Characterization of Human Glycogenin-2, a Self-glucosylating Initiator of Liver Glycogen Metabolism*

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Glycogenin-2 is a recently described self-glucosylating protein potentially involved in the initiation of glycogen biosynthesis (Mu, J., Skurat, A. V., and Roach, P. J. (1997) J. Biol. Chem. 272, 27589–27597). In human liver extracts, most of the glycogenin-2 was only detectable after treatment with a-amylase. Similarly, purified high M₆ glucogenin was only detected after release by a-amylase treatment. Based on analysis by polymerase chain reaction, the predominant isoform in liver was glycogenin-2P. Glycogenin-2 was found in Ewing’s sarcoma RD-ES cells where, however, it was not associated with high M₆ carbohydrate. Both human liver and human RD-ES cell extracts also contained glycogenin-1. Glycogenin-1 and glycogenin-2 interact with one another, based on in vitro interactions and co-immunoprecipitation from liver and cell extracts. Mutation of Tyr-196 in glycogenin-2 to a Phe residue abolished the ability of the reaction, the predominant isoform in liver was glycogenin-2P. Glycogenin-2 was found in Ewing’s sarcoma RD-ES cells where, however, it was not associated with high M₆ carbohydrate. Both human liver and human RD-ES cell extracts also contained glycogenin-1. Glycogenin-1 and glycogenin-2 interact with one another, based on in vitro interactions and co-immunoprecipitation from liver and cell extracts. Mutation of Tyr-196 in glycogenin-2 to a Phe residue abolished the ability of glycogenin-2 to self-glucosylate but not to interact with glycogenin-1. Stable overexpression of glycogenin-2 in Rat-1 fibroblast cells resulted in a 5-fold increase in the level of glycogen present in the low speed supernatant but little change in the low speed supernatant. This result is important since it indicates that the level of glycogenin-2 can determine glycogen accumulation and hence has the potential to control glycogen synthesis.

EXPERIMENTAL PROCEDURES
Detection of Glycogenin-2 Protein in RD-ES Cells and Human Liver—RD-ES cells, which are derived from human Ewing’s sarcoma, were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS and 25 mM glucose. To test the effect of glucose on glycogenin-2, 80% confluent cells were switched to DMEM without glucose and cultured for 24 h before being assayed or refed with medium with 25 mM glucose for several hours. Cells harvested in lysis buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 2 mM EGTA, 100 mM NaF, 0.1 mM N-5-tosyl-L-lysine chloromethyl ketone, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) were frozen in liquid nitrogen and thawed cells were homogenized by repeated pipetting. A 16,000 × g supernatant was obtained and glycogenin-2 signals detected by Western analysis as described previously (15). To study the interaction between glycogenin-2 and glycogenin-1, immunoprecipitation was performed using anti-glycogenin antibodies followed by in vitro self-glucosylation assay under conditions described in the next paragraph.

Frozen human liver samples were obtained at autopsy were kindly provided by Dr. William F. Bosron, Indiana University School of Medicine. For Western analysis and glycogen preparation, frozen liver samples were homogenized by Polytron in 5 volumes of lysis buffer. Homogenates were centrifuged at 700 × g for 10 min at 4 °C, and the supernatants were filtered through four layers of cheesecloth before being centrifuged again at 12,000 × g for 10 min. Pellets were resuspended in the same volume as the supernatants, and both fractions were analyzed by Western analysis. Prior to immunoblot analysis, some samples were digested with 80 µg/ml α-amylase (Worthington) for 5 min at 37 °C in buffer

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1 A. Lin and P. J. Roach, unpublished results.
2 The abbreviations used are: EST, expressed sequence tag; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; RT, reverse transcription; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.
containing 5 mM CaCl₂. To purify glycogen, 10% cold trichloroacetic acid was mixed with liver homogenates. After centrifugation for 10 min at 12,000 × g, a clear supernatant was obtained and was added to 4 volumes of cold ethanol to precipitate glycogen. Glycogen was collected by another 10-min 12,000 × g centrifugation. The glycogen pellet was washed twice with the corresponding antibodies (1:1000 dilution) for 1 h. Protein A-agarose (20 μl) was then added and samples rocked on a Nutator at 4 °C for 1 h. Samples were washed first with 1 ml of buffer containing 1× phosphate-buffered saline, 0.1% Triton X-100, and 0.3 M NaCl and then with 1× phosphate-buffered saline, 0.1% Triton X-100, and 0.1 M NaCl. The resulting pellets were analyzed further. Where desired, immunoprecipitates were allowed to self-glucosylate for 30 min with 77 μM UDP-[1-¹⁴C]glucose (specific activity 265 mCi/mmol) in buffer containing 50 mM HEPES, pH 7.5, 5 mM MnCl₂, 2 mM dithiothreitol, at 30 °C.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis of Glycogenin-2 Transcripts—RT-PCR was used to analyze glycogenin-2 transcripts. RNA extraction from frozen human liver was performed with TRIzol Reagent (Sigma) following the manufacturer's instructions. Briefly, liver samples were homogenized by Polytron in 10 volumes of TRI Reagent. After mixing with chloroform (0.2 volume of TRI Reagent), the aqueous phase containing RNA was transferred to a new tube and precipitated with isopropanol (0.5 volume of TRI Reagent). The pellet was washed twice with 75% ethanol and air-dried before being resuspended in water. For RT-PCR, 10 μg of total RNA was used for first strand synthesis utilizing the Superscript II reverse transcriptase system (Life Technologies, Inc.). The oligonucleotides used for first strand cDNA synthesis were CCCTTACTTGTATGGTGCCAGTC and TTCTTGCACAGGCTTCTAGG. Two μl of product from the first strand reaction were used as a template for the following PCR, which was performed utilizing Taq DNA polymerase (Promega). To detect the presence of different NH₂-terminally splice glycogenin-2 isoforms, PCR reactions were conducted using primers straddling the splicing region: CGGCCAATCATCAGGTCACTGT (+) and TTGGTGAGGTTGACGCCCAGG (-). Glycogenin-2 cDNA clones provided control templates to generate RT-PCR products corresponding to the different splice variants. The PCR conditions used were denaturation at 95 °C for 30 s and annealing at 57 °C for 1 min, followed by a 2-min extension at 72 °C. PCR products were separated on a 1.5% agarose gel.

Analysis of Mutated Glycogenin-2 Proteins—Site-directed mutagenesis was used to mutate residues in glycogenin-2 with the QuickChange site-directed mutagenesis kit (Stratagene) using pcDNAs-glycogenin-2β as a template. The mutagenic oligonucleotides for Y196F were: GTAGTAACAC-GATGTGTCATTACAGGCCTCGGTTCTC (+) and GAGGAGCTGCTGA-ATGAGCACTGTTTATTTAAC (-). The mutagenic oligonucleotides for Y198F were: CACGATGTACACTTTCAGCCCTGCCTTCAAG (+) and GAAGGCAGGGCTGTA-GTGCGCAGTTTATTTAAC (-). The mutagenic oligonucleotides for Y198F were: CACGATGTACACTTTCAGCCCTGCCTTCAAG (+) and GAAGGCAGGGCTGTA-GTGCGCAGTTTATTTAAC (-). The mutagenic oligonucleotides for Y198F were: CACGATGTACACTTTCAGCCCTGCCTTCAAG (+) and GAAGGCAGGGCTGTA-GTGCGCAGTTTATTTAAC (-). The DNA fragments that were manipulated in the course of the mutagenesis were sequenced (16) to confirm the mutation.

Wild type and mutated glycogenin-2β inserted in the mammalian expression vector pcDNA3 were expressed in COS-1 cells. Conditions for transient transfection, cell extract preparation, self-glucosylation assay, and Western analysis were essentially as described before (15). Glycogenin-2 signals were visualized by enhanced chemiluminescence assay, and Western analysis were essentially as described before (15).

RESULTS

Glycogenin-2 Is Covalently Associated with Human Liver Glycogen—In our initial characterization of glycogenin-2 (15), we detected glycogenin-2 in a rat liver fraction and in H4IIEC3 cells. However, in the rat hepatoma cells, most of the glycogenin-2 was present as a free protein and only a small fraction required α-amylase treatment for its release. Since this result implied a potentially important difference between glycogenin-1 and glycogenin-2, it was important to analyze glycogenin-2 in human liver tissue. Analysis of 12,000 × g supernatants from human liver extracts by Western analysis clearly indicated the presence of species of ~66 kDa, the expected size of glycogenin-2 based on analysis of recombinant proteins (Fig. 1A). This species was only present after digestion of the sample with α-amylase. There was also a strong signal from a species with lower mobility than glycogenin-2 that was present whether the sample was treated with α-amylase or not (marked by an asterisk in Fig. 1A). However, this signal is nonspecific and was also present when pre-immune serum was used for blotting (data not shown). Glycogenin-2 was also detected in the pellet fraction after prior treatment with α-amylase (data not shown). By using recombinant glycogenin-2 as a standard (Fig. 1B), the Western analysis was performed using anti-glycogenin-2 antibodies. The Western analysis was performed using anti-glycogenin-2 antibodies. The Western analysis was performed using anti-glycogenin-2 antibodies. The Western analysis was performed using anti-glycogenin-2 antibodies.
Glycogenin-2 and Glycogen Synthesis

Glycogenin-2 in the soluble fraction of human liver extracts. A, samples from three different frozen human livers were homogenized by Polytron in lysis buffer (see “Experimental Procedures”). Supernatants (∼50 µg of protein/sample) after a 10-min 12,000 × g centrifugation were treated or not with α-amylase and then analyzed by Western blotting using anti-glycogenin-2 antibodies (panel A). Recombinant glycogenin-2a (38 ng) was run as a standard (lane C). The asterisk indicates the migration of a species that interacts nonspecifically with the antibodies. B, glycogen purified from two different human liver samples (see “Experimental Procedures”) was treated or not with α-amylase prior to analysis by SDS-PAGE and Western analysis using anti-glycogenin-2 antibodies.

The preceding results led us to conclude that, in human liver, essentially all of the glycogenin-2 is covalently linked to glycogen, a substantial proportion of which is of high Mr.

Glycogenin-2 in Cultured Cell Lines—Since human liver has inconveniences as an experimental system, we surveyed several human cell lines for expression of glycogenin-2. No glycogenin-2 signal was detected by Western analysis in hepatoblastoma HepG2 cells, melanoma SK-MEL-1 cells, and breast adenocarcinoma Sk-Br-3 cells. The last mentioned cell line was selected because it was reported to contain glycogen granules (24). The only cell line with which we observed a strong signal was human Ewing’s sarcoma-derived RD-ES cells, which have been reported to contain glycogen granules (25). Using cells cultured in medium containing 25 mM glucose, anti-glycogenin-2 antibodies detected a major species at mass ∼66 kDa as well as a “smear” of species up to ∼120 kDa (Fig. 2, lane 1). When the cells were switched to medium without glucose for 24 h (lane 2) or were treated with α-amylase, the smear disappeared. The higher Mr species were restored when the glucose-starved cells were exposed to 25 mM glucose for 1 or 3 h (lanes 3 and 4). We conclude that the higher Mr species represent glycogenin-2 with intermediate levels of glucosylation, as can be seen for glycogenin-1 under appropriate circumstances (18). Thus, although we see glucose-dependent changes in glycogenin-2 glucosylation, we do not see stoichiometric conversion into high Mr glycogen. In fact, the glycogen content in this cell line under our conditions of culture is relatively low. 8.2 µg of glycogen/mg of soluble protein, suggesting that the prevailing cellular conditions do not favor glycogen synthesis even at high glucose concentrations. Therefore, although the RD-ES cells are a useful source of glycogenin-2, they have limitations for the study of the role of glycogenin-2 in glycogen biosynthesis.

Glycogenin Isoforms Present in Human Liver—Given the earlier results (14, 15) suggesting that glycogenin-1 was present in rat and rabbit liver, it was of interest to analyze human liver for the presence of this protein. However, our anti-glycogenin-1 antibodies were generated with recombinant rabbit skeletal muscle protein and do not detect human glycogenin-1 in liver extracts by Western blotting. We were able to overcome this problem, at least qualitatively, because the antibodies are effective in immunoprecipitating human glycogenin-1 and the glycogenin can then be detected after self-glucosylation. With this analysis, we visualized a glucosylated species of ∼38 kDa corresponding to glycogenin-1 (data not shown; see similar experiments in Fig. 5). We also observed glucosylation of a species of ∼66 kDa with the same size as glycogenin-2. First, we conclude that there is glycogenin-1 in the liver extract. In addition, this result suggests that glycogenin-1 and glycogenin-2 can interact with one another. The self-glucosylation assay is in fact a very sensitive way to detect glycogenin-1 because of its relatively high self-glucosylating activity.

From cDNA cloning, there are at least three different liver isoforms. The anti-glycogenin-2 antibodies available are not isoform-specific, and the predicted size differences among the isoforms are too small to assign isoforms based on protein size. Thus, we developed an RT-PCR method to distinguish the glycogenin-2α, -β, and -γ messages. Basically, a pair of primers straddled the regions of alternate splicing at the NH2 terminus so that products of slightly different size are generated (see standards in Fig. 3). Using RNA prepared from human liver, a single discrete PCR fragment was obtained with size corresponding to glycogenin-2β (Fig. 3). Although not a quantitative measurement, this result suggests that glycogenin-2β is the major form expressed in human liver.

**Tyra-196 Is Responsible for Carbohydrate Attachment to Glycogenin-2 in Vitro—**Sequence alignment with glycogenin-1 indicates that glycogenin-2 has a tyrosine residue (Tyr-196 in glycogenin-2β) in correspondence with Tyr-194 of glycogenin-1, the residue known to be the site of carbohydrate attachment (14, 26). We individually mutated Tyra-196, as well as the adjacent Tyr-198, to Phe using site-directed mutagenesis. Similar levels of expression were achieved when wild type or mutated glycogenin-2β were transiently expressed in COS-1 cells (Fig. 4). Self-glucosylation assays using COS cell extracts showed
that mutation of Tyr-196 totally eliminated labeling of glycogenin-2. Mutation of Tyr-198 did not abolish the ability to self-glucosylate, even though the level of labeling was somewhat reduced. Similar results were obtained with purified, recombinant glycogenin-2 produced in E. coli except that in this case mutation of Tyr-198 had no effect on activity (data not shown). We conclude that, as in glycogenin-1, there is a single site of glucose attachment to glycogenin-2.

Interaction between Glycogenin-2 and Glycogenin-1—Several lines of evidence suggest that glycogenin-1 and glycogenin-2 might interact with each other. As noted above, antibodies to glycogenin-1 immunoprecipitated a self-glucosylating species from human liver extracts with the size of glycogenin-2. Using H4IEC3 cells, we were able to show that glycogenin-1 could be co-immunoprecipitated with anti-glycogenin-2 antibodies and vice versa (data not shown). Similar results were obtained with extracts from RD-ES cells, which express both glycogenin-1 and glycogenin-2. The interaction of glycogenin-2 with glycogenin-1 was also demonstrated in vitro using purified recombinant proteins. Independently purified recombinant GST-tagged glycogenin-1 and His8-tagged glycogenin-2 were mixed before being applied to glutathione-agarose column, which should bind the GST tag specifically. The proteins were eluted with glutathione. Analysis of the eluted proteins indicated the presence of glycogenin-2 as well as glycogenin-1, even though only glycogenin-1 had the GST tag (Fig. 7A). In a control, His8-tagged glycogenin-2 incubated with GST did not bind to the GST-agarose. Similar co-elution of glycogenin-1 and glycogenin-2 was observed with wild type glycogenin-1 or a mutant with Tyr-194 replaced with Phe. This mutation destroys the ability of the protein to act as a glucose acceptor even though activity toward exogenous acceptors such as maltose is not affected (10, 26, 27). In both cases, the glucosylation of glycogenin-2 was enhanced by the presence of glycogenin-1. Either the binding of glycogenin-1
allosterically activated glycogenin-2 or else the glycogenin-1 was able to transfer glucose to glycogenin-2.

Overexpression of Glycogenin-2 in Rat-1 Fibroblast Cells

Causes Overaccumulation of Glycogen—Rat-1 fibroblast cells stably expressing glycogenin-1 (GN23 cell line) have been described previously by Skurat et al. (18). Using a similar strategy, cells expressing wild type glycogenin-2 or glycogenin-2β with Tyr-196 (GN2b-Y196F) mutated to Phe were generated. Western analysis with anti-glycogenin-2 antibodies confirmed the expression of both glycogenin-2 proteins (Fig. 8). Although a fraction of the wild-type glycogenin-2 was present as a free protein, the majority was associated with glycogen and required α-amylase treatment in order to be detected. In contrast, the mutant glycogenin-2, lacking the glucosylation site, was present entirely as a free protein, regardless of α-amylase treatment. Glycogen levels in these cells were compared with cells expressing pcDNA3 vector alone or the GN23 line that expresses glycogenin-1 (Fig. 9). Consistent with previous studies (18), expression of glycogenin-1 in Rat-1 cells led to some increase in glycogen accumulation in the soluble fraction but did not provoke overall any large increase in the total glycogen levels. Cells expressing glycogenin-2 had a significant, 5-fold increase in the glycogen accumulated in the pellet fraction while the glycogen level in the soluble fraction increased modestly, if at all. Expression of the mutant glycogenin-2 also caused an ~2-fold increase in glycogen in the pellet fraction. The above results imply that, in contrast to case with glycogenin-1, glycogenin-2 overexpression in Rat-1 fibroblasts caused a substantial increase in the total glycogen accumulation.

DISCUSSION

One of the most important outcomes of this work is the demonstration that glycogenin-2 protein is present in human liver and is covalently linked to carbohydrate. Furthermore, a substantial proportion of the attached carbohydrate is high molecular weight material that is ethanol-precipitable and trichloroacetic acid-soluble. About half of the total glycogenin-2 was recovered in this fraction, but we do not know if the shortfall is due to degradation of the protein during processing or its association with smaller glycogen molecules that do not precipitate in ethanol. These findings differ from our previous results with cultured rat hepatoma cells in which most of the glycogenin-2 was present as a free, low Mr protein and only a relatively small fraction was bound to carbohydrate. Cells in culture may differ significantly in their ability to store glycogen, as is discussed further below. By using recombinant glycogenin-2 as a control in Western analysis, we estimate that the glycogenin-2 content in human liver is about 0.15 mg/g of liver.
Calculation of the amount of glycogenin-2 is based on Western analysis of human samples from the present study, by reference to the signal from known amounts of purified recombinant glycogenin-2. Other data are derived from Alonso et al. (5). The glycogen values are for resting, fed rats, and the glycogenin-1 content is for rabbit. The estimates involve a number of assumptions and must be treated as fairly crude approximations. For example, molarity for glycogen is calculated on the assumption of a monodisperse compound with a single molecular weight. One gram of tissue is taken as 1 g of solvent in calculating molarity.

| Glycogen (Mg) | Muscle | Liver |
|--------------|--------|-------|
| Per wet weight of tissue | 13 mg/g | 95 mg/g |
| Concentration | 1.3 μM | 0.95 μM |

### Glycogenin-1

| Glycogenin-1 | Per wet weight of tissue | Concentration |
|--------------|--------------------------|---------------|
| 0.045 mg/g  | 0.0025 mg/g | 0.07 μM |

| Glycogenin-2 | Per wet weight of tissue | Concentration |
|--------------|--------------------------|---------------|
| -0 mg/g     | 0.15 mg/g  | 2.7 μM |

The tissue distribution of glycogenin isoforms is still under investigation. As reported previously (15), the message for glycogenin-2 in human liver, was unable to quantitate the amount. In rabbit liver, the level of glycogenin-1 has been estimated to be 0.0025 mg/g of tissue (5) or 70 nM, some 40 times lower than our value for glycogenin-2. Our estimate of the amount of glycogenin-2 in liver is in the same range as has been reported for glycogenin-1 in skeletal muscle (Table I; Ref. 5). Previously, the low level of glycogenin-1 in rabbit liver had been rationalized by the fact that liver glycogen molecules, so-called α-particles, are larger, with $M_\alpha 1-3 \times 10^8$, than the β-particles of muscle, with $M_\beta 10^7$. Even so, Smythe et al. (14) noted that there would still be fewer glycogenin-1 than glycogenin molecules. Ercan et al. (28) had also suggested that in rat liver some glycogenin molecules are not attached to protein. This scenario prompted the suggestion (14, 29) that glycogenin-1 might be recycled so that each molecule could participate in the synthesis of multiple glycogen molecules. The number of glycogenin-2 molecules that we estimate to be present in liver is sufficient to allow one per α-particle of $M_\alpha 10^8$ and, as noted, we found that essentially all of the glycogenin-2 was attached to carbohydrate. These calculations are not accurate enough to preclude the existence of glycogenin molecules free of glycogenin, as discussed by Ercan et al. (28, 29). Nonetheless, we conclude that there is sufficient glycogenin-2 in liver to have a major impact on glycogen synthesis in that organ.

The tissue distribution of glycogenin isoforms is still under investigation. As reported previously (15), the message for glycogenin-2 was most prominent in liver but was also found in heart and pancreas. It was not detected in muscle (15), consistent with the inability to visualize glycogenin-2 by Western analysis of human muscle tissue. Efforts are under way to test for the presence of glycogenin-2 protein in human heart and pancreas. The currently available antibodies do not distinguish glycogenin-2 isoforms, but, based on PCR analysis, the predominant glycogenin-2 isoform in human liver is spliced at the NH2 terminus like glycogenin-2β. The size of the protein detected by Western analysis is consistent with utilization of all the COOH-terminal exons and so most likely the dominant isoform is glycogenin-2β. This is the isoform that most resembles glycogenin-1 at the NH2 terminus and which is the most active form. Glycogenin-1 is widely expressed and, as discussed above, is present in liver. However, whether the low level expression of glycogenin-1 is in the same cells as glycogenin-2 has yet to be formally proven. From analysis of cultured cell lines, we do have evidence for the co-expression of glycogenin-1 and glycogenin-2 in rat H4IIEC3 hepatoma cells and human RD-ES cells. However, it can be argued that such cultured cells do not have normal patterns of gene expression. For example, unlike the rat H4IIEC3 cells, the human hepatoblastoma HepG3 cells did not express glycogenin-2. Additionally, expression of glycogenin-2 in RD-ES cells may be related to a defect characteristic of Ewing's sarcoma cells. We have localized the human glycogenin-2 gene to the X chromosome at band p22.3 by FISH analysis, and confirmation appeared subsequently in the UniGene data base (Hs. 58589). Glycogenin-2 is located at a telomeric region of the X chromosome in what has been termed a pseudoautosomal region with a high rate of recombination (30). Interestingly, the MIC2 gene, which is localized to this same pseudoautosomal region, is also overexpressed in Ewing's sarcoma cells (31). One could ask whether the enhanced expression of glycogenin-2 in RD-ES cells might involve a mechanism common to that resulting in overexpression of the MIC2 gene.

Several results suggest that glycogenin-1 and glycogenin-2 are capable of interacting with each other. The two-proteins co-immunoprecipitate from extracts of human liver, human RD-ES cells, and rat H4IIEC3 cells. Similar co-immunoprecipitation is observed from COS cells transiently overexpressing glycogenin-1 and glycogenin-2. Finally, purified glycogenin-1 and glycogenin-2 co-purified through affinity chromatography specific for glycogenin-1. These results would imply the formation of relatively stable complexes, but their exact nature has yet to be determined. There is good evidence that glycogenin-1 forms dimers and perhaps larger oligomers (9, 10). Regions of the molecule important for dimerization have not been established, but it is interesting that glycogenin-1 and glycogenin-2, as well as the yeast Glg1p and Glg2p homologs, contain a minimal leucine zipper motif (32) that is also conserved in many other glycogenin-like sequences (4). One possibility would be that the leucine zippers permit formation of homo- or heterodimers, much as happens with some transcription factor families (33–35). An alternative is that a larger complex, such as a tetramer, is formed between glycogenin-1 and glycogenin-2 dimers.

What could be the rationale for the formation of heteromeric complexes? One possibility is the combinatorial generation of a greater variety of initiation complexes whose different properties might specify size, organization, or location of the resulting glycogen molecules. Glycogenin-1 and glycogenin-2 have different kinetic properties, glycogenin-1 being significantly more active as an enzyme. In addition, glycogenin-2 is larger, and has sequences that could determine functions or interactions not available with glycogenin-1. We also found that glycogenin-2 self-glucosylation was increased in the presence of glycogenin-1. There are several possibilities to explain the latter observation. First, glycogenin-1 could allosterically activate glycogenin-2 so that its inherent capacity for self-glucosylation is enhanced. Another possibility is that glycogenin-2 acts as the acceptor for glycogenin-1 action in an oligomeric complex. Then, the more active glycogenin-1 could glucosylate glycogenin-2 in an intramolecular, intersubunit reaction. Despite some

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3 J. Mu and P. J. Roach, unpublished results.

4 In Fig. 5 of Ref. 15, the legend incorrectly switched the identification of glycogenin-2α and glycogenin-2β, making glycogenin-2α seem more active whereas the reverse is true.

5 L. Zhai, J. Mu, A. A. DePaoli-Roach, and P. J. Roach, unpublished results.
interesting possibilities, however, we should emphasize that the physiological significance of glycogenin-1/glycogenin-2 interactions remains to be understood.

Ever since its discovery as a protein that mediates the initiation of glycogen synthesis, glycogenin has been an attractive potential candidate to control the rate of glycogen formation. To date, however, evidence that regulation of glycogenin-1 determines the rate of glycogen synthesis is sparse. Simple overexpression of glycogenin-1 in COS cells does not increase the accumulation of glycogen (36). COS cells, however, do not accumulate much glycogen, perhaps because the synthetic pathway is opposed strongly by glycogenolysis, and, even in the presence of high glucose levels, most of the expressed glycogenin-1 remains as a free protein and not bound to carbohydrate. In this regard, Rat-1 fibroblasts stably expressing glycogenin-1 provide a better model since, in the presence of high glucose levels, all of the glycogenin-1 becomes attached to glycogen (18). However, Rat-1 fibroblasts that overexpressed glycogenin-1 did not accumulate substantially more glycogen in terms of the number of polymerized glucose equivalents than control cells although the proportion of glycogen in the low speed supernatant was enhanced (Ref. 25; see also Fig. 11). Perhaps the most exciting result of the present study is the observation that stable overexpression of glycogenin-2 in Rat-1 fibroblasts resulted in a significant increase, of 5-fold, in glycogen accumulation, and this result is the first clear indication, to our knowledge, of such a function for any glycogenin. Interestingly, the increase is specific to the pellet fraction, perhaps implying interaction of glycogenin-2 with some larger cellular structure that is easily centrifuged. One recent study suggested that glycogenin-1 might associate with the actin cytoskeleton (37). In any event, glycogenin-2 becomes a good candidate for regulation, such as by hormones like insulin and glucagon, that control liver glycogen storage and blood glucose homeostasis. Our efforts to investigate this possibility have been seriously hampered by the poor cross-reactivity between our anti-human glycogenin-2 antibodies and rodent glycogenin-2. Although we had previously detected glycogenin-2 in rat cells and liver, we realized after analyzing human sources how poor the signal was, and our success could be explained by a very high level of glycogenin-2 expression in these cells. Furthermore, no rodent EST sequences for glycogenin-2 are present in the data bases, consistent with the low frequency of human glycogenin-2 ESTs. We are currently attempting to clone rodent glycogenin-2 cDNAs to provide reagents to analyze the hormonal and metabolic control of glycogenin-2 in animal models.

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