Identification of the substrate recruitment mechanism of the muscle glycogen protein phosphatase 1 holoenzyme

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Glycogen is the primary storage form of glucose. Glycogen synthesis and breakdown are tightly controlled by glycogen synthase (GYS) and phosphorylase, respectively. The enzyme responsible for dephosphorylating GYS and phosphorylase, which results in their activation (GYS) or inactivation (phosphorylase) to robustly stimulate glycogen synthesis, is protein phosphatase 1 (PP1). However, our understanding of how PP1 recruits these substrates is limited. Here, we show how PP1, together with its muscle glycogen–targeting (GM) regulatory subunit, recruits and selectively dephosphorylates its substrates. Our molecular data reveal that the GM carbohydrate binding module (GmCBM21), which is amino-terminal to the GM PP1 binding domain, has a dual function in directing PP1 substrate specificity: It either directly recruits substrates (i.e., GYS) or recruits them indirectly by localization (via glycogen for phosphorylase). Our data provide the molecular basis for PP1 regulation by GM and reveal how PP1-mediated dephosphorylation is driven by scaffolding-based substrate recruitment.

INTRODUCTION

The Ser/Thr protein phosphatase 1 (PP1) regulates diverse cellular processes, including neuronal plasticity, cell division, and protein synthesis (1). However, PP1 was originally discovered for its ability to direct glycogen metabolism in skeletal muscle. Specifically, PP1 dephosphorylates glycogen synthase, phosphorylase kinase, and glycogen phosphorylase, essential enzymes that are regulated by insulin and together control glycogen synthesis and breakdown (2). The dephosphorylation of glycogen synthase and phosphorylase α (glyco- gen phosphorylase phosphorylated on Ser14 is commonly referred to as phosphorylase a) by PP1 has opposing effects, namely, the activation of glycogen synthase and the inactivation of phosphorylase a. As a consequence, glycogen synthesis is robustly stimulated (3, 4).

During the last 30 years, it has become apparent that PP1 associates with scores of different PP1 regulatory subunits to form distinct, heterodimeric holoenzymes (5, 6). These PP1 regulatory subunits function to both target PP1 to its cellular point of action and selectively recruit specific substrates for PP1-mediated dephosphorylation. This latter function, substrate recruitment, is typically achieved by protein domains outside the primary PP1-anchoring domain. The ability of PP1 to regulate glycogen synthesis also requires its association with a specific regulatory subunit, the glycogen-targeting subunit in muscle, GM. Although GM was the first PP1-specific regulatory protein discovered, it is now known that it is one of seven genes in mammalian genomes that constitute the glycogen-targeting PP1 family (G-subunits): GM (RGL) is expressed in skeletal and cardiac muscle (4, 7); Gα (FLJ14005) is most abundantly expressed in the liver (8); and Gc (PTG, R5) and Gd (R6) are ubiquitously expressed (9). A comprehensive molecular understanding of how these G-subunits direct both PP1 targeting and dephosphorylation of glycogen synthase and phosphorylase a is currently missing.

All G-subunits include a highly conserved RVxF motif, which is essential for PP1 binding, and a family 21 carbohydrate binding module [CBM21; also known as starch binding domains (SBDs)], which is responsible for binding glycogen (10–12). Typically, SBDs have two sugar binding sites, known as sites 1 and 2 (13). It is currently unknown whether only one or both of these sites in GM CBM21 are necessary for glycogen targeting. Further, it was suggested that the GM CBM21 domain may also facilitate glycogen synthase substrate recruitment (14). However, whether or how this occurs is unknown. The first structure of PP1 bound to any of its regulators was that of PP1 bound to a short RVxF peptide from GM (15). This structure identified the RVxF binding pocket in PP1, which showed that it is more than 20 Å away from the PP1 active site. This structure also revealed that RVxF binding does not alter the conformation of the PP1 active site, explaining why it does not affect PP1 catalytic activity. However, it did not provide any insights into whether and how GM binds to PP1 beyond the RVxF motif, how GM binds glycogen, or how these interactions facilitate glycogen-specific substrate recruitment.

Here, we used nuclear magnetic resonance (NMR) spectroscopy, x-ray crystallography, and enzymatic studies to determine how GM recruits and targets PP1 to phosphorylase α and glycogen synthase. Unexpectedly, we found that PP1 interacts with GM outside its RVxF sequence via an extended ΦΦ motif and the GM CBM21 domain; this results in extremely tight binding. After determining the structure of the GM CBM21 domain, we then used NMR chemical shift perturbation (CSP) mapping to show that only one of its two carbohydrate sites binds directly to glycogen. This led to the discovery that the second site has a different function, namely, binding and recruiting the PP1-specific substrate glycogen synthase. We then showed that while holoenzyme formation with GM does not enhance phosphorylase α dephosphorylation, the simultaneous recruitment of both GM:PP1 and phosphorylase α to glycogen does. Together, these structural and enzymatic data reveal how, at a molecular level, GM targets PP1 to its glycogen-specific substrates phosphorylase α and glycogen synthase to...
specifically and robustly dephosphorylate both enzymes. Thus, this study provides the most comprehensive molecular understanding of how a specific PP1 holoenzyme, GM:PP1, mediates the rapid and selective dephosphorylation of its specific substrates.

RESULTS

**GM has two PP1 interaction sites**

The skeletal muscle glycogen–binding subunit [PP1 regulatory subunit 3A (PPP1R3A); hereafter referred to as GM] is a 1109–amino acid protein (124 kDa) (Fig. 1A). It was previously shown that residues 1 to 240 are required for PP1 binding and regulation [if not otherwise noted, PP1α residues 7 to 330 (PP1α7–330) are used in all experiments]. Bioinformatics predicts that residues 1 to 100 are intrinsically disordered (IDR; typical for PP1 regulatory/binding regions), while residues 102 to 237 form a well-folded domain that is a member of the CBM21 family. To define the GM residues that bind directly to PP1, we used NMR spectroscopy, isothermal titration calorimetry (ITC), and surface plasmon resonance (SPR). ITC showed that GM residues 2 to 64 do not bind PP1, as both GM^{2–237} and GM^{64–237} bind PP1 with the same affinities [statistically identical dissociation constant (K_D) values of 27 and 21 nM, respectively; Fig. 1, B and C, and Table 1]. Next, we used NMR spectroscopy to investigate the solution behavior of GM^{64–237}. A 2D [1H,15N] HSQC spectrum confirmed that GM^{64–93} is unstructured in solution, based on the limited chemical shift dispersion in the 1H dimension (Fig. 1D). In contrast, GM^{102–237} (hereafter referred to as GM^{CBM21}) has a 2D [1H,15N] HSQC spectrum that is typical for a folded protein (Fig. 1E). Furthermore, overlaying the NMR spectra of GM^{64–93} and GM^{CBM21} with that of GM^{64–237} shows that both domains are independent in solution, as their individual spectra overlap nearly perfectly with that from GM^{64–237} (Fig. 1F). Last, using SPR, we showed that GM^{64–105} binds PP1α7–330 with a K_D of 114 ± 4 nM (fig. S1, A to D), revealing that GM^{CBM21} directly contributes to PP1 binding (~4-fold increase in PP1 binding with GM^{64–237} versus GM^{64–105}) and thus PP1 and GM bind one another via two distinct interaction sites.

We then performed ITC measurements to test whether GM^{64–237} interacts with PP1 in an isoform-specific manner. ITC showed that...
GM$_{64-237}$ binds PP1$_{a7-330}$ (26.5 ± 2.3 nM) and PP1$_{g7-323}$ (21.4 ± 11.6 nM; PP1$_{g1}$) with statistically identical $K_D$ values, demonstrating that the interaction of GM is not PP1 isoform specific (fig. S1, E to H).

The GM PP1–anchoring domain

To determine how GM$_{64-237}$ binds PP1 at atomic resolution, we used x-ray crystallography. Despite extensive efforts, crystallization of the GM$_{64-93}$:PP1$_{a7-330}$ holoenzyme was unsuccessful. However, we were able to determine the 3D structure of the GM$_{64-93}$:PP1$_{a7-300}$ holoenzyme (hereafter referred to as GM:PP1) to a resolution of 1.45 Å (table S1). The structure shows that GM residues 64 to 85 become ordered when bound to PP1 (Fig. 2A). They bind in a largely extended manner at multiple sites across the top of PP1, including the RVxF motif binding site and the FF motif binding site (Fig. 2B), both of which are used by a large number of PP1 interactors. The interaction between PP1 and GM is extensive, with the complex burying 2194 Å$^2$ of a solvent-accessible surface area. As expected for a targeting protein, the PP1 catalytic site is accessible, and PP1 is catalytically active.

**Table 1. Thermodynamic and dissociation constants for the GM interaction with PP1 and cyclodextrins derived from ITC experiments at 25°C.** n.d., not determined.

| Interaction                  | $K_D$ (nM) | $\Delta H$ (kcal·mol$^{-1}$) | $-\Delta S$ (kcal·mol$^{-1}$) | $\Delta G$ (kcal·mol$^{-1}$) | Repeats |
|------------------------------|------------|-------------------------------|-------------------------------|-------------------------------|---------|
| GM$_{64-237}$:PP1$_{a7-330}$ | 26.5 ± 2.3 | -20.2 ± 1.0                   | -9.9 ± 0.9                    | -10.3 ± 0.1                   | 5       |
| GM$_{64-237}$:PP1$_{g7-323}$ | 21.4 ± 11.6| -22.6 ± 0.1                   | -12.1 ± 0.2                   | -10.5 ± 0.3                   | 2       |
| GM$_{64-237}$:PP1$_{g7-308}$ | 51.0 ± 5.1 | -25.1 ± 0.9                   | -15.2 ± 0.9                   | -10.0 ± 0.1                   | 3       |
| GM$_{64-237}$:PP1$_{a6-327}$ | 17.3 ± 4.0 | -15.4 ± 0.8                   | -4.8 ± 0.9                    | -10.6 ± 0.1                   | 3       |
| GM$_{64-237}$:PP1$_{a7-300}$ | 51.0 ± 5.1 | -25.1 ± 0.9                   | -15.2 ± 0.9                   | -10.0 ± 0.1                   | 3       |
| GM$_{64-237}$:PP1$_{a7-330}$ | 20.8 ± 2.4 | -34.5 ± 2.4                   | -26.1 ± 2.4                   | -8.4 ± 0.1                    | 4       |
| GM$_{64-237}$:PP1$_{g7-323}$ | 21.4 ± 11.6| -22.6 ± 0.1                   | -12.1 ± 0.2                   | -10.5 ± 0.3                   | 2       |
| GM$_{64-237}$:PP1$_{g7-308}$ | 55.7 ± 5.2 | -13.3 ± 5.2                   | -3.4 ± 5.1                    | -9.9 ± 0.1                    | 2       |
| GM$_{64-237}$:PP1$_{a7-300}$ | 51.0 ± 5.1 | -25.1 ± 0.9                   | -15.2 ± 0.9                   | -10.0 ± 0.1                   | 3       |
| GM$_{64-237}$:PP1$_{a7-330}$ | 20.8 ± 2.4 | -34.5 ± 2.4                   | -26.1 ± 2.4                   | -8.4 ± 0.1                    | 4       |
| GM$_{64-237}$:PP1$_{g7-323}$ | 21.4 ± 11.6| -22.6 ± 0.1                   | -12.1 ± 0.2                   | -10.5 ± 0.3                   | 2       |
| GM$_{64-237}$:PP1$_{g7-308}$ | 55.7 ± 5.2 | -13.3 ± 5.2                   | -3.4 ± 5.1                    | -9.9 ± 0.1                    | 2       |
| GM$_{64-237}$:PP1$_{a7-330}$ | 26.5 ± 2.3 | -20.2 ± 1.0                   | -9.9 ± 0.9                    | -10.3 ± 0.1                   | 5       |
| GM$_{64-237}$:PP1$_{g7-323}$ | 21.4 ± 11.6| -22.6 ± 0.1                   | -12.1 ± 0.2                   | -10.5 ± 0.3                   | 2       |
| GM$_{64-237}$:PP1$_{g7-308}$ | 51.0 ± 5.1 | -25.1 ± 0.9                   | -15.2 ± 0.9                   | -10.0 ± 0.1                   | 3       |
| GM$_{64-237}$:PP1$_{a6-327}$ | 17.3 ± 4.0 | -15.4 ± 0.8                   | -4.8 ± 0.9                    | -10.6 ± 0.1                   | 3       |
| GM$_{64-237}$:PP1$_{a7-300}$ | 51.0 ± 5.1 | -25.1 ± 0.9                   | -15.2 ± 0.9                   | -10.0 ± 0.1                   | 3       |
| GM$_{64-237}$:PP1$_{a7-330}$ | 20.8 ± 2.4 | -34.5 ± 2.4                   | -26.1 ± 2.4                   | -8.4 ± 0.1                    | 4       |
| GM$_{64-237}$:PP1$_{g7-323}$ | 21.4 ± 11.6| -22.6 ± 0.1                   | -12.1 ± 0.2                   | -10.5 ± 0.3                   | 2       |
| GM$_{64-237}$:PP1$_{g7-308}$ | 55.7 ± 5.2 | -13.3 ± 5.2                   | -3.4 ± 5.1                    | -9.9 ± 0.1                    | 2       |
| GM$_{64-237}$:PP1$_{a7-330}$ | 20.8 ± 2.4 | -34.5 ± 2.4                   | -26.1 ± 2.4                   | -8.4 ± 0.1                    | 4       |
| GM$_{64-237}$:PP1$_{g7-323}$ | 21.4 ± 11.6| -22.6 ± 0.1                   | -12.1 ± 0.2                   | -10.5 ± 0.3                   | 2       |
| GM$_{64-237}$:PP1$_{g7-308}$ | 55.7 ± 5.2 | -13.3 ± 5.2                   | -3.4 ± 5.1                    | -9.9 ± 0.1                    | 2       |
active in the G_{M}^{64–237}:PP1α_{2–300} holoenzyme, as it is capable of dephosphorylating model substrates, such as p-nitrophenyl phosphate (fig. S2, A and B).

G_{M} residues^{65} RVSF^{68} form the RVxF motif, which binds the PP1 RVxF binding pocket (Val^{66}_{GM} and Phe^{67}_{GM} are the anchoring hydrophobic residues that bind deeply in this pocket). This interaction is highly similar to those observed in other PP1 holoenzyme complexes, as well as the previously determined PP1-GM_{peptide} structure (G_{M}^{peptide} includes residues 63 to 75 with density visible only for residues 63 to 68; fig. S2, C and D) (15). As has been shown for other RVxF containing PP1 regulatory subunits, phosphorylation by protein kinase A (PKA) of the “x” residue in the G_{M} RVxF motif, Ser^{67}_{GM}, inhibits PP1 binding (16). Val^{79}_{GM} and Lys^{80}_{GM} form the G_{M} {ΦΦ} motif, which binds the PP1 {ΦΦ} binding pocket. Like the RVxF interaction, the {ΦΦ} interaction is highly similar to those observed in other PP1 holoenzyme complexes. Further, these residues are part of a short β strand (G_{M} residues 78 to 81) that hydrogen bonds with PP1 β strand β14 to extend one of PP1’s two central β sheets (fig. 2C).

Notably, a distinctive feature of the G_{M}:PP1 holoenzyme structure is the “extended kink” between the RVxF and {ΦΦ} motifs (fig. 2, B and C). In G_{M}, these two motifs are separated by 12 residues, the longest insert observed to date for any PP1 regulator. It is long because it forms an ~8-residue “Greek key” turn. This structural feature is stabilized by two anchoring interactions: (i) a strong intermolecular bi-dentate salt bridge between PP1 residue Arg^{261}_{PP1} and Asp^{70}_{GM} and (ii) hydrophobic interactions made by the G_{M} “lid” residue (Leu^{76}_{GM}), with the hydrophobic ΦΦ pocket adjacent to the RVxF binding pocket (Fig. 2D). As a consequence, the side chains of G_{M} residues Phe^{74}_{GM} and Phe^{75}_{GM} point up away from the holoenzyme surface, where they bury the side chain of PP1 residue Met^{90}_{PP1}.

A second distinctive feature of the G_{M}:PP1 complex is that the G_{M}:PP1 interaction extends beyond the {ΦΦ} motif, with Phe^{80}_{GM} binding in a deep pocket immediately adjacent to Pro^{299}_{PP1} (Fig. 2D). Comparison with other PP1 holoenzyme structures reveals that, similar to the RVxF and {ΦΦ} binding pockets, this pocket is also frequently used by regulators to bind PP1. Namely, spinophilin (Thr^{165}_{GM}, Trp^{168}_{GM}, Gln^{169}_{GM}, Thr^{170}_{GM}, Leu^{196}_{GM}, Val^{197}_{GM}, Asn^{225}_{GM}, and Thr^{230}_{GM}) (20). Mapping these residues onto the G_{M}^{CBM21} structure shows that the largest changes correspond to residues within the G_{M}^{β3-β4} loop (Fig. 3, B and C).

Structural comparison of G_{M}^{CBM21} with the CBM21 family

Unlike the majority of SBDs, the N terminus of G_{M}^{CBM21} contains an additional β strand, β0, and an α helix, α1. The ^{15}N[^1H]-NOE data of G_{M}^{CBM21} showed that this loop is very rigid, showing no differences in dynamics when compared to the rest of G_{M}^{CBM21} (fig. S3E). Unexpectedly, variants of these loops fail to express solubly, making ITC measurements to further confirm the interaction impossible. ITC data testing a direct interaction of G_{M}^{CBM21} with PP1 showed no binding isotherm, confirming that G_{M} residues 64 to 85 are strictly required for the interaction (fig. S11).

The G_{M} carbohydrate binding domain also interacts with PP1

As shown by ITC, G_{M}^{64–237} binds PP1—4-fold more tightly than the primary PP1-anchoring domain (G_{M}^{64–105}). To understand this in more detail, we determined the 3D structure of G_{M}^{CBM21} (16 kDa) using solution NMR spectroscopy. The NMR data of G_{M}^{CBM21} are of outstanding quality, allowing for the sequence-specific backbone assignment of 128 out of an expected 132 residues and a 98% completeness for the side-chain assignment (fig. S3A). A total of 2709 nuclear Overhauser effect (NOE)—based unambiguous distance restraints (~20 restraints per residue) and 110 dihedral angle restraints were used for the final structure refinement of 200 structures (table S2). The 20 lowest-energy formers show excellent stereochemistry, with a backbone root mean square deviation of 0.56 Å (secondary structure).

G_{M}^{CBM21} like all known SBD structures, adopts an immunoglobulin-like β-sandwich fold (fig. S3B) (13, 20, 21). The β-sandwich consists of nine antiparallel β strands: β0 (G_{M} Residues 108 to 110), β1 (130 to 137), β2 (144 to 151), β3 (158 to 164), β4 (174 to 178), β5 (187 to 193), β6 (208 to 215), β7 (218 to 221), and β8 (229 to 235) (fig. S3C). At its N terminus, G_{M}^{CBM21} has an additional α helix, α1 (118 to 127). SBDs adopt one of two topologies, type I or type II, which differ in the order and orientation of a single β strand (fig. S3D) (22). The G_{M}^{CBM21} structure adopts a type II topology; this is consistent with the observation that the majority of N-terminal SBDs adopt a type II topology, whereas most C-terminal SBDs adopt a type I topology (23).

We then used NMR spectroscopy and ^{2}H,^{15}N-labeled G_{M}^{64–237} (here, only ^{1}H attached to carbons are exchanged to ^{2}H; nitrogen-bound ^{1}H are rapidly back exchanged) to test the interaction with PP1. Two results were readily detected. First, peaks corresponding to G_{M}^{64–85} are missing as they move to their folded PP1-bound state. This demonstrates that the residues observed in the G_{M}:PP1 holoenzyme structure also bind PP1 in solution (Fig. 3A). Second, several peaks corresponding to residues in G_{M}^{CBM21} have CSPs or intensity changes, including Leu^{165}_{GM}, Trp^{168}_{GM}, Gln^{169}_{GM}, Thr^{170}_{GM}, Leu^{196}_{GM}, Val^{197}_{GM}, Asn^{225}_{GM}, and Thr^{230}_{GM}. Mapping these residues onto the G_{M}^{CBM21} structure shows that the largest changes correspond to residues within the G_{M}^{β3-β4} loop (Fig. 3, B and C).
curvature on the sugar molecule (fig. S3F) (20). Site 1 in GM

CBM21 and GL

CBM21 is flatter (fig. S3G), which may affect the binding affinity of these proteins for glycogen/β-CD. In RoGACBM21, sugar binding site 2 forms a small narrow groove, with two protruding aromatic residues that clamp down over the sugar molecule (fig. S3G) (20). GM

CBM21 has a similar "closed clamp" architecture at site 2, whereas GL

CBM21 adopts an "open clamp" conformation. This may simply reflect the structural plasticity of site 2 (21), a site known to undergo large conformational rearrangements upon β-CD binding. These data, in addition to the presence of β0 and α1 in only GM

CBM21, suggest that these domains may differ in function, despite being in the same CBM21 family.

G

M contains a single glycogen binding site

In GM

CBM21, the two sugar binding sites are formed by Trp168

GM,

His171

GM, Cys173

GM, Trp221

GM, Asn223

GM, and Asn228

GM (site 1), and by Asn152

GM, Phe155

GM, Glu156

GM, Lys157

GM, Tyr178

GM, and Asp188

GM (site 2; fig. S4A). Site 1 is a shallow binding pocket whose curvature is defined by two to three aromatic rings. Site 2 is generally formed by the β2-β3 and β4-β5 loops and is typically dominated by two aromatic residues that undergo large conformational rearrangements upon β-CD binding, acting as a clamp (fig. S4B). Unlike site 1, site 2 is highly conserved in sequence among GM

CBM21, GL

CBM21, and RoGACBM21 (fig. S4A).

To examine the sugar binding properties of GM

CBM21, we performed NMR CSP experiments titrating 15N-labeled GM

CBM21 with α-CD or β-CD at 1:0.5, 1:1, 1:2, 1:5, 1:20, and 1:40 (protein:ligand) molar ratios, achieving saturation of binding at a ratio of 1:20 (fig. S4, C and D). In general, both α-CD and β-CD caused similar CSPs, with the largest CSPs observed for Phe155

GM, Glu156

GM, and Glu186

GM in site 2, and for Thr215

GM and Ser216

GM in the β6-β7 loop immediately adjacent to site 2 (Fig. 3, D and E). Unexpectedly, no CSPs were
observed for the residues that form site 1, with an average Δδ of 0.057 parts per million (ppm) for site 1 and 0.333 ppm for site 2 when bound to β-CD. Consistent with this observation, ITC of Gm\textsubscript{CBM21} with both α-CD and β-CD showed a binding stoichiometry of 1 and \( K_D \) values of 27.6 ± 5.8 μM for α-CD (fig. S1I) and 8.2 ± 0.1 μM for β-CD (fig. S1K). Together, our data show that soluble cyclodextrins bind Gm\textsubscript{CBM21} at a single binding site, centered on Phe\textsubscript{155}Gm.

In the presence of either α-CD or β-CD, most 2D [\( ^{1}H,^{15}N \)] HSQC cross-peaks shift “linearly,” such that the beginning, middle, and end titration points can all be connected with a straight line in the overlaid spectra (fig. S4, C and D). However, a subset of perturbed cross-peaks deviate from linearity and are curved (fig. S4E). Curved cross-peak patterns generally represent a convolution of two or more processes that occur as a result of ligand binding. In Gm\textsubscript{CBM21}, these residues map to the central β-sandwich, not to the sugar binding sites (fig. S4F), suggesting that cyclodextrin binding to site 2 is accompanied by a conformational change that is translated across the center of the protein, but does not allosterically perturb/activate site 1; i.e., these sites are functionally independent.

To confirm that the same CSPs are observed between the Gm\textsubscript{CBM21} domain and β-CD when Gm\textsubscript{M} is bound to PP1, we repeated the CSP NMR titration experiments of β-CD with the Gm\textsubscript{M}–PP1 holoenzyme (fig. S5, A and B). Site 2 showed the same CSPs as identified for Gm\textsubscript{CBM21} alone. These data show that Gm\textsubscript{M} is able to bind glycogen and PP1 simultaneously, further supporting its function as a scaffolding protein.

**Glycogen enhances dephosphorylation of phosphorylase a by scaffolding**

Substrate recruitment is poorly understood for most PP1 holoenzymes, mainly because very few substrates have been identified. This is different for the Gm\textsubscript{M}-PP1 holoenzyme, whose primary substrates are phosphorylase a and glycogen synthase. This enables a detailed study of the mechanism(s) by which these substrates are recruited to PP1. Phosphorylase a is phosphorylated on Ser\textsuperscript{14}, which is the specific substrate of PP1. To understand the recruitment of the substrate phosphorylase a by the Gm\textsubscript{M}–PP1 holoenzyme, we measured the dephosphorylation of phosphorylase a by PP1 alone or by the Gm\textsubscript{M}–PP1, Gm\textsubscript{M}–PP1, Gm\textsubscript{M}–PP1, and spinophilin (fig. S6A). Different Gm\textsubscript{M} constructs were used to determine how Gm\textsubscript{M}-interacting domains influence substrate recruitment, while the PP1-specific regulators nuclear inhibitor of protein phosphatase 1 (NIPP1) (24) and spinophilin (25) were used as negative controls [phosphorylase a is not an endogenous substrate of either the NIPP1:PP1 or the spinophilin:PP1 holoenzyme; further, previous studies showed that both holoenzymes inhibit phosphorylase a dephosphorylation (17, 26)]. The data show that PP1 alone and all Gm\textsubscript{M} holoenzymes were equally effective at dephosphorylating phosphorylase a (fig. 4A and fig. S6B). This demonstrates that Gm\textsubscript{CBM21} does not alter PP1’s activity toward phosphorylase a. In contrast, and consistent with previous data, the PP1 binding domains from both NIPP1 and spinophilin inhibit phosphorylase a dephosphorylation.

Because phosphorylase a, like Gm\textsubscript{M}, binds directly to glycogen (via phosphorylase residues 397 to 437), we hypothesized that the dephosphorylation of phosphorylase a by the Gm\textsubscript{M}–PP1 holoenzyme would be enhanced in the presence of glycogen. To test this, we measured phosphorylase a dephosphorylation at multiple time points in the presence and absence of glycogen. The data show that glycogen greatly enhances phosphorylase a dephosphorylation (fig. S6C). Thus, this leads to a model where the recruitment of both phosphorylase a and the Gm\textsubscript{M}–PP1 holoenzyme to glycogen is necessary to achieve the highest dephosphorylation. To test this, we performed a glycogen pelleting assay using glycogen-bound concanavalin A (Con A)–Sepharose beads (27). The data showed that phosphorylase a, Gm\textsubscript{M}–PP1, and the Gm\textsubscript{M}–PP1 holoenzyme (both alone and in combination with phosphorylase a and/or Gm\textsubscript{M}–PP1) robustly bind glycogen. In contrast, PP1 alone does not (fig. 4C). This demonstrates that glycogen serves as the scaffold that recruits both phosphorylase a and the Gm\textsubscript{M}–PP1 holoenzyme to facilitate the Gm\textsubscript{M}–PP1 holoenzyme–mediated dephosphorylation of phosphorylase a.

**PP1 regulator mediated inhibition of phosphorylase a dephosphorylation**

PP1 has ~200 confirmed distinct regulators, including Gm\textsubscript{M}. However, while the Gm\textsubscript{M}–PP1 holoenzyme is fully capable of dephosphorylating its endogenous substrate, phosphorylase a, other PP1 regulators can inhibit PP1-mediated dephosphorylation. Previously, we found the molecular mechanism by which spinophilin prevents PP1 from dephosphorylating phosphorylase a (17). Namely, our crystal structure of the spinophilin:PP1 holoenzyme showed that spinophilin binds the PP1 C-terminal binding groove, blocking access to Asp\textsuperscript{71}PP1 (28). This residue is essential for phosphorylase a dephosphorylation. Thus, spinophilin and other PP1 regulators that bind the same substrate binding groove on PP1 (i.e., PNUSTS) inhibit phosphorylase a dephosphorylation by steric exclusion. That is, the regulators block phosphorylase a from binding PP1 in the C-terminal groove and, as a consequence, it is not dephosphorylated. While it is well documented that NIPP1 also inhibits the dephosphorylation of phosphorylase a, the molecular mechanism by which this is achieved is still unknown (our crystal structure of the NIPP1:PP1 holoenzyme revealed that, unlike spinophilin, NIPP1 does not bind the C-terminal groove and thus Asp\textsuperscript{71}PP1 is accessible) (26).

To further understand the molecular basis of NIPP1-mediated inhibition, we generated two chimeric PP1 regulators: NG1: NIPP1\textsubscript{158–198}Gm\textsubscript{M}–PP1 and NG2:NIPP1\textsubscript{175–198}Gm\textsubscript{M}–PP1. NG1 includes the NIPP1\textsubscript{helix} (residues 158 to 174), the NIPP1\textsubscript{connector} (residues 175 to 198), not visible in the NIPP1:PP1 complex crystal structure, and the full primary PP1-anchoring domain from Gm\textsubscript{M} (Gm\textsubscript{M}–PP1) (29). NG2 is identical to NG1, with the exception that it does not include the NIPP1\textsubscript{helix} (fig. S6A). The data show that the NG1:PP1 complex inhibits phosphorylase a dephosphorylation to the same extent as NIPP1:PP1, showing that the inhibition is due to either the NIPP1\textsubscript{helix} or the NIPP1\textsubscript{linker}, or both, but not the RVxF or ΦΦ interaction (i.e., Gm\textsubscript{M} or NIPP1 is exchangeable) (fig. 4A and fig. S6D). By comparison, the NG2:PP1 complex inhibits phosphorylase a dephosphorylation poorly and allows for rapid dephosphorylation similar to Gm\textsubscript{M}–PP1. We have previously shown that mutating positively charged residues within the NIPP1\textsubscript{linker} to a poly-A stretch (NIPP1 residues K\textsubscript{193}K\textsubscript{194}K\textsubscript{195}K\textsubscript{196}K\textsubscript{197} mutated to 193AAA AAAA (19)) in the NIPP1\textsubscript{linker} does not change inhibition significantly (26). Thus, these data demonstrate that the NIPP1\textsubscript{helix} is likely critical for the ability of NIPP1-mediated inhibition of PP1’s ability to dephosphorylate phosphorylase a. These data correlate with those obtained for the PP1 regulator MYPT1, which has an MYPT1\textsubscript{helix} that binds PP1 in an area similar to that of the NIPP1\textsubscript{helix} (29). Together these data provide insight into how the substrate phosphorylase a engages PP1 and highlights that Gm\textsubscript{M} does not alter PP1’s activity toward phosphorylase a itself; rather, it
forms a glycogen recruitment platform that allows phosphorylase $\alpha$ recruitment via glycogen scaffolding.

**G$_M$ enhances the dephosphorylation of glycogen synthase**

A second confirmed substrate of G$_M^{64-237}$:PP1 is GYS1 (muscle specific glycogen synthase; 84 kDa) (14). GYS1 catalyzes the conversion of glucose to glycogen and is activated by two mechanisms: (i) allosterically, as it is positively allosterically regulated by glucose-6-phosphate, and (ii) dephosphorylation, as the dephosphorylation of specific Ser/Thr residues increases GYS1 activity (GYS1 is phosphorylated by glycogen synthase kinase 3, AMP-activated protein kinase (AMPK), PKA, and casein kinase 2, which inhibit GYS1 activity). Dephosphorylation, which activates GYS1, is mainly controlled by PP1 via the recruitment by the G$_M$:PP1 holoenzyme. To understand how G$_M$:PP1 dephosphorylates GYS1, we produced functional human muscle GYS1 by coexpressing it in complex with human glycogenin-1 (GYG1; muscle specific; 66 kDa; glycogenin-1 binds directly to glycogen). Production of GYS1 in this manner results in an enzyme that is phosphorylated at both well-characterized and uncharacterized Ser/Thr residues (14). Incubation of GYS1 with PP1 shows a significant shift in the migration of the GYS1 band in SDS-PAGE (polyacrylamide gel electrophoresis), as often seen when an extensively phosphorylated protein is dephosphorylated (fig. S6, Fig. 4. Dephosphorylation of phosphorylase $\alpha$ and GYS1 by G$_M$:PP1. (A) Relative phosphorylation level of phosphorylase $\alpha$ following incubation alone (brown circle) or with PP1 (coral square), various G$_M$:PP1 holoenzymes (green triangle, cyan triangle, blue diamond), NIPP1$^{158-216}$:PP1 (orange circle), spinophilin$^{417-602}$:PP1 (pink circle), NG1:PP1 (green circle), or NG2:PP1 (light blue circle). Only NIPP1$^{158-216}$, spinophilin$^{417-602}$, and NG1 inhibit the PP1-mediated dephosphorylation of phosphorylase $\alpha$ (error bar corresponds to the SD with an $n$ between 4 and 12; statistical significance was determined by one-way analysis of variance (ANOVA): **** $P < 0.0001$, Tukey's multiple comparisons test); n.s., not significant. (B) Glycogen enhances the G$_M^{64-237}$:PP1-mediated dephosphorylation of phosphorylase $\alpha$. Time course of the relative phosphorylation of phosphorylase $\alpha$ in the presence of PP1 (black circle), G$_M^{64-237}$:PP1 (red square) or G$_M^{64-237}$:PP1, and glycogen (glycogen (4 mg/ml); purple triangle; two-way ANOVA, $P < 0.0001$). (C) Phosphorylase $\alpha$ and the G$_M^{64-237}$:PP1 holoenzyme bind directly to glycogen. Glycogen pelleting assay using Con A beads incubated with the biomolecules indicated ($n = 3$). (D) Gel shift analysis shows that G$_M^{64-237}$:PP1 causes the largest shift in the migration of the GYS1 band (error bar corresponds to the SD, $n = 5$; one-way ANOVA with post hoc Tukey test); n.s., not significant. (E) Time course of dephosphorylation measured by gel shift. G$_M^{64-237}$ (red square) enhances the dephosphorylation of GYS1 by PP1 alone compared to PP1 alone (black circle; two-way ANOVA, $P = 0.0017$). The single-point mutation G$_M^{CBM21}$N228A (blue triangle) eliminates this enhancement.

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E and F). The same shift is also detected when GYS1 is incubated with G_{M}^{64–237:PP1} and G_{M}^{64–105:PP1}, showing that the G_{M} anchoring PP1 binding domain does not specify or influence the PP1 activity toward GYS1. However, G_{M}^{64–237:PP1}, which includes G_{M}^{CBM21}, showed a significantly larger shift, indicating that the CBM21 domain facilitates the recruitment and dephosphorylation of substrate GYS1 (Fig. 4D).

To identify the residues selectively dephosphorylated by the G_{M}^{64–237:PP1} holoenzyme, we used liquid chromatography–tandem mass spectrometry (LC-MS/MS). It is well known that the phosphorylation state of multiple residues of GYS1 is directly correlated with its activity, including residues 8 and 11 (sites 2 and 2a) and residues 641, 645, and 649 (sites 3a/b/c) (31). We focused our analysis on Ser^{641}_{GYS1} (3a) and Ser^{645}_{GYS1} (3b), as the dephosphorylation of both residues is essential for GYS1 activity (sites 2 and 2a were not identified by LC-MS/MS and thus were not analyzed) (fig. S7, A and B). The MS data showed that only singly (3a) or doubly (3a/b) phosphorylated peptides were detected for GYS1; no unphosphorylated or singly phosphorylated (3b) peptide was observed. However, incubation with PP1 or any of its holoenzymes [G_{M}^{64–98:PP1}, G_{M}^{64–105:PP1}, G_{M}^{64–237:PP1}, and spinophilin:PP1 (used as control)] resulted in the complete disappearance of the single phosphorylated 3a peptide (fig. S7C). This demonstrates that this residue is rapidly dephosphorylated by all versions of PP1. In contrast, the doubly phosphorylated 3a/b peptide was most effectively dephosphorylated by the G_{M}^{64–237:PP1} holoenzyme compared to either PP1 alone or any other PP1 holoenzymes (fig. S7D). This enhanced dephosphorylation by G_{M}^{64–237:PP1} PP1 was also observed for the singly phosphorylated 3b peptide (note that this peptide is only present after incubation with PP1 or one of its holoenzymes due to the dephosphorylation of 3a) (fig. S7E). Further, the amount of unphosphorylated 3a/b peptide present after incubation is highest for the G_{M}^{64–237:PP1} holoenzyme (fig. S7F). Last, the data also show that GYS1 phosphorylated residues that are not correlated with GYS1 activity, such as Thr^{278} and Ser^{112}, were not dephosphorylated by either PP1 or any of the PP1 holoenzymes (fig. S7, G and H). Together, these data show that recruitment of PP1 to the G_{M}^{CBM21} via G_{M}^{64–85} creates a specific enzyme that activates GYS1 and that G_{M}^{CBM21} functions as the specifier, but not any G_{M} residue that is directly interacting with PP1.

To confirm these results, we performed two additional experiments. First, we repeated the experiment with glycogen (fig. S8). In contrast to phosphorylase a, no dephosphorylation enhancement is detected upon the addition of glycogen, showing that the enhancement comes solely from G_{M}^{CBM21}. Second, we generated a G_{M}^{CBM21} N228A variant, a residue that is part of a highly conserved patch of residues and was previously speculated to be involved in GYS1 recruitment (14). Repeating the dephosphorylation assay with G_{M}^{64–237:PP1} N228A showed a significant reduction in dephosphorylation, confirming that N228A is essential for the recruitment of GYS1 to PP1 (Fig. 4E).

**DISCUSSION**

The balance between glucose storage in the form of glycogen and its subsequent breakdown is controlled by phosphorylation. A single phosphatase, the G_{M}:PP1 holoenzyme, specifically dephosphorylates three of the key enzymes that control glycogen synthesis and breakdown: phosphorylase kinase, phosphorylase a, and glycogen synthase. While many insights into PP1 activity and function of PP1 have been obtained by studying G_{M}:PP1, a molecular understanding of how PP1 generally and G_{M}:PP1 in particular recruits and selectively dephosphorylates its specific substrates is still largely missing. G_{M}:PP1 is uniquely positioned to answer these questions, as its substrates are well described. Phosphorylase a is the canonical substrate for measuring PP1 activity, and thus a detailed molecular understanding of substrate recruitment will have a profound impact on understanding PP1 regulation.

Our NMR spectroscopy, crystallography, enzymatic, and molecular binding data show that G_{M} binds PP1 via a much longer domain than previously thought, including the canonical RVxF motif and an unusually extended ΨΦ motif that is connected by a highly structured, kinked linker. As is typical for PP1 holoenzymes, these interactions do not alter the conformation of PP1, nor do they block its active site. Thus, G_{M} binding does not alter the catalytic activity of PP1 toward its substrates. Yet, the G_{M}:PP1 holoenzyme is highly selective for its endogenous substrates. Previous data suggested this specificity resides in G_{M} residues 102 to 240, which include the G_{M}^{CBM21} domain. Thus, we determined the 3D structure of G_{M}^{CBM21}, which showed that it belongs to the family of SBDs. Unexpectedly, using CSP mapping, we found that G_{M}^{CBM21} has only one starch binding site, unlike many of its closest family members, which commonly have two (20). This site is necessary and, as we have now shown, sufficient for the effective recruitment of glycogen. In G_{M}, however, we show that the second starch binding site has evolved to (i) interact with PP1 and (ii) bind glycogen synthase (Fig. 5A). These data, coupled with multiple enzymatic assays, led to the discovery that G_{M} achieves G_{M}-mediated substrate recruitment via two distinct mechanisms. In the first mechanism, G_{M} functions as a scaffold to localize PP1 near its substrates; i.e., the G_{M} PP1 binding domain binds PP1, while the G_{M}^{CBM21} domain binds glycogen. Glycogen binding, in
turn, targets G\textsubscript{M};PP1 to one of its glycogen-specific substrates, phosphorylase \textit{a} (Fig. 5B). This is because ~70\% phosphorylase \textit{a} is always glycogen bound (32). The phosphorylation of Ser\textsubscript{67}GM by PKA releases PP1 from G\textsubscript{M} (16), leading to a model in which G\textsubscript{M} stays localized near its substrate via glycogen binding, while PP1 (via the phosphorylation state of Ser\textsubscript{67}GM) is recruited only when needed. The second mechanism is direct binding between G\textsubscript{M} and a G\textsubscript{M};PP1-specific substrate. Namely, the G\textsubscript{M} \text{CBM21} domain binds directly to GYS1 to recruit this substrate to the G\textsubscript{M};PP1 holoenzyme for the PP1-mediated dephosphorylation (Fig. 5C).

As previously highlighted, phosphorylase \textit{a} is also the canonical substrate used to measure PP1 activity. This has led to the discovery that many PP1-specific regulatory proteins potently inhibit PP1-mediated dephosphorylation of phosphorylase \textit{a}. One of these is the PP1-specific regulator spinophilin. Using x-ray crystallography and enzymatic assays, we previously showed that spinophilin inhibits the dephosphorylation of phosphorylase \textit{a} by blocking its access to the PP1 C-terminal substrate binding groove (17). This demonstrates that regulators can achieve substrate selectivity, in part, by sterically excluding binding site for subsets of substrates. A second regulator that inhibits dephosphorylation of phosphorylase \textit{a} is NIPP1. However, its structure showed that the C-terminal substrate binding groove is fully accessible, and thus, that it inhibits phosphorylase \textit{a} dephosphorylation by a distinct mechanism (26). Here, we used NIPP1-G\textsubscript{M} chimeras to show that the NIPP1\textsubscript{helix} which binds at the entrance of a second substrate binding groove in PP1, the hydrophobic substrate binding groove, is important for NIPP1’s ability to inhibit the dephosphorylation of phosphorylase \textit{a}. Thus, the NIPP1\textsubscript{helix} likely sterically blocks the access of phosphorylase \textit{a} to the PP1 hydrophobic groove. Together, these data reveal that robust dephosphorylation of phosphorylase \textit{a} requires its ability to bind both the hydrophobic and the C-terminal substrate binding grooves.

This study provides the most comprehensive molecular understanding of how a specific PP1 holoenzyme, G\textsubscript{M};PP1, mediates the rapid and selective dephosphorylation of its specific substrates. In particular, it highlights the essential role of distinct recruitment domains present in PP1-specific regulatory proteins for directing the specificity of PP1. These domains (G\textsubscript{M} \text{CBM21} in G\textsubscript{M};PP1 recruit PP1 to their cellular points of action (glycogen) that, in turn, convert PP1 into an exemplar for a specific substrate, either specifically or by its localization to PP1 to its specific substrates (i.e., phosphorylase \textit{a}, which, like G\textsubscript{M}; PP1, also binds directly to glycogen) or directly by binding to the substrate itself (i.e., GYS1, which binds directly to G\textsubscript{M} \text{CBM21}). Similar mechanisms for substrate recruitment have also been observed in other PP1-specific regulators. For example, the PSD95/Disc large/ZO-1 (PDZ) domain of the PP1-specific regulator spinophilin binds to the C termini of GluR2/3 subunits of the AMPA (\textit{a}-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor, and thus functions to localize PP1 to its specific substrate, Ser\textsubscript{845} of the GluR1 (33). Likewise, the forkhead associated (FHA) domain in the regulator NIPPP1 binds directly to specific substrates of the NIPP1;PP1 holoenzymes (34). Thus, these data show that to fully understand the function of a particular PP1 holoenzyme, and especially its specificity toward distinct substrates, it is essential to understand the functions of all the domains in cognate PP1 regulatory protein. As we and others have shown, substrate recruitment sites on PPP holoenzymes are functional drug binding sites (e.g., as seen for PP2B/PP3/calcineurin and FK-506) (35). Thus, the identification and characterization of these specific and unique interactions, especially in PP1 holoenzymes, will lead to the design of potent, effective PP1-selective drugs.

**MATERIALS AND METHODS**

**Protein expression**

The coding sequences of rabbit G\textsubscript{M} \text{CBM21} \text{GYS1}, G\textsubscript{M} \text{CBM21} \text{GYS1}, and G\textsubscript{M} \text{CBM21} were subcloned into a pET-M30-MBP vector containing an N-terminal His\textsubscript{6}-tag followed by maltose binding protein (MBP) and a tobacco etch virus (TEV) protease cleavage site. G\textsubscript{M} \text{CBM21} (residues 102 to 237) was subcloned into pPRP1B containing an N-terminal His\textsubscript{6}-tag followed by a TEV protease cleavage site. *Escherichia coli* strain BL21-Codon-Plus (DE3)-RII (Agilent) cells were transformed with the G\textsubscript{M} expression vectors. Freshly transformed cells were grown at 37°C in LB medium containing selective antibiotics until they reached an OD\textsubscript{600} (optical density at 600 nm) of 0.8 to 1.0. Protein expression was induced by addition of 1 mM β-d-thigalactopyranoside to the culture medium, and cultures were allowed to grow overnight (18 to 20 hours) at 18°C. Cells were harvested by centrifugation (6000g, 15 min, 4°C) and stored at ~80°C until purification.

Expression of uniformly \textsuperscript{13}C- and/or \textsuperscript{15}N-labeled protein was carried out by growing freshly transformed cells in M9 minimal media containing [\textsuperscript{13}C]-d-glucose (4 g/liter) and/or [\textsuperscript{15}N]NH\textsubscript{4}Cl (1 g/liter) (Cambridge Isotopes Laboratories) as the sole carbon and nitrogen sources, respectively. Expression of uniformly \textsuperscript{2H,15N}-labeled G\textsubscript{M} \text{CBM21} was achieved by growing cells in D\textsubscript{2}O-based M9 minimal media containing [\textsuperscript{15}N]NH\textsubscript{4}Cl (1 g/liter) as the sole nitrogen source. Multiple rounds (0, 30, 50, 70, and 100%) of D\textsubscript{2}O adaptation were necessary for high-yield expression. Cloning, expression, and purification of PP1\textsubscript{GluR2–300}, PP1\textsubscript{GluR2–330}, PP1\textsubscript{GluR2–327}, PP1\textsubscript{GluR2–308}, and PP1\textsubscript{GluR2–323} were performed as previously described (18); human and rabbit PP1 are 100\% identical. G\textsubscript{M} \text{CBM21} \text{GYS1}, G\textsubscript{M} \text{CBM21} \text{GYS1}, and G\textsubscript{M} \text{CBM21} \text{GYS1} peptides were purchased from Bio-Synthesis Inc. G\textsubscript{M} \text{CBM21} \text{N228A} was generated by site-directed mutagenesis and expressed using the same methods as those used for wt-G\textsubscript{M} \text{CBM21}.

The plasmid of human glycogen synthase (pFastBacDual GST-GYG1+GYS1) was a gift from E. Zeqiraj, University of Leeds. Expression and purification were carried out according to previously published methods (30). Recombinant bacmid was generated in DH10Bac cells (Thermo Fisher Scientific) and purified using the PureLink HiPure Plasmid Maxiprep Kit (Thermo Fisher Scientific). SF9 cells were cultured as suspension in SF-900 III SFM medium (Thermo Fisher Scientific; 110 rpm at 26°C). P1 baculovirus was produced in monolayer cultures by transfecting the recombinant bacmid using Cellfectin II Reagent (Thermo Fisher Scientific). P2 virus was generated by infecting SF9 cultures (1.6×10\textsuperscript{6} cells/ml) with P1 virus at 400 ml per 100 ml of cells. The supernatant from P2 was harvested 3 days after infection, and 3 ml was used to infect 600 ml of SF9 suspension culture to produce the P3 culture. P3 was used at a 1:10 ratio to infect 3 liters of SF9 cell culture at 2.0×10\textsuperscript{6} cells/ml for protein production. Cells were grown in suspension for 3 days, and the cell pellets were washed in phosphate-buffered saline before they were frozen and stored at ~80°C until used.

**Protein purification**

G\textsubscript{M} \text{CBM21}, G\textsubscript{M} \text{CBM21}, G\textsubscript{M} \text{CBM21}, and G\textsubscript{M} \text{CBM21} N228A cell pellets were resuspended in ice-cold lysis buffer [50 mM tris (pH 8.0), 500 mM NaCl, 5 mM imidazole, 0.1\% Triton X-100, and an EDTA-free protease inhibitor tablet (Roche)] and lysed by high-pressure homogenization (Avestin Emulsiflex C3). Lysate was clarified by centrifugation (45,000g, 45 min, 4°C), and the supernatant was loaded onto a HisTrap column (GE Healthcare) pre-equilibrated with 50 mM tris (pH 8.0), 500 mM NaCl, and 5 mM imidazole. Protein was eluted using a
To purify the GM peptide was prepared by dissolving 2 mg of peptide in 1% NH4OH (pH 8.0), 50 mM NaCl, and 0.5 mM tris(carboxyethyl)phosphine (TCEP) to cleave the Hisa-tag. The cleaved protein was incubated with the Ni2+-NTA (nitritolatrictic acid) beads (GE Healthcare) to remove the TEV protease and cleaved His-tag. The flow-through was collected, concentrated, and further purified using size exclusion chromatography (SEC; Superdex 75 26/60 (GE Healthcare)) equilibrated in NMR buffer [20 mM sodium phosphate (pH 6.5), 50 mM NaCl, and 10 mM dithiothreitol (DTT)] or 20 mM bis-tris (pH 6.8), 150 mM NaCl, and 0.5 mM TCEP] or ITC buffer [20 mM tris (pH 8.0), 0.5 M NaCl, 0.5 mM TCEP, and 1 mM MnCl2]. Fractions were pooled, concentrated, and stored at −20°C. 15N-labeled GM64–93 was purified identically except that the protein was heat purified at 95°C (15 min), and the supernatant was collected and concentrated before SEC. PP1 was purified as previously described (18).

The GST-GYG1-GYS1 complex was purified using glutathione agarose beads (Pierce). The Sf9 cell pellet (8 g) was lysed in ice-cold lysis buffer [50 mM tris (pH 8.0), 150 mM NaCl, 5% glycerol, 1 mM EDTA, and 0.1% Triton X-100] with Complete mini protease inhibitor cocktail tablets (Roche). Lysate was clarified by centrifugation (40,000g, 45 min) and filtered through a 0.22-μm syringe filter. The supernatant was incubated on a rolling platform for 1 hour at 4°C with 1-ml bed volume of glutathione agarose resin pre-equilibrated in low-salt buffer [50 mM tris (pH 8.0), 150 mM NaCl, 5% glycerol, and 1 mM EDTA]. The beads were then washed with 10 column volumes (CVs) of low-salt buffer, followed by 50 CVs of high-salt buffer [50 mM tris (pH 8.0), 500 mM NaCl, 5% glycerol, and 1 mM EDTA], and followed again by 10 CVs of low-salt buffer. The complex was eluted with 10 mM fresh reduced glutathione, concentrated to 1 mg/ml, flash frozen in liquid nitrogen, and stored at −80°C until needed.

**Gm64–237; PP1α7–330 complex formation**

To purify the Gm64–237; PP1α7–330 complex for NMR spectroscopy analysis, PP1α7–330 was lysed in PP1 Lysis Buffer [25 mM tris (pH 8.0), 700 mM NaCl, 5 mM imidazole, 1 mM MnCl2, and 0.1% Triton X-100], clarified by ultracentrifugation, and immobilized on Nip32+–NTA resin. Bound His6a–PP1 was washed with PP1 Buffer A [25 mM tris (pH 8.0), 700 mM NaCl, 5 mM imidazole, and 1 mM MnCl2], followed by a stringent wash containing 6% PP1 Buffer B [25 mM tris (pH 8.0), 700 mM NaCl, 250 mM imidazole, and 1 mM MnCl2] at 4°C. The protein was eluted using PP1 Buffer B and purified using SEC [Superdex 200 26/60 (GE Healthcare)] pre-equilibrated in ITC Buffer [20 mM tris (pH 8.0), 500 mM NaCl, 0.5 mM TCEP, and 1 mM MnCl2]. Peak fractions were incubated overnight with TEV protease at 4°C. The cleaved protein was incubated with Nip32+–NTA beads (GE Healthcare), and the flow-through was collected. The flow-through was combined with excess 2H,15N-labeled Gm64–237 and concentrated, and the complex was purified using SEC (pre-equilibrated in 20 mM bis-tris (pH 6.8), 150 mM NaCl, and 0.5 mM TCEP). Fractions containing the holoenzyme complex were concentrated to 0.1 mM for NMR studies.

To generate the Gm64–93; PP1α7–330 complex for crystallization, purified PP1 was incubated with microcin-3 LR (MC-LR), and Gm64–93 was added to a final ratio of 1:1.5 in crystallization buffer [20 mM tris (pH 8.0), 50 mM NaCl, 0.5 mM TCEP, and 1 mM MnCl2]. The Gm64–93 peptide was prepared by dissolving 2 mg of peptide in 1% NH4OH before diluting with buffer [20 mM bicine (pH 9.0) and 5 mM DTT]. Final complex concentration was ~8 mg/ml for crystallization trials using vapor diffusion (sitting drop).

**NMR spectroscopy**

All NMR experiments were acquired at 298 K on Bruker Avance 500 or 800 MHz spectrometers, both equipped with a TCI HCN-z cryo-probe. The following spectra were used to complete the sequence-specific backbone assignment (recorded at 500-MHz 1H Larmor frequency): 2D [1H,15N] HSQC, 3D HNCA,CB, 3D CBCA(CO)NH, 3D HNCA, 3D (H)CC(CO)NH, and 3D HBHA(CO)NH. Together with these spectra, 3D HC(C)H−total correlation spectroscopy (TOCSY) (Tm = 11.3 ms) was used for the assignment of aliphatic side-chain 1H and 13C resonances. Aromatic side chains were assigned using 2D [1H,1H] NOE spectroscopy (NOESY) (Tm = 70 ms), 2D [1H,1H] TOCSY (Tm = 60 ms), and 2D [1H,1H] correlation spectroscopy (COSY) spectra of Gm64–93; CBM21 in 20 mM sodium phosphate (pH 6.5), 50 mM NaCl, 10 mM DTT, and 100% D2O. A 2D 15N-[1H]−NOE (heteronuclear NOE) experiment was recorded at 500 MHz 1H Larmor frequency with a saturation delay of 5 s and evaluated using the Dynamics Center 2.0 software (Bruker).

All spectra were processed using Topspin 2.1/3.0/3.1 (Bruker, Billerica, MA), and chemical shift assignments were achieved using Cara (http://cara.nmr.ch). NMR spectra of Gm64–93; CBM21 were acquired using either 15N- or 13N,C-labeled protein at a final concentration of 0.8 mM in 20 mM sodium phosphate (pH 6.5), 50 mM NaCl, 10 mM DTT, and 90% H2O/10% D2O. The interaction of Gm64–93; CBM21 with carbohydrates was tested by NMR titration experiments using α-CD (Thermo Fisher Scientific) and β-CD (Acros Organics).

For Gm64–93; CBM21, only Asn224, Asn228, and two cloning artifacts (His2 and Met4) have no sequence-specific backbone assignment. The high-quality spectral data also enabled a 98% completeness of the side-chain assignment, except Pro110 Cα/Cβ resonances and all of the side-chain resonances for Gly4, His5, Phe92, Phe230, and Tyr198. Aromatic side-chain assignment was more challenging owing to the large number of aromatic residues in Gm64–93; CBM21—a total of 16—a characteristic feature of SBDs.

The interaction between Gm64–237 and PP1α7–330 was studied by direct comparison of 2D [1H,15N] TROSY spectra of free and PP1α7–330-bound (2H,15N)-labeled Gm64–237. The final concentration used was 0.1 mM Gm64–237; PP1α7–330 complex in 20 mM bis-tris (pH 6.8), 150 mM NaCl, 0.5 mM TCEP, and 90% H2O/10% D2O. The spectra were processed using Topspin 4.0.3 (Bruker, Billerica, MA) and analyzed using Sparky. The NMR spectra were acquired on a Bruker Avance NEO 800 MHz 1H Larmor frequency NMR spectrometer equipped with a TCI-active HCN-cooled z-gradient cryo-probe at 298 K.

**Structure calculation of Gm64–93; CBM21**

The following spectra were used for structure calculation: 3D 15N-resolved [1H,1H] NOESY (Tm = 70 ms, 800 MHz 1H Larmor frequency), 3D 13C-resolved [1H,1H] NOESY (Tm = 70 ms, 800 MHz 1H Larmor frequency), and 2D [1H,1H] NOESY (Tm = 70 ms, 100% D2O solution, 500 MHz 1H Larmor frequency). Automated NOE peak picking and NOE assignment were carried out using ATNOS/CANDID (automated NOE peak picking/combined automated NOE assignment and structure determination module) (36). A total of 2709 unambiguous NOE-derived distance restraints along with 110 dihedral angle restraints derived from 13C-chemical shifts were used in the initial structure calculations performed using CYANA (combined assignment and dynamics algorithm for NMR applications). Final energy minimization and structure refinement were performed in explicit solvent using CNS 1.3 (Crystallography and NMR system).
along with the RECOORD (Recalculated Coordinates Database) script package. A total of 200 structures were generated, and the 20 conformers with the lowest restraint violation energies were selected as the final representative model. The quality of the structures was assessed by the programs WHATCHECK, AQUA (Analyzing the Quality), NMR-PROCHECK, and MOLMOL. Ramachandran analysis showed that the final bundle of 20 lowest-energy conformers of G\textsubscript{M}\textsubscript{GM}\textsubscript{CBM21} has excellent stereochemistry, with 97.8% of residues in the most favored and allowed region, 2.0% in the generously allowed region, and 0.2% in the disallowed region.

**CSP experiments with \(\alpha\)-CD and \(\beta\)-CD**

The interaction of G\textsubscript{M}\textsubscript{GM}\textsubscript{CBM21} with carbohydrates was tested by NMR titration experiments using \(\alpha\)-CD (Thermo Fisher Scientific) and \(\beta\)-CD (Acros Organics). To this end, 2D \([^{1}H,^{15}N]\) HSQC spectra at 298 K were recorded at 500 MHz \(^1H\) Larmor frequency. Experiments were performed in 20 mM phosphate (pH 6.5), 50 mM NaCl, 10 mM DTT, and 90% H\textsubscript{2}O/10% D\textsubscript{2}O, with \(\alpha\)-CD and \(\beta\)-CD titrated into \(^{15}N\)-labeled G\textsubscript{M}\textsubscript{GM}\textsubscript{CBM21} at 1:1, 1:2, 1:5, 1:10, 1:20, and 1:40 (protein: sugar) molar ratios. \(^{15}N\)-G\textsubscript{M}\textsubscript{GM}\textsubscript{CBM21} was used at concentrations of 300 \(\mu\)M for the 1:0.5, 1:1, and 1:2 titrations, 190 \(\mu\)M for the 1:20 titration, 100 \(\mu\)M for the 1:40 titration, and 40 \(\mu\)M for the 1:5 titration. In the 1:20 and 1:40 titration points, the concentration of G\textsubscript{M}\textsubscript{GM}\textsubscript{CBM21} was limited by the maximum solubility of \(\beta\)-CD in this buffer (less than 10 mM). Saturation of sugar binding by G\textsubscript{M}\textsubscript{GM}\textsubscript{CBM21} was achieved at a ratio of 1:20 for both \(\alpha\)-CD and \(\beta\)-CD. Chemical shift differences (\(\Delta\delta\)) between free G\textsubscript{M}\textsubscript{GM}\textsubscript{CBM21} (no \(\alpha\)-CD or \(\beta\)-CD) and sugar-bound G\textsubscript{M}\textsubscript{GM}\textsubscript{CBM21} (1:20 molar ratio) spectra were calculated using

\[
\Delta\delta(ppm) = \sqrt{\left(\Delta\delta_H\right)^2 + \left(\Delta\delta_N\right)^2} \times \frac{10}{M}
\]

To test the interaction of carbohydrates with G\textsubscript{M}\textsubscript{64-237}:PP1\textsubscript{a\textgreek{a}7-330}:MC-LR a 2D \([^{1}H,^{15}N]\) TROSY spectrum was recorded with \(\beta\)-CD (1:20 molar ratio) for G\textsubscript{M}\textsubscript{CBM21} alone, saturation was observed at this ratio. \(\beta\)-CD was chosen because of its stronger binding affinity to G\textsubscript{M}\textsubscript{GM}\textsubscript{CBM21} when compared to \(\alpha\)-CD. Chemical shift differences between G\textsubscript{M}\textsubscript{64-237}:PP1\textsubscript{a\textgreek{a}7-330} and G\textsubscript{M}\textsubscript{64-237}:PP1\textsubscript{a\textgreek{a}7-330}\(\beta\)-CD were calculated as described above. The data were recorded on a Bruker Avance NEO 800MHz \(^1H\) Larmor frequency equipped with a cryoprobe at 298 K.

**Crystallization and structure determination of the G\textsubscript{M}\textsubscript{64-93}:PP1\textsubscript{a\textgreek{a}7-300}:MC-LR complex**

G\textsubscript{M}\textsubscript{64-93}:PP1\textsubscript{a\textgreek{a}7-300}:MC-LR holoenzyme crystallized as clusters or single rod-shaped crystals in 0.4 M magnesium formate dihydrate and 0.1 M sodium acetate trihydrate (pH 4.6) at 4°C. For x-ray diffraction, crystals were cryoprotected in 30% glycerol and immediately flash frozen in liquid N\textsubscript{2}. Diffraction data were collected at the Stanford Synchrotron Radiation Lightsource beamline 12-2 at 100 K using a Dectris PILATUS 6M detector. The structure of G\textsubscript{M}\textsubscript{64-237}:PP1 was obtained in space group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1}. The model was completed using iterative rounds of refinement in PHENIX and manual building using Coot (Ramachandran statistics: 95.8% favored and 4.2% allowed).

**Isothermal titration calorimetry**

ITC experiments testing the interaction between G\textsubscript{M}\textsubscript{CBM21} and \(\alpha\)-CD and \(\beta\)-CD were performed at 25°C using a VP-ITC microcalorimeter (Malvern). Both \(\alpha\)-CD and \(\beta\)-CD were dissolved in 20 mM sodium phosphate (pH 6.5), 50 mM NaCl, and 10 mM DTT. Concentrations of G\textsubscript{M}\textsubscript{CBM21} between 14.5 and 16 \(\mu\)M were used in the sample cell. Ligand was titrated in 10-\(\mu\)l increments over 20 s at concentrations of 410, 440, and 1030 \(\mu\)M for \(\alpha\)-CD and 450 \(\mu\)M for \(\beta\)-CD (performed in duplicate). Twenty-eight injections were delivered during each experiment, with a 250-s interval between titrations to allow for complete equilibration and baseline recovery, and the solution in the sample cell was stirred at 307 rpm to ensure rapid mixing. To determine the thermodynamic parameters (\(\Delta H\), \(\Delta S\), and \(\Delta G\)) and binding constant (\(K_b\)), data were analyzed with one-site binding model assuming a binding stoichiometry of 1:1 using the Origin 7.0 software.

H\textsubscript{isc}\textsubscript{-tagged} PP1s (PP1\textsubscript{a\textgreek{a}7-300}, PP1\textsubscript{a\textgreek{a}7-330}, PP1\textsubscript{b\textgreek{b}6-327}, PP1\textsubscript{7-308}, and PP1\textsubscript{7-332}) were purified as described for ITC analysis (18, 19). G\textsubscript{M} (30 or 40 \(\mu\)M) was titrated into PP1 (3 or 4 \(\mu\)M) using a VP-ITC microcalorimeter (Malvern) or an Affinity SV ITC (TA Instruments) at 25°C. Data were analyzed using NITPIC, SEDPHAT, and GUSSI for a one-site binding model.

**Surface plasmon resonance**

Measurements were conducted using a BI-4500A five-channel SPR with autosampler and degasser pump (Biosensing Instrument Inc.) and a Ni-NTA chip. H\textsubscript{isc}\textsubscript{-tagged} PP1\textsubscript{a\textgreek{a}7-330} (62.5 nM) in 20 mM tris (pH 8.0), 500 mM NaCl, 0.5 mM TCEP, 1 mM MnCl\textsubscript{2}, and 0.005% Tween-20 was loaded onto a Ni\textsubscript{2+}-NTA chip (Biosensing Instrument Inc.) using different loading times (20, 40, 60, and 80 s) to achieve different PP1 densities on the H\textsubscript{isc}\textsubscript{-}sensor chip in four different channels (channel 1 was the reference channel). G\textsubscript{M}\textsubscript{64-105} was prepared in the same buffer as PP1\textsubscript{a\textgreek{a}7-330} using 1:3 serial dilutions (31.25 to 500 nM). Kinetic parameters were determined by curve fitting using Scrubber (BioLogic Software).

**Dephosphorylation assay**

Rabbit liver glycogen (Sigma-Aldrich, G8876) and rabbit phosphorylase a (Sigma-Aldrich, P1261) were purchased. All assays were carried out at 30°C for 30 min if not otherwise stated. Dephosphorylation of phosphorylase a was performed at a final concentration of 2 \(\mu\)M in reaction buffer [50 mM tris (pH 7.8), 150 mM NaCl, and 1 mM TCEP]. To investigate the effects of the PP1 interactors G\textsubscript{M} (49–86), G\textsubscript{M}\textsubscript{64-105}, NIPPP1 (158–216), spinophilin (417–602), NG1, or NG2 toward phosphorylase a dephosphorylation, the interactors were added at a 10 M excess to PP1 and incubated for 30 min at room temperature before the initiation of the assay. The final concentration of PP1 for the steady-state experiments was 0.2 \(\mu\)M, except for the time point experiment for which 0.04 \(\mu\)M PP1 was used. To test the effect of glycogen on the dephosphorylation of phosphorylase a, glycogen (4 mg/ml) was added to phosphorylase a before the addition of the G\textsubscript{M}\textsubscript{64-237}:PP1 holoenzyme. The reactions were terminated by 5x SDS loading buffer, and the samples were boiled (95°C) for 5 min. The samples were analyzed using SDS-PAGE, fixed, and stained with Pro-Q Diamond and Sypro Ruby (Thermo Fisher Scientific) to quantify the phosphor-protein (phosphorylase a) and total proteins, respectively. Gel images were captured using a ChemiDoc MP Imaging system or a Pharos FX Imager (Bio-Rad), and the densitometry of protein bands was analyzed using Image Lab 6.0 (Bio-Rad).
The GST–GYG1:GYS1 complex (final concentration of 0.1 mg/ml) in assay buffer [50 mM tris (pH 7.8), 150 mM NaCl, 1 mM TCEP, and 5% glycerol] was used in all assays. A 10 M excess of Gm$_{49–86}$, Gm$_{64–105}$, Gm$_{64–237}$, or spinophilin$_{417–602}$ was incubated with PP1 (0.2 μM) for 30 min before the initiation of the assay. For the time point study, reactions were initiated by the addition of 0.2 μM His$_6$PP1α$_{230–330}$ with or without Gm$_{64–237}$ or Gm$_{64–237}$ N228A. To test the effect of glycogen, glycogen (4 mg/ml) was added to GYS1 before the addition of the Gm$_{64–237}$:PP1 holoenzyme. The reactions were carried out at 30°C and were terminated at different time points (3, 6, 9, 12, 15, and 30 min) by the addition of 5× SDS loading buffer and boiling at 95°C for 5 min. Samples were analyzed using SDS-PAGE and stained with Sypro Ruby. Dephosphorylation of GYS1 can be readily visualized as a band shift in SDS-PAGE (30). The relative distance between the GYS1 bands relative to GST–GYG1 was used to assay the dephosphorylation of GYS1. Last, Pro-Q Diamond (Invitrogen), which stains for total phosphorylation, and mass spectrometer analysis were used to confirm the dephosphorylation states of GYS1.

Con A–Sepharose glycogen binding assay

Con A–Sepharose (GE Healthcare) beads were used to assay glycogen binding. Fresh Con A beads were washed in Con A buffer [67 mM Hepes (pH 6.8), 0.2 mM CaCl$_{2}$, 10 mM MgCl$_{2}$, 500 mM NaCl, and 4 mM DTT] and incubated with rabbit liver glycogen (50 mg/ml; Sigma–Aldrich, G-8876, 1:1 volume) at 4°C for 1 hour. Glycogen-conjugated Con A beads were washed three times with Con A buffer and resuspended as 50% slurry. Con A–glycogen Sepharose beads (30 μl) were incubated with 15 μg of phosphorylase α, Gm$_{64–237}$, and PP1α$_{230–330}$ alone and in various combinations thereof in a total volume of 250 μl for 1 hour at 4°C under gentle mixing. The beads were washed three times with Con A buffer (750 μl) and recovered by centrifugation (2000g, 1 min). The supernatant was removed using gel-loading tips, leaving behind the beads that were then incubated with 25 μl of 1 M α-D-methylglucoside (in Con A buffer) to elute all proteins bound to glycogen. Eluate (10 μl) was carefully transferred to new tubes to avoid contamination with residual beads and analyzed by SDS-PAGE. SDS-PAGE was stained using Sypro Ruby (Invitrogen) for visualization of total proteins.

MS analysis

Samples were separated by SDS-PAGE, and bands were excised and digested with trypsin in 50 mM ammonium bicarbonate overnight at 37°C. Peptides were extracted using 5% formic acid/50% acetonitrile (v/v) for 30 min. The supernatant was removed using gel-loading tips, leaving behind the beads that were then incubated with 25 μl of 1 M α-D-methylglucoside (in Con A buffer) to elute all proteins bound to glycogen. Eluate (10 μl) was carefully transferred to new tubes to avoid contamination with residual beads and analyzed by LC-MS/MS.

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