Identification of Protein Phosphatase-1-binding Proteins by Microcystin-Biotin Affinity Chromatography

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Biotinylated microcystin was used to affinity purify over avidin-Sepharose the entire cellular content of active forms of protein phosphatase (PP) 1 and 2A holoenzymes present in three subcellular fractions of skeletal muscle. Biotinylated microcystin displayed IC50 values in the nM range against PP-1C (1.58 ± 0.6 nM S.E., n = 3), PP-2AC (0.63 ± 0.2 nM S.E., n = 3) and SMPP-1M (5.9 ± 1.3 S.E., n = 3). Subsequent anion-exchange chromatography and SDS-polyacrylamide gel electrophoresis of the microcystin-biotin eluates of the three fractions revealed a complex pattern of proteins associated with PP-1C and PP-2AC. Far Western analysis and the rebinding interaction with recombinant PP-1C distinguished proteins in the eluates that bound PP-1C from those that bound PP-2AC. In Far Western analysis, 29 distinct proteins were identified to bind PP-1C. Significantly, these same proteins, plus seven others, were also recovered from the isothiocyanate eluates from microcystin-Sepharose by a rebinding interaction with PP-1C-microcystin-biotin. The number of proteins and range of novel molecular masses (18–125 kDa) identified to interact with PP-1C by these two techniques cannot be accounted for by the previously characterized subunits of PP-1. Our findings further support the concept that PP-1C is regulated in vitro by multiple and distinct substrate-targeting subunits.

Although there are many hundreds of phosphoserine- and phosphothreonine-containing proteins in cells, their dephosphorylation is regulated by only four major classes of serine/threonine protein phosphatases whose catalytic subunits show broad and overlapping substrate specificity in vitro, namely PP-1,1 PP-2A, PP-2B, and PP-2C (for reviews see Refs. 1–4 and references therein). Studies with inhibitors such as okadaic acid and microcystin have shown that most of the measurable serine/threonine phosphatase activity in cell extracts is likely to be accounted for by PP-1 and PP-2A (5–7). Earlier research showed that these two phosphatases have significant regulatory roles in most of the major metabolic pathways including glycolysis, glycogen synthesis, fatty acid synthesis, cholesterol biosynthesis, protein synthesis, and lipolysis (1–4). More recent studies with the phenotypes of genetic mutants in which these enzymes have been deleted in fungi (8), fission yeast (9), and fruit flies (10) have implicated PP-1 and PP-2A as essential in the regulation of processes as diverse as the transport of ions and nutrients into cells, gene transcription, and the cell cycle. The involvement of PP-1 and PP-2A in all of these important cellular events raises the question as to how all of these processes can be regulated independently of one another. This question is even more apparent when one considers that both PP-1 and PP-2A are expressed at micromolar concentrations in cells and share a 49% sequence homology within their catalytic core (1–4). In the case of PP-1 the key to this paradox is the finding that the functions of the phosphatase are closely linked to the subcellular localization of regulatory targeting subunits that confer substrate specificity to a common catalytic subunit (11).

To date 11 subunits have been identified that specifically interact with PP-1C. Six potently inhibit the activity of the enzyme toward all substrates (3, 12, 13), while the others target PP-1C toward myosin or glycogen synthase in smooth muscle, skeletal muscle, and liver (14–23). Significantly many of the PP-1C subunits are the targets of serine/threonine protein kinases activated by insulin or adrenaline. For example, I1 and its close relative DARPP-32, specifically inhibit PP-1 when they are phosphorylated by cyclic A kinase (24, 25). Similarly, the 124-kDa skeletal muscle G (GSK) subunit of PP-1 can be phosphorylated both in vitro and in vivo at two regulatory sites. Phosphorylation at site 2 by cyclic AMP-dependent protein kinase in vitro causes dissociation of PP-1C from the G subunit and the glycogen particle (26–28). In contrast, phosphorylation of the G subunit at a second site (site 1) by the insulin-stimulated protein kinase ISP1 selectively increases the activity of PP-1GSK toward glycogen synthase (29). Based on the unique relationship between PP-1C and its G subunit, Cohen and co-workers have proposed a model to explain how insulin and adrenaline might control the synthesis and degradation of glycogen through the association/dissociation of the catalytic subunit with its glycogen targeting subunit (11). A liver equivalent to Gsk1, G1w was also recently purified, which although homologous to Gsk is of smaller molecular weight (23, 30). In the case of regulation of smooth muscle contraction, the M110 subunit in conjunction with its M21 regulatory component binds PP-1C to myosin and selectively alters its substrate specificity toward myosin (17–21). We and others have shown that alterations in the activity of SMPP-1M in vivo profoundly effects the contractile state of smooth muscle. Phosphorylation of M110 and allosteric regulation appear to be two mechanisms by which the activity of SMPP-1M is controlled (31–34). Homologous forms of M subunit have also been purified from skeletal muscle, where it has been proposed to play a role in the development and maintenance of muscle tension (35).

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1 The abbreviations used are: PP, protein phosphatase; MC, microcystin LR; EDT, ethanedithiol; TCEP, Tris-(2-carboxyethyl)phosphine hydrochloride; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; AKAPS, A kinase anchoring protein.
The regulation of glycogen metabolism and muscle contraction by the G and M subunits represent only two cellular events that are known to be regulated by PP-1. Clearly these two subunits alone and the inhibitor proteins cannot account for all the intracellular actions of PP-1. To examine the possibility that other regulatory subunits exist in cells we used biotinylated microcystin LR (MC-biotin) to specifically affinity purify over avidin-Sepharose native PP-1 and PP-2A holoenzyme complexes from skeletal muscle extracts. Following affinity purification, PP-1 and PP-2A could be separated into many distinct holoenzyme forms by anion-exchange chromatography. To specifically identify PP-1C binding proteins from those that interact with PP-2A a combination of Far Western analysis and re-binding interaction with recombinant PP-1C was utilized. Using these approaches we report the identification of up to 36 novel PP-1C binding proteins in the particulate and cytosolic fractions of skeletal muscle. This finding strongly supports the hypothesis that the catalytic subunit of PP-1 is regulated by multiple targeting subunits that specifically localize the phosphatase intracellularly.

EXPERIMENTAL PROCEDURES

Materials

MC-Sepharose was prepared following the method of Moorhead et al. (16). Antibodies to the subunits of PP-2A were a gift from Dr. Marc Mumbly (University of Texas, Dallas). Antibodies to PP-1C and the plasmid pTacTac-CS1a for the expression of recombinant PP-1Csa were generous gifts from Dr. Anna DePaoli-Roach (Department of Biochemistry and Molecular Biology, University of Indiana). Antibody to the G subunit was a gift from Dr. David Brautigan (Markey Center for Cell Signaling, University of Virginia). Microcystin was purchased from LC services (Boston, MA) or was a gift from Dr. Geoffrey Codd (University of Dundee, Scotland). Iodoacetyl-LC-biotin was purchased from Pierce.

Methods

Synthesis of Microcystin-Biotin—The N-methyldehydroalanine residue of MC was derivatized with ethanethiol (EDT) to form the product EDT-MC using a modification of the reaction conditions described by Moorhead et al. (16). Approximately 5.0 mg of MC were dissolved in 90 µl of ethanol and mixed with 200 µl of water, 65 µl of 5 M NaOH, 6 µl of 250 mM EDTA, 245 µl of dimethyl sulfoxide, and 15 µl of EDT. All components were gassed thoroughly with nitrogen prior to mixing. After 60 min at 56°C, 0.60 ml of 17.4 M glacial acetic acid was added and the mixture diluted 10-fold with 0.1% trifluoroacetic acid containing 1 mM Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP). HPLC analysis showed that reaction of MC with EDT resulted in a shift in the retention time of MC from 34.12 min to 28.00 min, consistent with the formation of the product EDT-MC (Fig. 1, A and B). Quantitative analysis showed that conversion of MC to EDT-MC was 95 ± 1.2% (S.E., n = 3). The products of the reaction were applied to a C18 Sep-Pak (Waters) cartridge equilibrated in 0.1% trifluoroacetic acid, washed with 0.1% trifluoroacetic acid, 1 mM TCEP solution, and EDT-MC eluted in a minimal volume of acetonitrile. Following evaporation to dryness, the EDT-MC was dissolved in 1.0 ml of 50 mM borate, pH 9.2, 1 mM TCEP and incubated in the dark with 6 µmol of iodoacetetyl-LC-biotin/µmol of EDT-MC for 90 min at room temperature. Analysis by HPLC showed that the reaction of EDT-MC with iodoacetetyl-LC-biotin resulted in a dramatic shift in retention time of EDT-MC to 40.5 min (Fig. 1C), consistent with the formation of MC-biotin. Spectral analysis of the 40.5-min peak indicated that, in addition to an extinction maximum at 238 nm, the product also showed increased absorbance at 200 nm consistent with the addition of LC-0.1% trifluoroacetic acid to the free sulfhydryl groups of EDT-MC. This result was further confirmed by mixing the isolated 40.50 peak with avidin-Sepharose which quantitatively bound the product (Fig. 1D). The efficiency of conversion of EDT-MC to MC-biotin was 94 ± 5.6% (S.E., n = 3). MC-biotin was purified by preparative reverse phase HPLC, evaporated to dryness and stored in ethanol at –20°C.

Purification of Recombinant PP-1C and Preparation of PP-1C-MC-Biotin Complex—The plasmid pTacTac-CS1a (0.5 µg) was transfected into bacterial strain DH5a and individual colonies grown at 30°C in LB (2 liters) containing ampicillin (100 µg/ml) until an absorbance of 0.3 was reached at 600 nm. Isopropyl-β-D-thiogalactopyranoside (0.25 mM final) and 0.5 mM MnCl2 were added to induce expression and proper folding of the recombinant protein. After 16 h, cells were harvested by centrifugation at 6000 x g for 10 min. Following freezing at –20°C, the cells were thawed and lysed in 20 ml of buffer D (50 mM Tris-HCl, pH 8.0, 0.5 mM MnCl2, 150 mM NaCl, 1 mM phenylmethlysulfon fluoride, 6 µg/ml leupeptin, 1 mM benzamidine) containing 0.5 mg/ml lysozyme. Following complete lysis, Nonidet P-40 (10 ml/liter) was added, and the lysate was centrifuged at 30,000 x g for 30 min. The supernatant was passed over MC-Sepharose (5 x 10 cm column) and washed extensively with buffer A containing 1 mM salt and 0.5 mM MnCl2. The bound phosphatase was eluted in this buffer and dialyzed immediately into buffer A containing 150 mM NaCl and 0.5 mM MnCl2. SDS-PAGE and Coomassie Blue staining showed that the eluted phosphatase was essentially 95% pure (Fig. 2). Against 32PPhosphorylase a, the recombinant enzyme had a specific activity of 15 ± 4 µmol/min/mg (n = 3, SE), which is close to the specific activity of native PP-1C purified from rabbit skeletal muscle (36).

For the preparation of PP-1C bound to MC-biotin (PP-1C-MC-biotin), the bacterial extracts were applied to avidin-Sepharose previously saturated with MC-biotin. The column was washed extensively with buffer A containing 1 mM NaCl, 0.5 mM MnCl2, and the bound protein was eluted with 1 mM biotin in buffer A.

Affinity Purification of PP-1 and PP-2A Holoenzymes and Anion-exchange Chromatography—The rectus abdomenus muscle of a single euthanized male Wistar rat (300 g) was removed and frozen in liquid nitrogen. The frozen muscle was powdered in liquid N2, weighed, and 30 g (wet weight) of tissue were homogenized in 10 ml of buffer B (25 mM Hepes, pH 7.0, 25 mM NaF, 0.15 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 0.1% β-mercaptoethanol plus the protease inhibitors 4 µg/ml leupeptin, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM tosylphenylalanil chloromethyl ketone, 10 µg/ml soya bean trypsin inhibitor, and 0.1 µg/ml apronitin) and centrifuged at 15,000 x g for 10 min at 4°C. The pellet was kept (particular fraction). The supernatant, containing cytosolic proteins and glycogen particles, was removed and centrifuged at 100,000 x g for 90 min at 4°C to isolate the glycogen fraction. The 100,000 x g supernatant (citosolic fraction)
was precleared of endogenous biotinylated proteins by passing over avidin-Sepharose (2 × 5 cm column) equilibrated in buffer A. The precleared extract was mixed with 10 μM MC-biotin and applied to fresh avidin-Sepharose. Note that this concentration of MC-biotin was sufficient to completely inhibit all detectable PP-1 and PP-2A activity present in the extract as determined by phosphorylase phosphatase assay (36). The avidin column was washed extensively with buffer A containing 1 M salt and then buffer A. The bound proteins were eluted with buffer A containing 1 M biotin, and the eluate was applied to an AP-1Q 8HR (Waters) anion-exchange column (2.5 × 10 cm) equilibrated in buffer A. The column was washed extensively and developed with a linear 120 min salt gradient from 0 to 1 M NaCl. Column fractions were analyzed by SDS-PAGE and silver staining. To characterize the glycoprotein fraction, a 100,000 × g pellet was resuspended in 10 ml of buffer A and recentrifuged at 100,000 × g for 90 min at 4 °C. The supernatant was discarded, and the washed glycoprotein pellet was resuspended and homogenized in buffer B (buffer A containing 0.5% Triton X-100). The resuspended pellet was digested with 20 μg/ml a-amylase for 120 min at 30 °C (11), then precleared through avidin-Sepharose. The precleared extract was mixed with MC-biotin (10 μM), reapplied to fresh avidin-Sepharose, and characterized as described above. For characterization of the particulate fraction, the 15,000 × g pellet was homogenized in 25 ml of buffer B and recentrifuged at 15,000 × g for 10 min at 4 °C. The supernatant was decanted, and the pellet was homogenized in 10 ml of buffer C (buffer A containing 0.6 M NaCl and 0.5% Triton X-100). The homogenate was incubated on ice for 30 min, then an equal volume of buffer C was added. Following centrifugation (15,000 × g for 30 min) the supernatant was dialyzed against 20 volumes of buffer A for 6 h, precleared of endogenous biotinylated proteins, then treated with MC-biotin for affinity purification over avidin-Sepharose.

**For Western Analysis and Rebinding Interaction with PP-1C**—For Far Western analysis, the MC-biotin eluates were transferred to polyvinylidene difluoride membrane (Millipore) following SDS-PAGE. The blots were first incubated overnight with 100 mg/ml ovalbumin and 100 mg/ml bovine serum albumin in TBST (150 mM NaCl, 10 mM Tris-HCl, 0.1% Tween 20, pH 7.4), then washed three times with TBST. The blots were incubated for 2 h at room temperature with 2.5 μg/ml purified recombinant PP-1C in TBST containing 0.5 mM MnCl₂, washed extensively with TBST, and incubated with antibody to PP-1C for 2 h. Following washing, the blots were incubated with anti-rabbit antibody coupled to horseradish peroxidase. PP-1C binding proteins were detected by ECL (Amersham Corp.) according to the manufacturer’s instructions. For control blots, recombinant PP-1C was omitted from the experiment. For Identification of PP-1C binding proteins by rebinding interaction, particulate and cytosolic fractions were prepared and passed separately over a column of MC-Sepharose (5 × 10 cm) equilibrated in buffer A. The column was washed extensively with buffer C to disrupt any nonspecific or nonionically bound proteins. The column was incubated for 2 h with 3 mM isothiocyanate in buffer A, and the proteins were eluted in this buffer. This procedure was found to separate PP-1C and its associated proteins from PP-2AC which remained bound to the Sepharose. Although, under these conditions the regulatory subunits of PP-2A were eluted from the column. The isothiocyanate eluate was dialyzed into buffer A containing 150 mM NaCl and 0.5 mM MnCl₂, and the recombinant PP-1C-MC-biotin complex (10 μM) was added. Incubation of the dialyzed isothiocyanate eluate with PP-1C-MC-biotin served two purposes. First, it ensured optimal reassociation with the eluted PP-1C binding proteins. Second, it reduced the possibility that any PP-2AC that did elute from the MC-Sepharose would be recovered from subsequent application to avidin-Sepharose. The dialyzed mixture was applied to an avidin-Sepharose column (10 × 5 cm) and washed extensively with buffer C and then buffer A. After incubating the column for 2 h with 10 mM biotin in buffer A, the bound proteins were eluted and analyzed by SDS-PAGE.

**RESULTS**

**Characterization of Microcystin-Biotin**—The ability of MC-biotin to inhibit purified PP-2AC, PP-1C and SMPP-1M was compared with microcystin (Fig. 3). Fig. 3 shows that MC-biotin displayed similar IC₅₀ values toward PP-1C and PP-2AC (PP-2AC IC₅₀ = 0.630 ± 0.2 μM; PP-1C IC₅₀ = 1.58 ± 0.6 μM S.E., n = 3), although these were approximately 4–5-fold higher than values obtained for free microcystin in the same assay (PP-2AC IC₅₀ = 0.16 ± 0.02 μM; PP-1C IC₅₀ = 0.25 ± 0.01 μM S.E., n = 3). Reduction in the efficiency of MC-biotin binding is consistent with modification of the N-methyldehydrodionaline moiety of microcystin, which participates in a covalent bond with conserved cysteine residues in the catalytic clefs of both PP-1C (Cys-273) and PP-2AC (Cys-266) (37, 38). In contrast to the free catalytic subunits, when assayed against purified SMPP-1M, MC-biotin displayed similar IC₅₀ values to free microcystin (MC-biotin; IC₅₀ = 5.9 ± 1.3; MC; IC₅₀ = 6.0 ± 0.5 μM S.E., n = 3) (Fig. 3C). The finding that microcystin was generally less potent against SMPP-1M compared with PP-1C may reflect steric hindrance effects due to the presence of its two regulatory subunits, M110 and M20 (17–21).

**Affinity Purification of PP-1 and PP-2A Holoenzyme Complexes from Skeletal Muscle and Characterization by Anion-Exchange Chromatography**—To test the hypothesis that multiple PP-1 and PP-2A holoenzymic forms exit in cells, we utilized MC-biotin to rapidly affinity purify over avidin-Sepharose active forms of these phosphatases present in the particulate, cytosolic, and glycogen fractions of rat skeletal muscle. Recently, MacKintosh and co-workers (16, 30, 35) described the preparation of MC-Sepharose for the affinity purification of enriched fractions of PP-1GSK and PP-1G₁ from rat liver and skeletal muscle. However, two problems arise with the use of MC-Sepharose. First, denaturing conditions are required to elute PP-2AC from MC-Sepharose. Second, kaotroptic agents are used to elute the bound phosphatases resulting in dissociation of the regulatory subunits from their catalytic subunits. Two major advantages are gained by linking microcystin to biotin. First, mild conditions are used to elute the bound proteins, and second, the holoenzyme complexes remain intact. The entire compliment of active forms of cytosolic and particulate associated PP-1/PP-2A holoenzymes were affinity-purified over avidin-Sepharose and subsequently separated into their various holoenzymic forms by anion-exchange chromatography. SDS-PAGE and silver staining of the column fractions reveals that a complex pattern of proteins associate with PP-1C and PP-2AC in these fractions (Fig. 4). Several lines of evidence indicate that these associations are specific. First, microcystin has been shown to be completely selective for the catalytic subunits of PP-1 and PP-2A (for review, see Ref. 7). Second, pretreatment of extracts with a saturating amount of free microcystin (10 μM) completely abolishes all recovery of these proteins including the catalytic subunits of PP-1 and PP-2A (data not shown). Furthermore, stringent column washing conditions are employed throughout (1 M NaCl and 0.1% Triton X-100) to break nonspecific ionic and hydrophobic interactions prior to elution of PP-1/PP-2A holoenzymes from avidin-Sepharose with biotin. More direct evidence that MC-biotin selectively recovers PP-1 and PP-2A holoenzymes was obtained.
following Western analysis of the anion-exchange column fractions with a series of antibodies to known subunits of PP-2A and PP-1 (Fig. 5). Western analysis with antibodies to PP-1C \( \alpha \) (37 kDa) and PP-2AC \( \alpha \) (36 kDa) showed both proteins to be present in many of the fractions eluting from the anion-exchange column of the particulate and cytosolic fractions (Fig. 5, A and B). Note that PP-1C\( \alpha \) antibody showed only weak cross-reactivity to PP-1C\( \delta \); therefore, this isoform may not have been detected in some of the column fractions. Significantly, no cross-reactivity to PP-1C or PP-2AC was detected in regions of the column profile where the free catalytic subunits were found to elute (fractions 20–30, data not shown). This finding strongly suggests that both phosphatases are eluting throughout the column profile because they are tightly associated with other proteins. Consistent with this hypothesis, invariably, in regions where PP-2AC was detected, cross-reactivity with its 65-kDa regulatory \( \alpha \) subunit was also observed (Fig. 5C).

In the case of PP-1C-associated proteins, Western analysis with antibody to the G subunit of PP-1GSK \( \alpha \) (G\( \alpha \)) of the MC-biotin eluates from the glycogen fraction revealed cross-reactivity at 160 kDa and with its proteolytic fragments at 90 and 60 kDa (Fig. 6, A and B). These results are consistent with previous work by others localizing the G subunit to the sarcoplasmic reticulum and glycogen particle in skeletal muscle (11). In all cases, where cross-reactivity was detected with G\( \alpha \), PP-1C was also present (Fig. 6, C and D).

To further verify that MC-biotin recovers holoenzymic forms of PP-2A and PP-1, column fractions containing proteins that were relatively pure as judged by SDS-PAGE and silver staining were treated with trypsin (1:50 w/w) and peptides isolated by HPLC for amino acid sequencing (Table I). For example, fraction P40 in the particulate profile contains three proteins at 36, 54, and 65 kDa (Fig. 4A), whereas in the cytosolic profile, fraction C54 contains two heavily staining proteins at 36 and 65 kDa (Fig. 4B). Densitometer measurements of the intensity of silver staining of the proteins in these column fractions showed that they were recovered with equal stoichiometry relative to one another. A data base search of amino acid sequence obtained from selected peptides from these fractions identified PP-2AC (36 kDa), B' (54 kDa), and the \( \alpha \) subunit (65 kDa) in
column fraction P40 and PP-2AC and the A subunit in column fraction C54 (Table I). These findings demonstrate that P40 contains the heterotrimeric form of PP-2A, PP-2Ao, and that C54 contains the heterodimer, PP-2A2 (for review, see Ref. 4). The presence of PP-1C was also identified in column fraction C40 of the cytosolic fraction when this was similar treated with trypsin and peptides isolated for amino acid sequencing by HPLC (Table I). The amino acid sequence data, combined with Western analysis, provide additional evidence that MC-biotin specifically affinity purifies holoenzymic forms of PP-2A and PP-1.

Significantly, not all of the proteins in the anion-exchange column profiles detected by silver staining can be accounted for by known PP-1 and PP-2A regulatory subunits or their proteolytic fragments. Many are of novel molecular weight and elute throughout the column profiles with varying stochiometry relative to PP-1C or PP-2AC. The stringency of the conditions employed during affinity chromatography, combined with the appearance of PP-1C and PP-2AC throughout subsequent anion-exchange chromatography, strongly suggests that these are novel PP-1C- or PP-2AC-binding proteins and that many holoenzymic forms of these phosphatases exist in cells.

Identification of Novel PP-1C-binding Proteins by Western Analysis and Rebinding Interaction with PP-1C—SDS-PAGE and silver staining of the MC-biotin eluates of the cytosolic, particulate, and glycogen fractions reveals them to contain over 50 distinct proteins (Fig. 7, A, E, and I). Far Western analysis and re-binding interaction with recombinant PP-1Cα was used to distinguish PP-1C binding proteins from those that bound PP-2AC. In Far Western analysis 15 distinct PP-1C binding proteins were detected in the cytosolic fraction (Fig. 7B) and a further 14 others in the particulate fraction (Fig. 7F). In control incubations, in which recombinant PP-1C was omitted from the analysis, cross-reactivity was detected only at 37 kDa (Fig. 7, C and G), consistent with the presence of endogenous skeletal muscle PP-1C. These results demonstrate that the interactions with PP-1Cα shown in Fig. 7, B and F, are specific. Importantly, the number of proteins and range of molecular masses (125–25 kDa) detected by Far Western analysis with PP-1Cα cannot simply be accounted for by the previously characterized subunits of PP-1, GSK, and MSK or their proteolytic fragments. No evidence of I1 or I2 was detected by Western analysis with antibodies to these proteins in the MC-biotin eluates from either fraction. This finding is consistent with work by MacIntosh and co-workers showing that microcystin and I1 and I2 compete at the same sites for binding to PP-1C (7, 39). Far Western analysis of the MC-biotin eluate from the glycogen fraction was less complicated and identified four distinct proteins at 160, 90, 60, and 37 kDa, respectively (Fig. 7, I and J). As shown in Fig. 6, A and B, Western analysis of this fraction with antibody to GSK identified the 160-, 90-, and 60-kDa proteins as GSK and its proteolytic fragments.

Rebinding interaction with PP-1Cα was used as an alternative means of identifying PP-1C binding proteins in the cytosolic and particulate fractions. SDS-PAGE and silver staining of the rebinding-biotin eluates shows that rebinding interaction with PP-1Cα recovers 16 proteins from the cytosolic and a further 20 proteins from the particulate fractions (Fig. 7, D and H). The molecular masses of these proteins range from 124 to 18 kDa. Importantly, with the exception of seven proteins, the majority of the proteins recovered by rebinding interaction also cross-react with PP-1Cα in Far Western analysis (compare Fig. 7, B and F, with D and H). Recovery of proteins by rebinding interaction that do not cross-react with PP-1C in Far Western analysis (e.g. the 124-, 122-, 33-, 24-, 22-, and 18-kDa proteins in the particulate fraction, and the 18-kDa in the cytosolic fraction) may reflect differences in the sensitivity of the two techniques. Far Western analysis is carried out on denatured proteins transferred to polyvinylidene difluoride following SDS-PAGE; whereas, in the rebinding method, the proteins remain in their folded state. Importantly, comparison of Fig. 7, D with H, shows that the rebinding interaction recovers a set of distinct proteins in the cytosol that are not present in the particulate fraction, and vice versa. A similar result is also
Regulation of Protein Phosphatase 1

The indicated column fractions from anion-exchange chromatography of the cytosolic and particulate fractions were characterized by SDS-PAGE and stained with Coomassie Blue. Individual proteins at the indicated molecular masses were excised from the gel and peptides extracted for amino acid sequencing as described previously (46).

| AP-IQ fraction | Molecular mass (kDa) | Sequence and position in protein | Protein |
|----------------|----------------------|---------------------------------|---------|
| Cytosolic fraction, tube 40 | 37 | pllpatyppgrm (304–319); gsetxllayk (98–101) | PP-1C (β) |
| Particulate fraction, tube 40 | 36 | qlesaqyk (22–29); lftk (5–8); ysfidpp (284–293); spdnay (75–80) | PP-2AC (α) |
| Particulate fraction, tube 40 | 65 | igpilndst (546–555); tdvpafqnl (271–391); ewhaak (476–542) | A subunit of PP-2A |
| Particulate fraction, tube 40 | 54 | evnlvexexv (332–345); lqaxvmxd (80–88) | B (α) subunit PP-2A |
| Particulate fraction, tube 54 | 38 | gyscetttll (90–100); gephvtt (296–300); spdnayf (75–82) | PP-2AC |
| Cytosolic fraction, tube 54 | 65 | efksxadxren (308–320); lagdwhfs (134–143) | A subunit of PP-2A |

**Fig. 7. Identification of PP-1C binding proteins by Far Western analysis and rebinding interaction with PP-1C.** Lanes A, E, and I show silver stains of the MC-biotin eluates from the cytosolic (A), particulate (E), and glycogen fractions (I). In lanes B, F, and J, the MC-biotin eluates were transferred to polyvinylidene difluoride and probed in Far Western analysis with PP-1C. Lanes C and G represent control Far Western analyses in which exogenous PP-1C was omitted. Lanes D and H show silver stains of proteins recovered from the particulate and cytosolic fraction following rebinding interaction with PP-1C. Arrows indicate the position of PP-1C at 37 kDa as identified by Western analysis. Results shown are from a single experiment, although this was repeated several times with identical results.

observed when comparing the blots from Far Western analysis (Fig. 7, B and F). This finding is consistent with the hypothesis that the subunits of PP-1 are highly compartmentalized. Thus, one would not necessarily expect to pattern the PP-1C binding proteins identified in the particulate and cytosolic fractions to be identical. Importantly, rebinding interaction experiments with PP-1C did not recover PP-2AC and its A subunit as judged by Western analysis of the rebinding-biotin eluates. This finding confirms that the method recovers proteins that only bind PP-1C. Consistent with the data shown in Fig. 6B, Western analysis of the rebinding eluates from the particulate fraction identified the presence of GSK. Western analysis of the rebinding biotin eluates with antibodies to inhibitor 1 or 2 did not identify either of these proteins.

**DISCUSSION**

Traditional approaches to characterizing the cellular content of PP-1 and PP-2A holoenzymes have relied on enzymatic assay of column fractions following chromatographic separation of tissue extracts (2). Although this approach has been successful in the identification of several holoenzymic forms of PP-2A and PP-1, typically nonspecific substrates have been used. This in part explains the modest number of PP-1 subunits identified thus far. PCR screening of cDNA libraries has been very successful in identifying subtypes of PP-2A subunits (40), however, this approach has not yielded any new PP-1 subunits. Two hybrid screening with PP-1C as the bait has been successful in identifying at least two PP-1C-binding protein, ribosomal protein L5 and P53BP2 (12, 13). However, in a more direct approach, we developed MC-biotin to isolate native forms of PP-1 and PP-2A from tissue extracts. Using MC-biotin we have identified at least 36 proteins that specifically interact with PP-1C. Seven separate lines of evidence demonstrate that these proteins specifically interact with PP-1C: 1) Microcystin is completely selective for the catalytic subunits of PP-1 and PP-2A, therefore only these proteins and their associated regulatory subunits would be expected to be recovered with MC-biotin. 2) Pretreatment of extracts with free microcystin completely abolishes all recovery of PP-1C, PP-2AC, and their associated subunits from avidin-Sepharose. 3) The MC-biotin eluates of the particulate and cytosolic fractions separate into specific PP-1 and PP-2A holoenzymic forms upon anion-exchange chromatography. 4) Stringent column washing conditions are employed throughout (1 M NaCl and 0.1% Triton X-100) to break nonspecific ionic and hydrophobic interactions prior to eluting of PP-1/PP-2A holoenzymes from avidin-Sepharose with biotin. 5) In addition to isolating novel PP-1C binding proteins, amino acid sequence and Western analysis identifies known holoenzymic forms of PP-1 and PP-2A. 6) Rebinding interaction studies with PP-1C recovers a pattern of proteins from the particulate and cytosolic fraction that are also independently identified by Far Western analysis of the MC-biotin eluates. 7) Far Western analysis and a rebinding interaction identify separate patterns of PP-1C binding proteins in the MC-biotin eluates of the glycogen, particulate, and cytosolic fractions. This later finding is consistent with the hypothesis that the holoenzymic forms of PP-1 are highly compartmentalized in the intact cell.

The PP-1C-binding proteins we have identified in the present study could either be substrates for PP-1 or regulatory subunits. However, by example with PP-1GSK or PP-1M5 it is
likely that any potential substrates for PP-1 would be lost during affinity purification because of the stringent column washing conditions employed. In the case of the PP-1GSK or PP-1MSM, both glycogen synthase and myosin readily dissociate from these complexes at ionic strengths greater than 0.5M NaCl (14, 17–21). In contrast, high concentrations of chaotropic agents are required to dissociate GSK and MSM from PP-1C (17–21). Therefore, with the exception of the G subunit in the particulate and glycogen fractions, it is our hypothesis that the majority of the 36 or so PP-1C binding proteins characterized in the present study are likely represent novel PP-1 regulatory subunits. We are currently utilizing MC-biotin to characterize the complement of PP-1C (and PP-2AC)-binding proteins in skeletal muscle and other tissues. The high affinity of this probe for both catalytic subunits should allow sufficient quantities of these proteins to be co-purified from tissues for direct identification by amino acid sequencing.

Despite the fact that two PP-1 regulatory subunits have been purified and their amino acid sequence determined, no information concerning the mechanisms by which these subunits bind and alter PP-1C activity has been elucidated. The mechanism of action of these subunits is made even more intriguing following the recent structural determination of PP-1C, which shows the enzyme to have an open shallow Y-shaped active site (38). The accessible nature of the active site explains the broad substrate specificity of PP-1C in vitro and clearly illustrates the requirement of regulatory subunits to control activity. The finding that there are no obvious homologous regions between the M and G regulatory subunits has hampered identification of the regulatory subunits. The requirement of regulatory subunits to control activity. The substrate specificity of PP-1C (38). The accessible nature of the active site explains the broad substrate specificity of PP-1C in vitro and clearly illustrates the requirement of regulatory subunits to control activity.

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