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The hepatic PP1 glycogen-targeting subunit interaction with phosphorylase *a* can be blocked by C-terminal tyrosine deletion or an indole drug

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Abstract The inhibition of hepatic glycogen-associated protein phosphatase-1 (PP1-G_L) by glycogen phosphorylase *a* prevents the dephosphorylation and activation of glycogen synthase, suppressing glycogen synthesis when glycogenolysis is activated. Here, we show that a peptide (^{280}LGPYY^{284}) comprising the last five amino acids of G_L retains high-affinity interaction with phosphorylase *a* and that the two tyrosines play crucial roles. Tyr284 deletion abolishes binding of phosphorylase *a* to G_L and replacement by phenylalanine is insufficient to restore high-affinity binding. We show that a phosphorylase inhibitor blocks the interaction of phosphorylase *a* with the G_L C-terminus, suggesting that the latter interaction could be targeted to develop an anti-diabetic drug.

Keywords: Glycogen-targeting subunit; Protein phosphatase 1; Glycogen phosphorylase; Glycogen metabolism; CP-316819; Diabetes

1. Introduction

Type-2 diabetes is characterised by hyperglycaemia, the inability of insulin to stimulate plasma glucose uptake into peripheral tissues, defects in insulin secretion and excessive endogenous glucose production [1]. A key objective in treating diabetes is to lower plasma glucose levels. The liver is a major organ regulating glucose homeostasis and when plasma glucose levels decline postprandially, increased hepatic glycogenolysis and gluconeogenesis are the major routes of endogenous glucose production [2]. Glycogenolysis predominates initially after fasting, while gluconeogenesis is the major route after glycogen stores are depleted. It may be advantageous in diabetes to counteract hyperglycaemia by stimulating hepatic GS, so that conversion of blood glucose to hepatic glycogen is accelerated. One possible way of achieving this would be to increase the activity of the hepatic GS phosphatase, which activates GS.

Hepatic GS is regulated both allosterically by glucose-6-phosphate and by phosphorylation of multiple serine residues [3,4]. Phosphorylation is catalysed by several protein kinases that inactivate GS, while dephosphorylation by glycogen-targeted PP1 activates GS. The major form of glycogen-targeted PP1 in the liver is PP1-G_L, which is controlled allosterically by the active '*a*' form of glycogen phosphorylase [5,6]. When glycogenolysis is stimulated, glycogen phosphorylase *b* is converted to phosphorylase *a* by phosphorylation of Ser14. Phosphorylase *a* binds to the G_L regulatory subunit of PP1-G_L, preventing the activation of GS, without affecting the inactivation of phosphorylase *a* by PP1-G_L. This allosteric regulation of PP1-G_L allows glycogen synthesis to be switched off when glycogenolysis is stimulated. Phosphorylase *a* must be converted back to phosphorylase *b* almost completely before glycogen synthesis ensues [5]. Previous studies have demonstrated that the C-terminal 16 residues of G_L are sufficient for interaction with phosphorylase *a* [7]. Here, we have identified the key amino acids within the 16mer that are essential for binding. We also show that CP-316819, a member of the indole-2-carboxamide series of phosphorylase inhibitors [8] is capable of modulating the interaction of the G_L 16mer peptide with phosphorylase *a*.

2. Materials and methods

2.1. Materials

Glycogen phosphorylase *a* was prepared by phosphorylation of rabbit skeletal muscle phosphorylase *b* with phosphorylase kinase (both purified by Dr. James Hastie) to a stoichiometry of ~1 mol of phosphate/mol phosphorylase subunit [9]. Peptides were synthesised by Dr. Graham Bloomberg, University of Bristol, UK. 5-Chloro-*N*-[(1*R*,2*S*)-2-hydroxy-3-(methyloxymethylamino)-3-oxo-1-(phenylmethyl)propyl]-1H-Indole-2-carboxamide (CP-316819) was synthesised by Pfizer Global Research and Development [10].

2.2. Isothermal titration calorimetry

ITC measurements were performed in degassed 50 mM Tris–HCl, pH 7.0, 1 mM DTT at 20 °C using a VP-ITC instrument (MicroCal, LLC, Northampton, MA). Unless otherwise stated, titrations

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Abbreviations: PP1, protein phosphatase 1; G_L, hepatic glycogen-targeting subunit (encoded by gene PPP1R3B); GS, glycogen synthase; ITC, isothermal titration calorimetry

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consisted of a preliminary 5 μl injection of 0.32 mM G_L peptide solution followed by 19 injections of 10 μl peptide into the reaction cell, which contained 1.4 ml of 0.025 mM phosphorylase *a* monomer solution. Typically, 180–240 s equilibration time was allowed between injections to allow heat measurements to return to baseline. The heat of dilution in separate titrations of both peptide into buffer and buffer into phosphorylase solutions was determined to be negligible. In order to determine the effect of CP-316819 on binding, 0.025 mM phosphorylase *a* monomer was pre-incubated with 0.3–10 μM of drug for 45 min at 30 °C with shaking in 50 mM Tris–HCl, pH 7.0, 1 mM DTT, 0.1% (v/v) DMSO. Titrations were then performed as before but with the addition of 0.1% (v/v) DMSO to all solutions. Calorimetric data analysis were carried out with ORIGIN 7.0 software. Integration of the raw heat data using a one-binding-site model yielded a differential thermal binding curve, the equilibrium constant for association of phosphorylase *a* with the G_L-derived peptides (K_a), the enthalpy (ΔH) and entropy (ΔS) of binding.

3. Results

3.1. Analysis of the interaction of G_L with phosphorylase a

Initial experiments showed that [^{32}P] labelled skeletal muscle glycogen phosphorylase *a* interacted with bacterially-expressed glutathione-*S*-transferase-G_L [7] on membranes in the absence or presence of 50 mM glucose or 5 mM caffeine (data not shown) but not in the presence of 3 mM AMP as noted previously [6]. The binding of a peptide comprising the 16C-terminal residues of G_L (Fig. 1A) to phosphorylase *a* was determined by ITC to be exothermic and saturating at a stoichiometry (*n*) of 1:1 (Fig. 1B; Table 1). The dissociation constant K_d (the reciprocal of K_a) is in the nanomolar range (218 ± 111 nM for four experiments). The binding reaction is enthalpy-driven ($\Delta H = -11.8$ kcal/mol) and entropically unfavourable ($T\Delta S = -2.5$ kcal/mol) displaying entropy/enthalpy compensation effects typical of biomolecular interactions.

ITC with a series of truncated peptides (Table 1 upper half) showed that the K_d was largely unaffected when the 16 residue peptide was truncated to the C-terminal 13 or 11 amino acids of G_L. Although the binding affinity was decreased (K_d 860 nM) when Tyr274 and Leu 275 were removed, a K_d value of 854 nM was still observed for a peptide comprising only the 5C-terminal amino acids ^{280}LGPYY^{284} (rodent numbering).

Alanine scanning analysis of the peptide comprising the nine C-terminal amino acids (^{276}GYEKLGPYY^{284}) showed that substitution of Leu280 or Pro282 by Ala weakened the binding affinity of the peptide (K_d 1.1 and 1.5 μM, respectively), while substitution of Lys279 or Gly281 actually increased the affinity to that seen for the wild-type 16mer peptide (Table 1, lower half). The enthalpical component of the binding energy was increased, although this was counterbalanced by an unfavourable reduction in entropy. Replacement of Tyr283 by Ala completely abolished binding, as did deletion of Tyr284 in the 11mer and 13mer, indicating the critical importance of these two C-terminal tyrosine residues in the interaction with phosphorylase *a*. Substitution of Tyr283 by the structurally similar amino acid Phe severely decreased the binding affinity of the peptide ($K_d = 3.2 \pm 0.8$ μM). Substitution of the C-terminal Tyr284 by phenylalanine was sufficient to completely disrupt the interaction of the 8mer peptide with phosphorylase *a* (Fig. 2B), as was substitution of both Tyr283 and Tyr284 by Phe. Replacement of Tyr284 by Phe within the 16mer did not abolish binding but severely decreased the K_d, which was estimated at 24.5 ± 11.6 μM (Table 1). Overall the data indicate a critical role for the C-terminal Tyr284 of G_L in the interaction with phosphorylase *a*.

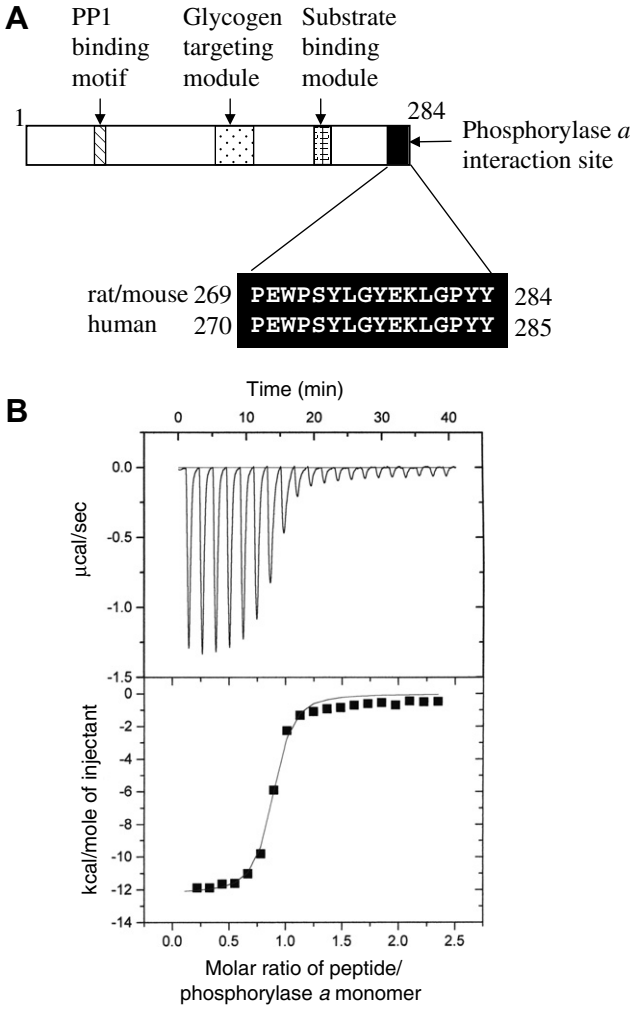

Fig. 1. Interaction of the 16 C-terminal amino acids of G_L with phosphorylase *a*. (A) Schematic representation of G_L showing the C-terminal phosphorylase *a* binding site. The PP1, glycogen and substrate binding domains of G_L are indicated. The sequence of the phosphorylase *a* binding region of G_L, which is identical in humans, mouse and rat, is shown [7]. (B) Titration of phosphorylase *a* by the G_L peptide ^{269}PEWPSYLGYEKLGPYY^{284}. The upper panel shows the raw data for one experiment, generated by titration of 1.8 ml of 0.025 mM rabbit skeletal muscle phosphorylase *a* by 20 injections (12 μl) of 0.32 mM G_L 16mer. The lower panel shows the integrated area within each peak (from the baseline shown) plotted against the molar ratio of the G_L 16mer to phosphorylase *a* monomer. The smooth line represents the best fit of the experimental data to a model with one set of sites. The K_d for the binding of the G_L 16mer to phosphorylase *a* in this experiment was 0.20 μM.

3.2. Effect of the glycogen phosphorylase inhibitor CP-316819 on the interaction of G_L with phosphorylase a

The development of inhibitors of phosphorylase *a*, such as CP-316819 an indole-2-carboxamide [10], capable of decreasing hepatic glucose output raised the question of whether any of these compounds affect the binding of G_L to phosphorylase *a*. We found that CP-316819 inhibited phosphorylase *a* in the presence of 7.5 mM glucose with an IC_{50} of 57 nM as found previously [8]. Calorimetric analysis of the interaction

Table 1
Thermodynamic parameters for the association of various G_L-derived peptides with glycogen phosphorylase *a*

Peptide sequence	*n*	K_d (nM)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)[a]
PEWPSYLGYEKLGPYY	0.95 (±0.10)	218 (±111)	−11.8 (±0.7)	−2.5 (±0.4)	−9.3 (±0.8)
PSYLGYEKLGPYY	0.80 (±0.02)	374 (±48)	−13.5 (± 0.5)	−4.9 (±0.5)	−8.6 (±0.7)
YLGYEKLGPYY	0.80 (±0.31)	297 (±97)	−13.4 (± 4.7)	−4.6 (±2.9)	−8.8 (±5.5)
GYEKLGPYY	0.92 (±0.01)	860 (±38)	−12.3 (±0.05)	−4.1 (±0.1)	−8.2 (±0.1)
YEKLGPYY	0.88 (±0.03)	825 (±56)	−12.2 (±0.05)	−4.0 (±0.1)	−8.2 (±0.1)
EKLGPYY	1.08 (±0.04)	801 (±44)	−9.7 (±0.1)	−1.5 (±0.05)	−8.2 (±0.1)
KLGPYY	1.07 (±0.003)	655 (±71)	−12.3 (±0.1)	−4.0 (±0.2)	−8.3 (±0.2)
LGPYY	1.00 (±0.05)	854 (±87)	−11.1 (±0.2)	−2.9 (±0.2)	−8.2 (±0.3)
GYEKLGPAY	NB	NB	NB	NB	NB
GYEKLGAYY	0.93 (±0.01)	1141 (±53)	−11.8 (±0.1)	−3.8 (±0.1)	−8.0 (±0.1)
GYEKLAPYY	0.59 (±0.03)	305 (±97)	−17.6 (±0.04)	−8.8 (±0.2)	−8.8 (±0.2)
GYEKAGPYY	1.03 (±0.03)	1527 (±255)	−10.7 (±0.5)	−2.9 (±0.6)	−7.8 (±0.8)
GYEALGPYY	0.59 (±0.03)	177 (±59)	−17.3 (±0.5)	−8.2 (±0.7)	−8.3 (±0.9)
GYAKLGPYY	1.02 (±0.03)	968 (±117)	−11.5 (±0.1)	−3.4 (±0.1)	−8.1 (±0.1)
PSYLGYEKLGPY_	NB	NB	NB	NB	NB
YLGYEKLGPY_	NB	NB	NB	NB	NB
YEKLGPFY	0.80 (±0.004)	3202 (±759)	−10.7 (±0.4)	−3.3 (±0.5)	−7.4 (±0.6)
YEKLGPFF	NB	NB	NB	NB	NB
YEKLGPYF	NB	NB	NB	NB	NB
PEWPSYLGYEKLGPYF	0.75 (±0.09)	24476 (± 11644)	−11.0 (±1.7)	−4.5 (±1.9)	−6.5 (±2.5)

All experiments were performed in triplicate, with the exception of the PSYLGYEKLGPY_ peptide, the result of which is from a single experiment only, and the PEWPSYLGYEKLGPYY and the PEWPSYLGYEKLGPYF peptides, the data for which is derived from four separate binding exotherms. The errors given correspond to the standard error of the mean unless otherwise indicated. *n* is the stoichiometry of binding of each peptide to phosphorylase *a* monomer. NB means that the experiments have been performed, but no binding was detected.
[a]Calculated from the equation $\Delta G = \Delta H - T\Delta S$. The errors given are the square root of the sum of the squares of ΔH and $T\Delta S$.

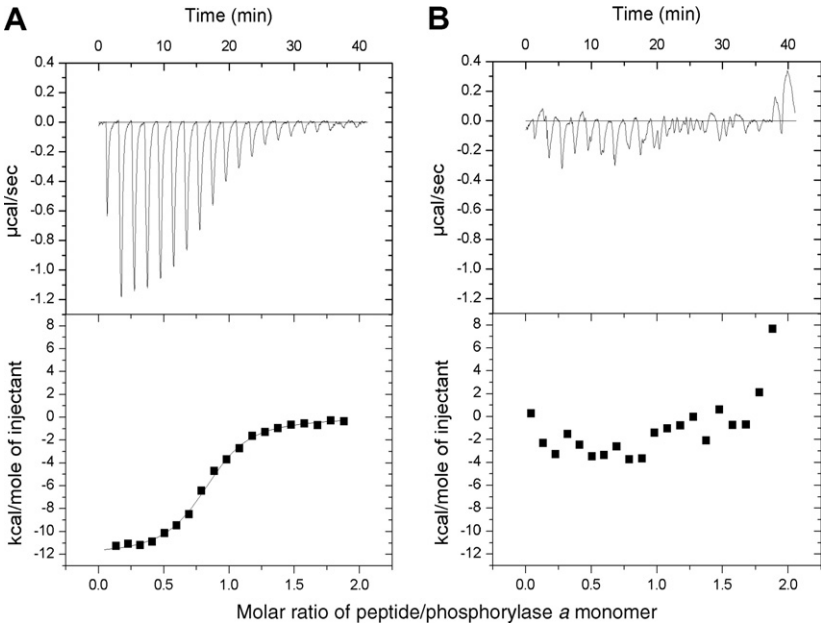

Fig. 2. Titration of phosphorylase ***a*** by the G_L (277–284) peptide and the Y284F mutant peptide. Phosphorylase *a* was titrated with 20 injections of (A) YEKLGPYY and (B) YEKLGPYF at 20 °C. The upper traces show the raw data and the lower traces show the binding isotherms in terms of heat per injected peptide vs. molar ratio of peptide to phosphorylase *a* monomer. The mutation of the extreme C-terminal tyrosine residue to phenylalanine completely abolishes binding to phosphorylase *a*.

of the G_L 16mer peptide ^{269}PEWPSYLGYEKLGPYY^{284} with phosphorylase *a*, in the presence of increasing concentrations of CP-316819, showed that the binding affinity of the peptide was decreased at 1 μM CP-316819 and above (Table 2). 5 μM CP-316819 decreased the overall reaction enthalpy and modified the integrated binding isotherm to a more shallow curve (Fig. 3B, lower panel). 10 μM CP-316819 resulted in raw data (Fig. 3C, upper panel) that was not significantly different from heats of dilution and mixing determined in controls, indicating that 10 μM CP-316819 was sufficient to completely block the interaction of the 16mer G_L peptide with 25 μM phosphorylase *a*. Although in vivo muscle phosphorylase *a* is a dimer, in vitro at 25 μM phosphorylase *a* may be mainly tetrameric [11,12]. The ratio, CP-316819: phosphory-

Table 2
Thermodynamic parameters for the association of the PEWPSYLGYEKLGPYY peptide with 25 μM phosphorylase *a* monomer in the presence of the indicated concentrations of CP-316819

CP-316819 concentration (μM)	*n*	K_d (nM)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
0	0.95 (±0.10)	218 (±111)	−11.8 (±0.7)	−2.5 (±0.4)	−9.3 (±0.8)
0.3	0.59 (±0.04)	135 (±20)	−10.9 (±0.4)	−1.2 (±0.2)	−9.7 (±0.4)
1	0.90 (±0.03)	888 (±122)	−11.0 (±0.4)	−2.8 (±0.5)	−8.2 (±0.6)
5	1.15 (±0.17)	1917 (±423)	−7.9 (±1.3)	−0.2 (±1.2)	−7.7 (±1.8)
10	NB	NB	NB	NB	NB

Experiments at CP-316819 concentrations 0 and 10 μM were performed in quadruplicate. All other experiments were performed in triplicate.

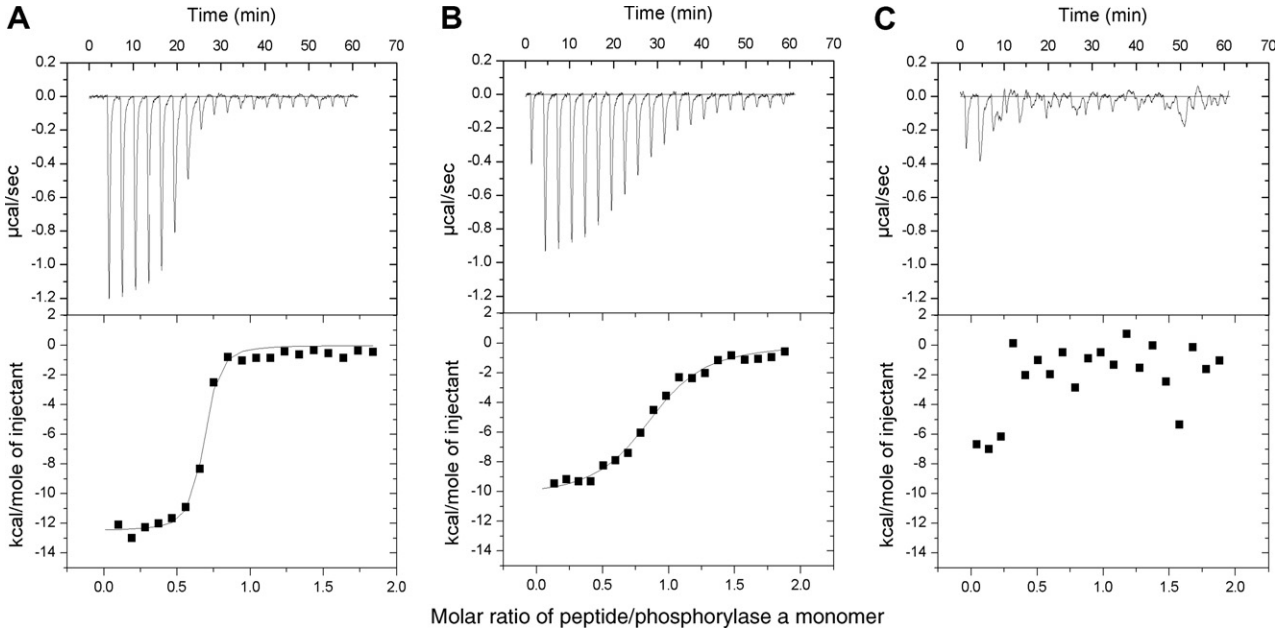

Fig. 3. ITC data for phosphorylase *a* binding to the PEWPSYLGYEKLGPYY peptide (A) in the absence of CP-316819, (B) following pre-incubation of phosphorylase with 5 μM CP-316819, (C) following pre-incubation of phosphorylase with 10 μM CP-316819. The upper traces show the raw heat data and the lower traces show the fit of a 1:1 binding model after integration of the raw data. 10 μM CP-316819 blocks the interaction of the peptide with 25 μM phosphorylase *a* monomer.

lase *a* monomer of 0.4:1 that blocks interaction with G_L suggests that homotropic cooperative effects between the subunits of phosphorylase *a* allow inhibition of approximately two subunits in the tetramer (or one subunit in the dimer) to allosterically change the conformation of the other subunits(s) to the inhibited structure [13].

4. Discussion

We show here that the site of interaction of G_L with phosphorylase *a* is even smaller than the 16 C-terminal residues of G_L previously determined [7]. A peptide comprising the five C-terminal amino acids ^{280}LGPYY^{284} formed a complex with phosphorylase *a*, with only slightly lower affinity than the 16mer peptide. The two C-terminal tyrosines play critical roles in binding; replacement of either by the structurally similar amino acid Phe is insufficient to retain the nanomolar affinity of the G_L peptides for phosphorylase *a*, suggesting that the tyrosine hydroxyls are crucial for interaction with phosphorylase *a*. The marginally increased dissociation constants when Leu280 or Pro282 are replaced by Ala raise the possibility that Pro282 may allow favourable presentation of the tyrosines to phosphorylase *a*, and Leu280 may provide hydrophobic interactions. Thus, the consensus sequence for initiation of the interaction is LXPYY, where *x* may be Gly, Ala or possibly other amino acids. The crucial interaction of G_L with phosphorylase *a* involves the two tyrosine residues at the C-terminus, with the C-terminal Tyr284 playing the major role.

The K_d of 218 nM for the binding of phosphorylase *a* to the G_L 16mer determined by ITC is consistent with the earlier analyses employing immobilised GST-G_L [7,14] and glycogen-bound GS phosphatase [15]. Our data indicate that each phosphorylase *a* dimer binds two molecules of G_L (Table 1). Since G_L binding occurs in the presence of glucose and caffeine, which bind to distinct sites that are homologous in muscle and liver phosphorylases [16,17], G_L is not binding at these sites. The interaction sites for AMP in liver and muscle phosphorylase *a* are homologous, lying across the dimeric subunit interface, where each AMP binds between a loop from one subunit (containing the phosphorylated Ser14) and a helix of the other subunit [18,19]. Since AMP prevents the binding of G_L to muscle phosphorylase *a*, AMP binding may either cause a conformational change that prevents the G_L interaction or the binding sites for AMP and the G_L C-terminus overlap.

Inhibitors of phosphorylase have been developed as hypoglycaemic agents that may be useful in the treatment of diabetes to counteract elevated hepatic glucose output. The indole-2-carboxamide series of phosphorylase inhibitors reached phase II clinical trials [23] and have been shown to bind to muscle and liver phosphorylases at a novel allosteric site located at the dimeric subunit interface [20,21]. Interestingly, CP-316819 is able to completely block the binding of G_L to muscle phosphorylase *a*, raising the possibility that G_L binds at or near the indole-2-carboxamide binding site on phosphorylase *a*. However, the interaction sites for indole-2-carboxamide inhibitors and AMP on phosphorylase *a* are distinct, and the G_L C-terminus could not contact both binding sites. More likely, conformational changes induced by the binding of indole-2-carboxamide drugs prevent the binding of G_L to phosphorylase *a*. These inhibitors can interact with the active and inactive conformations of phosphorylase *a*, but are likely to favour the inactive conformation [20].

The finding that CP-316819 blocks the binding of G_L to phosphorylase *a* suggests that the indole-2-carboxamide drugs (used in the μM range) may exert their effects on hepatic glucose output not only by inhibition of liver phosphorylase *a* but also by lowering the affinity of phosphorylase *a* for PP1-G_L. While the inhibition of phosphorylase *a* is a valid strategy for attenuating hyperglycaemia in type 2 diabetes, the drugs developed to date inhibit both liver and muscle phosphorylases and inhibition of the latter may be detrimental because it impairs aerobic muscle function during prolonged contraction [22]. A drug that inhibits the interaction of G_L with phosphorylase *a* and not phophorylase *a* itself avoids this problem. The discovery that a small region of G_L binds to phosphorylase *a* indicates that it should be possible to develop a small molecule drug capable of disrupting the interaction. A drug binding to G_L should be effective at far lower concentrations in vivo than a phosphorylase *a* inhibitor [23] due to the lower concentration of G_L in the liver. Disruption of the allosteric regulation of PP1-G_L by phosphorylase *a* may also create an energy-consuming glycogenolytic–glycogen synthetic cycle, which could be advantageous. In addition, such a drug may operate in human muscle, where PP1-G_L is also expressed [24].

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