Targeting of the Catalytic Subunit of Protein Phosphatase-1 to the Glycolytic Enzyme Phosphofructokinase†

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ABSTRACT: In this study, we demonstrate that the catalytic subunit of rabbit muscle protein phosphatase-1 (PP1) binds to muscle phosphofructokinase (6-phosphofructo-1-kinase, PFK). A protein of 85 kDa was isolated from rat muscle by affinity chromatography on PP1−Sepharose and was identified as phosphofructokinase by partial amino acid sequence analysis. This novel finding of a protein−protein interaction between PP1 and PFK was confirmed by reciprocal experiments in which the binding of PP1 to PFK−agarose was demonstrated. Elution of PP1 from PFK−agarose was maximal at ca. 0.4 M NaCl. The specificity of binding was demonstrated by isolation of PP1 from a partially purified rabbit muscle PP1 preparation. All four known isoforms of PP1 (PP1α, PP1γ1, PP1γ2, and PP1δ) were shown to bind to PFK−agarose. The activity of PP1 was only partially inhibited by PFK. The preformed complex between PP1 and PFK did not bind to inhibitor-2−Sepharose. The stoichiometry of binding of PP1 to the PFK monomer was found to be 1:1 in the isolated PP1·PFK complex. An interaction between PP1 and PFK in muscle extracts was demonstrated by their coimmunoprecipitation. Our findings raise the interesting possibility that PP1 may be targeted to PFK, and may be physiologically relevant in the context that PFK and other glycolytic enzymes have been shown to be micro-compartmentalized by binding to F-actin. This in turn points to a role for PP1 in control of glycolytic flux by protein phosphorylation−dephosphorylation mechanisms.

Protein phosphatase-1, also known as phosphorylase phosphatase, is one of the four major classes of Ser/Thr protein phosphatases (Brautigan, 1994; Bollen & Stalmans, 1992; Shenolikar & Nairn, 1991; Lee, 1995). The catalytic subunit of rabbit muscle protein phosphatase-1 (PP1) is a protein of 38 kDa and has been extensively studied. In addition to a key role in regulation of glycogen metabolism, protein phosphatase-1 activity has been shown to be important in other aspects of cellular regulation, including the control of the cell cycle (Brautigan et al., 1994). Molecular cloning of PP1 and the development of a bacterial expression system (Zhang et al., 1992) have enabled the preparation of recombinant PP1 in amounts that have allowed determination of its crystal structure (Goldberg et al., 1995; Egloff et al., 1995). The enzymology of PP1 is complex, as it is found in association with different regulatory/targeting subunits. This has been best demonstrated for the glycogen binding subunit, which targets PP1 to glycogen, and serves as a regulatory subunit through a mechanism involving both modulation of substrate specificity and loss of activity through dissociation from glycogen (Hubbard & Cohen, 1991). A yeast PP1 mutant, in which the ability of PP1 to bind to the glycogen binding subunit is lost, was shown to exhibit a phenotype in which there is a loss of glycogen accumulation, consistent with a failure to dephosphorylate and activate glycogen synthase, despite the presence of active PP1 in the cell (Stuart et al., 1994). This evidence provides strong support for the concept that the functional expression of PP1 activity can be dictated by localization to the molecular proximity of its target substrates. PP1 has been shown to be targeted to myosin by specific targeting subunits that bind both myosin and PP1 (Chen et al., 1994). PP1 is also associated with at least three polypeptide inhibitors; inhibitor-2 (Bollen & Stalmans, 1992); NIPP-1, a nuclear protein (Jagiello et al., 1995; Van Eynde et al., 1995); and RIPP-1, a ribosomal protein (Beullens et al., 1996).

In addition, PP1 has been found to interact with the retinoblastoma gene product, Rb (Durfee et al., 1993), and the ribosomal S5 protein (Hirano et al., 1995) by use of the yeast two-hybrid system, and has also been found to associate with heat shock protein HSP78 (Chun et al., 1994). Thus, the issue arises as to the generality of the targeting of PP1, if molecular association is a paradigm for directing both the specificity and activity of PP1 for its target molecules. Similar paradigms have been developed with other signaling systems, as with the protein kinases where a family of PKA anchoring proteins (AKAP'S) have been described (Coghlan et al., 1995; Rubin, 1995), and in the growth-related signaling pathways, where the roles of SH2 and SH3 domains in bringing regulatory proteins into molecular apposition are now well established (Pawson, 1994; Schlessinger, 1994). We have recently developed an affinity chromatography support for PP1 binding proteins by the use of PP1 immobilized on Sepharose (Zhao et al., 1996), and have demonstrated that it can be effectively used for the isolation of several of the known regulatory subunits of PP1, including inhibitor-2, the glycogen binding subunit, and the NIPP proteins. In this study, we report the novel finding that a key enzyme of glycolysis (Van Shaftingen, 1993), muscle phosphofructokinase, is a protein ligand for PP1.

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MATERIALS AND METHODS

Materials. Recombinant PP1α (Zhang et al., 1992), the PP1 isoforms γ1, γ2, and δ (Zhang et al., 1993a), and deletion mutants of PP1 (Zhang et al., 1994a) were expressed in *E. coli* and purified as previously described. PP1−Sepharose was prepared by immobilization of recombinant PP1α on CH-Sepharose as described by Zhao et al. (1996). Inhibitor-2−Sepharose was prepared by immobilization of recombinant inhibitor-2 on CH-Sepharose (Pharmacia-LKB Biotech) as described by Zhang et al. (1994b). Rabbit muscle (PFK) and PFK−agarose were obtained from Sigma Chemical Co. Disuccinimidyl suberate, dimethyl suberimidate, and ethylene glycol bis(succinimidyl succinate) were obtained from Pierce Chemical Co. A guinea pig antibody against rabbit PFK was a generous gift from Dr. R. G. Kemp, University of Chicago. The catalytic subunit of cAMP-dependent protein kinase was a generous gift of Dr. E. M. Reimann, Medical College of Ohio. Rabbit polyclonal IgG against the synthetic peptide corresponding to residues 316−330 of PP1α was obtained from Upstate Biotechnology Inc. Horseradish peroxidase coupled anti-rabbit IgG and reagents for chemiluminescent Western blotting were obtained from Amersham Life Science. Protein standards for SDS−PAGE were obtained from New England Biolabs.

Assay of PP1. PP1 activity was assayed using phosphorylase *a* or *p*-nitrophenyl phosphate as previously described (Zhang et al., 1992).

Isolation of an 85 kDa Protein from Rat Muscle. Rat muscle was homogenized in buffer and an extract prepared as described previously (Zhao et al., 1996). Rat muscle was homogenized (3 mL/g of muscle) in 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1 mM dithiothreitol, 0.25 M sucrose, 0.5 mM phenylmethanesulfonyl chloride, 5 μg/mL leupeptin, 5 μg/mL pepstatin, and 0.01 unit/mL aprotinin. The homogenate was centrifuged for 30 min at 10000*g*. The supernatant was precipitated by addition of ammonium sulfate to 50% saturation. The precipitate was resuspended in IED buffer (50 mM imidazole chloride, pH 7.4, 1 mM EDTA, 2 mM dithiothreitol, and 2 mM $MnCl_2$) containing 0.025% Tween 20 and 10% glycerol, and dialyzed against the same buffer. A sample of the extract (40 mL, 800 mg of protein) was loaded onto a heparin−Sepharose column (15 mL), and washed with 100 mL of 50 mM NaCl in the same buffer. The column was then eluted with 0.6 M NaCl. The 0.6 M NaCl eluate was then concentrated by precipitation with 10% poly(ethylene glycol) (MW 6000−7000) and resuspended in IED buffer. All the preceding procedures were performed at 4 °C. The preparation was then subjected to affinity chromatography on PP1−Sepharose as described previously (Zhao et al., 1996). The column (15 mL) was equilibrated in IED buffer. The sample (50 mL, 50 mg of protein) was loaded onto the column and washed with 10 bed volumes of IED/50 mM NaCl. The column was then eluted with 1 M NaCl. The flow rate was 60 mL/h, and 1 mL fractions were collected. The fractions were assayed for protein by the Coomassie blue dye procedure (Bradford, 1976) and analyzed by SDS−PAGE. The 85 kDa protein was concentrated by precipitation with 20% poly(ethylene glycol). Amino acid sequencing of the 85 kDa protein was performed by the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville. The 85 kDa rat muscle protein was fragmented, and the N-terminal sequence of an internal peptide was obtained. This contained the sequence RFDEAIKLRGRSFMNN.

Chromatography of PP1 on PFK−Agarose. Purified recombinant PP1 (1.3 mg) was diluted to a volume of 40 mL in IED buffer and loaded onto a PFK−agarose column (1.5 mL). The column was washed with 15 mL of IED/50 mM NaCl. The column was then eluted with IED/1 M NaCl. Fractions of 0.5 mL were collected and assayed for PP1 activity and for protein.

Partial Purification of Rabbit Muscle PP1. Rabbit skeletal muscle was subjected to partial purification as described by Lee and DeGuzman (1986) up to the ethanol precipitation stage. The material was then extracted with IED buffer and dialyzed as described by Lee and DeGuzman (1986). The preparation (4 mg of protein, 140 units of PP1) was chromatographed on PFK−agarose (2 mL) as described above.

Phosphorylation of PFK. PFK (12 μg) was incubated in a reaction mixture (50 μL) containing 4 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP (1 μCi), and recombinant catalytic subunit of cAMP-dependent protein kinase (0.2 μg) in IED. Phosphorylation of the PFK·PP1 complex (12 μg) was performed in the same way. The preformed PFK·PP1 complex was prepared by passage of a 1:1 mixture by weight of PP1 and PFK through inhibitor-2−Sepharose to remove excess PP1 (see above). Microcystin, when added, was at a final concentration of 1 nM. The reaction mixtures were incubated at 30 °C for 30 min. Reactions were terminated by the addition of 20 μg of bovine serum albumin and precipitated with trichloroacetic acid (final concentration 20%). The pellets were washed with cold 95% ethanol and resuspended in SDS−PAGE sample buffer and subjected to SDS−PAGE. The gels were dried, and the labeled protein bands were examined by autoradiography.

Immunoprecipitation/Western Blotting. A rat skeletal muscle extract was prepared as described above and diluted to a protein concentration of 1 mg/mL in phosphate-buffered saline (PBS). Protein A−Sepharose (50 μL) was mixed with the diluted extract (1 mL) at room temperature for 30 min and then removed by centrifugation to preclean the extract. A rabbit IgG against PP1 (4 μg) was then added, followed by incubation at room temperature for 60 min. Protein A−Sepharose (50 μL) was then added and the suspension mixed at room temperature for 30 min. The beads were then washed 5 times with PBS, and the protein was extracted with SDS−PAGE sample buffer. SDS−PAGE was performed in gels of 8% acrylamide, and transferred to nitrocellulose. The blots were visualized using a guinea pig antibody against PFK. The second antibody was a horseradish peroxidase coupled donkey anti-rabbit antibody, and blots were visualized using a chemiluminescence procedure (Amersham Life Sciences). The donkey anti-rabbit antibody exhibited sufficient cross-reactivity to guinea pig antibody to permit blotting of PFK in tissue extracts.

RESULTS

Binding of an 85 kDa Protein to PP1−Sepharose and Its Identification as Phosphofructokinase. In the course of identifying PP1 binding proteins by affinity chromatography on PP1−Sepharose, we isolated a protein of 85 kDa from

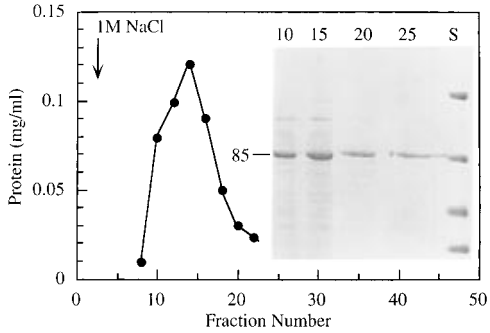

FIGURE 1: Isolation of a 85 kDa rat muscle protein that binds to PP1. The 0.6 M NaCl eluate of a rat muscle extract that had been chromatographed on heparin−Sepharose (Materials and Methods) was subjected to affinity chromatography on PP1−Sepharose (Materials and Methods). The diagram shows the elution of protein (solid circles) in the 1 M NaCl eluate. Fifty milligrams of protein was loaded onto the PP1−Sepharose column (15 mL), and 0.8 mg of protein was recovered in the 1 M salt fractions. The inset shows the protein-stained SDS−PAGE (10% acrylamide) of the peak fractions. "S" represents the protein standards (175, 83, 62, and 47 kDa, in descending order of size).

rat skeletal muscle (Figure 1). The starting material for the isolation was the 0.6 M NaCl eluate from a rat muscle extract that had been precipitated with 50% ammonium sulfate and chromatographed on a heparin−Sepharose column (Materials and Methods). The amount of protein isolated from the 1 M NaCl eluate from PP1−Sepharose was 0.8 mg, starting from 50 mg of protein. In order to determine if this was a known muscle protein, it was subjected to amino acid sequencing after proteolytic cleavage to obtain an internal sequence. The sequence RFDEAIKLRGRSFMNN was obtained from one of these peptides. This was found to match residues 365−380 (RFDEAMKLRGRSFMNN) of rabbit muscle phosphofructokinase (6-phosphofructo-1-kinase) (Lee et al., 1987) with one conservative replacement (underlined), in a search against the Genbank database. The molecular size of the protein was also consistent with the size of rabbit muscle phosphofructokinase (85.2 kDa) (Lee et al., 1987).

Binding of PP1 to PFK−Agarose. The reciprocal experiments were performed, in which the binding of purified recombinant PP1 to rabbit muscle PFK immobilized on agarose was examined (Figure 2). The SDS−PAGE of the material bound to PFK−agarose and eluted with 1 M NaCl revealed two major components: a 85 kDa band corresponding to PFK itself and a 38 kDa band corresponding to PP1. Assay of the column fractions (Figure 2) confirmed the presence of PP1 activity in the 1 M NaCl eluate. Since PFK is oligomeric, and has been observed as a dimer or tetramer as well as larger species (Pavelich & Hammes, 1973; Le Bras et al., 1995), it appeared likely that the presence of PFK was due to its leaching from the column. This was confirmed in additional experiments in which the PFK−agarose was washed with 1 M NaCl. PFK was readily detected in the eluate from the gel, but after extensive washing, only a small trace of PFK was present in the 1 M NaCl eluate (not shown). Experiments with a control agarose gel were performed, and the results for PP1 binding were negative. The allosteric effectors of PFK, fructose 2,6-bisphosphate, AMP, and ATP, were tested and found to have no effects on PP1 binding to PFK−agarose. The elution of PP1 from the washed PFK−agarose was examined using stepwise increases in [NaCl] to determine the salt concentration required to disrupt the PP1−PFK interaction. Purified PP1 bound to PFK−Sepharose was eluted between 0.1 and 0.6 M NaCl, with the maximum amount eluted at salt concentrations of 0.4 M NaCl (Figure 3). Approximately 80 μg of PP1 was recovered from a column of 1.5 mL gel.

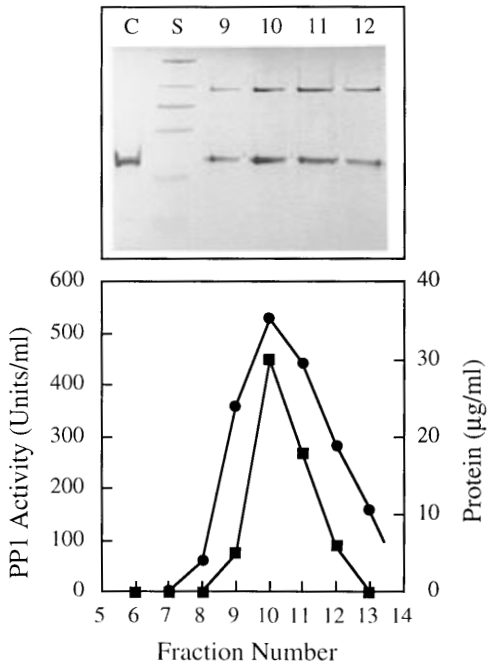

FIGURE 2: Binding of purified PP1 to PFK−agarose. Recombinant rabbit muscle PP1 (1.3 mg) was chromatographed on a PFK−agarose column (Materials and Methods). The column (1.5 mL) was washed with 10 bed volumes of IED buffer/50 mM NaCl until the absorbance at 280 nm was near base line. The column was then eluted with 1 M NaCl/IED buffer. Fractions of 0.8 mL were collected and assayed for phosphatase activity against *p*-nitrophenyl phosphate (circles) and for protein (squares). The upper panel shows the SDS−PAGE of the peak fractions. Lane "C", purified PP1; S, protein standards (175, 83, 62, 47, and 32 kDa, in descending order of size); 9−12, column fractions as shown in the lower panel.

In order to examine the specificity of the interaction of PP1 and PFK, a partially purified preparation of rabbit muscle PP1 was chromatographed on PFK−agarose. The SDS−PAGE of the 1 M NaCl eluate is shown in Figure 4. The results show that PP1 can be extensively purified by this method. The presence of PP1 activity in the 1 M NaCl eluate was confirmed by enzyme assay (Figure 4). The PP1 appears as a doublet; this may be due to partial proteolysis of PP1 (Lee & DeGuzman, 1988). Also present in the preparation was a 58 kDa band which was not further identified. Demonstration of the binding of native PP1 from muscle is important since it shows that the binding is not a selective property of recombinant PP1. The recombinant enzyme differs from native PP1 in that its activity is Mn^{2+}-dependent (Zhang et al., 1992), and it may represent a different conformer of PP1 (Zhang et al., 1993b), or a form in which the metal ion composition is different (Chu et al., 1996).

Binding of PP1 Isoforms to PFK. Since PP1 is known to exist in four isoforms (Sasaki et al., 1990), we tested PP1γ1, PP1γ2, and PP1δ for their ability to bind PFK−agarose and found that all three, like PP1α, were able to bind (not shown). Thus, the binding of PP1 to PFK is not isoform-specific, and since these isoforms differ mainly in the C-terminus, it seemed unlikely that the latter was involved. This was confirmed by examining the ability of a deletion mutant in

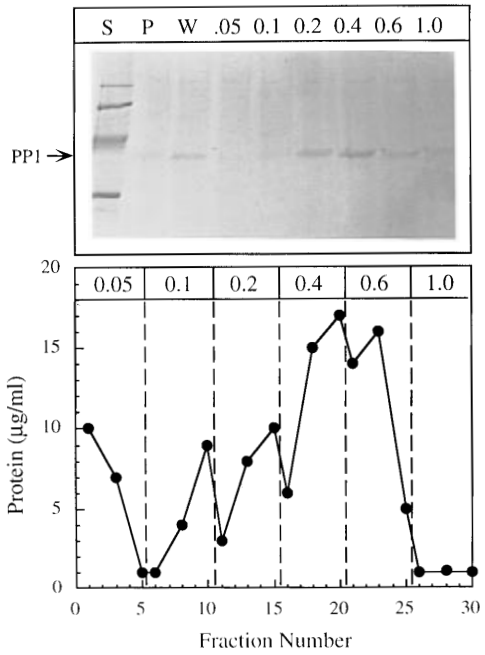

FIGURE 3: Elution of PP1 from PFK−agarose. Recombinant PP1 (1 mg) was loaded onto a 2.5 mL column of PFK−agarose which had previously been washed with 1 M NaCl in IED buffer (Materials and Methods). The column was washed with successively 5 mL of IED buffer containing 0, 0.05, 0.1, 0.2, 0.4, and 0.6 M NaCl in IED buffer. Fractions of 1 mL were collected. The column fractions were then analyzed for protein content (lower panel). Peak fractions from each salt elution were examined by SDS−PAGE followed by protein staining (upper panel). Lane P, pass-through fraction from loading of the sample; lane W, buffer wash of the column; other lanes are labeled according to the NaCl concentration used. Lane S, protein standards (83, 62 47, and 32 kDa, in descending order of size).

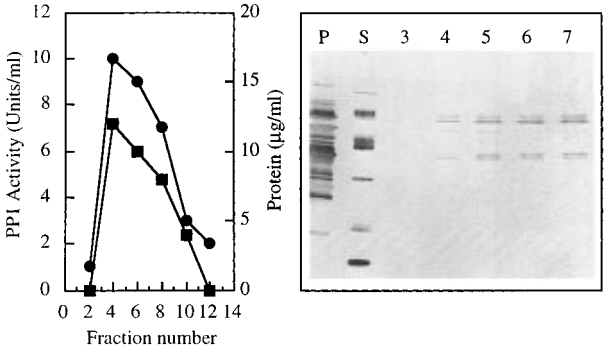

FIGURE 4: Affinity purification of rabbit muscle PP1 on PFK−agarose. A partially purified preparation of the catalytic subunit of rabbit muscle PP1 (0.4 mg of protein) which had been purified to the heparin−Sepharose stage (Materials and Methods) was chromatographed on PFK−agarose (2 mL) which had previously been washed with 1 M NaCl (Materials and Methods). The left panel shows the elution of protein (squares) and PP1 activity (circles) in the 1 M NaCl eluate. The right panel shows the SDS−PAGE of the column fractions after silver staining for protein. Lane "P", material prior to chromatography. "S", protein standards (83, 62, 47, 32, 25, and 16 kDa, in descending order of size); 3−7, column fractions. The larger band was estimated to have a molecular mass of 58 kDa. The molecular mass of the lower band which is a doublet was estimated to be 36−38 kDa.

which PP1α (330 amino acid residues) was truncated by 33 residues at the C-terminus (Zhang et al., 1994a). This truncated mutant of PP1α was found to bind to PFK−agarose (not shown).

Binding of a Mixture of PFK and PP1 to Inhibitor-2−Sepharose. A mixture of PFK and PP1 was applied to an inhibitor-2−Sepharose column, which we have previously shown to function well as an affinity support for the binding of PP1 (Zhang et al., 1994b). A mixture of PP1 and PFK was eluted from the column, while the bound material contained only PP1 (Figure 5). Rechromatography of the unbound material (Figure 5) confirmed that this was not due to overloading of the column. These results show that the PFK−PP1 complex does not bind to inhibitor-2, nor is there any apparent exchange of the PP1 between PFK and inhibitor-2, so that binding of inhibitor-2 and PFK to PP1 is mutually exclusive. The effects of PFK on PP1 activity were tested, and it was found that PFK had little effect on PP1 activity at molar ratios between 1 and 2, but that there was a partial inhibition of PP1 activity at high molar ratios of PFK (Figure 6). The same results were obtained with PP1 to which inhibitor-2 had been added so that inhibition was

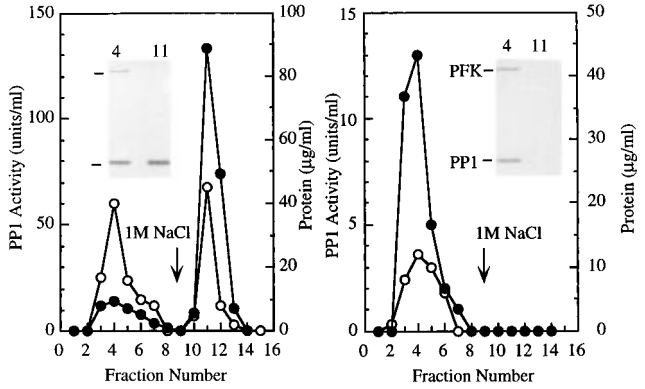

FIGURE 5: Binding of PP1 and PFK on inhibitor-2−Sepharose. A mixture of PP1 and PFK (50 μg each in 2 mL) was chromatographed on a column (1.5 mL) of inhibitor-2−Sepharose in IED buffer. Fractions of 0.5 mL were collected. The column was washed with 15 mL of 50 mM NaCl/IED buffer and eluted with 1 M NaCl/IED buffer. The diagram shows the elution pattern of the fractions from the sample loading as fractions 1−8 and from the 1 M salt elution as fractions 9−15. The fractions containing the 50 mM NaCl wash are not shown as no protein or activity was detected in these fractions. Left panel: Assays for PP1 activity using *p*-nitrophenyl phosphate (solid circles) and protein (open circles). The peak fractions (4 and 11) were subjected to SDS−PAGE and stained for protein by silver staining; the two bars show the elution of PFK and PP1, respectively (inset). Right panel: Fractions 2−7 which contained the unbound material were pooled and analyzed as for the left panel.

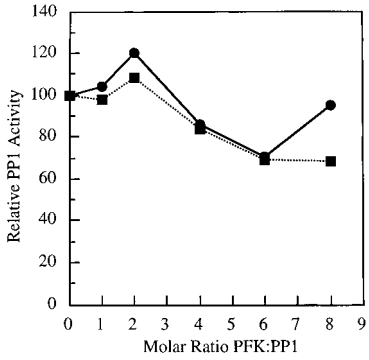

FIGURE 6: Effect of PFK on PP1 activity. Rabbit muscle PFK was mixed with PP1 in the molar ratios indicated, and then diluted and assayed for protein phosphatase-1 activity using phosphorylase *a* as the substrate (circles, solid line). In a parallel experiment, inhibitor-2 was added to PP1 to yield approximately 50% inhibition of enzyme activity. The effect of addition of PFK on the residual activity was then determined by addition of PFK at the molar ratios to PP1 indicated (squares, dotted line). Data are shown as percentage of the control activities.

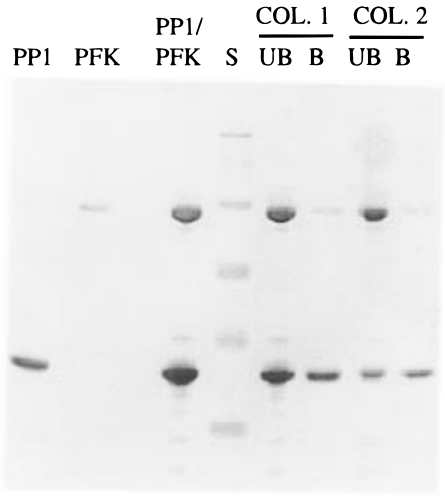

FIGURE 7: Preparation of the PFK·PP1 complex by passage through inhibitor-2−Sepharose. A 1:1 mixture by weight (0.1 mg of each) of PP1 and rabbit muscle PFK was passed through an inhibitor-2−Sepharose column as described for Figure 5. The unbound protein was then rechromatographed. The bound and unbound fractions were subjected to SDS−PAGE, and stained for protein with Coomassie blue. The first three lanes show PP1 and PFK, and the mixture of PP1 and PFK that was chromatographed. The next two lanes (COL. 1) show the SDS−PAGE of unbound (KB) and bound fractions (B) from the first chromatography. The unbound fractions were pooled and rechromatographed. The unbound and bound fractions are shown in the last two lanes (COL. 2). The relative intensities of the PP1 and PFK bands for the unbound material of the second column were determined by using image analysis using a UVP gel documentation system (UVP Inc.).

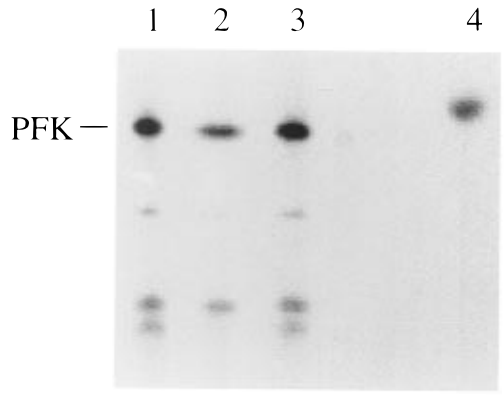

FIGURE 8: Phosphorylation of PFK and the PFK·PP1 complex. Rabbit muscle PFK or a preformed PP1·PFK complex was phosphorylated with recombinant catalytic subunit of cAMP-dependent protein kinase and [γ-^{32}P]ATP as described under Materials and Methods, and the products were examined by SDS−PAGE and autoradiography. Lane 1, PFK + PKA; lane 2, PFK·PP1 + PKA; lane 3, PFK·PP1 + microcystin + PKA; lane 4, ^{32}P-labeled phosphorylase *a* (93 kDa). The amount of PFK or PFK·PP1 used was 12 μg per reaction mixture. The PFK·PP1 complex was prepared by passage of a mixture of PFK and PP1 through inhibitor-2−Sepharose to remove excess free PP1 as illustrated in Figure 5. When microcystin was added, it was precincubated overnight with the PFK·PP1 complex at 4 °C. The mixture was tested for PP1 activity which was found to be completely inhibited.

only partial (Figure 6). This result is again consistent with the inability of PP1 to exchange between inhibitor-2 and PFK.

Stoichiometry of the PP1·PFK Complex. The stoichiometry of PP1 to PFK monomer in the complex was assessed. A 1:1 mixture by weight of PFK and PP1 was passed through inhibitor-2−Sepharose twice, to remove free PP1 (Figure 7). The relative amount of PFK and PP1 in the complex was then determined after SDS−PAGE and staining with Coomassie blue by image analysis. The weight ratio of PP1 to PFK was found to be 1:1.8 which corresponds to a molar ratio of 1:0.8. In other experiments, mixtures of PP1 and PFK were treated with different cross-linking agents. Those tested were dimethyl suberimidate, ethylene glycol bis(succinimidyl succinate), and disuccinimidyl suberate (DSS). Only the latter was found to produce suitable cross-linking for our purposes. The cross-linking was analyzed by an immunoblot procedure, as analysis of the products was complicated by the fact that PFK itself undergoes dimer/tetramer formation (Bock & Frieden, 1976; Lad & Hammes, 1974; Telford et al., 1975), so that a number of potential products might be expected. In the presence of PFK and PP1, DSS led to formation of a discrete species of ca. 240 kDa which was the major product containing PP1 (not shown). We did not detect a 123 kDa species corresponding to a 1:1 PP1/PFK cross-link with any of the cross-linking agents. The size of the complex we did observe is consistent with one containing two PFK monomers and two PP1 molecules, which would arise if cross-linking of the PFK dimer is very rapid. This is consistent with the ability to PFK to undergo dimer/tetramer interconversions (Lad & Hammes, 1974; Pavelich & Hammes, 1975), as it is possible that the subunit interfaces involved in dimer formation are more susceptible to cross-linking than those involved in dimer−dimer interaction to form the tetramer.

Phosphorylation of PFK and the PFK/PP1 Complex by PKA. PFK has been reported to be phosphorylated by cAMP-dependent protein kinase (Foe & Kemp, 1982; Valaitis et al., 1989; Zhao et al., 1991). The ability of PKA to phosphorylate PFK and the PP1/PFK complex was examined. It was observed that the rat muscle PFK isolated using PP1−Sepharose and purified commercial rabbit muscle PFK were readily phosphorylated (not shown). However, much less incorporation of ^{32}P was observed in the PFK/PP1 complex, suggesting that dephosphorylation was extremely rapid, as might be expected considering the ratio of PP1 to PFK. This is illustrated in the experiment in Figure 8, in which commercial rabbit muscle PFK and the PFK·PP1 complex were phosphorylated with PKA. Labeling of PFK is greatly reduced in the PFK·PP1 complex. When microcystin was added to inhibit PP1 (Zhang et al., 1992), an increase in phosphorylation of PFK in the PFK·PP1 complex could be observed. These results show that PP1 is able to dephosphorylate PFK even when in the complex.

Presence of PFK−PP1 Complexes in Muscle Extracts. Rat muscle extracts were immunoprecipitated with an antibody against PP1. The immunoprecipitate was then Western-blotted using an antibody against muscle PFK (Figure 9). The results show that PFK is present in the immunoprecipitates, indicating that PP1−PFK complexes were present in the muscle homogenates. Also shown in Figure 9 is an immunoblot of the 85 kDa rat muscle protein isolated by affinity chromatography on PP1−Sepharose with the antibody against PFK; this result provides additional confirmation of the identity of the protein as PFK.

DISCUSSION

The results presented here provide the first evidence for the novel observation that PFK is a protein ligand for PP1. The experimental findings establish quite clearly the ability

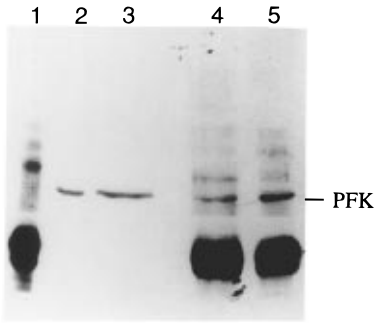

FIGURE 9: Coimmunoprecipitation of PP1 and PFK from rat muscle extracts. A rat muscle extract was immunoprecipitated with a rabbit antibody against PP1 (Materials and Methods). The immunoprecipitate was resuspended in buffer and subjected to SDS−PAGE and immunoblotted with an antibody against rabbit muscle PFK. Lane 1, PP1 antibody alone; lane 2, crude rat muscle extract; lane 3, rat muscle PFK isolated by affinity chromatography on PP1−Sepharose as in Figure 1; lane 4, immunoprecipitate of rat muscle extract with PP1 antibody without precleaning with protein A−Sepharose; lane 5, immunoprecipitate of rat muscle extract with PP1 antibody after precleaning with protein A−Sepharose. A control experiment was performed to confirm that the results obtained were not due to nonspecific binding of PFK to the antibody. The rabbit IgG against PP1 was mixed with a 25-fold excess by weight of pure rabbit muscle PFK and subjected to the same procedure; no PFK immunoreactive protein could be detected by immunoblotting (not shown).

of the catalytic subunit of PP1 to bind to muscle PFK *in vitro*. Thus, PFK joins Rb (Durfee et al., 1993), HSP78 (Chun et al., 1994), and ribosomal S5 protein (Hirano et al., 1995) as proteins (other than those already described as subunits) that interact with PP1. PFK itself, in addition to its association with F- actin (see below), has also been shown to interact with a phospholipase A2 in myocardial and pancreatic islet cells (Hazen & Gross, 1993; Ramanadham et al., 1996). While our studies were performed with the purified proteins *in vitro*, additional support for the possibility that this association may be of functional significance was obtained. First, the interaction appears strong enough to take place at physiological ion strengths; PFK was also able to compete with inhibitor-2, which has an IC_{50} in the nanomolar range (Zhang et al., 1992), for binding to PP1. Second, immunoprecipitation/Western blot analysis using antibodies to PP1 and PFK establishes that these two proteins can be found in association in muscle extracts. Third, PFK phosphorylated with PKA is a substrate for PP1. It is unlikely that PFK *in vivo* would be entirely in a complex form with PP1, simply based on considerations of the relative abundance of the two proteins and the strength of their interactions, since neither protein has been reported to copurify with each other.

The *in vivo* significance of the interaction of PP1 with PFK is unknown, and has to be considered within the role of phosphorylation of PFK itself, or of other proteins that may be in association with PFK, since there is considerable evidence for the dynamic association of glycolytic enzymes to subcellular structures [see Parkhouse (1992) for a review]. PFK, as a key pacemaker enzyme of glycolysis, has been extensively studied and is subject to complex regulation through allosteric effectors (Kemp & Foe, 1983) and by fructose 2,6-bisphosphate (Claus et al., 1984; Rousseau & Hue, 1993). Mammalian muscle is phosphorylated by PKA (Foe & Kemp, 1982; Valaitis et al., 1989; Hofer, 1985), but its activity is not affected, so that the phosphorylation of the mammalian muscle isoform is not considered a key regulatory factor (Hofer, 1985). However, phosphorylation of mammalian PFK from nonmuscle tissues [reviewed in Su and Storey (1994)] and of muscle PFK of other species does affect PFK activity, as in the mollusc, *A. suum*, and fish enzymes (Biethinger et al., 1991; Hofer et al., 1982; Su & Storey, 1995).

The second complex aspect of PFK function that is relevant to our findings arises from the well-documented association of PFK (and other glycolytic enzymes) to subcellular structures both in muscle and in nonmuscle cells. There is an extensive literature on the binding of PFK to muscle F-actin (Offer et al., 1988; Roberts & Somero, 1987, 1989; Su & Storey, 1995). The association of other glycolytic enzymes including aldolase, pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglucomutase, and lactate dehydrogenase to muscle contractile elements has supported the concept of their spatial localization to the muscle fibers (Parkhouse, 1992; Brooks & Storey, 1991), such that a micro-compartmentalization of the glycolytic enzymes could provide a direct delivery of energy to the muscle contractile system. There is also evidence for the binding of PFK to the cytoskeleton (Knull & Walsh, 1992) and to erythrocyte membranes (Uyeda, 1992; Harris & Winzor, 1990). Furthermore, the association of PFK to subcellular structures may be dynamic; there are rapid and reversible changes in the association of PFK with myocardial membranes during ischemia (Hazen *et al.*, 1994), and also during extended contraction in fast twitch muscle (Parra & Pette, 1995). Phosphorylation of PFK also affects its association to F-actin, so that an effect of PFK phosphorylation on glycolysis that does not directly involve activity changes in PFK itself is possible (Luther & Lee, 1986). 6-Phosphofructo-2-kinase (PFK-2), which is responsible for the synthesis and hydrolysis of fructose 2,6-bisphosphate, is regulated by cAMP-dependent protein kinase (Rousseau & Hue, 1993). A recent finding that is relevant to the potential significance of the interaction of PP1 to PFK is that in yeast PFK and PFK-2 have been shown to interact by use of the yeast two-hybrid system (Muller et al., 1996). Thus, there is another possible rationale for the targeting of PP1 to the PFK, as the prime target of regulatory significance could be PFK-2 rather than PFK itself.

In summary, we have established that PP1 and muscle PFK behave as ligands for each other and that there is a significant *in vitro* protein−protein interaction that exists between them. The physiological significance of this association is not known, and further investigation will be needed to establish a cellular function for this interaction. Nevertheless, as discussed above, there are several possibilities which include dephosphorylation of PFK itself, or of its associated regulatory proteins, that suggest that the targeting of PP1 to PFK could have a functional significance in the regulation of glycolysis.

REFERENCES

Beullens, M., Stalmans, W., & Bollen, M. (1996) *Eur. J. Biochem. 239*, 183−189.

Biethinger, M., Hoffmann, R., & Hofer, H. W. (1991) *Arch. Biochem. Biophys. 287*, 263−267.

Bock, P. E., & Frieden, C. (1976) *J. Biol. Chem. 235*, 5673−5643.

Bollen, M., & Stalmans, W. (1992) *Crit. Rev. Biochem. Mol. Biol. 27*, 227−281.

Bradford, M. (1976) *Anal. Biochem. 72*, 248−254.
Brautigan, D. L. (1994) *Recent Prog. Horm. Res. 49*, 197−198.
Brooks, S. P., & Storey, K. B. (1991) *FEBS Lett. 278*, 135−138.
Chen, Y. H., Chen, M. X., Alessi, D. R., Campbell, D. G., Shanahan, C., Cohen, P., & Cohen, P. T. W. (1994) *FEBS Lett. 356*, 51−55.
Chu, Y., Lee, E. Y. C., & Schlender, K. K. (1996) *J. Biol. Chem. 271*, 2574−2577.
Chun, Y. S., Shima, H., Nagasaki, K., Sugimura, T., & Nagao, M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3319−3323.
Claus, T. H., El-Maghrabi, M. R., Regen, D. M., Stewart, H. B., McGrane, M., Kountz, P. D., Nyfeler, F., Pilkis, J., & Pilkis, S. J. (1984) *Curr. Top. Cell. Regul. 23*, 57−86.
Coghlan, V. M., Perrino, B. A., Howard, M., Langeberg, L. K., Hicks, J. B., Gallatin, W. M., Scott, J. D. (1995) *Science 267*, 108−111.
Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y., Kilburn, A. E., Lee, W. H., & Elledge, S. J. (1993) *Genes Dev. 7*, 555−569.
Egloff, M. P., Cohen, P. T. W., Reinemer, P., & Barford, D. (1995) *J. Mol. Biol. 254*, 942−959.
Foe, L. G., & Kemp, R. G. (1982) *J. Biol. Chem. 257*, 6368−6372.
Goldberg, J., Huang, H., Kwon, Y., Greengard, P., Nairn, A., & Kuriyan, J. (1995) *Nature 376*, 745−753.
Harris, S. J., & Winzor, D. J. (1990) *Biochim. Biophys. Acta 1038*, 306−314.
Hazen, S. L., & Gross, R. W. (1993) *J. Biol. Chem. 268*, 9892−9890.
Hazen, S. L., Wolf, M. J., Ford, D. A., & Gross, R. W. (1994) *FEBS Lett. 339*, 213−216.
Hirano, K., Ito, M., & Hartshorne, D. J. (1995) *J. Biol. Chem. 270*, 19786−19790.
Hofer, H. W. (1985) in *Regulation of Carbohydrate Metabolism* (Beitner, R., Ed.) Vol. I, pp 105−142, CRC Press, Boca Raton, FL.
Hofer, H. W., Allen, B. L., Kaeini, M. R., & Harris, B. G. (1982) *J. Biol. Chem. 257*, 3807−3810.
Hubbard, M. J., & Cohen, P. (1991) *Methods Enzymol.*, 414−427.
Jagiello, I., Beullens, M., Stalmans, W., & Bollen, M. (1995) *J. Biol. Chem. 270*, 17257−17263.
Kemp, R. G., & Foe, L. G. (1983) *Mol. Cell. Biochem. 57*, 147−154.
Knull, H. R., & Walsh, J. L. (1992) *Curr. Top. Cell. Regul. 33*, 15−30.
Lad, P. M., & Hammes, G. G. (1974) *Biochemistry 13*, 4530−4537.
Le Bras, G., Auzat, I., & Garel, J. R. (1995) *Biochemistry 34*, 13203−13210.
Lee, C.-P., Kao, M.-C., French, B. A., Putney, S. D., & Chang, S. H. (1987) *J. Biol. Chem. 262*, 4195−4199.
Lee, E. Y. C. (1995) *Zool. Stud. 34*, 149−163.
Lee, E. Y. C., & DeGuzman, A. (1988) *Methods Enzymol. 159*, 356−368.
Luther, M. A., & Lee, J. C. (1986) *J. Biol. Chem. 261*, 1753−1759.
Muller, S., Boles, E., & Zimmermann, F. K. (1996) *Eur. J. Biochem. 236*, 626−631.
Offer, G., Starr, R., & Trinick, J. (1988) *Adv. Exp. Med. Biol. 226*, 61−73.
Parkhouse, W. S. (1992) *Can. J. Physiol. Pharm. 70*, 150−156.
Parra, J., & Pette, D. (1995) *Biochim. Biophys. Acta 1251*, 154−160.
Pavelich, M. J., & Hammes, G. G. (1973) *Biochemistry 12*, 1408−1414.
Pawson, T. (1994) *Adv. Cancer Res. 64*, 87−110.
Ramanadham, S., Wolf, M. J., Ma, Z., Li, B., Wang, J., Gross, R. W., & Turk, J. (1996) *Biochemistry 35*, 5464−5671.
Roberts, S. J., & Somero, G. N. (1987) *Biochemistry 26*, 3437−3442.
Roberts, S. J., & Somero, G. N. (1989) *Arch. Biochem. Biophys. 269*, 284−294.
Rousseau, G. G., & Hue, L. (1993) *Prog. Nucleic Acid Res. Mol. Biol. 45*, 99−127.
Rubin, C. S. (1994) *Biochim. Biophys. Acta 1224*, 467−479.
Sasaki, K., Shima, H., Kitagawa, Y., Irino, S., Sugimura, T., & Nagao, M. (1990) *Jpn. J. Cancer Res. 81*, 1272−1280.
Schlessinger, J. (1994) *Curr. Opin. Genet. Dev. 4*, 25−30.
Shenolikar, S., & Nairn, A. (1991) *Recent Prog. Adv. Second Messenger Phosphoprotein Res. 23*, 1−123.
Stuart, J. S., Frederick, D. L., Varner, C. M., & Tatchell, K. (1994) *Mol. Cell. Biol. 14*, 896−905.
Su, J. Y., & Storey, K. B. (1994) *Biochem. Mol. Biol. Int. 33*, 1191−1200.
Su, J. Y., & Storey, K. B. (1995) *Biochem. Mol. Biol. Int. 35*, 781−791.
Telford, J. N., Lad, P. M., & Hammes, G. G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3054−3056.
Uyeda, K. (1992) *Curr. Top. Cell. Regul. 33*, 31−46.
Valaitis, A. P., Foe, L. G., Kwiatkowska, D., Latshaw, S. P., & Kemp, R. G. (1989) *Biochim. Biophys. Acta 995*, 187−194.
Van Eynde, A., Wera, S., Beullens, M., Torrekens, S., Van Leuven, F., Stalmans, W., & Bollen, M. (1995) *J. Biol. Chem. 270*, 28068−28074.
Van Schaftingen, E. (1993) *Diabetologia 36*, 581−588.
Zhang, Z., Bai, G., Deans-Zirattu, S., Browner, M. F., & Lee, E. Y. C. (1992) *J. Biol. Chem. 267*, 1464−1490.
Zhang, Z., Bai, G., Shima, S., Zhao, S., Nagao, M., & Lee, E. Y. C. (1993a) *Arch. Biochem. Biophys. 303*, 402−406.
Zhang, Z., Bai, G., Zhao, S., & Lee, E. Y. C. (1993b) *Adv. Protein Phosphatases 7*, 183−197.
Zhang, Z., Zhao, S., Deans-Zirattu, S., Bai, G., & Lee, E. Y. C. (1994a) *J. Biol. Chem. 269*, 13766−13770.
Zhang, Z., Zhao, S., Zirattu, S. D., Bai, G., & Lee, E. Y. C. (1994b) *Arch. Biochem. Biophys. 308*, 37−41.
Zhao, S., Xia, W., & Lee, E. Y. C. (1996) *Arch. Biochem. Biophys. 325*, 82−90.
Zhao, Z. Z., Malencik, D. A., & Anderson, S. R. (1991) *Biochemistry 30*, 2204−2016.

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