A Protein Phosphatase-1-binding Motif Identified by the Panning of a Random Peptide Display Library*

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An unusually large number of regulatory or targeting proteins that bind to the catalytic subunit of protein phosphatase-1 have been recently reported. This can be explained by their possession of a common protein motif that interacts with a binding site on protein phosphatase-1. The existence of such a motif was established by the panning of a random peptide library in which peptide sequences are displayed on the *Escherichia coli* bacterial flagellin protein for bacteria that bound to protein phosphatase-1. There were 79 isolates containing 46 unique sequences with the conserved motif VXF or VXW, where X was most frequently His or Arg. In addition, this sequence was commonly preceded by 2–5 basic residues and followed by 1 acidic residue. This study demonstrates that binding to protein phosphatase-1 can be conferred to a protein by the presentation of a peptide motif on a surface loop. This binding motif is found in a number of protein phosphatase-1-binding proteins.

Protein phosphatase-1, originally studied in the context of glycolysis metabolism, is the enzyme that converts phosphorylase a to phosphorylase b (1). It has been implicated in the regulation of a number of important cellular processes (for reviews, see Refs. 2 and 3). Biochemical studies have revealed that it plays a role in the regulation of a number of important cellular processes (for reviews, see Refs. 2 and 3). Biochemical studies have revealed that it plays a role in the regulation of a number of diverse cellular processes.

Few, if any, of the PP1 proteins share any major sequence identity, although examination of different glycol-binding subunits has revealed the presence of two small regions of sequence similarity that is shared between several glycogen-binding proteins (20–22). The unusually large number of PP1-binding proteins that have been described suggests either that PP1 contains a motif that is recognized by a common binding structure on this diverse group of proteins or that the latter contain a protein motif that is recognized by a single binding site on PP1. To test the latter possibility, we have used a panning technique to isolate PP1-binding peptides using a random peptide display library and found that there is a consensus binding motif for PP1.

**EXPERIMENTAL PROCEDURES**

**Preparation of PP1**—Recombinant PP1a was expressed in *Escherichia coli* (26) and purified as described previously (27). The preparation was further purified by gel filtration on a Sephacryl S-200 column (1.5 × 60 cm).

**Growth of the *E. coli* Peptide Library**—The FliTrx random peptide library was obtained from Invitrogen (San Diego, CA) and is based on the system described by Lu et al. (28). Growth of the cultures and general panning methods were essentially as described in the manufacturer's protocol or as by Lu et al. (28). *E. coli* strain G286 harboring the pFliTrx plasmids was grown in IMC medium (6 g/liter Na2HPO4, 3 g/liter KH2PO4, 0.5 g/liter NaCl, 1 g/liter NH4Cl, pH 7.4, 0.2% casamino acids, 0.5% glucose, and 1 mM MgCl2) containing 100 μg/ml ampicillin.

Expression of the thioredoxin-flagellin fusion proteins containing the peptide inserts was induced by growth in IMC medium containing 100 μg/ml ampicillin and 100 μg/ml tetracyclin at 25 °C for 6 h.

**Immobilization of PP1 on Culture Plates**—PP1 was immobilized on 60-mm plastic Petri dishes by adsorption from a solution of PP1 (1 ml, 100 μg of PP1/plate) with gentle agitation for 1 h. Adsorption was confirmed by testing for activity of the bound enzyme toward phosphorylase a. The plates were washed with 10 ml of sterile water and agitated for 1 h with 10 ml of blocking solution (1% nonfat dry milk, 150 mM NaCl, 1% α-methylmannoside, and 100 μg/ml ampicillin in IMC medium).

**Panning of the Random Peptide Display Library**—The following were added to 50 ml of the induced *E. coli* culture after 6 h of growth: 0.5 g of nonfat dry milk, 1.5 ml of 30% NaCl, and 2.5 ml of 20% α-methylmannoside. The culture (10 ml) was used in a “panning” technique by addition to the plate containing immobilized PP1. The plate was rotated gently for 1 min at 50 rpm and allowed to incubate for 60 min at room temperature. The bacterial culture was decanted, and the plate was washed by gentle agitation for 5 min with 10 ml of IMC medium containing 100 μg/ml ampicillin and 1% α-methylmannoside. After an additional four washes, the bound bacteria were detached by simple mechanical shearing (vortexing the plates for 30 s). The detached bacteria were decanted from the plate and grown as described above. The culture was then induced by growth with tryptophan-containing medium, and the cycle of panning was repeated. Six rounds of panning were performed, after which the cultures were plated out, and individual colonies were selected for further study. The pFliTrx plasmids were then isolated and sequenced using the primer 5'-ATTACCTGTGAT-3'.
Procedures”) were centrifuged, and the cells were resuspended in 60 μl of SDS-PAGE loading buffer and boiled for 5 min. Ten μl of the samples were then subjected to SDS-PAGE and transferred to nitrocellulose. The blots were exposed to digoxigenin-conjugated PP1, and bound digoxigenin-conjugated PP1 was then detected using an anti-digoxigenin antibody (see “Experimental Procedures”). A blot for 13 isolates is shown in the upper panel (lanes 1-13), and the corresponding gel stained for protein with Coomassie Blue is shown in the bottom panel. Lane S refers to the prestained protein standards, which, in order of descending size, were 83, 62, 45, 32.5, 25, and 16 kDa. The position of the 68-kDa thioredoxin-flagellin fusion protein is indicated in the bottom panel. Note that the isolate in lane 9 harbors peptide D2, whereas lanes 1 and 2 harbor peptide D1 (Table I).

Digoxigenin-conjugated PP1 Blotting of PP1-binding Proteins—PP1 (0.25 mg/ml) was dialyzed overnight in 50 mM sodium borate and 1 mM dithiothreitol, pH 8.5, and concentrated on a Centricon-10 filter. One ml of the samples was reacted with 25 μl of digoxigenin carboxymethyl-N-hydroxysuccinimide ester (2 mg/ml in dimethyl sulfoxide; Boehringer Mannheim) for 2 hours at room temperature and then passed through a Sephadex G-25 column equilibrated in phosphate-buffered saline. The conjugated enzyme was then diluted 1000-fold in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM bovine serum albumin and stored at 4°C. Polyclonal sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Boehringer Mannheim) were used for detection of PP1-binding proteins after SDS-PAGE and transfer to nitrocellulose (29, 30).

Assay of PP1 Activity—PP1 activity was assayed using 32P-labeled rabbit muscle phosphorylase a as the substrate (26). PP1 was diluted in 50 mM imidazole chloride, pH 7.4, 1 mM EDTA, and 0.2 mg/ml bovine serum albumin immediately before assay. The synthetic peptides RPKKKRRKRRSP, SKFERVDPRRSRTDK, CGPVHRKRVRFEADVGPC, CGPSTRHVVĐWDREDAGPC, and CGPVRVSRRHVHWDLEGPC were synthesized by GENEMED Synthesis Inc. (San Francisco, CA). The peptides were dissolved in water and diluted in 50 mM imidazole chloride, pH 7.4.

RESULTS

The FliTrx library displays random dodecapeptides flanked by the sequences CPG and GPC at their N and C termini, respectively, and has a diversity of 1.77 × 10^6 individual peptides. The peptides are inserted into the active-site loop of thioredoxin, which is itself fused into the major flagellin protein of E. coli. The peptides are thus displayed on the flagella. Six rounds of panning on Petri dishes to which PP1 was adsorbed were performed (see “Experimental Procedures”), after which the plasmids from individual E. coli isolates were isolated and analyzed. To confirm that these isolates were indeed binding to PP1, they were examined for the expression of thioredoxin-flagellin fusion proteins that would bind to PP1. This was done by SDS-PAGE of the E. coli proteins and transfer to nitrocellulose, after which PP1 binding was determined using digoxigenin-conjugated PP1. A typical set of results are shown in Fig. 1. Of 13 isolates examined, it can be seen that there are 10 positives for a protein of 68 kDa. This is consistent with the expected size of the fusion protein since E. coli thioredoxin has a molecular mass of ~12 kDa (31) and E. coli flagellin has a molecular mass of ~60 kDa (32).

DNA sequence analysis of the encoded peptides from a total of 104 isolates was performed. Seventy-nine of these isolates harbored peptide sequences that could be aligned as shown in Table I. These represent 46 unique sequences that share the common motif V(F/W)X(F/W)X(F/W) and acidic residues in the first and second positions after the motif are underlined.

Alignment of peptide sequences that bind to PP1

| Sequence | No. Occurrences |
|----------|-----------------|
| A1       | VEHVZVVFDAEV    |
| A2       | VEHVZVVFADD     |
| A3       | LKKVVFZDGP      |
| A4       | VVEVFVFZEOGAA   |
| A5       | VVEVFVFZEOGAA   |
| A6       | RKKVVRKGRVNS    |
| A7       | RKKVVRKVPVPCQ   |
| A8       | RKKVVRSPNIVRQR  |
| A9       | AKKSVKVRGDLR    |
| A10      | BAKKOVFAQLR     |
| A11      | SBKKOVOFAQLR    |
| A12      | BBKKOVOFAQLR    |
| A13      | VEEVOFAADKR     |
| A14      | BAEKVVOFAADLR   |
| A15      | LNHVHRFNGAVS    |
| ...      |                 |

TABLE I

Shown is an alignment of 79 peptide sequences that were obtained by analysis of the pFliTrx plasmids obtained after six rounds of panning against immobilized PP1 (see “Experimental Procedures”). Basic residues that occur N-terminal to the VX/F/W motif and acidic residues in the first and second positions after the motif are underlined.
by 2 basic residues immediately preceding the conserved valine. The residue immediately N-terminal to the conserved valine was frequently Arg or Lys, although Gln and His were also well represented. On the C-terminal side of the V/(F/W) motif, it was also evident that an acidic residue was present in 1 of the next 2 residues. Thus, the consensus motif that emerges is (R/K)(R/K)0–2V(R/H)(F/W/X/D/E). The frequency of amino acids at various positions is shown diagrammatically in Fig. 2. These findings demonstrate the existence of a peptide binding motif for PP1.

We tested several synthetic peptides for their ability to inhibit PP1 since a number of the proteins that contain the binding motif are inhibitors of PP1. The peptide sequence derived from the NIPP sequence (11), which contains the motif RVTFSE, and a scrambled sequence of the same amino acids were tested for the ability to inhibit phosphorylase phosphatase activity. The NIPP peptide was inhibitory, with an IC50 of −3 μM (Fig. 3). That this inhibition is specific is supported by the failure of the scrambled sequence to inhibit PP1 activity at concentrations up to 10 μM. Above this value, the scrambled NIPP peptide was inhibitory, with an IC50 of 100 μM. We also tested a peptide (Table I, A1) that harbors the VRF motif. This peptide was inhibitory, with an IC50 of −10 μM. This is consistent with the finding that a N-terminal peptide (residues 349–430) of the splicing factor PSF, which harbors an RVRF motif, is inhibitory to PP1 (15). Two peptides (Table I, D1 and D2) containing the most frequently encountered VHW motif were tested for the ability to inhibit phosphorylase phosphatase activity. The NIPP peptide was inhibitory, with an IC50 of 100 μM. This is consistent with the finding that a N-terminal peptide (residues 349–430) of the splicing factor PSF, which harbors an RVRF motif, is inhibitory to PP1 (15). Two peptides (Table I, D1 and D2) containing the most frequently encountered VHW motif were not inhibitory at low concentrations and behaved in a similar way to the scrambled NIPP peptide in that inhibition was observed at high concentrations. Because of the basic nature of these peptides, it is possible that this inhibition is similar to the inhibition of phosphorylase phosphatase activity by polyamines, which has previously been shown to be likely a substrate-directed effect (33).

**DISCUSSION**

In this study, we have used a random peptide display library in which peptides are inserted into a surface loop at the active site of thioredoxin (31), which itself is fused into a nonessential domain of the bacterial flagellin protein (28). Our results clearly demonstrate that a short peptide sequence containing the general motif VXF or VXW preceded by 1 or more basic residues is sufficient to generate a capacity for the fusion proteins to bind to PP1. These results strongly support the thesis that the binding of proteins to PP1 can be specified by presentation of this binding motif. During the course of this work, the crystal structure of PP1δ in a complex with a peptide corresponding to the region on the muscle glycogen-binding subunit, Gm, that had previously been implicated in binding to PP1 was determined (34). The crystal structure reveals that the sequence RRVSFA of Gm binds in an extended conformation to a hydrophobic groove, adjacent to a region of acidic residues that accommodates the N-terminal basic residues. This site is located on a side of PP1 that is nearly opposite to the active site and is thus ideally situated as a site for the binding of targeting subunits. Taken together with our findings that binding to this region can be specified by a relatively short consensus peptide sequence, there is now a strong structural basis for the concept that PP1 contains a site that recognizes a novel peptide motif that can confer PP1-binding ability to a given protein.

An examination of the coding regions of a number of yeast genes as well as the sequences of mammalian PP1-binding proteins for the presence of the binding motif is shown in Table II. Table II includes sequences encoded by the yeast genes GAC1, REG1, REG2, SCDS, GIP1, SHP1, and GIP2 and several yeast open reading frames, most of which were identified by a two-hybrid screen (20, 21), and the mammalian proteins DARPP-32, GIP1, SHP1, and GIP2 and several yeast open reading frames, most of which were identified by a two-hybrid screen (20, 21), and the mammalian proteins p53bp2, NIPP, splicing factor PSF, the M110 subunit and several variants of the glycogen-binding subunits, and the herpes simplex virus protein γ34.5 (12, 14–18, 22–24). With one exception, these all exhibit the VXF motif, and only one occurrence of the VXW sequence was found. Both inhibitor-1 and DARPP-32 contain sequences at their N termini (KIQF) that are similar to the RVF motif and that are required for inhibition of PP1 (35, 36). Interestingly, the results of the random peptide screen did not yield any substitution of Val for Ile. It should also be noted that there are several sequences that fit the consensus in GIP2, SCDS, and YFL023W (Table II), but that are inconsistent with the results of the two-hybrid screen in that the fusions are one in which these sequences are deleted. Clearly, identification of any of these sequences as a PP1-binding motif needs to be experimentally confirmed by mutagenesis studies.

Our data indicate that binding of the peptides containing the NIPP sequence and a peptide containing a VRF motif was inhibitory in the micromolar range. This is much weaker than the inhibition observed with several inhibitory proteins of PP1,
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Table II

| Gene/protein | Sequence | Reference |
|--------------|----------|-----------|
| S. cerevisiae genes | GACI | TSEKSNVRPAE | 76 X63941 |
| | GIP | KEEKCVFNPKN | 452 Z35914 |
| | GIP | DESSYVRFSD | 497 |
| | GIP2 | LBSXSVHPDAO | 227 U18813 |
| | GIP2/W | LGDSVWHPDRV | 202 Z46861 |
| | YIP26/W | EYVFRFSD | 422 |
| | SCD5 | GLPPFSWSPFD | 37 U03492 |
| | SCD1b | NTSEKYSFSD | 279 |
| | YFL023W | DIESTLVRFND | 42 D50617 |
| | YAL014 | ESELKVYRFNDK | 106 L01546 |
| | YFR003c | TPTENRVEWEN | 46 D50617 |
| | SNP1 | AKELDQOFQG | 271 Z25819 |
| | SHP1 | CNDSTDVFYLF | 355 |

Mammalian

| Rabbit Gm | GSRRGEVSP | 71 M65109 |
| Human Gm | GSRRGEVSP | 68 X75787 |
| PTG | NAOKKESVFRDSP | 67 U08992 |
| Human Gm | TTVLSNQYFVD | 155 X75787 |
| PTG | TVLSVHNFSD | 160 U08992 |
| Gm | KEKAVTNVR | 67 U08992 |
| M110 | KETEYVYPDFD | 41 Z74907 |
| NIPP1 | KEKAVTVSFSD | 206 Z50748 |
| p35 | TAHGMYVNPFS | 804 U58334 |
| p35 | AKELDQOFQG | 271 Z25819 |
| Splicing factor PSIF | DGQCLEWFP | 369 Z70944 |
| Rib. protein L5 | YFLRFFQVPVR | 22 U14966 |
| HSV y734.5 | PTAPAEYRFFP | 187 M33701 |
| Hamster GADD44 | PLARAENVPFS | 511 L28147 |
| DARPP12 | KEKAVTNVR | 67 U08992 |
| Inhibitor-1 | DSNPUEQTP | 14 M23594 |

See Tu et al. (20). The following Saccharomyces cerevisiae genes were examined and found not to contain sequences resembling the binding motif: GLC8, Orf06032, RED1, SLA1, YTA6, YTF7, D9461.18, and SDS22. Both REG1 and REG2, however, contain the motif K/V/I/F (34).

b Numbering and/or sequence differs slightly from that given by Egloff et al. (34).

c GIP2 fusions were found at codons 192 and 349 in the two-hybrid system (30); this would eliminate residues 216–227 as a binding sequence. Similarly, SCD5 fusions were found at codons 53 and 239, eliminating sequence 26–37, and YFL023W were found at codon 106, eliminating sequence 31–42 as candidates for PP1 binding.
d Both the PTG and human Gm glycogen-binding sequences have two sequences that could fit the consensus. The more C-terminal of the two falls in a region of sequence similarity between the glycogen-binding proteins and glucocarnylase, and it is a putative carbohydrate-binding sequence (20, 24). Wu et al. (25) have also suggested that this region might be involved in PP1 binding.

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which have IC50 values in the nanomolar range (2, 3, 10). On the other hand, two other peptides containing the VHW motif were not inhibitory. The differential effects on the inhibition of phosphorylase phosphatase activity observed with the peptides were not surprising, given that the binding of the Gm subunit has been shown to partially inhibit phosphorylase phosphatase activity, but to increase activity toward glycogen synthase (15, 22, 34). It remains a possibility that binding of variants of the PP1-binding motif could produce differential effects on either activity or substrate specificity. In this regard, it is of interest to note that both inhibitor-1 and DARPP-32 contain sequences at their N termini (KIQF) that are similar to the RVXF motif and that are required for inhibition of PP1 (35, 36). Irrespective of whether occupancy of the VXF binding site can affect either activity or substrate specificity, there is support for a model in which a high affinity interaction with PP1 involves at least two binding sites of lower affinity, as has been suggested for inhibitor-1 and DARPP-32 (34–36).

The analysis ofputative PP1-binding proteins for motifs that might represent a binding sequence is obviously facilitated by the information provided by the availability of the family of peptides that are shown in Table I. The structural information that only a small peptide motif is involved in the interaction with the PP1 binding site (34) and the demonstration that presentation of peptides conforming to a consensus motif on a surface loop is sufficient to endow PP1 binding to the thio-doxin-flagellin fusion protein have other implications for the identification of PP1-binding proteins. The motif K/V/I/F occurs in 10% of known sequences, so that the occurrence of the motif is very common, and the issue of whether such a sequence functions to bind PP1 may depend on its structural context (34). Thus, caution is needed in the sole use of the yeast two-hybrid system for the identification of PP1-binding proteins, particularly since this commonly involves the ability of partial protein sequences to interact with PP1. Expression of a fusion protein with a partial sequence or even fusion of a full-length sequence may lead changes in the structural context of sequences that have the potential for PP1 binding, but that are cryptic in the native protein, such that these are now surface-accessible and functionally active in PP1 binding. The identification of putative PP1-binding proteins that are of physiological relevance by the two-hybrid system needs to be confirmed experimentally by the biochemical demonstration of binding of the native protein with PP1. For example, it was found that the PSF protein inhibited PP1 with an IC50 that is 30-fold higher than for a partial fragment, and it was concluded that PSF, at least in an unmodified form, was unlikely to function to inhibit PP1 in vivo (15). The same caveat holds for the use of digoxigenin-conjugated PP1 for the detection of PP1-binding proteins following SDS-PAGE. Nevertheless, it is evident that mammalian PP1-binding proteins is likely to be quite large, as demonstrated by the use of immobilized microcystin affinity chromatography (37).

Recent findings have now provided a basis for understanding the complex enzymology and regulation of PP1, which have been an enigma for half a century (1–3). First, the primary structure of the catalytic subunit of PP1 is one of the most highly conserved in evolution (38). Second, PP1 exhibits a broad specificity that is seemingly inconsistent with its multiple regulatory functions. Third, targeting of PP1 to the molecular proximity of its substrates appears to be essential for the expression of its function. This is a critical concept, first shown for the effect of mutations of PP1 in yeast that prevent its association with the yeast glycogen-binding subunit (39, 40) and also by the deletion of yeast genes for the targeting subunits (20). These considerations suggest that the evolutionary route for PP1 took a different direction from that of the protein kinases. Rather than the evolution of multiple catalytic subunits with different functions (41), the route taken was the acquisition of multiple regulatory subunits that individually define both specificity and function. This process would have been facilitated through the agency of the PP1-binding motif that is the focus of this study. A new perspective of PP1 and its regulatory proteins emerges, in which PP1 heterodimers can be viewed as a family of protein phosphatases of far more diversity than previously could be considered from the standpoint of the existence of PP1 as a single catalytic entity. This evolutionary strategy provides a novel paradigm for the development of an enzyme family that encompasses diverse cellular functions. It
is also noteworthy that a parallel in the use of targeting proteins exists for the protein kinases such in the AKAP family of proteins (42, 43).

Finally, a knowledge of the binding site of PP1 and of its specificity for variations of the binding motif provides the foundation for design of peptide inhibitors that could target this site, with the possibility of selective interference with PP1 functions. The idea that PP1 may be a useful target for future therapeutic purposes has recently been enhanced by the observations that targeting of PP1 by a herpesvirus protein is part of the viral mechanism for overcoming host defenses (17) and by the observation that HOX11, which is oncogenic in human T-cell leukemia, interacts with PP1 and PP2A (6).

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