Mechanism of glycogen synthase inactivation and interaction with glycogenin

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Glycogen is the major glucose reserve in eukaryotes, and defects in glycogen metabolism and structure lead to disease. Glycogenesis involves interaction of glycogenin (GN) with glycogen synthase (GS), where GS is activated by glucose-6-phosphate (G6P) and inactivated by phosphorylation. We describe the 2.6 Å resolution cryo-EM structure of phosphorylated human GS revealing an autoinhibited GS tetramer flanked by two GN dimers. Phosphorylated N- and C-termini from two GS protomers converge near the G6P-binding pocket and buttress against GS regulatory helices. This keeps GS in an inactive conformation mediated by phospho-Ser641 interactions with a composite “arginine cradle”. Structure-guided mutagenesis perturbing interactions with phosphorylated tails led to increased basal/unstimulated GS activity. We propose that multivalent phosphorylation supports GS autoinhibition through interactions from a dynamic “spike” region, allowing a tuneable rheostat for regulating GS activity. This work therefore provides insights into glycogen synthesis regulation and facilitates studies of glycogen-related diseases.

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Glycogen is a branched polymer of glucose that functions as the primary energy store in eukaryotes. In its mature form, the glycogen particle can comprise up to ~50,000 glucose units that are rapidly utilised when glucose levels are low. Glycogen is stored predominantly in the muscle and liver cells, and to a lesser extent in other organs and tissues including kidney, brain, fat and heart.

Glycogen is synthesised through the cooperative action of three enzymes: glycogenin (GN), glycogen synthase (GS) and glycogen branching enzyme (GBE). GN initiates the process via auto-glucosylation of a conserved tyrosine residue, producing a primer glucose chain of 8–12 residues connected by α,1,4-linkages. This glycogen initiating particle is further extended by GS after its recruitment by the GN C-terminus, allowing the addition of glucose residues using α,1,4-linkages. GBE introduces α,1,6-linkages every 6–8 residues to the growing glycogen molecule, thus creating the final globular structure containing GN at the centre.

Glycogen synthesis and breakdown are tightly regulated processes, and thus dysregulation of the enzymes involved in glycogen metabolism contributes to glycogen storage diseases (GSDs), diabetes, neuroinflammation, neurodegeneration and muscle damage. Excessive and/or abnormal glycogen is a common characteristic in most GSDs. Pompe disease (GSDII) is caused by deficiency of acid-α-glucosidase, resulting in accumulation of lysosomal glycogen and consequent lysosomal destruction and dysfunction. Lafora disease is a fatal neurodegenerative condition, characterised by Lafora bodies that contain hyper-phosphorylated and poorly branched, insoluble glycogen deposits. In addition, loss of GS-GN interaction results in muscle weakness and cardiomyopathy.

Studies using mouse models have found inhibition of glycogen synthesis, particularly by reducing GS activity, to be beneficial for multiple GSDs. To date there is no structure of the GS-GN complex and no structure of human GS. Since inhibition of GS activity is potentially beneficial for GSD patients, obtaining a human GS-GN structure and understanding how GS is regulated is instrumental in developing new therapeutics.

GN is found in two isoforms, GN1 and GN2, encoded by the GYG1 and GYG2 genes respectively. While GYG1 is widely expressed, GYG2 is restricted to the liver, pancreas and heart. GN belongs to the GT8 family of glycosyltransferases, containing a glycosyltransferase A (GT-A) fold with a single Rossmann fold domain at the N-terminus, which is essential for binding of the glucose donor uridine diphosphate glucose (UDP-G) to a single Rossmann fold domain at the N-terminus, which is essential for binding of the glucose donor uridine diphosphate glucose (UDP-G).

The C-term comprises a highly conserved region of ~34 residues (GN3). Other interaction interfaces have been suggested, but further investigation into the full-length complex is required to precisely define any additional interface interfaces. The area between the N-terminal catalytic domain and C-terminal GS binding motif is a linker region that is variable in sequence and in length.

GS is also found as two isoforms, GS1 and GS2, encoded by the GYS1 and GYS2 genes respectively. These are differentially expressed, with GYS1 being expressed predominantly in skeletal muscle and most other cell types where glycogen is present, while GYS2 is expressed exclusively in the liver.

The complex interplay between allosteric activation and inhibitory phosphorylation is not yet fully understood, at least in part because of the lack of structural data for the full GS-GN complex. Although a binary GS-GN complex was co-purified over 30 years ago, we have yet to confirm the stoichiometry of this complex and identify precisely how the two proteins cooperate to make glycogen.

Here, we report the structural and functional analysis of the full-length human GS-GN complex and the cryo-EM structure of phosphorylated human GS. The structure reveals that phosphoregulatory elements form a flexible inter-subunit “spike” region emanating from two GS protomers. GS is kept in an inactive conformation via interactions of phosphorylated Ser641 with arginine residues from GS regulatory helices, which we have termed the arginine cradle. Moreover, low resolution maps of GN bound to GS reveal two flexible GN dimers coordinating a GS tetramer, providing insights into the stoichiometry and the conformational plasticity of this enzyme complex. Collectively, these results shed light on the regulation of glycogen biosynthesis and the inner workings of how GS and GN cooperate to synthesise glycogen.

Results

GS-GN forms an equimolar 4:4 complex. To characterise the synthesis of glycogen by the GS-GN complex, we expressed and purified human full-length GS1 and GN1 in insect cells. Consistent with previous reports, co-expression of GS with GN resulted in improved production yields over the expression of GS alone. Purification of the wild-type (WT) complex resulted in a highly glycosylated sample, as evidenced by a smear by SDS-PAGE corresponding to glucosylated GN detected by Coomassie stain, periodic acid-Schiff (PAS) staining and immunoblotting.

In-gel protease digestion of different molecular weight regions (encompassing mass ranges from 43 to 55 kDa, 55 to 72 kDa, 95 to 130 kDa and >130 kDa) combined with tandem mass spectrometry confirmed the presence of GN1 in all these higher molecular weight species. In addition, treatment of GS-GN preparations with α-amylase (endo-α-1,4-d-glucan hydrolase) resulted in the disappearance of the smeared bands revealing a single, sharp band migrating at the expected molecular weight for GN (~37.5 kDa) and also absence of glycosylated species after PAS staining.

To determine the stoichiometry of the GS-GN complex, we first performed mass photometry analysis of GS-GN and GS-GN(Y195F) mutant complexes, which enables mass
measurements of single molecules in solution. Mass photometry measurements of the GS-GN(Y195F) complex showed a predominant species with an average molecular weight of 473 kDa, which is suggestive of a 4:4 stoichiometry (calculated mass of 485 kDa) (Fig. 1f). Analysis of the GS-GN(WT) sample identified a species with an average molecular weight of 534 kDa and the measured peak was broader than the non-glucosylated species (Fig. 1d). While mass photometry measurements lack the resolution to ascertain the precise molecular mass of heterogeneously glucosylated species, the observed increase in average molecular mass and overall distribution of the WT complex when compared to the Y195F complex is consistent with the observed higher molecular weight of WT GN1 glucosylated species (Fig. 1d and Supplementary Fig. 3b).
To understand how GS and GN interact and to reveal the overall shape of the GS-GN complex we performed negative stain electron microscopy (nsEM) of the WT and Y195F complexes. 2D class averages show two GN dimers, one on either side of a GS tetramer, for both WT and mutant complexes (Fig. 1g). Final 3D maps for both complexes are consistent with the 2D classes, and the reconstructed 3D EM density map can accommodate a GS tetramer flanked by two GN dimers (Fig. 1h). This nsEM confirms a 4:4 stoichiometry and is consistent with previous findings showing that GS can interact with four GN C-terminal peptides simultaneously.4,5,17 Surprisingly, GN dimers do not engage the GS tetramer in an identical fashion, with one GN dimer tilted slightly towards GS and bringing it closer to one of the GS subunits (Fig. 1h). Collectively, these results provide a glimpse of the glycogen initiating particle, where two GN dimers can engage a single GS tetramer.

Phosphorylated human GS is in the inactive state. GS is regulated by both allosteric activation by G6P and inhibition via phosphorylation of its N- and C-terminal tails2 (Fig. 1c). Mechanistic and structural studies of yeast GS have elegantly dissected its allosteric activation by G6P30,34. However, GS structures to date were from protein preparations produced in bacterial expression systems and thus could not provide insights into the phospho-regulatory apparatus. Our GS-GN preparations are from eukaryotic expression systems and therefore provide an opportunity to study the inactive GS form. We confirmed that GS was phosphorylated at sites 2 (S8) and 3a (S641) and the enzyme preparation was inactive unless stimulated by G6P or dephosphorylation (Fig. 2a, b). Protein phosphatase 1 (PP1) and lambda protein phosphatase (lambda PP) treatment resulted in faster migration of GS by SDS-PAGE and also a reduction in signal detected by specific phosphorylation site antibodies (Fig. 2a and Supplementary Fig. 4a). Notably, we see only minor dephosphorylation by mass spectrometry for the GS-GN(Y195F) complex with PP1 alone, which is higher than the 73%35 and 65%36 sequence coverage which is indicated. Not to scale.

High-resolution structure of human GS. Previous attempts to crystallise full-length GS in complex with full-length GN were unsuccessful leading us to pursue structural analysis using cryo-electron microscopy (cryo-EM). NsEM indicated that the position of each GN dimer is different suggesting flexibility of GN in the complex (Fig. 1g, h). Cryo-EM analysis of the GS-GN(Y195F) complex confirmed this GN flexibility as evidenced from the lack of GN signal in 2D class averages (Supplementary Fig. 5a) and subsequent 3D maps. Although we could detect the presence of GN after data processing without the application of symmetry averaging (Supplementary Fig. 6c), it was not possible to trace any connecting residues between the GN globular domain and the C-terminal GN34 region that binds GS. To gain a higher resolution structure for the human GS, we applied D2 symmetry and achieved a global resolution of 2.6 Å (EMDB-14587; PDB 7ZBN) (Fig. 2c, Supplementary Fig. 5 and Supplementary Table 1). The 3D reconstruction revealed a tetrmeric arrangement of human GS in agreement with the crystal structures of the C. elegans GS and yeast GS enzymes, with root mean square deviation (RMSD) values of 1.1 Å (between 484 Ca atom pairs) and 0.9 Å (between 522 Ca atom pairs) respectively (Fig. 2d, Supplementary Fig. 7a, b). Structural analysis of the human GS-GN(WT) complex revealed a 6 Å map of the GS tetramer and comparing this to the GS structure from human GS-GN(Y195F) complex reveals no differences at this resolution (Supplementary Fig. 6d, e). Density for the C-terminal GS interacting region of GN allows for model building of residues 300–332 (human GN34). Four GN peptides bind to the GS tetramer, and these residues form a helix-turn-helix, where the first helix is denoted binding helix 1 (BH1) and the second as BH2 (Fig. 2d). This is consistent with the C. elegans GS-GN34 crystal structure22, with an RMSD value of 0.8 Å (between 30 Ca atom pairs) (Supplementary Fig. 7c). The interaction interface between human GS, namely α4, α9 and α10, and human GN34 is mediated by a combination of hydrophobic and hydrogen bonding interactions and is consistent with the interactions observed for GS-GN34 from C. elegans22 (Fig. 2d and Supplementary Fig. 7c).

Mechanism of GS inactivation. A unique feature of metazoan GS is that both N- and C-terminal tails are phosphorylated, but the mechanism by which they participate in enzyme inactivation has remained elusive. We were able to build a model for the N-terminus starting from residue 13, and of the C-terminus up to residue 625, and then from 630–639 (chain A/C) and 630–642 (chain B/D), that could help understand the mechanisms of GS inactivation. The N- and C-terminal tails of each GS protomer lie almost parallel to each other, and travel side by side along the GS tetrameric core to reach the centre (Fig. 3a, right panels). Here, the C-terminal tail (chain A) meets the C-terminal tail from an
adjacent GS protomer (chain B), which has travelled from the opposite direction (Fig. 3a, right panels). A 2.8 Å cryo-EM map of GS generated without the application of D2 symmetry averaging (EMDB-14587) (Supplementary Fig. 6a, b), suggests that one C-terminal tail disengages with the GS core earlier than the other C-terminal tail from the adjacent chain. The C-terminal tail from chain B continues to travel further across the regulatory helices than chain A, prior to traversing away from the core (Fig. 3a).

This allows chain B to engage with the regulatory helices α22, specifically phosphorylated S641 interacting with residues R588 and R591, which come from two GS protomers to form a positively charged pocket we have termed the “arginine cradle” (Fig. 3a and Supplementary Fig. 7d). This is consistent with our phosphorylation mapping and immunoblotting data showing S641 is phosphorylated in our preparations (Table 1, Supplementary Fig. 4b and Fig. 2a).

**Fig. 2** Cryo-EM structure of human GS-GN34 complex. a Immunoblot for the indicated human GS phosphorylation sites and total GS. Data are representative of two independent experiments carried out in technical duplicates. b Activity of GS-GN(Y195F) with and without the addition of lambda protein phosphatase (lambda PP) and protein phosphatase 1 (PP1) (left) and −/+ G6P activity ratio (right). Upon G6P saturation, GS reaches similar activity levels regardless of phosphorylation state. Data are mean ± S.E.M. from n = 2 and representative of two independent experiments. One-way analysis of variance (Tukey’s post hoc test); exact p values are shown. Source data for (a and b) are provided as a Source Data file. c 2.6 Å cryo-EM map of the GS tetramer coloured by corresponding chain. Density corresponding to the GN34 C-terminal region is shown in green. d Human GS-GN34 cartoon model shown in ribbons coloured by corresponding chain (left). Interaction between GS and GN34 (right). e Unsharpened cryo-EM map shown at a lower threshold to visualise the “spike” region depicted in grey (left). The N- and C-terminal tails of two protomers converge and form the “spike” region (right).
S641 is a major phosphorylation site involved in the regulation of GS activity, and interaction of pS641 with the arginine cradle in helix α22 shows the mechanism of inactivation of human GS through constraining the GS tetramer in a “tense state”. This interaction therefore provides a crucial activity switch mechanism from a tense (phosphorylated) state to a relaxed (G6P-bound) state. The involvement of helices α22, which also interact with G6P via the nearby arginine residues R582 and R586 (Supplementary Fig. 8a), provides a possible link between G6P-binding and its ability to override inactivation by phosphorylation.

The Rossmann fold domains of human GS were predicted to a high level of accuracy by AlphaFold (RMSD 1.0 Å between 575 Ca atoms), although the position of the N- and C-terminal tails does not agree entirely (Supplementary Fig. 8c). However, the position of S641 is consistent and overlays well with the phospho-human GS, where phosphorylation appears to contribute to the closing of the regulatory helices and its ability to override inactivation by phosphorylation.

GS contains a dynamic “spike” region. Notably, the EM structure maps show density for an inter-subunit region that extends from the N- and C-termini of two adjacent GS protomers. These N- and C-termini contain the GS phosphorylatable apparatus which meet and traverse away from the GS core (Fig. 2e). Analysis of this ~25 kDa region by focussed 3D classification (without applying symmetry) reveals that the region is highly flexible, as seen by the various different conformations (Supplementary Fig. 5f, g). Interestingly, these “spike” regions were present in all the refined classes, and suggests that GS exists as a continuum of structures with a core inactive tetramer and “dynamic spikes” buttressed on either side, thus preventing GS from adopting an open, active conformation.

To explore the flexibility and mobility of GS, we performed 3D variability analysis using cryoSPARC. The dynamic movements of the “spike” region and concurrent movements of the GS tetramer are highlighted in Supplementary Movie 1. Consistent with the focussed 3D classification, the “spike” is highly mobile, whereas only slight flexibility was observed within each GS protomer. This suggests a role of the “spike” region in constricting a tense state of the GS tetramer, and subsequently contributing to the GS regulation.

Cross species comparison of GS structures. When comparing human GS to previous crystal structures of yeast GS, the distance between regulatory helices (α22) in adjacent monomers changes according to the activity state of GS (Fig. 3b). In the phosphorylated human GS structure, helices α22 lie 7.9 Å apart when measuring Ca-Ca distances from Arg591 on chain A and Arg580 on chain B (Fig. 3b). A similar measurement of the corresponding residues in the yeast proteins shows that helices α22 are furthest apart, at 16 Å, when G6P is bound and GS is in its high activity state, and this translates into better access for accepting the substrate. When no G6P is bound and there is no phosphorylation, GS is in the basal state and the helices lie 11 Å apart. In a yeast GS structure of a mimic of the inhibited state, produced in bacteria, the helices are closest together at 8 Å. This is similar to the phospho-human GS, where phosphorylation appears to contribute to the closing of the regulatory helices constraining the GS tetramer and thus locking it in a tense, inactive state (Fig. 3b).

The position of the extreme N-terminus is noticeably different in human and C. elegans GS structures compared to yeast (Supplementary Fig. 7e). The majority of the first β-sheet in all structures is in a similar orientation, however human residues before 26 (residue 7 in yeast) move in the opposite direction to yeast (Supplementary Fig. 7e). This positioning of the human GS N-terminus is directed towards the regulatory helices α22.
Previous structural investigation of *C. elegans* GS-GN34 suggested a hypothesis where phosphorylation could enable the N-terminus to engage with regulatory helices, as the N-terminus is also situated towards the regulatory helices (Supplementary Fig. 7e). Our structure of the human, phosphorylated enzyme supports this hypothesis, although the current density does not allow model building before residue 13. However, using LAFTER denoised maps to aid model building and electron density interpretation, some density for the N-terminus is present next to the regulatory helices, near residues R579 and R580. This suggests that perhaps the N-terminal phosphorylation sites can also interact with the regulatory helices and/or nearby residues (Fig. 3a and Supplementary Fig. 7f).

**Comparisons between human, *C. elegans* and yeast GS structures are consistent with the human structure in the inactive state. Each human GS protomer shows a closed conformation of its active site, and a regulatory loop that only becomes ordered upon G6P binding is disordered in the human structure (Supplementary Fig. 8a, b). Previous studies have suggested that phosphorylated tails may be able to engage the G6P binding site and directly compete with G6P. However, our EM density maps show no extra density within the G6P binding site (Supplementary Fig. 8a). Thus, we see no evidence to support the hypothesis that the phosphorylated tails interact with residues lining the G6P pocket to directly compete with G6P binding. Instead, we posit that the phosporegulatory regions indirectly affect G6P binding by constraining the opening and closing of the GS tetramer. Collectively, our structural analyses support a model by which phosphorylated N- and C-terminal tails inhibit the GS tetramer by constraining a tense conformation through inter-subunit interactions.

**Dislodging the GS phosphoregulatory region.** Due to the flexibility evident in the N- and C-terminal tails, we were unable to build phosphorylated residues in the cryo-EM map other than phosphorylated S641. However, we can see the beginning of the flexible phosphoregulatory “spike” region and residues from the GS core tetramer which interact with this regulatory region (Fig. 3a, bottom right panel). To investigate the relationship between allosteric regulation and inhibitory phosphorylation and elucidate the mechanism of inactivation, we mutated residues in GS that contact the beginning of the phosphoregulatory region. We selected residues which are not involved in G6P binding and mutated these in order to “dislodge” the regulatory tails (Fig. 3a and Supplementary Fig. 8e). If the phosphorylated tails are indeed

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**Fig. 3 The phosphoregulatory region of human GS.** a Human (Hs)GS-GN34 structure shown in ribbons (top left). The N- and C-terminal tails of one GS protomer (chain A) lie next to one another and move towards the adjacent protomer, meeting the N- and C-terminal tails from chain B. Arrows indicate continuation of cryo-EM density (top right). Electron density (C1 symmetry) for phosphorylated S641 (pS641) interacting with R588 and R591 on the regulatory helices α22 (bottom left). Residues that are interacting with the N- and C-terminal tails that are mutated in this study are shown (bottom right).

b Comparison of distances between regulatory helices of adjacent monomers of HsGS (reported here), low activity inhibited mimic (PDB ID 5SUL), basal state (PDB ID 3NAZ) and G6P activated (PDB ID 5SUK) yeast GS (yGS) crystal structures. Quoted distances were measured from Ca of Arg591 (chain A) and -Cα of Arg580 (chain B) of HsGS and corresponding yeast residues.
holding GS in an inactivated state, weakening the interaction between the core tetramer and the N- and C-termini inhibitory regions should create an enzyme with higher basal activity in comparison to the WT. Consistent with our hypothesis, we observed a marginal increase in basal (−G6P) GS activity in R588A + R591A, Y600A, R603A, H610E and W18A mutants, that was reflective of the phosphorylated state at residues S8, S641 and S645 (Fig. 4a, b). These mutants were unaffected in terms of GN co-purification and with the exception of R588A + R591A mutant, they had similar melting (T_m) profiles and oligomeric state to the WT GS complexes (Supplementary Fig. 3e, Fig. 4c, Supplementary Fig. 9). All mutants except Y600A could still be activated to similar levels to the WT upon addition of G6P (Fig. 4a).
Upon addition of PP1 and lambda PP, the GS mutants R588A + R591A, R603A and W18A were more robustly dephosphorylated at S641 and S8 than WT GS (Fig. 4d), suggestive of increased exposure of the phospho-tails to phosphatases. For the W18A mutant, this dephosphorylation by both lambda PP and PP1 resulted in over a 20-fold increase in basal activity, and also an approximately threefold increase in comparison to WT GS (Fig. 4e). The GS R603A-GN(Y195F) mutant has a basal activity similar to WT GS upon dephosphorylation. However, the robust dephosphorylation at S641 and S8 in GS R588A + R591A was not associated with an increase in activity (Fig. 4e). As described above, R588 and R591 lie on the regulatory helices and are also involved in inter-subunit interactions and form the arginine cradle that interacts with phospho-S641 (Fig. 3a). In addition, we noticed some dissociation of the GS R588A + R591A double mutant complex in mass photometry (Supplementary Fig. 9c). Therefore, the role of these residues in stabilising the GS tetramer may be the cause for the lack of rescue of activity upon dephosphorylation (Fig. 4e). Moreover, dephosphorylated GS had a markedly lower Tm (48 °C) than WT or mutant GS (Fig. 4c) for most of the observable interactions with helix α22 via R588 and R591 residues, however, these residues are not conserved in C. elegans (Supplementary Fig. 2), perhaps explaining the positional differences between human and C. elegans GS tails. Interestingly, S641 (site 3a) is also not conserved in C. elegans GS, suggesting an evolutionary divergence, and hinting at additional mechanisms for C. elegans GS inactivation where the N-terminus interacts with helix a22. This potential exchange of interactions between N- and C-termini suggests a functional redundancy between the multiple phosphorylation sites.

The non-identical engagement of the C-terminal tails and the proximity of the N-terminus to the regulatory helices, as well as the flexibility of the “spike” region indicates coordination between the N- and C-termini of a single GS protomer, as well as between protomers (Fig. 3a and Supplementary Fig. 7i, g). Having one tail buttressed against the regulatory helix and the other steering away from the core may allow interchanging of the tails based on their level of phosphorylation, perhaps explaining why multiple phosphorylation sites are required. It may also aid/allow rapid dephosphorylation of GS, leading to an increase in GS activity and thus promoting glycogen synthesis (Fig. 5).

Studying the role of the inter-subunit domain that house the phosphorylation sites through mutations that weaken the interactions between the core tetramer and the “spike” regions, resulted in GS basal activity equal to or higher than the WT, yet retaining activation by G6P (Fig. 4a). One GS mutant (Y600A) was not activated by G6P to the same extent as WT (Fig. 4a), and although Y600 does not directly bind to G6P, UDP or sugars, it is possible that this hydrophobic residue is important for interdomain movements which are required for full GS activation.

Dephosphorylation of the GS-GN(Y195F) complex resulted in an increase in basal activity, yet there is little difference in the
Fig. 5 GS and GN cooperate to synthesise glycogen. Glucose is converted into glycogen through the action of glycogenin (GN), glycogen synthase (GS) and glycogen branching enzyme (GBE). GN interacts with GS to feed the initial glucose chain into the GS active site for elongation. GS is regulated by allosteric activation and inhibitory phosphorylation. Phospho-S641 (pS641) from one C-terminal tail interacts with the regulatory helices of GS and glycogen branching enzyme (GBE). GN interacts with GS to feed the initial glucose chain into the GS active site for elongation. GS is regulated by allosteric activation and inhibitory phosphorylation. Phospho-S641 (pS641) from one C-terminal tail interacts with the regulatory helices of GS, resulting in low basal activity, yet it could still be fully consistent with the requirement of this interaction for glycogen synthesis. The structure presented here will therefore provide a valuable resource to understand disease mutations. In addition, this structure and increased understanding of GS regulation facilitates GS studies and its relevance in GSD, particularly Pompe and Lafora diseases where a reduction of glycogen levels could be beneficial. The high resolution achieved here (2.6 Å) would undoubtedly be beneficial in efforts to design GS inhibitors that block G6P, substrate binding and/or GS-GN interaction.

GS has evolved a mechanism by which the phosphorylated N- and C-terminal “spike” regions hold GS in an inactive conformation that is relieved by dephosphorylation and/or G6P binding. We propose that the dynamic nature of these regulatory regions provides a functional redundancy mechanism and serves the purpose of exposing phosphorylated residues to phosphatases, thus allowing a “tuneable rheostat” instead of an on/off switch for regulating GS activity. Collectively, our analyses of the human GS-GN enzyme complexes reveal important mechanistic and structural details that could improve our understanding of GSDs.
Cloning, protein expression and purification of GS-GN complex. Genes encoding human GS1 (HsGS-NM 002103) and human GN1 (HsGN-NM 001840) were cloned into pFfastBac vectors, both with a 6x His purification tag followed by a cleavable site was engineered at the N-terminus of WT GN or WT Y195F mutant. For co-expression of WT GS and mutants the genes encoding human GS1 and human GN1 (Y195F) were cloned in pFfastBac vectors, both with a 6x His purification tag followed by a T7 EVI site at the N-terminus. Recombinant baculoviruses were generated in DH10Bac cells. Virus amplification and protein expression, in Spodoptera frugiperda (Sf9) cells and Trichoplusia ni (Tni) cells respectively, were carried out using standard procedures. For co-infection of pFfastBac clones, a 1:1 ratio of the GN-P2 virus ratio was used. A PCR-based site directed mutagenesis was used to introduce the following mutants from the pFfastBac GS1 construct: W18A, R588A ↔ R591A, Y600A, R630A, H610E, S141R. All of the alterations were confirmed by DNA sequencing.

Cell pellets containing HsGS-GN, HsGS-GN(Y195F) and mutants were resuspended in lysis buffer (50 mM Tris-HCl pH 7.6, 300 mM NaCl, 20 mM imidazole, 10% glycerol, 0.075% β-mercaptoethanol, 1 mM benzamidine), followed by four CV washes of high salt buffer (50 mM Tris-HCl pH 7.6, 500 mM NaCl, 20 mM imidazole, 10% glycerol, 0.075% β-mercaptoethanol, 1 mM benzamidine) and final CV washes in low salt buffer. The column was then attached to the AKTA system (GE Healthcare) and washed with elution buffer. The protein was then eluted by applying an imidazole gradient (20 mM imidazole, 10% glycerol, 0.075% β-mercaptoethanol, 1 mM benzamidine), followed by dialysis against buffer containing 10% glycerol, 0.075% β-mercaptoethanol, 1 mM benzamidine). The fractions containing protein were analysed by SDS-PAGE and then pooled and dialysed overnight (10,000 MWCO MCO dialysis tubing (MerckMillipore). Filtered lysate was loaded onto a pre-equilibrated 1 mL or 5 mL Ni2+ resin column. The protein was then then pooled and dialysed overnight (10,000 MWCO MCO dialysis tubing (Thermo Scientific)).

In vitro dephosphorylation of GS-GN. PP1 and lambda PP2 were obtained from MRC PPU. Both have an N-terminal GST tag and lambda visualised by Coomassie blue staining, and glucosylated species were detected using μ6mBar pressure (PELCO easiGlow, Ted Pella). Grids were incubated for 1 min with 0.01184720) mutant were cloned into pFL a vector encoding human GS1 (HsGS:NM 002103) and human GN1 (HsGN:NM 001840). A single 6 × His puri tag followed by a cleavable site was engineered at the N-terminus of GN WT or GN Y195F. Grids were incubated for 1 min with 0.001184720) mutant were cloned into pFL a vector encoding human GS1 (HsGS:NM 002103) and human GN1 (HsGN:NM 001840). A single 6 × His puri tag followed by a cleavable site was engineered at the N-terminus of GN WT or GN Y195F. Grids were incubated for 1 min with

Negative stain electron microscopy—data processing. RELION 3.0 was used for processing of negative stain-EM data. Real-time contrast transfer function (CTF) parameters were determined using gCTF. Approximately 2000 particles were manually picked, extracted with a box size of 104 Å2, then subjected to reference-free 2D classification to produce initial references to be used for auto-picking. The parameters for auto-picking were optimised and 92,580 particles were extracted. The extracted particles were used for iterative rounds of reference-free 2D classification. Based on visual inspection, best quality 2D average classes were selected to generate a de novo 3D initial model, which was used as a reference in unprocessed 3D classification. These classes were then subjected to 3D refinement to generate a final EM density map.

Cryo-electron microscopy—grid preparation and data collection. Quantifoil R2/2 Cu300 or Quantifoil R1.2/1.3 Cu300 (Quantifoil Micro Tools) grids were grid preparation and data collection. RELION 3.0 was used for processing of negative stain-EM data. Real-time contrast transfer function (CTF) parameters were determined using gCTF. Approximately 2000 particles were manually picked, extracted with a box size of 104 Å2, then subjected to reference-free 2D classification to produce initial references to be used for auto-picking. The parameters for auto-picking were optimised and 92,580 particles were extracted. The extracted particles were used for iterative rounds of reference-free 2D classification. Based on visual inspection, best quality 2D average classes were selected to generate a de novo 3D initial model, which was used as a reference in unprocessed 3D classification. These classes were then subjected to 3D refinement to generate a final EM density map.

Cryo-electron microscopy—data processing. For GS-GN(Y195F), a FEI Fialon IV direct electron detector with an energy per electron (eV) was used (Supplementary Fig. 2). A dose per physical pixel of 4.71 was used resulting in a total dose of 34.8 e2Å−2, fractionated across 128 EPU frames. This was then grouped into 21 frames, resulting in a dose per frame of 0.8 e2Å−2. Magnification was ×16000 resulting in a pixel size of 0.71 Å/pixel. Eight exposures per hole was taken and the defocus values ranged from −1 μm to −3.1 μm. 20,841 movies were recorded using the EPU automated acquisition software (v2.13).

For GS-GN, a FEI Fialon III direct electron detector was used in integrating mode. The total electron dose was 85 e2Å−2, a magnification of ×750 was used and a final calibrated object sampling of 1.065 Å/pixel. Each movie had a total exposure time of 1.6 s, collected 47 fractions with an electron dose of 1.8 e2Å−2 per fraction. One exposure per hole was taken and the defocus values ranged from −1 μm to −3.1 μm. 3009 movies were recorded using the EPU automated acquisition software.

Cryo-electron microscopy—data processing. For GS-GN(Y195F), movies were motion corrected using RELION’s implementation of the MotionCor2 algorithm and real-time CTF parameters were determined using gCTF. Motion correction and CTF estimation were carried out on the φ-t (Ref. 3), 1,883,188 particles were picked using the PhosaurusNet general model in cryoYLO57 v1.6.1. Particles were imported into RELION 3.1 and extracted and binned by 2. These particles were subjected to 2D classification. 1,188,332 particles selected after 2D classification were subjected to 3D classification, applying D2 symmetry. Carrying all “good”/unambiguous classes forward, 739,232 particles were un-binned to a box size of 288 pixels and subjected to 3D refinement and postprocessing, generating a map at 2.92 Å. Followed by iterative rounds of per particle CTF refinement and Bayesian particle polishing to generate a map at 2.62 Å (Supplementary Fig. 5).

To elucidate the movement of phosphoregulatory regions, an alignment free 3D classification with a mask containing the “spike” density was performed, using a regularisation parameter T of 6058 (Supplementary Fig. 5f, g). Local resolution was estimated using the local resolution feature in RELION.

To prevent interpretation of any artefacts created by applying D2 symmetry, the data were also processed in C1 symmetry (Supplementary Fig. 6). The same particles after 2D classification were subjected to 3D classification applying D2 symmetry. Carrying all “good”/unambiguous classes forward, 739,232 particles were un-binned to a box size of 288 pixels and subjected to 3D refinement and postprocessing, to generate a 3.1 Å map. Following iterative rounds of CTF refinement and Bayesian particle polishing to generate a 2.8 Å map. 

To explore the heterogeneity in the dataset, the 3D variability analysis tool in cryoSPARC v3.2.044 was used. The 739,232 particles after 3D classification were subjected to 3D variability analysis43 tool in cryoSPARC v3.2.044 was used. The 739,232 particles after 3D classification were subjected to 3D variability analysis43 tool in cryoSPARC v3.2.044 was used. The 739,232 particles after 3D classification were subjected to 3D variability analysis43 tool in cryoSPARC v3.2.044 was used. The 739,232 particles after 3D classification were subjected to 3D variability analysis43 tool in cryoSPARC v3.2.044 was used. The 739,232 particles after 3D classification were subjected to 3D variability analysis43 tool in cryoSPARC v3.2.044 was used.
**Model building and refinement.** A preliminary model of human GS was generated by AlphaFold42 (accessed 1 October 2021) and a preliminary model of last 34 residues of human GN was created by Phyre260. These preliminary models used rigid body fitting into the cryo-EM density in UCSF Chimera39. The model was then built using iterative rounds of manual building in COOT v0.9.2-pre39 and real space refinement in PHENIX v1.1939.

**Visualisation, structure analysis and sequence alignments.** Visualisation and structure analysis were performed using UCSF Cimera25 or Chimera59. Multiple sequence alignments were performed using MUSCLE24 and displayed and edited using ALINE v1.0.0259.

**Mass photometry.** Mass photometry experiments were performed using a Refn One® mass photometer. Immediately prior to mass photometry measurements, proteins were diluted in 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP to a final concentration of 0.5 nM. For each measurement, (16 μL) buffer was added to a well and the focus point was found and adjusted when necessary. Protein (4 μL) was then added to the buffer droplet, the sample was mixed and movies of 60 s were recorded using AcquireMP. Data were analysed using DiscoverMP.

**Differential scanning fluorimetry.** Thermal shift assays were performed using an Applied Biosystems QuantStudio 3 Real-Time PCR system. SYPRO™ Orange (Invitrogen) was used as a fluorescence probe. Proteins were diluted in 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP to a final concentration of 1 μM. Varied concentrations of G6P were added and the reaction was incubated at room temperature for 30 min. SYPRO Orange was diluted in 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP to a final concentration of 2.5 X, in a total reaction volume of 20 μL. The temperature was raised in 0.018 °C intervals from 20°C to 95°C. Data were analysed using Protein Thermal Shift v1.4.

**Tandem mass spectrometry.** Concentrated purified protein complexes (6.75 μg) were diluted 30-fold in 25 mM ammonium bicarbonate pH 8.0 before being subject to reduction with dithiothreitol and alkylation with iodoacetamide, as previously described39. The eluent was equally divided into three for digestion with either: 33:1 (w/w) trypsin gold (Promega), 25:1 (w/w) chymotrypsin (Promega), or 10:1 (w/w) elastase (Promega), using the manufacturer’s recommended temperatures for 18 h with 600 rpm shaking. Digests were then subject to in-house packed, strong cation exchange stage tip clean-up, as previously described by69. Dried peptides were solubilized in 20 μL of 3% (v/v) trypsin and 0.1% (v/v) TFA in water, sonicated for 10 min, and centrifuged at 13,000 g for 15 min at 4 °C being separated using an Ultimate 3000 nano system (Dionex) by reversed-phase HPLC; over a 60 min gradient, as described in66. All data acquisition was performed using a Thermo Orbitrap Fusion Lumos Trivid mass spectrometer (Thermo Scientific), with higher-energy C-trap dissociation (HCD) fragmentation set at 32% normalized collision energy for 2+ to 5+ charge states. MS1 spectra were acquired in the Orbitrap (30 K resolution at 200 m/z) over a range of 150–1400 m/z. AGC target = standard, maximum injection time = auto, with an intensity threshold for fragmentation of 2e4. MS2 spectra were acquired in the Orbitrap (30 K resolution at 200 m/z), AGC target = standard, maximum injection time = dynamic. A dynamic exclusion window of 20 s was applied at a 10 ppm mass tolerance. Data were analyzed by Proteome Discoverer 1.4 using the UniProt Human reviewed database (updated April 2020) with fixed modification = carboxymethylation (C), variable modifications = oxidation (M) and phospho (S/T/Y), instrument type = electrospray ionisation–Fourier-transform ion cyclotron resonance (ESI–FTICR), MS1 mass tolerance = 10 ppm, MS2 mass tolerance = 0.01 Da, and the pteR8S node; on set to a score > 99.0.

**Protein identification mass spectrometry.** 10 μg of purified protein was separated by SDS-PAGE (10% resolving, 4% stacking) before colloidal Coomassie staining overnight and thorough washing in milliQ water. A scalpel was used to excise the major band at ~85 kDa, and incremental bands spanning 43–60 kDa were analysed by Proteome Discoverer 1.4 using the UniProt Human reviewed database. Mass photometry data were acquired using an Applied Biosystems QuantStudio 3 Real-Time PCR system. SYPRO™ Orange (Invitrogen) was used as a fluorescence probe. Proteins were diluted in 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP to a final concentration of 1 μM. Varied concentrations of G6P were added and the reaction was incubated at room temperature for 30 min. SYPRO Orange was diluted in 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP to a final concentration of 2.5 X, in a total reaction volume of 20 μL. The temperature was raised in 0.018 °C intervals from 20°C to 95°C. Data were analysed using Protein Thermal Shift v1.4.

**Data availability**

The cryo-EM maps have been deposited in the Electron Microscopy Data Bank under the accession code EMDB-14587. Coordinates have been deposited in the Protein Data Bank with accession code 6VCI.

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Author contributions
L.M. performed molecular biology, protein production, electron microscopy, differential scanning fluorimetry and mass photometry experiments, D.B. performed glycogen synthase activity and western blot assays, L.A.D. performed phosphorylation mapping and mass spectrometry experiments and C.B., S.C.M.V. and D.P.M. provided support with structural biology and protein production. L.M. and E.Z. drafted the paper with input from D.B., K.S., L.A.D. and C.E.E. and all authors revised it. J.P., C.E.E., C.H., J.A.B., N.A.R., H.K., K.S. and E.Z. provided supervision and project management. E.Z., K.S., C.E.E., C.H. and J.A.B. designed and interpreted data in consultation with all authors.

Competing interests
The authors declare no competing interests.

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