Molecular Recognition of the Protein Phosphatase 1 Glycogen Targeting Subunit by Glycogen Phosphorylase

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Disrupting the interaction between glycogen phosphorylase and the glycogen targeting subunit (G1) of protein phosphatase 1 is emerging as a novel target for the treatment of type 2 diabetes. To elucidate the molecular basis of binding, we have determined the crystal structure of liver phosphorylase bound to a G1-derived peptide. The structure reveals the C terminus of G1 binding in a hydrophobically collapsed conformation to the allosteric regulator-binding site at the phosphorylase dimer interface. G1 mimics interactions that are otherwise employed by the activator AMP. Functional studies show that G1 binds tighter than AMP and confirm that the C-terminal Tyr-Tyr motif is the major determinant for G1 binding potency. Our study validates the G1–phosphorylase interface as a novel target for small molecule interaction.

Diabetes is one of the major public health problems. Approximately 194 million people worldwide, or 5.1%, in the age group 20–79 were estimated to have diabetes in 2003. This estimate is expected to increase to some 333 million, or 6.3% of the adult population, by 2025 (1). Type 2 diabetes, the most common form of diabetes is characterized by defects in insulin secretion, insulin resistance, and elevated hepatic glucose production. Both increased gluconeogenesis and increased glycogenolysis contribute to excessive hepatic glucose output despite hyperglycemia (2). Several novel pharmacological strategies are aiming to treat hyperglycemia by normalizing or increasing depleted glycogen stores (3). For example, drug discovery has focused on competitive as well as allosteric inhibition of glycogen phosphorylase activity (4).

Glycogen phosphorylase (GP) is an important allosteric enzyme in carbohydrate metabolism that catalyzes phosphorylisis of an α-1,4-glycosidic bond of glycogen to glucose-1-phosphate. In humans there are three GP isoforms (liver, muscle, and brain GP), which are named after the tissues where they are predominantly expressed. Glycogen phosphorylase is a homodimer that cycles between two conformations: active (R) and inactive (T) state. Phosphorylation of Ser14 by phosphorylase kinase and active site as well as allosteric binders modulate the equilibrium between both states (5), but the isozymes differ in their responsiveness to regulatory mechanisms. In the liver, phosphorylation is the major regulator of GP activation. Conversion of unphosphorylated liver GPa to phosphorylated GPa fully activates the enzyme. AMP stimulates liver GPa by 10–20%, whereas it does not further activate GPa (6). In contrast, AMP activates the unphosphorylated muscle isoform to 80% of the maximal activity and increases the activity of phosphorylated muscle GPa by a further 10%. Crystallographic studies have shown endogenous and synthetic modulators bound to four major sites (see Fig. 1a): active site (7), purine site (8), central cavity (9), and allosteric AMP site (10, 11).

Important for glycogen metabolism is the strong reciprocal control between GPa and glycogen synthase activity. Activation of glycogen synthase via its phosphatase (protein phosphatase 1 (PP1)) can be allosterically inhibited by binding of GPa (12) to G1 (13), a glycogen targeting subunit of PP1. PP1 in turn suppresses GP and phosphorylase kinase activities through dephosphorylation. Glycogen targeting subunits bind to PP1, modulate its activity toward substrates, localize it to specific cellular sites, and are proposed to function as a scaffold for the assembly and regulation of glycogen metabolizing enzymes. There is an increasing number of glycogen-targeting subunits. So far, seven family members of glycogen-targeting subunits are described in humans: GM (PPP1R3A) (14), G1 (PPP1R3B) (15), R5/PTG (PPP1R3C) (16, 17), R6 (PPP1R3D) (18), PPP1R3E (19), and PPP1R3F and PPP1R3G (20). Mutational analysis of the rat liver targeting subunit G1 has identified three separate regions that are responsible for binding to PP1 (residues 59–94), glycogen (residues 94–257), and phosphorylase (residues 269–284 at the G1 C terminus) (21). The G1 C-terminal region is unique in G1 and absent in other glycogen targeting subunits but is conserved between rodent and human. Pharmacological inhibition of the interaction of phosphorylase a with G1 could provide a novel mechanism to lower blood glucose levels by inducing the dephosphorylation and activation of glycogen synthase (3).

Here we show that the G1 C-terminal region structurally and functionally mimics AMP binding to human liver glycogen phosphorylase (hlGP). Using x-ray crystallography we identify the C terminus of G1 bound in the allosteric regulator site of hlGP. Using functional assays we map binding...
contributes of the $G_L$ peptide and show that it activates phosphorylase $b$, in vitro.

**EXPERIMENTAL PROCEDURES**

**Protein Production**—Human liver glycogen phosphorylase was expressed as described (7). Briefly, full-length $hGPa$ was expressed in insect cells and purified using copper-chelating, anion exchange, and size exclusion chromatography. $hGpb$ was expressed in *Escherichia coli* and purified analogously.

**Crystallization and Structure Determination**—Crystals were obtained at 20 °C in 1 + 1 μl hanging drops from $hGPa$ concentrated to 8 mg/ml in 20 mM BES, pH 6.7, 1 mM EDTA, 0.5 mM dithiothreitol, 50 mM glucose, 0.5 mM AMP over a reservoir of 0.1 M Tris, pH 8.5, 7–8% (w/v) polyethylene glycol 8000. Macroseeding improved crystal size and reproducibility. The reservoir supplemented with 1 mM GL-Cterm peptide (see below). For cryoprotection the mother liquor was incrementally exchanged to 0.1 M Tris, pH 8.5, 20% glycerol, 20% polyethylene glycol 8000, 1 mM $G_{L−}$Cterm peptide. The crystals were then flash frozen in a 100 K nitrogen stream. The diffraction data were collected on the PX-1 beamline at the SLS (Villigen, CH) and processed with XDS (22) (see Table 1). The complex structure was solved using difference Fourier methods with the coordinates of $hGPa$-AMP (Protein Data Bank accession code 1FA9) as a template. Following rigid body refinement the $(F_o - F_c)$ electron density maps included easily interpretable electron density for the bound ligand. Restrained refinement was performed with REFMAC (23) and BUSTER (Global Phasing Ltd.) iterated with model building in COOT (24). The final model has been deposited in the Protein Data Bank (Protein Data Bank accession code 2QLL).

**Peptides**—Synthetic peptides were purchased from Thermol Electron (Ulmm, Germany). The peptides were delivered as 5-mg lyophilized aliquots and resuspended in dimethyl sulfoxide before use. The sequence of the 16mer $G_{L−}$C-terminal peptide is NH$_2$-PEWSPYLGYEKLGPYY-COOH ($G_{L−}$Cterm). A biotin-labeled 17-mer $G_{L−}$-peptide, biotin-FPEWPSPYLGYEKLGPYY-COOH ($G_{L−}$ probe) was synthesized by Interactiva (Ulm, Germany).

**SPA Assay for $G_L$/hGPa Interaction**—A SPA was used to measure the interaction between $^{33}$P-radio labeled hGPa and a biotin-labeled $G_L$-peptide ($G_L$ probe). A one-step phosphorylation reaction by phosphorylase kinase (Sigma) transformed hGpb into $^{33}$P-radio labeled hGPa. The incubation reaction contained test compound, 5 μg of $^{33}$P-hGPa, 50 pmol of $G_L$ probe, 0.4 mg SPA-Beads (streptavidin-SPA beads; Amersham Biosciences, RPNQ 0007) in test buffer (50 mM Tris, 5 mM EDTA, pH 7.5)/well in a 384-well format. Scintillation was measured after overnight incubation at room temperature. Triplicate determinations were made in all of the binding experiments.

**rmGPa Activation Assay**—Activation of enzymatic activity of rabbit muscle glycogen phosphorylase $b$ (rmGpb from Sigma) was measured in direction of glycogen degradation by coupling glucose-1-phosphate production to NADP consumption (25). Activation experiments were performed as triplicate determinations. All of the reagents and enzymes were purchased from Sigma.

**RESULTS**

**Structure of $G_L$ Bound to hGPa**—To identify the molecular basis of $G_L$ binding, we diffused a peptide comprising the last 16 residues of $G_L$ (GL-Cterm, 269GL-284GL; numbering refers to the sequence of rat $G_L$) into crystals of hGPa and determined the crystal structure of the resulting hGPa-$G_L$ complex (Fig. 1 and Table 1). Only the terminal four amino acids (residues 281GL–284GL, Gly-Pro-Tyr-Tyr) were visible in difference electron density maps (Fig. 2A), whereas the remainder of the peptide was disordered. The $G_L$-binding site is located at the subunit interface and overlaps with the binding site for the allosteric regulator AMP (Fig. 2C). The binding site is ~14 Å from the Ser14 phosphorylation site, 32 Å from the catalytic site, and 25 Å from the central cavity where the allosteric inhibitor CP-403700 binds (9). The allosteric site is lined by helices α2 and αβ, and a short strand, β7, and is closed by the cap’ region (residues 36’–47’; the prime symbol refers to residues from the second chain of the homodimer) from the other subunit. $G_L$ binds in an U-shaped, hydrophobically collapsed conformation where it exploits numerous polar and hydrophobic contacts to hGPa (Fig. 2, A and B). The $G_L$ peptide protrudes deep into the pocket, and 78% (542 Å$^2$) of its total solvent-accessible surface becomes buried upon binding.

The terminal carboxylate group mimics the AMP phosphate by addressing an arginine-rich region that also comprises the binding site for the allosteric effector phosphate. The Tyr$^{284}\_GL$ side chain points into the ribose binding region, where it forms hydrophobic contacts to Trp$^{67}$, Gln$^{71}$, Tyr$^{75}$, and Val$^{45}$ and a strong hydrogen bond to Asp$^{141}$. Tyr$^{284}\_GL$ protrudes into a region that is not contacted by AMP. Its phenol group is incorporated in a hydrogen bonding network with Asp$^{106}$ and Arg$^{242}$. We further observe an edge-to-face interaction with Phe$^{196}$ and stacking of the phenol ring against the Arg$^{159}$ guanidinium group. The pyrroline of Pro$^{282}\_GL$ is packed through hydrophobic stacking interactions between the two subunits: the phenolic side chain of Tyr$^{75}$ from subunit A and two residues from the subunit B cap region (carbonyl oxygen of Asn$^{44}$ and CG2 of Val$^{45}$). In this it partially mimics the stacking interactions of the AMP adenine between Tyr$^{75}$ and Asn$^{44}$ (Fig. 2C). With Gly$^{281}\_GL$, the $G_L$ peptide leaves the pocket and protrudes into the solvent. The last notable interaction is a van der Waals’ contact of its carbonyl oxygen to Cβ of Ala$^{313}$.

**Conformational Changes**—Prior to soaking with $G_L$, hGPa was crystallized in the active, AMP-bound conformation (7), and on the subunit level there are no large conformational changes upon $G_L$ binding. The backbone atoms of hGPa-$G_L$ and
hGL-AMP align with an root mean square deviation of 0.48 Å. However, the side chains of three residues undergo local reorganization to accommodate the Tyr^{283}_{GL} phenol (Phe^{306} and Arg^{280}) or adapt to the terminal carboxylic acid (Tyr^{151}). Moreover, there is a subtle change in quaternary structure as the two subunits rotate ~2° toward each other (not shown). Within the allosteric pocket this translates to a 0.7-Å shift of the cap’ loop (Fig. 2C) to improve contacts to P_{GL}.

Phosphorylase a is a potent allosteric inhibitor of the PP1-GL complex (12, 21), whereas the inactive phosphorylase b is not (12). To address the molecular basis of this finding we compared hGLPa-GL to a complex with the inhibitor N-acetyl-β-D-glucopyranosylamine (hGLPa-GlcNac), which defines the inactive (T state) conformation (7). Upon inhibitor binding the subunits rotate “outward” by ~7°, and the dimer interface (including the allosteric site) is remodeled (Fig. 2D). Consequently, several interactions that stabilize the G_{l} complex are lost (for example: cap’ interactions, stacking with Tyr^{151}, hydrogen bond to Tyr^{155}) or would lead to steric conflicts (for example: Arg^{280}, Phe^{196}, and Asp^{306}).

**Functional Analysis of G_{l} Recognition by Glycogen Phosphorylase**—After solving the complex structure, we sought to elucidate the determinants that are crucial for conferring binding potency. We developed a SPA to measure the competitive binding of a biotinylated GL peptide to 32p-labeled hGLPa. The dissociation constant (K_{D}) for the interaction of the G_{l} peptide with hGLPa was determined from a saturation binding curve (Fig. 3A) to be 145 nm. Next, we measured the competitive displacement of the G_{l} probe through several peptide variants as well as AMP. Representative binding curves for the displacement of the G_{l} probe by GL-Cterm and AMP are shown in Fig. 3B. The apparent IC_{50} values of the different peptides (Table 2) allow an assessment of the relative importance of individual amino acids for binding. We find that the hexa- to tetrapeptides inhibit the hGLPa-G_{l} interaction in a similar range as the complete GL-Cterm. This is in agreement with the structure, because residues 269_{GL}–280_{GL} are not ordered and therefore are not supposed to contribute strongly to binding. An approximately 8-fold drop in affinity is observed once truncation includes Gly^{281}_{GL}, and no binding could be detected when only the two terminal tyrosines were probed. Likewise, mutation of either tyrosine to alanine or truncation of Tyr^{284}_{GL} was deleterious for binding. Again, this is in good agreement to the numerous contacts these residues involve in the structure. AMP is not a good competitor and inhibits the hGLPa-G_{l} interaction only with an IC_{50} of 21 μM, roughly 10 times weaker than GL-Cterm.

We then tested the ability of G_{l} derivatives to activate the inactive rabbit muscle phosphorylase b (rmGPb; Table 2) and compared it with AMP-induced activation (6). Interestingly, G_{l} peptides are able to activate rmGPb. Activation through GL-Cterm matches that of AMP at 30 μM, which represents a maximally active concentration. Also the three shorter, tightly binding peptides still activate rmGPb between 20 and 30%.

### Table 1

| Data collection and refinement statistics | hGLPa-G_{l} |
|------------------------------------------|-------------|
| **Data set**                             | P_{5,21}    |
| Space group                             | P_{3,1}     |
| Cell dimensions a, c (Å)                | 124.0, 124.7|
| Resolution (Å)                          | 20.25 (2.71-2.56) |
| Observed reflections                    | 145576      |
| Unique reflections                      | 35387       |
| Completeness (%)                        | 98.6 (99.9) |
| R_{work} (%)                            | 7.5 (47.1)  |
| R_{free} (%)                            | 12.5 (3.1)  |
| No. of refined atoms                    | 20.3/27.6   |
| Average B-factor (Å²)                   | 67.34       |
| Root mean square deviation              | 0.008       |
| Bond length (Å)                         | 1.12        |

* The values in parentheses are for the highest resolution shell.

* R_{work} = \sum_{hkl} |F_{hkl}| - |F_{calc}| / \sum_{hkl} |F_{hkl}|

* R_{free} = \sum_{i=1}^{n} |F_{obs}| - |F_{calc}| / \sum_{i=1}^{n} |F_{obs}|

and R_{free} was calculated using 5% of data excluded from refinement.
DISCUSSION

GL as an AMP Mimic—In vitro binding studies have shown that AMP is able to inhibit the specific interaction between phosphorylase \( \alpha \) and recombinant G\(_L\) protein (15). The structural analysis of the G\( \alpha \)-G\(_L\) complex revealed that the very C terminus of G\(_L\) binds in an AMP-competitive fashion, mimicking many of the nucleotides interactions. Additional contacts (mostly provided by Tyr\(_{283}^{GL}\)) result in a 10-fold tighter binding compared with AMP. The improved potency might be necessary to counterbalance the high cellular AMP levels. Phosphorylase itself can accommodate G\(_L\) smoothly, without undergoing any larger conformational changes. A structural comparison between active and inactive states of h\( \alpha \)G explains why the latter cannot directly accommodate G\(_L\) and consequently is not effective as an inhibitor of PP1 (12). Nevertheless, G\(_L\) peptides are able to activate muscle GP\( \beta \). Because G\(_L\) cannot directly bind to GP\( \beta \) in its inactive (T state) conformation, we propose that the peptide acts analogous to AMP (5) and shifts the equilibrium between active and inactive conformations toward the R state.

The h\( \alpha \)G-G\(_L\) Interface as a Drug Target—Inhibiting the interaction of phosphorylase \( \alpha \) and G\(_L\) has the potential to block the allosteric inhibition of the PP1/G\(_L\) activity on glycogen synthase by phosphorylase \( \alpha \). It has been hypothesized that a stimulation of the glycogen synthase pathway via pharmacological dissociation of phosphorylase \( \alpha \) from G\(_L\) could help to normalize hyperglycemia (3). We identified the AMP site as a high affinity binding site for G\(_L\). Our data indicate that it might be feasible to antagonize binding of G\(_L\) protein to GP\( \alpha \) with small molecules. Because in humans G\(_L\) is expressed in liver and muscle cells, glycogen synthesis might be activated in both tissues. Increasing G\(_L\) activity by overexpression was found in cultured primary human myotubes (26) as well as in primary hepatocytes (27) to stimulate glycogen synthase activity and to exhibit a high glycogenic effect. The effects of increasing activity of different glycogen targeting subunits by hepatic overexpression have also been studied in ani-
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expression than with $G_l$ overexpression. Higher glycogen stores even in the fasted state following $G_l$ overexpression were discussed as indicative for reduced glycogenolytic sensitivity.

Therefore, the potential in vivo profile of an inhibitor of $G_l$ binding to GP with respect to effects on glycogenic and glycogenolytic pathways is hard to predict. It is clear that our small peptides might not exhibit the ideal profile, because they behave like AMP with respect to rmGPb activation. Increasing GP activity might counterbalance the stimulation of glycogen synthesis, thus neutralizing potential beneficial effects. Therefore, drug development strategies should aim for compounds lacking GP activating properties.

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Addendum—While this manuscript was under revision, an independent study was published (30) that employed calorimetric measurements on $G_l$-derived peptides to locate the interaction site to the last five residues. The authors also show that an indole-2-carboxamide drug (which binds to the central cavity and stabilizes the T state) can block the interaction. The study clearly supports our conclusions.

TABLE 2

Functional analysis of ligand binding

Table 2

| Ligand     | $G_l$ displacement (IC<sub>50</sub>)<sup>a</sup> | % control/30 μM ligand | rmGPb activation<sup>a</sup> |
|------------|---------------------------------|------------------------|-----------------------------|
| $G_l$-Cterm<sup>b</sup> | 2.3 ± 0.8 | 104 | |
| KLGPYY     | 5.8 ± 1.7 | 21 ± 5.2 | |
| LGPY       | 5.4 ± 1.0 | 23 ± 8.3 | |
| GPYY       | 4.1 ± 1.1 | 29 ± 7.2 | |
| PYY        | 18.7 ± 4.1 | 3 ± 0.0 | |
| YY<sup>b</sup> | >30     | 0      | |
| GPYA<sup>b</sup> | >30     | 0      | |
| GPAY<sup>b</sup> | >30     | 0      | |
| GPY<sup>b</sup> | >30     | 0      | |
| AMP<sup>b</sup> | 21.0 ± 3.9 | 100 | |

<sup>a</sup> The values are the means of three independent measurements unless noted otherwise. Standard deviations are shown as ±S.D. Activation of rmGPb through $G_l$-Cterm was performed as a single experiment only.

<sup>b</sup> The values are the means of two independent measurements.

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FIGURE 3. Functional analysis of $G_l$ binding. A, equilibrium binding curve of $G_l$ peptide against 33P-labeled hlGPb. Increasing concentrations of $G_l$ peptide were added to the binding reaction to develop a saturation curve. The calculated $K_d$ from half-maximal binding is 145 nM (95% CI = 106–185 nM). B, dose-dependent inhibition of $G_l$-hlGPb interaction by $G_l$-Cterm (triangles) or AMP (squares). Calculated IC<sub>50</sub> values are 2.3 μM for $G_l$-Cterm and 21 μM for AMP.
G_L-Phosphorylase Complex

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