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INCORPORATION OF PHOSPHATE INTO GLYCOGEN BY GLYCOGEN SYNTHASE

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Abstract

The storage polymer glycogen normally contains small amounts of covalently attached phosphate as phosphomonoesters at C2, C3 and C6 atoms of glucose residues. In the absence of the laforin phosphatase, as in the rare childhood epilepsy Lafora disease, the phosphorylation level is elevated and is associated with abnormal glycogen structure that contributes to the pathology. Laforin therefore likely functions *in vivo* as a glycogen phosphatase. The mechanism of glycogen phosphorylation is less well-understood. We have reported that glycogen synthase incorporates phosphate into glycogen via a rare side reaction in which glucose-phosphate rather than glucose is transferred to a growing polyglucose chain (Tagliabracci et al. (2011) *Cell Metab* **13**, 274-282). We proposed a mechanism to account for phosphorylation at C2 and possibly at C3. Our results have since been challenged (Nitschke et al. (2013) *Cell Metab* **17**, 756-767). Here we extend the evidence supporting our conclusion, validating the assay used for the detection of glycogen phosphorylation, measurement of the transfer of ^{32}P from [β-^{32}P]UDP-glucose to glycogen by glycogen synthase. The ^{32}P associated with the glycogen fraction was stable to ethanol precipitation, SDS-PAGE and gel filtration on Sephadex G50. The ^{32}P-signal was not affected by inclusion of excess unlabeled UDP before analysis or by treatment with a UDPase, arguing against the signal being due to contaminating [β-^{32}P]UDP generated in the reaction. Furthermore, [^{32}P]UDP did not bind non-covalently to glycogen. The ^{32}P associated with glycogen was released by laforin treatment, suggesting that it was present as a phosphomonoester. The conclusion is that glycogen synthase can mediate the introduction of phosphate into glycogen, thereby providing a possible mechanism for C2, perhaps C3, phosphorylation.

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Conflict of interest

The authors report no conflicts of interest.

Keywords

Glycogen; phosphorylation; glycogen synthase; laforin; Lafora

1. Introduction

Glycogen is a branched polymeric storage form of glucose present in many cell types. The polymerizing glucose residues are linked by α-1,4-glycosidic bonds formed by the action of glycogen synthase ([1]; Fig. 1). The branches are introduced as α-1,6-glycosidic linkages by the branching enzyme. Glycogen contains other minor constituents besides glucose, the most important of which is covalently attached phosphate [2-5]. Measurements of the abundance of the phosphate have ranged from ~1:500 to ~1:5000 phosphates per glucose residue and depend on the source of the glycogen. Recent studies suggest that the phosphate exists as monoesters at C2, C3 and C6 carbons of glucose residues within the glycogen [6, 7]. Plant amylopectin, which is a close relative of glycogen both chemically and functionally, also contains C3 and C6 phosphomonoesters of glucose [8-11]. No specific function for the phosphate in glycogen is known but its hyperaccumulation appears to disrupt normal glycogen structure [12]. The best evidence comes from studies of Lafora disease [13-17], a fatal teenage-onset myoclonic epilepsy in which an abnormal glycogen-like polymer forms insoluble deposits, Lafora bodies, in neurons, heart, skeletal muscle, skin and other tissues. Lafora disease has been linked, in about 90% of cases, to recessive mutations in one of two genes, *EPM2A* and *EPM2B/NHLRC1*, which encode respectively the proteins laforin [18, 19] and malin [20]. Mice with either locus disrupted accumulate Lafora bodies and have a number of the neurological defects seen in the human disease [13-17, 21]. Laforin, which by sequence can be placed in the sub-family of atypical dual specificity protein phosphatases [22], has been shown to dephosphorylate amylopectin [23, 24], glycogen [24], and phospho-oligosaccharides *in vitro* [25]. Furthermore, glycogen isolated from laforin or malin knockout mice has an elevated phosphate content [6, 24, 26] and, with aging, the glycogen becomes less branched and less water soluble, consistent with the formation of Lafora bodies [12]. Therefore, laforin appears to act as a glycogen phosphatase *in vivo* and its absence results in abnormal glycogen structure which may underlie the pathology of Lafora disease.

The origin of the phosphate present in glycogen is less well understood [5]. Nonetheless, the mechanism of glycogen phosphorylation is an important issue, not only for our fundamental understanding of glycogen metabolism but also because it could guide efforts to suppress glycogen phosphate accumulation as a therapy for Lafora disease. The phosphate present in plant starch is known to be introduced by specific dikinase enzymes [27-29]. However, neither bioinformatic nor biochemical studies have so far revealed comparable enzymes in mammals. Another possibility, that we have proposed, is that glycogen synthase itself can introduce covalently-linked phosphate into glycogen. By using [β-^{32}P]UDP-glucose as a substrate, we had shown that glycogen synthase could incorporate ^{32}P into newly synthesized glycogen. We attributed this result to the transfer of the β-phosphate of UDP-glucose into glycogen as a rare side reaction, once every ~10,000 normal catalytic cycles ([25]; Fig. 1). We proposed that the mechanism might involve a cyclic phosphate diester

intermediate that could explain phosphorylation at C2 and possibly at C3. It is difficult, however, to explain C6 phosphorylation by such a mechanism. Enzymological and structural studies did provide further support the involvement of cyclic phosphate [30]. First, a crystal structure of glycogen synthase with glucose-1,2-cyclic phosphate bound indicated that the catalytic site could accommodate the cyclic ester in a manner consistent with the proposed mechanism. Secondly, incubation of glycogen synthase with UDP-glucose resulted in the generation of the cyclic phosphate. Others have challenged this conclusion, suggesting that the ^{32}P signal detected was due to the normal reaction product [β-^{32}P]UDP binding non-covalently to glycogen [7]. In the present study, we describe more extensive investigation of the phosphorylation of glycogen and provide further evidence that glycogen synthase is capable of transferring the β-phosphate of UDP-glucose to glycogen.

2. Materials and methods

2.1 Reagents

[γ-^{32}P]ATP (NEG002A001MC; specific activity 3,000 Ci/mmol in 10 mM Tricine pH 7.6) and UDP-[U-^{14}C]glucose (NEC403V050UC; specific activity 250 mCi/mmol in ethanol:water 2:98) were from PerkinElmer. UDP for competition assays was from Sigma (#94330). High purity UDP (99.2%) used in laforin reactions was from Chem-Impex International, Inc. (#00310). [α-^{32}P]UDP (SCP230/37; specific activity 6,000 Ci/mmol in 50 mM Tricine pH 7.4) was from Hartmann Analytic. Materials for gel filtration experiments were Sephadex G-50 (Sigma, G-50-150) and Spin Columns (Promega, C1281). Glycogen was purified from the skeletal muscle of New Zealand White rabbits as described previously [6].

2.2 Enzymes

Recombinant yeast Gsy2p was produced in *E. coli* and purified as described by Baskaran et al. [31]. Recombinant human GYS1 glycogen synthase was produced in insect cells and purified as described by Khanna et al. [32]. Hexokinase (#1012765501) and pyrophosphatase (#91078329) were from Roche. Phosphoglucomutase (#46550003) was from Oriental Yeast Co., LTD. Recombinant mouse laforin, wild type and C266S mutant, were purified as described previously [33]. Recombinant *Leishmania* UDP-glucose pyrophosphorylase protein was produced and purified as described by Lamerz et al. [34]. Recombinant human soluble calcium-activated nucleotidase (hSCAN-1) was produced and purified as previously described [35].

2.3 Synthesis of [β-^{32}P]UDP-glucose

The synthesis of [β-^{32}P]UDP-glucose was similar to the protocol described by Heyen et al [36]. ^{32}P from the γ-phosphate of ATP is transferred to glucose by hexokinase, the resulting glucose-6-P isomerized by phosphoglucomutase to glucose-1-P which is converted to UDP-glucose by UDP-glucose pyrophosphorylase. [γ-32P]ATP (~1.5 mCi at a specific activity of 3,000 Ci/mol) was dried under nitrogen and then dissolved in 160 μl of a solution containing 62.5 mM Tris-HCl pH 7.6, 6.25 mM glucose, 7.5 mM $MgCl_2$, 1 mM UTP, 1.125 mM DTT, 16 U/ml pyrophosphatase, 38 U/ml phosphoglucomutase and 13 μg/ml of *Leishmania* UDP-glucose-pyrophosphorylase. The reaction was started by addition of 40 μl of hexokinase for

a final concentration of 30 U/ml in the 200 μl reaction volume. After incubation at 30°C for 2 hr, the reaction was terminated by boiling in a water bath for 5 min followed by cooling on ice and centrifugation for 10 min at 15,000 x g at 4°C to remove denatured proteins.

We also developed a two-step procedure in which the hexokinase reaction was conducted separately and the ATP concentration was increased by addition of unlabeled nucleotide. [γ-32P]ATP (~1.5 or 3 mCi) was dried as above, dissolved in 80 μl of 62.5 mM Tris-HCl pH 7.6, 6.25 mM glucose, 7.5 mM $MgCl_2$, 31.25 μM ATP and the reaction initiated by addition of 20 μl of hexokinase for a final concentration of 30 U/ml. After incubation at 30°C for 60 min, the reaction was terminated by boiling in a water bath for 5 min, cooled on ice and centrifuged for 10 min at 15,000 x g at 4°C. The supernatant was transferred to a new tube to which the remaining enzymes were added for final concentrations of 13 U/ml pyrophosphatase, 30 U/ml phosphoglucomutase and 13 μg/ml UDP-glucose pyrophosphorylase in a 200 μl reaction containing 50 mM Tris-HCl pH 7.6, 6 mM $MgCl_2$, 0.9 mM dithiothreitol (DTT), 0.8 mM UTP. After 60 min at 30°C, the reaction was terminated by boiling in a water bath for 5 min, cooled on ice, and centrifuged for 10 min at 15,000 x g at 4°C to remove denatured proteins.

In either procedure, the [β-^{32}P]UDP-glucose was purified by adsorption on activated charcoal. The supernatant was transferred to a tube containing ~7 mg charcoal, vortexed for 1 min and incubated on ice for 10 min. The activated charcoal was collected by centrifugation at 15,000 x g for 2 min at 4°C. The charcoal was then suspended in 1 ml ice cold water (from MilliQ), vortexed for 30 sec, and centrifuged at 15,000 x g for 2 min at 4°C. After three washes, [β-^{32}P]UDP-glucose was eluted by suspending the charcoal in 200 μl of 0.16 M NH_4OH in 50% (v/v) ethanol, vortexing for 30 sec and centrifuging at 15,000 x g for 2 min at 4°C, for a total of 4 elutions. The pooled eluates were centrifuged once more and the supernatant passed through a Costar Spin-X Centrifuge Tube Filter that had been equilibrated in 0.16 M NH_4OH, 50% (v/v) ethanol to remove any residual charcoal. The filtered eluate was dried extensively in a Speed Vac, dissolved in 100 μl of 10 mM Tris-HCl pH 7.5 and stored in aliquots at −80°C.

The radiochemical purity of the [β-^{32}P]UDP-glucose was assessed by TLC as in Heyen et al. [36] and judged to be ~99% pure. All radioactivity was converted to glucose-1-^{32}P by treatment with UDP-glucose pyrophosphatase [36]. Both synthetic protocols yielded [β-^{32}P]UDP-glucose of similar purity but the two-step procedure had higher yields, ~70% of the starting radioactivity compared to the one-step method, ~50%. [β-^{32}P]UDP-glucose purified by the two step method was used for most of the experiments reported.

2.4 Preparation and SDS-PAGE analysis of ^{32}P and ^{14}C glycogen

Purified rabbit skeletal muscle glycogen was incubated with recombinant yeast or human glycogen synthase enzymes essentially as described by Tagliabracci et al. [25] to produce ^{14}C- or ^{32}P-labeled glycogen[1]. Glycogen synthase was diluted into 50 mM Tris-

[1]For simplicity we sometimes refer to the polymer synthesized *in vitro* by incubation of glycogen and glycogen synthase as "glycogen" although technically this is not strictly correct since the elongated outer chains will not have the branching pattern of native glycogen.

HCl pH 7.8, 1 mg/ml glycogen,1mM DTT immediately prior to being added to start the reactions. The glycogen synthase reactions contained 50 mM Tris HCl pH7.8, 5 mM EDTA, 7.2 mM glucose-6-P, 6.9 mg/ml glycogen, 2-5 μM UDP-[U-^{14}C]-glucose (330-450 cpm/pmol) or [β ^{32}P]-UDP-glucose (75,000-260,000 cpm/pmol), and yeast (2-10 μg/ml) or human (20 μg/ml) glycogen synthase. Control reactions lacked glycogen synthase. At the indicated times, samples were removed, boiled in a water bath for 5 min, cooled on ice, and centrifuged to collect the reaction solution. In some cases, small aliquots were withdrawn for analysis by TLC. Glycogen was precipitated from the remainder by addition of 2 volumes of cold (−20°C or −80°C) 100% ethanol, as well as Na_2SO_4 and LiCl to final concentrations of 2.8 mM and 20 mM respectively to aid precipitation. Samples remained at −20°C for a minimum of 4 hr to allow precipitation, boiled for 2 min to aid glycogen aggregation, cooled on ice for 10 min, centrifuged at 15,000 x g for 30 min and the supernatant removed. The pellet was dried with a Speed Vac and then dissolved in water or buffer as needed, usually to the original sample volume. If further precipitations were to be performed, LiCl was added to a concentration of 2 mM followed by 2 volumes of cold ethanol and processed as described above. For SDS-PAGE, glycogen pellets were dissolved in 25 μl of water or buffer and 1/5 volume of 5X SDS loading buffer (60 mM Tris-PO_4 pH 6.8, 40% glycerol, 5% SDS, 0.08% Bromophenol Blue, and 5%(v/v) β-mercaptoethanol), boiled for 5 min and samples were loaded into wells on a 10% (w/v) acrylamide gel with a 4% (w/v) stacking gel. Loadings corresponded to 250-375 μg of the initial glycogen present in the reaction. Gels were developed with 180 volts for ~45 min. After electrophoresis, gels were dried and imaged with a FujiFilm FLA-5100 Phosphorimager.

2.5 Treatments of ^{32}P- and ^{14}C-labeled glycogen

^{32}P- and ^{14}C-labeled glycogen produced by glycogen synthase reactions was purified through ethanol precipitation(s) and subjected to various additional treatments prior to analysis by SDS-PAGE.

Gel filtration—After reaction with glycogen synthase, the glycogen underwent two rounds of ethanol precipitation, as described above except that the reaction was diluted two-fold initially and dissolved in two times the reaction volume after the first precipitation. The glycogen was finally dissolved in 150-160 μl of 10 mM Tris-HCl pH 7.5. A portion (70 μl) was subjected to gel filtration on a spin column (Promega) containing ~1 ml of packed Sephadex G50 resin (Sigma) which had been equilibrated with 10 mM Tris-HCl pH 7.5 and extensively washed with the same buffer. Immediately prior to loading the samples, excess buffer was removed by centrifugation (1,000 x g for 1 min at 16°C). Sample (70 μl) was applied to the spin column, the column centrifuged (1,000 x g for 1 min at 16°C), and the flow-through collected. The gel filtered glycogen and an equivalent unfiltered aliquot of 70 μl were dried in a Speed Vac, dissolved in 25 μl of 10 mM Tris-HCl pH 7.5 and aliquots (20 μl) were made 1X in SDS loading buffer and subjected to SDS-PAGE. From quantitation of the ^{14}C-labeled glycogen, recovery after gel filtration was ~95%.

Glucosidase and laforin treatment—After ethanol precipitation, glycogen was dissolved in water and adjusted to 20 mM sodium acetate, pH 4.8. For glucosidase treatment, the glycogen was incubated with or without 0.3 mg/ml of both α-amylase and

amyloglucosidase, unless noted otherwise, for 2 hr at 42°C. In other experiments, the glycogen was incubated with 25 or 50 μg/ml of either mouse wild type laforin or catalytically inactive C266S laforin at 37°C for 2 hr. Samples were boiled for 5 min, cooled on ice, and condensation collected by centrifugation prior to analysis by SDS-PAGE. For analysis of $^{32}P_i$ release, ^{32}P-glycogen was purified by ethanol precipitation, treatment with PiBind™ resin (Innova Biosciences, 501-0051) and gel filtration as described above. The PiBind™ resin binds and removes free inorganic phosphate from samples and is marketed for this purpose. This step was added to minimize inorganic phosphate contamination of the ^{32}P-glycogen. We observed that the resin also chelated and removed UDP and UDP-glucose, but not ^{32}P-glycogen, from our samples (data not shown).

Hydrolysis of UDP with recombinant human soluble calcium-activated nucleotidase (hSCAN-1)—hSCAN-1 is a nucleotidase that converts UDP to UMP but cannot hydrolyze UMP [35, 37] or UDP-glucose and glucose-6-P (data not shown). ^{32}P- and ^{14}C-labeled glycogen produced by glycogen synthase action was purified by three cycles of ethanol precipitation, dissolved in 50 mM Tris-HCl pH 7.5, 0.05% (v/v) Tween-20, and incubated at 37°C for 30 min with or without 1.2 μg/ml hSCAN-1 plus 2 mM $CaCl_2$. Reactions were terminated by boiling and processed for SDS-PAGE as described. In other experiments, glycogen synthase reactions were terminated by boiling, 2 mM UDP was added and the sample incubated at 37°C for 30 min with or without 1.2 μg/ml hSCAN-1 plus 2 mM $CaCl_2$ in excess of the EDTA concentration. Samples were then analyzed by TLC and/or SDS-PAGE.

Competition with unlabeled UDP—^{32}P- and ^{14}C-labeled glycogen produced by glycogen synthase action was purified by three cycles of ethanol precipitation and dissolved in 50 mM Tris-HCl pH 7.5, 0.05% (v/v) Tween-20. Once the glycogen was completely in solution, 2 mM UDP was added, so as to be at a 1000-fold excess compared to labeled UDP-glucose, and incubated at room temperature for 30 min. In other experiments, the UDP was added prior to ethanol precipitation. Samples were then processed and analyzed by SDS-PAGE.

2.6 Thin Layer Chromatography

Two chromatographic systems were used. High-performance thin layer chromatography (HPTLC) plates (Merk, Silica gel 60 F_{254}) were used for TLC analysis of nucleotides when reaction mixes were treated with hSCAN-1. One or two μl of sample was spotted on the plate which was developed by ascending chromatography with n-propanol:ethyl acetate:water (7:1:4). Plates were laid flat and allowed to air dry at room temperature. Radioactivity was visualized using either a Phoshorimager or by exposing the plates to X-ray film. For visualization of UV absorbing compounds, the plates were placed under a UV lamp (λ = 254 nm), the UV absorbing regions were marked, and a photograph of the plate was taken. For analysis of $^{32}P_i$ release from ^{32}P-glycogen, polyethyleneimine (PEI) cellulose plates (Merck, 1.05725.0001) were developed by ascending chromatography with 1M acetic acid: 3M LiCl (9:1). Plates were air dried and imaged as described above.

2.7 Analysis of laforin activity towards UDP and UDP-glucose

The ability of laforin to hydrolyze UDP was tested by incubating 2.5 mM UDP with 25 μg/ml wild type laforin in 20 mM sodium acetate, pH 4.8 for 2 hr at 37°C. A control reaction contained 25 μg/ml catalytically inactive C266S laforin. UDP-glucose, 2.5 mM, was also tested as a substrate. The wild type laforin hydrolyzed *p*-nitrophenolphosphate (specific activity 5.3 nmol/min/μg). The reactions were incubated at 37°C for 2 hr and terminated by boiling for 5 min. Samples, diluted to 50 μM with respect to the starting UDP or UDP-glucose concentrations, were passed through a centrifuge filter (Costar Spin-X, 8160) and 25 μl aliquots loaded onto a PA200 column for separation by high-performance anion exchange chromatography (HPAEC) using a Dionex ICS 3000 instrument and a gradient of sodium acetate. The starting mobile phase, eluent A, consisted of 1 mM NaOH and eluent B was 1 mM NaOH, 1M sodium acetate. The elution profile, in terms of percentage of eluant B, was 20-50% from 0-10 min, 55-85% from 10-35 min and 85-100% from 35-40 min with a flow rate of 0.30 ml/min. The nucleotides were detected by UV absorption at 262 nm.

2.8 Assessment of UDP binding to glycogen

Rabbit skeletal muscle glycogen at 8.3 mg/ml was incubated in 50 mM Tris-HCl pH 7.8, 5 mM EDTA, 7.2 mM glucose-6-P, 1 μM UDP-[U-^{14}C]glucose (specific activity ~450 cpm/pmol), with or without 4 μM [α-^{32}P]UDP(specific activity of ~100,000 cpm/pmol), and with or without 10 μg/ml yeast glycogen synthase for 60 min at 30°C. The incubation was initiated by addition of glycogen synthase or vehicle. Reactions were terminated by boiling for 5 min, cooled on ice, and precipitated with ethanol as already described. After drying in a Speed Vac, glycogen was dissolved in 1) 48 μl of water, 2) 5 mM UDP or 3) 20 mM sodium acetate pH 4.8 for glucosidase digestion. Amyloglucosidase and α-amylase, each at 60 μg/ml, were added and incubated overnight at 40°C. Control samples were kept at −20°C. All samples were boiled for 5 min, cooled on ice and analyzed by SDS-PAGE.

3. Results and discussion

3.1 Assay of the incorporation of the β-phosphate of UDP-glucose into glycogen

The assay for glycogen phosphorylation by glycogen synthase is based on a procedure developed for measurement of glucose-1-phosphotransferase activity involved in glycoprotein synthesis [38]. Central to the method is the use of [β-^{32}P]UDP-glucose as a substrate so that if the β-phosphate is transferred, ^{32}P-glycogen is formed by elongating unlabeled glycogen (Fig. 1, lower reaction). With this substrate, the normal transfer of glucose to glycogen is accompanied by the generation of [β-^{32}P]UDP as product. Separation of [β-^{32}P]UDP from glycogen after the reaction is achieved by 1) precipitation of glycogen with ethanol followed by re-dissolving in aqueous solution (one to three times) and 2) SDS-PAGE. By incubating with either UDP-[U-^{14}C]glucose or [β-^{32}P]UDP-glucose, time-dependent increases in both ^{14}C and ^{32}P signals were observed, using purified yeast glycogen synthase, as we reported previously (Fig. 2; [25]). High molecular weight glycogen is mostly retained at the bottom of the well, although signal often extends into the stacking gel, presumably smaller polysaccharide molecules that sometimes accumulate at the stacking-separating gel interface (just visible in Fig. 2, more prominent in some other figures). Negatively charged, small molecules like nucleotides would be expected to migrate

through the separating gel where no radioactivity was detected (Fig. 2). Note that even in control reactions lacking enzyme a detectable background at the bottom of the well is always observed when [β-^{32}P]UDP-glucose is used but less so with UDP-[U-^{14}C]glucose.

3.2 Evidence that phosphate incorporated by glycogen synthase is associated with glycogen

Gel filtration is frequently used as a means to remove small, non-covalently bound ligands from macromolecules. Therefore, we applied this technique to analyze glycogen elongated by incubation with [β-^{32}P]UDP-glucose and glycogen synthase to see whether the radioactive signal attributed to ^{32}P-glycogen could be eliminated or reduced by gel filtration. Parallel reactions contained UDP-[U-^{14}C]glucose. After the ethanol precipitation of glycogen, one aliquot of the re-dissolved samples was additionally subjected to gel filtration through Sephadex G50 prior to SDS-PAGE. Reactions with purified yeast (Fig. 3A) or human (Fig. 3B) glycogen synthase gave the expected time-dependent increase in both ^{32}P and ^{14}C labeling. Gel filtration, in our hands, had little effect on these signals when compared to samples that had not passed through Sephadex G50 (Fig. 3). Note that in controls (lanes “C”), incubations with [β-^{32}P]UDP-glucose present but lacking glycogen synthase, background was still detected even after gel filtration. However, we conclude that the bulk of the ^{32}P or ^{14}C radioactivity running with glycogen in SDS-PAGE is stable to gel filtration.

Evidence that the ^{32}P is covalently linked to the synthesized polysaccharide comes from the fact that catalytically active wild-type laforin, which has well documented glucan phosphatase activity, was able to reduce the ^{32}P-signal to the background level (Fig. 4A). Laforin had no effect on the ^{14}C-signal. In a control, the glycogen was exposed to a catalytically inactive C266S laforin mutant protein, unable to dephosphorylate glycogen [24], which caused no change in either the ^{32}P or the ^{14}C signal. As expected, treatment of the elongated glycogen with glucosidases (amyloglucosidase and α-amylase) to degrade the glucose polymer eliminated both the ^{32}P and the ^{14}C signals, implying their association with the synthesized glycogen, as has been previously shown.

The argument could be made that laforin removes the β-phosphate of [β-^{32}P]UDP non-covalently bound to glycogen. Although there have been no reports of such an activity for laforin, we tested this hypothesis by incubating active or C266S mutant laforin with UDP and analyzing the nucleotide composition by HPAEC. Even commercially available UDP of the highest purity (99.2%) had trace amounts of contaminating UMP by this analysis (Fig. 4B). However, exposure of the UDP to either wild-type or C266S mutant laforin had no effect on the UDP level and no increase in UMP was detected. Nor was UDP-glucose a substrate for hydrolysis by laforin (Fig. 4B), as expected for a monoesterase. The simplest conclusion from these experiments is that laforin hydrolyzes phosphomonesters present in the glycogen.

In other experiments, we showed that exposure of ^{32}P-glycogen to laforin led to the release of ^{32}P-labeled inorganic phosphate ($^{32}P_i$) (Fig. 5A). The ^{32}P-glycogen in this case was purified by ethanol precipitation, gel filtration and treatment with PiBind™ resin to eliminate phosphate contamination. The reaction products were analyzed by TLC using PEI-cellulose

plates. Labeled glycogen was concentrated at the origin with a “comet trail” extending into the chromatogram (Fig 5). The presence of active laforin resulted in the generation of $^{32}P_i$ and a reduction in the ^{32}P-glycogen signal (Fig. 5A) whereas the ^{14}C-glycogen signal was unchanged (Fig. 5B). When laforin was omitted (“C”) or heat inactivated (“HI”), no phosphate signal was visible. We conclude that the ^{32}P is covalently attached to glucose residues of glycogen by phosphomonoester linkages and that laforin can release inorganic phosphate from glycogen by hydrolysis.

3.3 Further controls that [^{32}P]UDP binding to glycogen is not responsible for the radioactivity associated with ^{32}P-glycogen

To address the possibility that the ^{32}P signal associated with the synthesized glycogen is caused by non-covalent binding of the [β-^{32}P]UDP produced in the reaction, we conducted three other types of control experiments. In the first, we added a 1000-fold molar excess of unlabeled UDP after the glycogen synthesis reactions but before analysis by SDS-PAGE (Fig. 6). Were the ^{32}P associated with glycogen due to the presence of [β-^{32}P]UDP, the unlabeled UDP should be able to compete with the radioactive nucleotide. However, no effect of cold UDP on the ^{32}P-glycogen signal was detected, whether added before (data not shown) or after the ethanol precipitation step (Fig. 6). As expected, the presence of unlabeled UDP had no effect on the ^{14}C-signal in the control reaction.

In a second approach, advantage was taken of hSCAN-1, a UDPase that hydrolyzes UDP to UMP [35, 37] but which does not hydrolyze UDP-glucose or UMP. In Fig. 7, glycogen synthase reactions were analyzed, using either [β-^{32}P]UDP-glucose or UDP-[U-^{14}C]glucose as substrate, with aliquots withdrawn at the indicated times, the reaction terminated by boiling for 5 min, exposed or not to hSCAN-1, separated by TLC and visualized under UV light and also quantitated with a Phosphorimager. During the glycogen synthase reaction, we observed a time dependent consumption of the radioactive UDP-glucose substrate; with [β-^{32}P]UDP-glucose, the concomitant generation of [β-^{32}P]UDP was detected and with UDP-[U-^{14}C]glucose there was sufficient signal to observe ^{14}C-glycogen accumulation at the origin of the plate. Small amounts of glucose-1,2-cyclic phosphate, the fast ester of Leloir [39], were also detected; it is a normal contaminant of UDP-glucose. The presence of hSCAN-1 correlated with the detection of inorganic $^{32}P_i$ whose accumulation matched the disappearance of [β-^{32}P]UDP-glucose. The amount of radioactive UDP-glucose was low enough (2 μM) that it was not detectable by UV on the TLC plate. Therefore, additional UDP was added before exposure to hSCAN-1. The essentially complete conversion of UDP to UMP by hSCAN-1 was evident by monitoring the UV absorbance on the TLC plate. Having established that hSCAN-1 could convert [β-^{32}P]UDP to $^{32}P_i$ and unlabeled UMP under relevant conditions, we went on to conduct glycogen synthetic reactions with [β-^{32}P]UDP-glucose or UDP-[U-^{14}C]glucose as substrates and exposed the products to hSCAN-1 after (Fig. 8) or prior to (not shown) ethanol precipitation followed by analysis by SDS-PAGE (Fig. 8). Exposure to hSCAN-1 did not decrease the incorporation of either ^{32}P or ^{14}C into glycogen with either protocol, providing further evidence that the observed ^{32}P signal was not due to [β-^{32}P]UDP.

A third control was to test whether non-covalent association of ^{32}P-UDP with glycogen could be detected after SDS-PAGE. For these experiments, we utilized commercially available [α-^{32}P]UDP. The amount of [α-^{32}P]UDP radioactivity present (550,000 cpm/μl) exceeded the amount of radioactivity in [β-^{32}P]UDP-glucose (180,000-400,000 cpm/μl) used in the glycogen labeling experiments described above. UDP-[U-^{14}C]glucose was present at 400-1,800 cpm/μl. After incubation with glycogen, the presence of [α-^{32}P]UDP contributed a slight increase in background corresponding to the region where glycogen runs at the bottom of the gel well when compared with reactions containing only UDP-[U-^{14}C]glucose (Figs. 9A and B, comparing lanes 4 and 8). A signal with a diffuse appearance was also seen above the stacking-separating gel interface, which was not observed in reactions lacking [β-^{32}P]UDP. The presence of a 1,000-fold molar excess of unlabeled UDP had no effect on either signal (Fig. 9B, lanes 5 and 9) and neither did treatment with glucosidases (Fig. 9A, compare lanes 5 and 9 to lanes 4 and 8, respectively). This background radioactivity is therefore associated with neither glycogen nor UDP. To mimic better the glycogen synthesis reactions with [β-^{32}P]UDP-glucose, in which [β-^{32}P]UDP is generated concomitant with chain elongation that might physically trap the UDP, parallel incubations were conducted in the presence of glycogen synthase. The glycogen synthase was active as indicated by the generation of a radioactive signal, due to ^{14}C incorporation, at the well bottoms (Figs. 9A and B, lane 6). In the presence of [α-^{32}P]UDP, there was no increase in the radioactive signal (Figs. 9A and B, compare lanes 2 and 6). The presence of cold UDP had no effect (Fig. 9A, compare lanes 3 and 7 to lanes 2 and 6, respectively) whereas treatment with glucosidases returned the signals to background (Fig. 9B, lanes 3 and 7). We therefore found no evidence under our conditions for non-covalent binding of ^{32}P-UDP to glycogen sufficient to account for the ^{32}P incorporation into glycogen observed when [β-^{32}P]UDP-glucose was incubated with glycogen synthase.

4. Conclusion

All enzyme assays that utilize a radioactive substrate to monitor the progress of a chemical reaction depend on the ability to physically separate product from substrate. The most common standard assay for glycogen synthase activity, as an example, monitors the transfer of [U-^{14}C]glucose from UDP-[U-^{14}C]glucose into glycogen, taking advantage of the insolubility of glycogen in ethanol to separate substrate and product [40]. Reaction aliquots are typically deposited on filter paper squares, immersed in 66% (v/v) ethanol, and washed extensively before quantitating the ethanol-insoluble radioactivity. In the present study, it is a minor side reaction that is of interest, [^{32}P]phosphate incorporation into glycogen, and so the task is to resolve ^{32}P-glycogen from unreacted [β-^{32}P]UDP-glucose as well as the main radioactive reaction product, [β-^{32}P]UDP. Furthermore, since the side reaction occurs at a rate about one ten thousandth of the rate of the main reaction, very high specific activity [β-^{32}P]UDP-glucose (75,000-260,000 cpm/pmol) is necessary to obtain sufficient sensitivity. This requires that even the most minor radioactive impurities must be considered. We found early in our studies that the standard filter paper assay was inadequate for monitoring ^{32}P incorporation into glycogen because of very high backgrounds. We therefore developed a protocol in which glycogen synthesis reactions were terminated, the polysaccharide was first separated from the bulk reactants by ethanol precipitation, the precipitate re-dissolved in

SDS-buffer, boiled and then subjected to SDS-PAGE [25]. The rationale was that non-covalent interactions would not survive this treatment. Indeed, SDS-PAGE is frequently and successfully used to separate ^{32}P-labeled proteins from high specific activity [γ-^{32}P]ATP in protein kinase reactions validating in principle the concept that radioactive nucleotides can be removed by gel electrophoresis. From the experiments described in this work, we have confirmed our previous findings and added several additional controls that further validate the analysis. The ^{32}P-signal that we attribute to ^{32}P-glycogen is stable to gel filtration, unaffected by inclusion of molar excesses of cold UDP, unaffected by exposure to a specific UDPase and susceptible to laforin treatment. In addition, we find no evidence for significant non-covalent binding of UDP to glycogen.

Since our first report that glycogen synthase could transfer phosphate to glycogen [25], Nitschke et al [7] concluded that the ^{32}P-signal, which we had interpreted to be ^{32}P-labeled glycogen, was in fact caused by contaminating [β-^{32}P]UDP bound tightly enough to glycogen to remain associated with the polymer through the SDS-PAGE separation. Their most compelling evidence was that gel filtration of samples significantly reduced the ^{32}P-signal seen after SDS-PAGE, although it was not entirely eliminated and a weaker signal remained that increased with reaction time. In this present study, we cannot corroborate their findings and show instead that the main ^{32}P-signal that we have studied is stable to gel filtration. The other principal difference between our results and those of Nitschke et al [7] concerns the susceptibility of synthesized ^{32}P-glycogen to hydrolysis by laforin. In our hands, we are able to reduce the ^{32}P signal of the glycogen to background level by exposure of the glycogen to active laforin. In addition, exposure of ^{32}P-glycogen to active laforin generated free $^{32}P_i$. These results we take as evidence that the ^{32}P is covalently attached to glycogen by a phosphomonoester bond, given the known substrate specificity of laforin. Thus, from the multiple lines of evidence presented in this study, we are confident that the large majority of the ^{32}P-signal associated with glycogen is indeed due to covalent phosphorylation of the polysaccharide by glycogen synthase.

Acknowledgments

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Highlights

- Glycogen synthase incorporates β-phosphate of UDP-glucose into glycogen
- ^{32}P-labeling of glycogen using [β-^{32}P]UDP-glucose as substrate monitors the reaction
- The ^{32}P associated with glycogen is not due to the non-covalent binding of [β-^{32}P]UDP

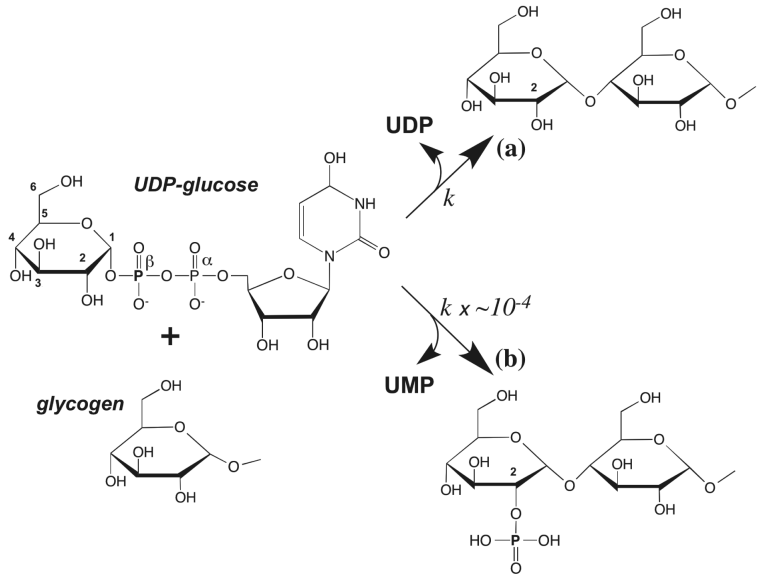

Figure 1.

Reactions of glycogen synthase. Shown are (a) the primary reaction catalyzed by glycogen synthase in which glucose is transferred from the substrate UDP-glucose to the non-reducing end of a polyglucose chain in glycogen and (b) the side reaction in which the β-phosphate of UDP-glucose is transferred to add a glucose-phosphate unit (see text). The rate constants are to indicate that the side reaction occurs at around one ten thousandth of the rate of the main reaction.

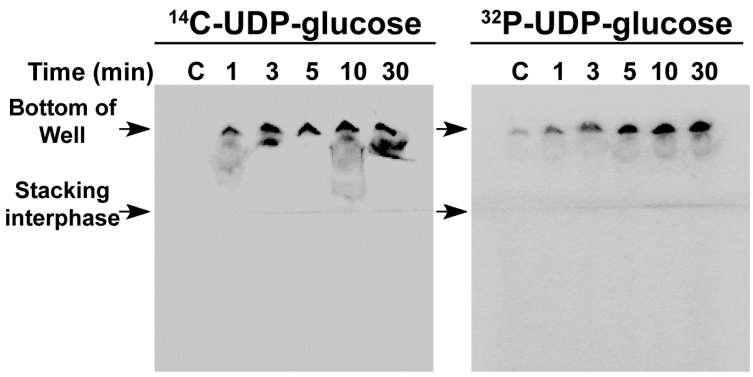

Figure 2.

Time-dependent incorporation of ^{32}P into synthesized glycogen by glycogen synthase. Glycogen synthase (yeast Gsy2p, 5 μg/ml) was incubated with glycogen, and 2 μM [β-^{32}P]UDP-glucose or UDP-[U-^{14}C]glucose, aliquots were removed at the indicated times, precipitated with ethanol and analyzed by SDS-PAGE (see Materials and Methods). C indicates a control reaction lacking enzyme. Dried gels were analyzed by a Phosphorimager. The bottom of the gel wells and the stacking/separating gel interfaces are indicated with arrows.

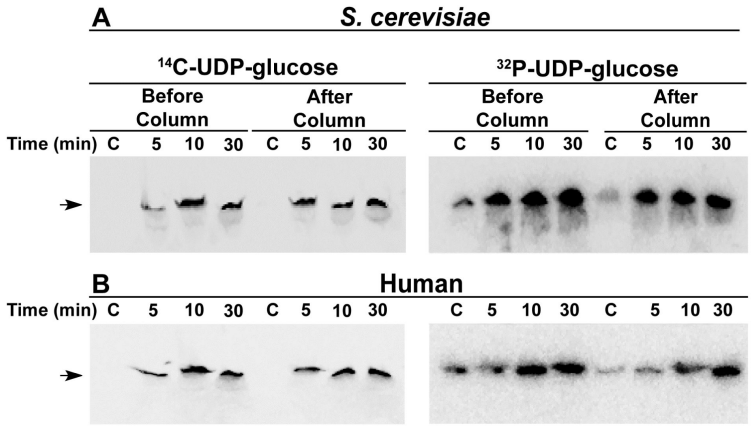

Figure 3.

Effect of gel filtration on ^{32}P-labeled glycogen. Reactions with 2 μM [β-^{32}P]UDP-glucose (right panels) or [U-^{14}C]glucose (left panels), glycogen and yeast (**A**) or human (**B**) glycogen synthase (2 μg/ml) were sampled at the indicated times, aliquots precipitated with ethanol two times, and treated or not by gel filtration over Sephadex G50 and analyzed by SDS-PAGE (see Materials and Methods). “C” indicates a control reaction lacking glycogen synthase. Dried gels were analyzed by a Phosphorimager.

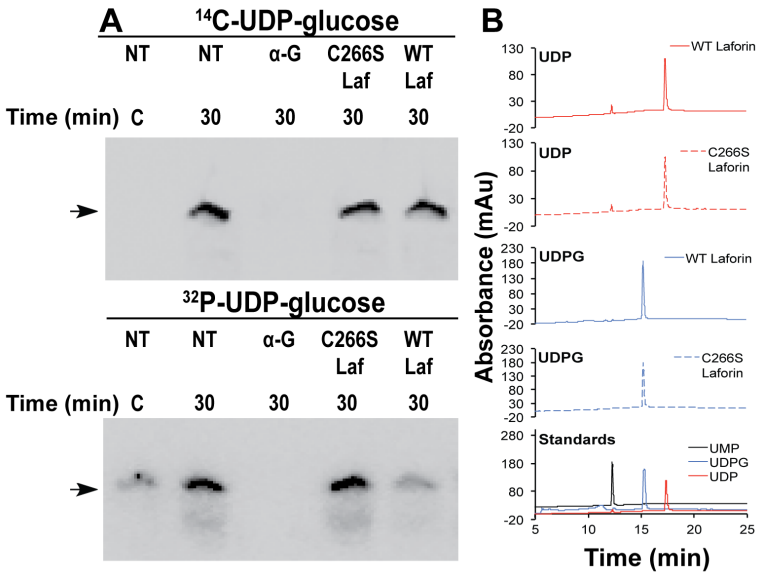

Figure 4.

Effect of treating ^{32}P-labeled glycogen with laforin or glucosidases. **A** Glycogen was labeled by incubation with 5 μM [β-^{32}P]UDP-glucose (lower panel) or [U-^{14}C]glucose (upper panel) and yeast glycogen synthase (2 μg/ml) for 30 min. Glycogen was precipitated with ethanol, dissolved in buffer and treated with α-glucosidases (α-amylase and amyloglucosidase) (α-G), inactive mutant laforin (C266S Laf) or wild type laforin (WT Laf) as indicated, and analyzed by SDS-Page (see Materials and Methods). Dried gels were analyzed by a Phosphorimager. C, control reaction lacking glycogen synthase; NT, not treated. **B** UDP-glucose and UDP were incubated with active (WT) or inactive (C266S) laforin as indicated and analyzed by HPAEC. Chromatograms of UDP, UMP and UDP-glucose standards are shown in the lowermost panel.

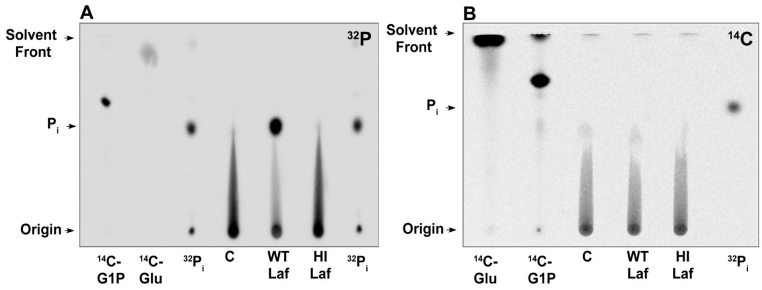

Figure 5.

Release of $^{32}P_i$ from ^{32}P-glycogen by laforin. Glycogen was labeled by incubation with 5 μM [β-^{32}P]UDP-glucose (**A**) or [U-^{14}C]glucose (**B**) and yeast glycogen synthase (5 μg/ml) for 30 min. Glycogen was precipitated three times with ethanol, treated with PiBind™ resin, purified by gel filtration and dissolved in buffer. The glycogen was incubated for 2 hr with 50 μg/ml laforin (WT Laf) or laforin inactivated by boiling for 5 min (HI Laf). A control (C) lacked laforin. The reaction mixtures were analyzed by TLC using PEI-cellulose plates. Standards of glucose-1-P (^{14}C-G1P), glucose (^{14}C-Glu) and inorganic phosphate ($^{32}P_i$), labeled with the indicated isotope, were also analyzed.

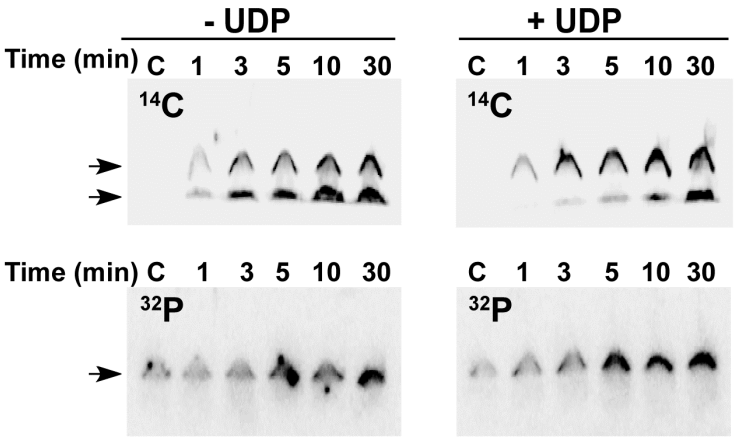

Figure 6.

Effect of unlabeled UDP on ^{32}P-labeling of glycogen. Glycogen was labeled by incubation with 2 μM [β-^{32}P]UDP-glucose (lower panel) or [U-^{14}C]glucose (upper panel) and yeast glycogen synthase (5 μg/ml) for the indicated times. Unlabeled UDP (2 mM, a 1000-fold excess of UDP-glucose) was added prior to SDS-PAGE (see Materials and Methods). C, control reaction lacking glycogen synthase.

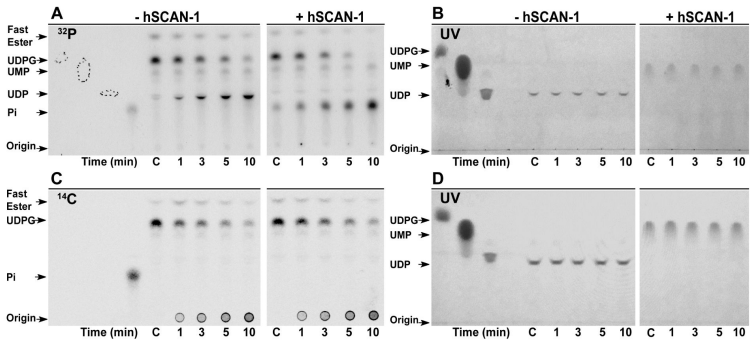

Figure 7.

Analysis of the progress of glycogen synthesis by glycogen synthase. Glycogen was synthesized by incubation with 2 μM [β-^{32}P]UDP-glucose (**A,B**) or [U-^{14}C]glucose (**C,D**) and yeast glycogen synthase (5 μg/ml), and aliquots removed at the indicated times. C corresponds to reactions lacking glycogen synthase. After addition of 2 mM UDP, the aliquots were analyzed by TLC and visualized either by Phosphorimager (**A,C**) or UV (**B, D**). Standards of UDP-glucose, UMP, UDP and [^{32}P]phosphate were present in the left most tracks and their migrations indicated. Prior to chromatography, the aliquots were treated or not with the UDPase hSCAN-1 for 30 min, as indicated (see Materials and Methods).

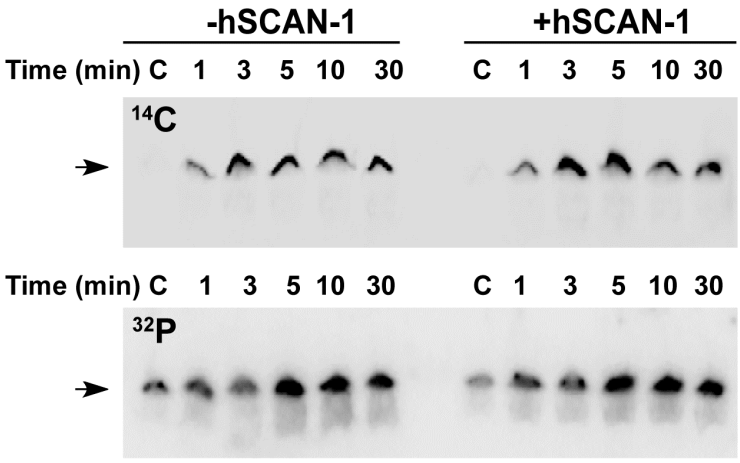

Figure 8.

Effect of hSCAN-1 treatment on ^{32}P-labeling of glycogen. Glycogen was labeled by incubation with 2 μM [U-^{14}C]glucose (upper panel) or [β-^{32}P]UDP-glucose (lower panel) and yeast glycogen synthase (5 μg/ml), and aliquots removed at the indicated times. After ethanol precipitation, samples were treated with hSCAN-1(+hSCAN-1) or without (−hSCAN-1) for 30 min prior to analysis by SDS-PAGE (see Materials and Methods). C corresponds to reactions lacking glycogen synthase.

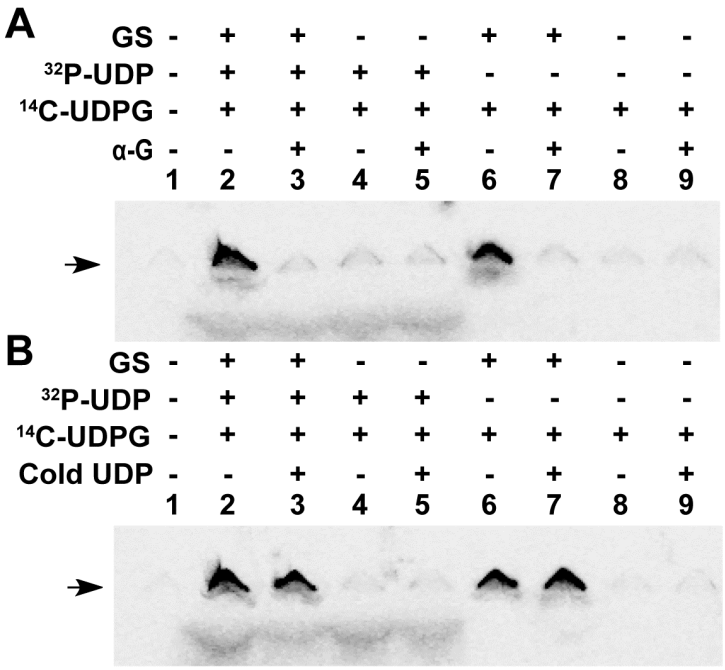

Figure 9.

Test for ^{32}P-UDP binding to glycogen. Glycogen was incubated with [α-^{32}P]UDP either with (lanes 2 and 3) or without (lanes 4 and 5) glycogen synthesis by 10 μg/ml glycogen synthase (GS) for 30 min. Samples were analyzed by SDS-PAGE and dried gels visualized with a Phosphorimager (see Materials and Methods). Controls lacked [α-^{32}P]UDP (lanes 6-9). In **A**, samples were treated or not with α-amylase and amyloglucosidase (α-G) and in **B**, unlabeled UDP (4 mM, a 1,000-fold excess over [α-^{32}P]UDP) was added to some samples prior to SDS-PAGE.