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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*.

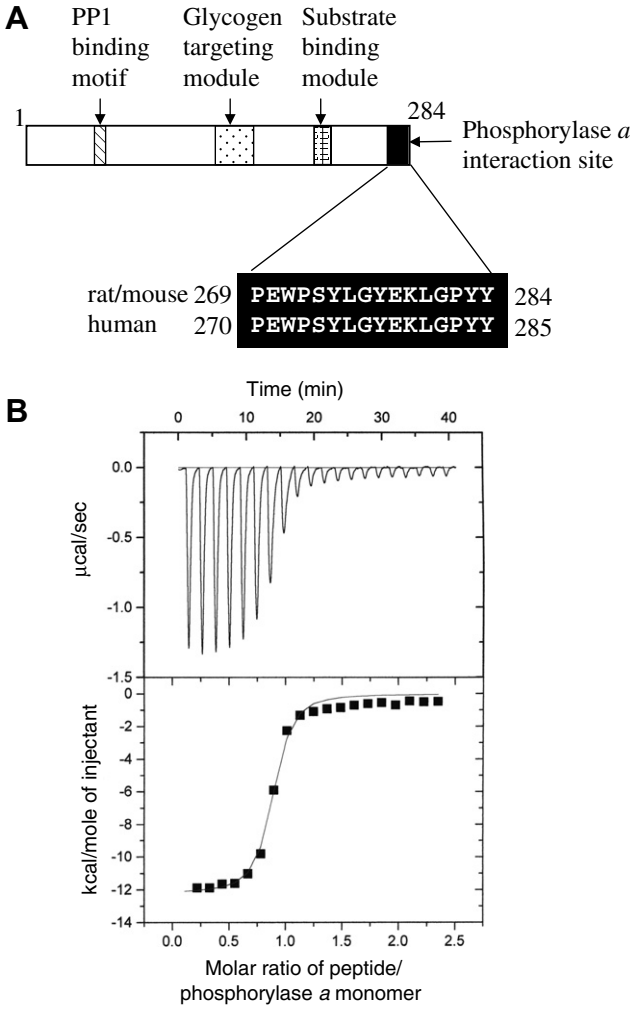

Fig. 1. Interaction of the 16 C-terminal amino acids of G_L with phosphorylase *a*. (A) Schematic representation of G_L showing the C-terminal phosphorylase *a* binding site. The PP1, glycogen and substrate binding domains of G_L are indicated. The sequence of the phosphorylase *a* binding region of 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