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Expression and purification of functional human glycogen synthase-1:glycogenin-1 complex in insect cells

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ABSTRACT

We report the successful expression and purification of functional human muscle glycogen synthase (GYS1) in complex with human glycogenin-1 (GN1). Stoichiometric GYS1:GN1 complex was produced by co-expression of GYS1 and GN1 using a bicistronic pFastBac™-Dual expression vector, followed by affinity purification and subsequent size-exclusion chromatography. Mass spectrometry analysis identified that GYS1 is phosphorylated at several well-characterised and uncharacterised Ser/Thr residues. Biochemical analysis, including activity ratio (in the absence relative to that in the presence of glucose-6-phosphate) measurement, covalently attached phosphate estimation as well as phosphatase treatment, revealed that recombinant GYS1 is substantially more heavily phosphorylated than would be observed in intact human or rodent muscle tissues. A large quantity of highly-pure stoichiometric GYS1:GN1 complex will be useful to study its structural and biochemical properties in the future, which would reveal mechanistic insights into its functional role in glycogen biosynthesis.

Introduction

Glycogen is a branched polymer of glucose that serves as a repository of rapidly accessible energy and, thus plays an important role in maintaining glucose and energy homeostasis at both cellular and organism levels [1]. In mammals, liver and muscle tissues are the major depots for glycogen storage, although to a much lesser extent most tissues contain non-negligible levels of glycogen. While the physiological importance of glycogen in muscle and liver has been extensively documented in the context of glucose homeostasis, recent evidence suggests a key role of glycogen in brain (in both astrocytes and neurons) for cognitive function [2] and also cancer cells for senescence and cellular growth [3].

Bulk biosynthesis of glycogen is mediated by glycogen synthase which catalyses the addition of α-1,4-linked glucose units from uridine diphosphate (UDP)[3]-glucose to a nascent glycogen chain, and branching enzyme which forms α-1,6-glycosidic branchpoints [1]. In mammals, there are two glycogen synthase isoforms: muscle glycogen synthase (GYS1; encoded by *GYS1*), which is ubiquitously expressed, but most abundantly expressed in skeletal and cardiac muscles, and the liver-specific isoform (GYS2; encoded by *GYS2*). Activity of both isoforms is regulated by a complex interplay between reversible phosphorylation at multiple sites and allosteric regulators of which glucose-6-phosphate (G6P), an activator, plays the most important role [1,4,5].

Besides glycogen synthase and branching enzyme, glycogenin is another key enzyme involved in biosynthesis of glycogen. Glycogenin (GN) initiates glycogen synthesis by generating a short linear (α-1,4-linked) chain of approximately 10 glucose units attached to a conserved tyrosine residue (i.e., Y194 in human) [1,6]. Cohen and colleagues first reported that glycogen synthase purified from rabbit skeletal muscle exists as a stoichiometric complex with glycogenin [7] and proposed a key interplay between the two enzymes in initiation of glycogen synthesis [6]. Subsequent studies have demonstrated that glycogenin interacts with glycogen synthase and enhances soluble overexpression of glycogen synthase (when ectopically co-expressed with glycogen synthase in cultured cell lines) [4,8]. This has led Khanna et al. [9] to make an attempt to co-express GYS1 and glycogenin using MultiBac system in insect cells, which resulted in a successful soluble expression and purification of large quantities of human GYS1 proteins. Direct

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[3] *Abbreviations used:* UDP, uridine diphosphate; G6P, glucose-6-phosphate; GSK3, glycogen synthase kinase-3; PKA, cAMP-dependent protein kinase/protein kinase A; CK, casein kinase; PP1, protein phosphatase-1.

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interaction of the C-terminus of GN with glycogen synthase was first reported by Skurat et al. [10] based on a yeast two-hybrid study followed by cellular overexpression experiments and more recently by an X-ray crystal structure of glycogen synthase in complex with a C-terminal 34-residue fragment of GN [11].

Given the implication that the glycogen synthase:glycogenin complex plays an important role in the initiation and elongation of glycogen particles [6,11], it would be important to further explore their molecular interaction and function as a complete full-length complex for X-ray crystallographic and biochemical property/functional studies. We here report a successful large-scale expression and purification of human GYS1 and human GN1 in insect cells and also show biochemical properties of the enzyme complex in cell-free assay systems.

Materials and methods

Materials

Rabbit liver glycogen and pancreatic α-amylase were from Sigma. Microcystin-LR was from Enzo Life Sciences. NADH was from Apollo Scientific. Antibodies against pS8 GYS1 and pS8/S11 GYS1 were kindly donated by D. Grahame Hardie (University of Dundee). pS641 GYS1 (#3891) and total GYS1 (#3893) antibodies were from Cell Signaling Technologies. Total GYS1 (sc-81173) and PP1c (sc-7182) antibodies were from Santa Cruz Biotechnology. pS641/645 GYS1 (S486A, 3rd bleed) and GN1 (S197C, 1st bleed) antibodies were generated by the Division of Signal Transduction Therapy (DSTT) at the University of Dundee as previously described [4]. Peroxidase conjugated secondary antibodies were from Jackson Immunoresearch. Glutathione Sepharose 4B, NHS-Sepharose FF, Superdex 200 3.2/300, Q-Sepharose HP and Enhanced Chemiluminescent (ECL) reagent were from GE Healthcare. Gel filtration standards were from Bio-Rad (151-1901). All other reagents, unless otherwise indicated, were from Sigma.

Expression and purification of GYS1:GN1 complex

Genes coding for human GYS1 (NM 002103) and GST-tagged GN1 (NM 004130) were cloned in a pFastBac™-Dual vector (Life Technologies). A PreScission protease site was added between GST and GN1. Recombinant bacmid was generated in DH10Bac™ cells (Life Technologies) and extracted using phenol:chloroform. Unless specified otherwise, *Spodoptera frugiperda* (Sf9) cells (Life Technologies) were cultured in suspension flasks on a rotating platform (110 RPM at 26 °C) using an incubator shaker (Infors HT) and Sf-900 II media following the supplier's manual. Recombinant progeny 1 (P1) baculovirus was produced in monolayer cultures by transfecting 5 ml Sf9 cells (1.0×10^6 cells/ml grown in 25 cm^2 flasks) and was harvested after 6 days, aliquoted and stored at −80 °C. P2 virus was generated by infecting Sf9 suspension cultures (1.5×10^6 cells/ml) with P1 virus (400 μl/100 ml cells). The supernatant from this culture (P2) was harvested 72 h post-infection, and 3 ml were used to infect 600 ml of Sf9 suspension culture (1.5×10^6 cells/ml), thus generating a P3 virus 72 h post-infection. This culture (P3) was used at a 1:10 ratio to infect 2 L of Sf9 cells ($1.5–2.0 \times 10^6$ cells/ml) for protein production. Cells were grown in suspension for 48 h and the cell pellets were washed in PBS prior to harvesting in ice-cold lysis buffer (50 mM tris–HCl pH 7.8, 150 mM NaCl, 5% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 0.2 mM PMSF, 5 μM leupeptin and 0.1% (v/v) 2-mercaptoethanol). Cells were lysed using a continuous flow cell disruptor at 18,000 psi and the lysate was clarified by centrifugation at 26,000*g* for 30 min. The supernatant was incubated on a rolling platform for 1 h at 4 °C with 1 ml of glutathione–Sepharose resin, pre-equilibrated in low salt buffer (50 mM tris–HCl pH 7.8, 150 mM NaCl, 5% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine and 0.1% (v/v) 2-mercaptoethanol). The beads were washed with 10 column volumes (CV) of low salt buffer and 50 CV of high salt buffer (low salt buffer containing 500 mM NaCl), followed by 10 CV of low salt buffer. Proteins were eluted by incubating with 0.2 mg of GST-tagged PreScission protease for 16 h at 4 °C. The eluate was concentrated to 0.7–1 ml and loaded on a Superdex 200 10/300 column (GE Healthcare), pre-equilibrated in 25 mM tris–HCl pH 7.8, 150 mM NaCl and 1 mM TCEP. Eluted peaks were analysed by SDS–PAGE, and fractions containing >95% pure GYS1-GN1 complex were combined, concentrated to 2–3 mg/ml, snap frozen in liquid nitrogen and stored at −80 °C.

Preparation of recombinant GST-PP1c

GST-tagged human PP1 catalytic subunit γ was cloned into pGEX6P-1 and expressed in BL21-CodonPlus (DE3)-RIL, cultured in LB media supplemented with 1 mM $MnCl_2$ at 18 °C post induction with 0.2 mM IPTG. Cultures were harvested and lysed in 50 mM tris pH 7.5, 300 mM NaCl, 0.1 mM EDTA, 10% (w/v) glycerol, 1 mM $MnCl_2$, 0.1% (v/v) 2-mercaptoethanol and 0.5 mM PMSF by sonication. GST-PP1c was purified sequentially using glutathione Sepharose 4B and Q-Sepharose HP and stored in 25 mM tris pH 7.5, 300 mM NaCl, 0.1 mM EDTA, 1 mM $MnCl_2$, 0.1% (v/v) 2-mercaptoethanol and 50% (v/v) glycerol at −20 °C.

Mass spectrometry analysis

GYS1:GN1 complex was separated by SDS–PAGE and stained with colloidal Coomassie staining solution. The GYS1 band was excised, destained, in-gel reduced and alkylated with iodoacetamide, then dried with acetonitrile followed by Speedvac. Samples were digested with 60 μl of 2 μg/ml trypsin in 50 mM triethylammonium bicarbonate (TEAB) pH 8 overnight. Peptides were extracted with an equal volume of acetonitrile, dried and redissolved in 60 μl 5% acetonitrile/10 mM TEAB. Samples were analysed by LC-MS on a 180 × 0.075 mm packed emitter coupled to an Orbitrap velos mass spectrometry system. Peptides were separated over a 60 min gradient and the 10 most abundant precursors detected in the Orbitrap at 60,000 resolution ($2–4^+$ charge state) were selected for MS/MS in the LTQ velos. To ensure the best fragmentation spectrum for any phosphopeptide was obtained, the precursors were fragmented using multistage activation (MSA) of the precursor mass (*m*/*z*) minus 49, 32.33 and 24.25 Da (this represents the loss of H_3PO_4 from a 2^+, 3^+ or 4^+ precursor ion). Raw files were processed using Proteome Discoverer 1.4 and searched using Mascot 2.3 against the Swissprot database (human only), allowing for carbamidomethyl modification of cysteine, oxidation of methionine and phosphorylation of Ser/Thr/Tyr.

Phosphate content

Covalent phosphate was estimated by alkaline hydrolysis followed by determination of released phosphate by complexometry with a malachite green/molybdate reagent [12].

Electrophoresis and Western blotting

Samples were denatured in Laemmli sample buffer, separated on 8–10% acrylamide tris–glycine gels and stained with Coomassie brilliant blue (CBB) G-250 [13] or transferred to polyvinylidene difluoride membrane in Towbin buffer for 1 h at 100 V constant. Membranes were blocked in 5% (w/v) milk prepared in tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 1 h at room temperature and incubated overnight in primary antibody.