaction with divalent metal ions that allowed expression of the highest levels of Me^{2+} -dependent activity. Exposure of this site after digestion provides an explanation for the apparent conundrum that association of PP-35K with peptide segments is not just the reversal of protease digestion.

The amino acid sequences from cDNA for PP-1 from *Drosophila melanogaster* [39] and *Saccharomyces cerevisiae* [31] are both truncated at their carboxyl termini, producing catalytic subunits which contain only the catalytic core, similar to PP-35K. Extracts from *S. cerevisiae* have unusually low phosphorylase phosphatase activity compared to extracts of mammalian tissues, even when the activity was normalized to the level of β -subunit specific phosphorylase kinase phosphatase (type-1) activity [40]. It will be interesting to determine the branch of the phylogenetic tree at which the sequence, Pro-Ile-Thr-Pro-Pro, is first present in the carboxyl terminus of PP-1.

Regulation of activity and/or specificity of PP-1 in 'larger' eukaryotes may involve modifications of the carboxyl terminus of PP-1. The active peptide segment, Pro-Ile-Thr-Pro-Pro, provides a potential phosphorylation site in the PP-1 catalytic subunit. Reportedly a brain phosphatase, recently classified as a type-3 phosphatase [41], is phosphorylated on threonine by protein kinase C [42]. We find that a peptide containing the core sequence and similar to P312-326 is phosphorylated by different protein kinases (Martin, B.L. et al., manuscript in preparation). The interaction of the carboxyl terminus with the catalytic core to modulate activity may be influenced by its

phosphorylation state. It will be interesting to explore the possible regulation of PP-1 by phosphorylation of its carboxyl terminal segment.

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