Identification of Two Essential Glutamic Acid Residues in Glycogen Synthase*

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The detailed catalytic mechanism by which glycosyltransferases catalyze the transfer of a glycosyl residue from a donor sugar to an acceptor is not known. Through the multiple alignment of all known eukaryotic glycogen synthases we have found an invariant 17-amino acid stretch enclosed within the most conserved region of the members of this family. This peptide includes an E-X₇-E motif, which is highly conserved in four families of retaining glycosyltransferases. Site-directed mutagenesis was performed in human muscle glycogen synthase to analyze the roles of the two conserved Glu residues (Glu-510 and Glu-518) of the motif. Proteins were transiently expressed in COS-1 cells as fusions to green fluorescence protein. The E510A and E518A mutant proteins retained the ability to translocate from the nucleus to the cytosol in response to glucose and to bind to intracellular glycogen. Although the E518A variant had approximately 6% of the catalytic activity shown by the green fluorescence protein-human muscle glycogen synthase fusion protein, the E510A mutation inactivated the enzyme. These results led us to conclude that the E-X₇-E motif is part of the active site of eukaryotic glycogen synthases and that both conserved Glu residues are involved in catalysis. We propose that Glu-510 may function as the nucleophile and Glu-518 as the general acid/base catalyst.

Glycosyltransferases and glycosidases catalyze the transfer of glycosyl residues from a donor sugar to an acceptor. The acceptor in glycosidases is water, the end result being hydrolysis of the glycoconjugate. For transferases the acceptor molecule is in most cases a growing carbohydrate chain, but it can also be a protein, a lipid, or a range of other compounds such as steroids, bilirubin, flavonones, carotenoids, etc., that are modified by glycosylation (1). Glycosyltransferases can be further divided into two groups depending on whether they use a nucleotide phosphosugar (Leloir-type) or an oligosaccharide as the glycosyl donor. In all cases, the reaction catalyzed is a substitution at the anomeric carbon of a sugar moiety and may occur with retention or inversion of the configuration at this center. Accordingly, enzymes that catalyze glycosyltransfer can be divided into retaining or inverting enzymes.

Glycogen synthase (GS)¹ catalyzes the key step of glycogen formation. In mammals, two major isoforms of the enzyme have been described, the muscle isoenzyme (2), which is expressed in several tissues (3), and the liver form (4), which appears to be tissue-specific (5). GS plays a crucial role in glucose metabolism and homeostasis, and its malfunction has been associated with several metabolic diseases such as diabetes mellitus (6, 7) and glycogen storage disease 0 (8). Mammalian GSs catalyze the transfer of a glucosyl moiety from UDP-glucose to a nascent chain of glycogen through an α₁→₄ linkage. The stereochemistry of the resulting glycosidic bond is the same as that of the donor sugar nucleotide, thus GS is classified as a retaining Leloir-type glycosyltransferase. The stereochromatic course of the reaction, analogously to what has been found for retaining glycosidases (9), determines the presence of two catalytic amino acids, which allow a double displacement mechanism. According to this model, these two essential residues must be close within the active center of the enzyme (10, 11).

Although many genes encoding glycosyltransferases have been sequenced and expressed, no structural information from x-ray crystallography or high resolution NMR spectroscopy is available for a retaining glycosyltransferase. The only structures known to date are those of the β-glucosyltransferase of T4 bacteriophage (12), the hypothetical nucleotide-diphosphosugar transferase SpsA from Bacillus subtilis (13), and the catalytic domain of the bovine β1,4-galactosyltransferase T1 (14). However, all these enzymes operate with inversion of configuration at the anomeric carbon and presumably have different active site geometry.

Almost all the studies of muscle and liver GS have focused on the covalent and allosteric regulation by hormonal and metabolic stimuli (15–17), and few attempts have been made to elucidate the catalytic mechanism (18). The aim of this study was to identify conserved regions and putative catalytic residues through the comparison of the amino acid sequences of mammalian GSs with those of other known retaining glycosyltransferases. Moreover, using site-directed mutagenesis and human muscle glycogen synthase (HMGS) as a model, we have...
probed the function of two conserved Glu residues in catalysis by this family of enzymes.

**EXPERIMENTAL PROCEDURES**

**Sequence Retrieval and Analysis**—Sequences were retrieved from the ExPASy or PubMed servers on the Web. The accession numbers (SWISS-PROT, TrEMBL, or Entrez) of the proteins studied are included in the figures below. BLAST and Ψ-BLAST (19, 20) were performed at the NCBI. Linear alignments were performed locally using ClustalW (21). Hydrophobic cluster analysis (HCA) (22) plots were obtained at the DrawHCA server. The secondary structure predictions were performed at the Jpred server (23). The classification of glycosyltransferases by Campbell et al. (24, 25) is accessible on the Web also.

**Site-directed Mutagenesis**—The plasmid pEGFP-HMGS (26), which encodes the fusion protein GFP-HMGS, was used as a template. The mutations in the coding sequence of HMGS were created using the QuikChange site-directed mutagenesis kit (Stratagene). The E510A mutation was generated with the oligonucleotide CACACCGGCTGcaTGCACGGTTATG and its exact complement, which introduced an NcoI restriction site (shown underlined) for diagnostic purposes. Similarly, the pEGFP-HMGS (E51A) plasmid was built with the oligonucleotide CACACCGGCACGGTTATG and its exact complement, which introduced a SphI restriction site. The mutant plasmids were purified by anion-exchange chromatography (Plasmid Maxi Kit, Qiagen), and the regions encoding the fusion proteins were sequenced in their entirety, using the ABI-PRISIM DNA sequencing kit and the ABI-PRISIM 377 automatic DNA sequencer (PE Applied Biosystems), to rule out spurious mutations.

**Cell Culture and Transfection—** COS-1 cells (ATCC no. CRL-1650) were grown on 60-mm dishes (for biochemical assays and immunoblotting) or on glass coverslips inside 35-mm dishes (for confocal microscopy analysis) in Dulbecco's modified Eagle's medium (DMEM; Whittaker), supplemented with 25 mM glucose, 10% fetal bovine serum (FBS; Biological Industries), and penicillin/streptomycin (Roche Molecular Biochemicals). Cells cultured on 60-mm dishes were transfected using 625 μg of plasmid DNA per dish in DMEM. After a 4-h incubation, cells were treated for 2 min in DMEM containing 10% dimethyl sulfoxide (Sigma) and 10% FBS. They were then washed with DMEM plus 10% FBS and maintained in this medium. Cells grown on coverslips were transfected at 70–80% of confluence using 4 μg of liposome suspension Clonfectin (CLONTECH) and 0.5 g of liposome suspension DEAE-dextran (Sigma), 0.5 μmol of chloroquine (Sigma), and 10 μg of plasmid DNA per dish in DMEM. After a 4-h incubation, cells were treated for 2 min in DMEM containing 10% dimethyl sulfoxide (Sigma) and 10% FBS. They were then washed with DMEM plus 10% FBS and maintained in this medium. Cells grown on coverslips were transfected at 70–80% of confluence using 4 μg of liposome suspension Clonfectin (CLONTECH) and 4 μg of plasmid DNA per 35-mm dish following the manufacturer's instructions. After transfection (4–5 h) at 37 °C in humidified 5% CO2/95% air, cells were washed in phosphate-buffered saline (PBS) and incubated in DMEM supplemented with 25 mM glucose and 10% FBS. Experiments were performed 48–52 h after transfection. Cells were preincubated overnight in DMEM without glucose, and on the day of the experiment they were incubated in DMEM with or without glucose for 4 h. At the end of the 4-h incubation, cells grown on 60-mm dishes were rinsed twice with PBS and frozen in liquid nitrogen. Cells grown on coverslips were fixed for 30 min at room temperature in PBS containing 4% paraformaldehyde (Pfluka) and washed several times with PBS. Alternatively, cells were permeabilized with digitonin (5 μg/ml) in a buffer containing 300 mM saccharose, 3 mM Hepes, 5 mM MgCl2 (Merck), 2 mM diethiothreitol (Sigma) for 8 min and were treated or not at 37 °C with α-aminase (22 units/ml, Sigma) and 1 mM CaCl2 in PBS for 30 min. Finally, cells were fixed with paraformaldehyde.

**Immunocytochemistry**—Coverslips were rinsed three times with PBS, and cells that had not been treated with digitonin were permeabilized for 20 min with PBS containing 0.2% Triton X-100 (Sigma) and blocked for 10 min with PBS containing 0.2% Triton X-100 and 3% bovine serum albumina (BSA; Sigma). Alternatively, before blocking, cells were treated for 30 min at 37 °C with α-aminase (22 units/ml, Sigma) and 1 mM CaCl2 in PBS. A monoclonal IgM antibody against glycogen, a generous gift from Dr. Otto Baba (27), was diluted in PBS containing 3% BSA and applied to the cells for 45 min at room temperature. Coverslips were then washed several times with PBS and subjected to a two-step immunogold procedure (TRITC, goat anti-mouse IgM secondary antibody (Chemicon) for 30 min. Finally, coverslips were washed, air-dried, and mounted onto glass slides using the Immuno Fluore mounting medium (ICN Biomedicals, Inc.).

**Confocal Microscopy**—Fluorescence images were obtained with a Leica TCS 4D (Leica Lasertechnik, Heidelberg, Germany) confocal scanning laser microscope adapted to an inverted Leica DMIRBE microscope and 63× (numerical aperture 1.4 oil) Leica Plan-Apo objective.

The light source was an argon/krypton laser (75 milliwatts). Green fluorescence from GFP and GFP recombinants was excited with the laser at 488 nm; red fluorescence of the TRITC secondary antibody was excited at 550 nm. Optical sections (0.1 μm) were obtained.

**Glycogen Synthase Activity Assays and Glycogen Content**—For the measurement of glycogen content, cell monolayers were scraped into 15 m M EDTA, 15 m M 2-mercaptooethanol, 10 μg/ml leupeptin, 1 μM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. Cell bursting was caused by sonication. Protein concentration was measured as described by Bradford (29) using the Bio-Rad protein assay reagent. GS activity was measured in the presence or absence of 6.6 mM Glu 6-P as described (30). The activity measured in the absence of Glu 6-P represents the active form of the enzyme (I or a form), whereas the activity tested in the presence of 6.6 mM Glu 6-P is a measure of total activity. The ratio of these two activities is an estimate of the activation state of the enzyme.

**Electrophoresis and Immunoblotting**—Samples from activity assays were boiled for 2 min with gel loading buffer 5× containing 250 μM Tris-HCl (pH 6.8), 1 μM diethiothreitol, 10% SDS, 0.5% bromphenol blue, and 50% glycerol. Electrophoresis was performed in a 10% SDS-polyacrylamide gel as described by Laemmli (31) in a Mini-Protein II cell (Bio-Rad) at 200 V, until the bromphenol blue dye front reached the end of the gel. Gel electrophoresis was performed from the gel to nitrocellulose (Protran; Schleicher & Schuell) was performed at room temperature for 1 h at 100 V (constant) in a Bio-Rad miniature transfer apparatus, as described by Towbin et al. (32). Nitrocellulose blots were incubated at room temperature in blocking buffer (3% BSA, 0.05% Tween 20 Sigma) in PBS) for 1 h, then with a rabbit antibody against GFP (CLONTECH) for 1 h, and finally with a secondary goat anti-rabbit horseradish peroxidase antibody for 45 min. Immunoreactive bands were visualized on Hyperfilm (Amer sham Pharmacia Biotech) films exposed to the membrane after incubation with ECL reagent (Amer sham Pharmacia Biotech).

**RESULTS**

**Sequence Analysis**—A linear multiple alignment of all known eukaryotic GSs (human muscle (2) and liver (33), rabbit muscle (34), rat liver (4), mouse muscle2 and brain (36), Drosophila melanogaster3 and Caenorhabditis elegans (38) open reading frames, Neur ospora crassa4, and Saccharomyces cerevisiae isofoms 1 (40) and 2 (41); not shown) revealed a 17-amino acid stretch with the sequence SYEWPWTAPAEC VTMYMG23 (the numbering corresponds to the HMGS sequence), which is strictly conserved and is enclosed within the region where homology among the members of this family is greatest. Ψ-BLAST searches using this 17-amino acid peptide showed that an E-X-E motif (two Glu residues separated by seven amino acids) is conserved among other glycosyltransferases that act with retention of the configuration at the reaction center. A similar E-X-E motif was described previously in a family of retaining bacterial α-mannosyltransferases (42). Through the multiple alignment of related glycosyltransferases different to eukaryotic GSs, Kapitovon and Yu (43) identified a conserved fragment, arbitrarily named nucleotide recognition domain 1a (NRD1a), which was characterized by the presence of two conserved Glu residues separated by seven amino acids.

Campbell et al. (24, 25) have classified glycosyltransferases in terms of sequence similarity and the retention or inversion of the configuration at the anomic carbon of the transferred sugar. Among the 43 families described, only 10 are known to

**Figure 1** 33615

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2 M. F. Seldin, Z. Xue, J. M. Rochelle, R. Debry, and R. Surwit, direct submission to the GenBank®, Accession number AA09457.


4 R. de Paula, H. F. Torenzi, and M. C. Bertolini, direct submission to the EMBL/GenBank®/DDBJ, Accession number O93869.
### Identification of Catalytic Amino Acids of Glycogen Synthase

**Figure 1.** Multiple sequence alignment of deduced amino acid sequences of selected glycosyltransferases. The alignment was performed using ClustalW and a blosum62mat matrix. Sequences were retrieved from the Entrez-protein server (NCBI) or Swiss Protein/TrEMBL (superscript 1) data bases. The glycosyltransferase families, according to the classification of Campbell et al. (24, 25), are indicated on the left and the accession numbers are shown on the right. The first aligned amino acid of each protein is indicated between brackets. The invariant Glu residue is shown on a black background and the conserved homologous residues on a gray background. ORF Y4655A:31: putative glycogen synthase from *C. elegans*; ORF C68904: putative glycogen synthase from *D. melanogaster*; UGS1_HUMAN: human muscle glycogen synthase; O93869: glycogen synthase from *N. crassa*; UGS1_YEAST: glycogen synthase isoform 1 from *S. cerevisiae*; VIPC_SALTI: VI polysaccharide biosynthesis protein VIPC/TVE1 from *Salmonella typhii*; ORF AF0045: putative mannosyltransferase A from *Archaeoglobus fulgidus*; GPI3YEAST: N-acetylgalactosamyl-phosphatidylglycerol biosynthetic protein from *S. cerevisiae*; SPS MAIZE: maize sucrose-phosphate synthase; SUS1 MAIZE: maize sucrose synthase 1; P78852: putative cell wall *a*-glucan synthase Ags1 from *Schizosaccharomyces pombe*; ORF PAB2292: putative glycogen synthase from *Pyrococcus abyssi*; GLGA_ECOLI: glycogen synthase from *E. coli*; O48899: maize starch synthase isoform zSTSII-1; BAA82346: granule-bound starch synthase I from *Phaeodurus vulgaris*; KRE2_CANAL: glycopid 2-α-mannosyltransferase MNT1 or KRE2 from *Candida albicans*; KRE2 YEAST: glycopid 2-α-mannosyltransferase MNT1 or KRE2 from *S. cerevisiae*; YUR1 YEAST: probable mannosyltransferase YUR1 from *S. cerevisiae*; KTR3 YEAST: probable mannosyltransferase KTR3 from *S. cerevisiae*; O60160: putative 2-α-mannosyltransferase ( locus SPBC19C7) from *S. pombe*.

![Multiple sequence alignment of deduced amino acid sequences of selected glycosyltransferases.](Image)

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<td>ORF C68904</td>
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<tr>
<td>11</td>
<td>O60160</td>
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To further assess the significance of this similarity, we performed hydrophobic cluster analysis (HCA) and secondary structure prediction of a 60-amino acid peptide spanning the E-X-E motif on a set of representative proteins of the aforementioned families (Fig. 2). Again, a number of features are conserved among the proteins analyzed, thus supporting the hypothesis that these four families are related. Both the shape of the hydrophobic clusters in the HCA profiles and secondary structure prediction anticipated the presence of an α-helix 12–15 amino acids before the E-X-E stretch. Both methods predicted two β-sheets, located 5–7 amino acids and 20–30 amino acids after this motif, respectively. Additionally, the profiles of the hydrophobic clusters just before the first Glu residue are compatible with a β-sheet, which is found by secondary structure prediction in all cases but one. These observations indicate that these proteins presumably present similarities at the level of secondary structure in the region encompassing the E-X-E motif and further suggest that the invariant Glu residue plays an essential role in the enzymatic activity of this class of enzymes. Although the second Glu of the motif is not strictly conserved, it must be noted that in all cases the amino acid that occupies this position can hypothetically act as a proton donor/acceptor.

**The GFP-HMGS Fusion Protein Is Catalytically Active**—One way to show that a given amino acid residue of an enzyme is essential for catalysis consists of mutating this particular amino acid and verifying that the mutant enzyme has a greatly decreased or null activity. This approach requires the use of a recombinant expression system that permits the production of active enzyme. Owing to the difficulties in obtaining reasonable amounts of soluble and active muscle GS by overexpression of...
the protein in *Escherichia coli* (44), we decided to use eukaryotic cells to express the chimerical protein constructed by fusing the green fluorescent protein (GFP) at the N-terminal end of HMGS. This system enables the ready observation of the intracellular localization of the GFP-HMGS chimera and thus represents an adequate means to verify the overall structural integrity of inactive mutants.

To study whether the GFP-HMGS fusion protein was catalytically active, COS-1 cells were transiently transfected with the pEGFP-C1 and pEGFP-HMGS plasmids, and homogenates from these cultures were assayed for GS activity. GFP-expressing COS-1 cells displayed endogenous GS activity, but total GS activity of cells overexpressing GFP-HMGS was approximately 8-fold that of control cells (Table I). Roach and co-workers obtained similar results when rabbit muscle GS was transiently expressed in COS M9 cells (45). The activity ratio of

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**FIG. 2.** HCA alignment of the region spanning the E-X7-E motif. The HCA plots of a 60-amino acid peptide spanning the E-X7-E motif are presented for one protein of each glycosyltransferase family analyzed. The regions showing similarity at the HCA level are boxed. Circles indicate the conserved residues of the motif. The protein sequences are written on a duplicated α-helical net, and the contour of clusters of hydrophobic residues is automatically drawn. The standard one-letter code for amino acids is used except for proline, glycine, serine, and threonine, which are represented by solid star, solid diamond, dotted square, and blank square, respectively. The secondary structure predicted by the JnetPret algorithm is shown below the HCA plot for each protein as a bar for an α-helix and an arrow for a β-sheet. SUS1_MAIZE: maize sucrose synthase 1; UGS1_HUMAN: human muscle glycogen synthase; GLGA_ECOLI: glycogen synthase from *E. coli*; KRE2_CANAL: glycolipid 2-α-mannosyltransferase MNT1 or KRE2 from *C. albicans*.

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5 J. C. Ferrer and J. J. Guinovart, unpublished results.
GFP-HMGS expressed in COS-1 cells increased from 0.13 ± 0.05, when determined in homogenates from cells incubated in a glucose-free medium, to 0.22 ± 0.09 in cells kept in the presence of 30 mM glucose for 4 h. This result further suggests that the fusion of GFP at the N terminus of HMGS does not significantly interfere with the normal function of the enzyme.

The GFP-HMGS Fusion Protein Binds to Intracellular Glycogen—In previous studies we have shown that the intracellular distribution of GFP-HMGS is dependent on the presence of glucose in the incubation medium. Thus, in the absence of glucose, GFP-HMGS was concentrated in the nucleus and translocated to the cytosol in response to the presence of the sugar. In both compartments, the fusion protein showed a punctuate pattern, and the size and the apparent complexity of the particles in the cytosol increased as incubation with glucose was prolonged (26), suggesting that most of the GFP-HMGS fusion protein was bound to glycogen particles. To test this hypothesis, immunocytochemical experiments were performed using a monoclonal antibody that has been shown to specifically bind to glycogen from chondrocytes, hepatocytes, and muscle cells, as well as to purified glycogen (27). First, we checked the ability of this antibody to bind to glycogen particles produced by COS-1 endogenous GS. Cells were transfected with the pEGFP-C1 vector and were incubated in a glucose-free medium. In these conditions, COS-1 cultures stored negligible amounts of glycogen, and no immunofluorescence arising from the anti-glycogen antibody could be detected (Fig. 3A). In contrast, cells incubated for 4 h in a medium containing 30 mM glucose accumulated 17 ± 10 μg of glycogen/mg of protein and showed a clear punctuate pattern in the confocal image, which was attributable to glycogen labeling (Fig. 3B). Furthermore, treatment of these cells with α-amylase after paraformaldehyde fixation and permeabilization completely abolished the fluorescence signal (not shown), thus confirming the specificity of the anti-glycogen antibody. This experiment also showed that the intracellular distribution of GFP was insensitive to the presence of glucose in the incubation medium and to the accumulation of glycogen (Fig. 3).

In another set of experiments, COS-1 cells were transfected with the pE0FP-HMGS plasmid and were also immunostained with the anti-glycogen antibody. In the absence of glucose, transfected cells did not accumulate measurable amounts of glycogen and no fluorescent signal arising from glycogen immunolabeling was detected (not shown). As previously reported (26), under these conditions green fluorescence from GFP-HMGS was mainly found in the nucleus (not shown). After 4 h incubation with 30 mM glucose, GFP-HMGS was almost exclusively found in the cytosol, mostly as round-shaped aggregates (Fig. 4, A and D). Surprisingly, the number of specks that were immunolabeled with the glycogen antibody was much lower in cells overexpressing GFP-HMGS than in non-transfected cells of the same preparation (Fig. 4, B and E). The percentage of transfection achieved in these experiments was always higher than 70%, and transfected and non-transfected COS-1 cultures, when incubated for 4 h with 30 mM glucose, reached similar levels of glycogen (170 ± 10 μg of glycogen/mg of protein).

Therefore, the decreased glycogen immunolabeling could not be attributed to the accumulation of lower amounts of the polysaccharide in the GFP-HMGS-expressing cultures. Rather, this finding suggests that the overexpressed fusion protein blocked the access of the antibody to glycogen particles. This hypothesis was supported by the observation that some very large GFP-HMGS aggregates, which were occasionally produced (Fig. 4D), were also immunolabeled with the glycogen antibody (Fig. 4E). However, the red fluorescence attributable to glycogen staining was mainly found in the center of the large round-shaped aggregates, whereas the green fluorescence from GFP-HMGS was concentrated in the perimeter, and both labels appeared to be mutually exclusive over the same particle (Fig. 4F).

To further corroborate the association between the GFP-HMGS fusion protein and intracellular glycogen, COS-1 cells transiently expressing GFP or GFP-HMGS were incubated in the presence of 30 mM glucose for 4 h and were permeabilized with digitonin before fixation and observation in the confocal microscope. This treatment was effective in releasing soluble proteins, as shown by the removal of GFP. However, in GFP-HMGS-expressing cells the fusion protein was not completely released by this treatment and the removal of GFP-HMGS was only achieved when digitonin-permeabilized cells were incubated with α-amylase to degrade glycogen before fixation (not shown). We conclude that the particulate pattern shown by the GFP-HMGS chimera is due to its close association with the glycogen particles produced when COS-1 cells are incubated in the presence of glucose.

Characterization of the GFP-HMGS (E510A) and GFP-HMGS (E518A) Mutant Proteins—To test the roles of Glu-510 and Glu-518 in the catalytic activity of HMGS, these two residues were mutated to Ala in the plasmid pEGFP-HMGS and the resulting mutant proteins were transiently expressed in COS-1 cells. Homogenates from these cultures were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting, using an anti-GFP antibody. The mutant proteins exhibited the expected molecular mass of ~110 kDa and were expressed at similar levels to the wild-type protein (Fig. 5). The integrity of the GFP-HMGS (E510A) and GFP-HMGS (E518A) proteins was further confirmed by confocal microscopy analysis of their intracellular distribution in transiently transfected COS-1, hepatocytes, and L6 myoblasts. In each cell type and in both the presence and absence of glucose in the incubation media, the two mutant enzymes exhibited an identical distribution to that of GFP-HMGS (not shown). The size and shape of the aggregates produced by the mutant proteins in the presence of glucose were very similar to those of the wild-type fusion enzyme. Moreover, glycogen immunolabeling of COS-1 cells was also partially blocked by the overexpression of both GFP-HMGS (E510A) and GFP-HMGS (E518A). The observation that the mutant proteins retained the ability to change their intracellular localization in response to glucose and to bind to glycogen strongly suggested that the mutations did not affect the overall structural integrity of the enzyme. Thus, changes in the activity of the mutants can be directly attributed to local disturbances at the active site region.

Detailed kinetic studies of the recombinant enzymes were prevented by the presence of endogenous GS activity. However, homogenates from COS-1 cells transiently expressing the wild-type and the mutant chimerical proteins were assayed for total GS activity. GFP-HMGS (E518A)-expressing cultures showed a slightly higher total GS activity than GFP-expressing cells.

### Table I

<table>
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<th>Total glycogen synthase activity</th>
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<tr>
<td>GFP</td>
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</tr>
<tr>
<td>GFP-HMGS</td>
<td>97.1 ± 7.9</td>
</tr>
<tr>
<td>GFP-HMGS (E510A)</td>
<td>11.8 ± 4.2</td>
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<td>GFP-HMGS (E518A)</td>
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Total GS activity in GFP and GFP-HMGS-expressing COS-1 cells

COS-1 cells were transfected following the DEAE-dextran method and were incubated for 42 h in DMEM supplemented with 25 mM glucose and 10% PBS to allow for protein expression. Cells overexpressing the indicated protein were then collected, and total GS activity was measured, as indicated under "Experimental Procedures." Data represent the mean ± S.E. for five independent experiments.
(Table I), indicating that the E518A mutant retained approximately 6% of the activity shown by the wild-type GFP-HMGS enzyme in the conditions of the assay. This small increase in GS activity over the control was consistently observed in all the individual experiments performed. In contrast, homogenates from cells expressing the E510A variant of HMGS did not exhibit a significant difference in activity when compared with control cells. We conclude that both Glu residues are involved in catalysis: Glu-510 is a critical residue, whereas Glu-518 plays a more secondary role.

**DISCUSSION**

In this study we have combined bioinformatic and experimental techniques to identify two Glu residues at the active site of eukaryotic GSs, using HMGS as a model. We have taken advantage of the classification of glycosyltransferases into 43 families proposed by Campbell et al. (24, 25), according to sequence similarity and the stereoechemical course of the reaction. Through the use of BLAST searches and multiple alignments we have found an E-X-E motif that is highly conserved among the members of families 3, 4, 5, and 15 of glycosyltransferases, all of which operate with retention of configuration at the anomeric carbon. Hydrophobic cluster analysis and secondary structure prediction of this region supported the hypothesis that these four families are related. In eukaryotic GSs, all belonging to family 3, this motif is enclosed within an invariant 17-amino acid stretch found roughly in the last third of the
corresponding coding sequences and in the region where these proteins exhibit the largest degree of similarity. This conserved core region has previously been assumed to contain the catalytic site, in contrast to the more variable N and C termini, which harbor the phosphorylation sites that regulate the enzyme activity (4).

The functional role of Glu-510 and Glu-518 in the E-X7-E motif of HMGS was probed by site-directed mutagenesis. The wild-type enzyme and two single point mutants, in which the conserved Glu residues were replaced by Ala, were transiently expressed in COS-1 cells as fusions to GFP. The structural integrity of the chimerical mutant proteins was shown in several members of the glycosyltransferases of family 4 differ-

Assuming that highly conserved regions in enzymes contain crucial residues for catalytic activity, the E-X7-E motif must be involved either in substrate recognition and binding or in catalysis. However, considering the large variety of glycoyl donors (GDP-mannose, ADP- and UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, etc.) and acceptors (mono- and polysaccharides, glycolipids, glycoproteins, etc.) used by the proteins of families 3, 4, 5, and 15, only the active site would be clearly conserved in all of them. The observation that both mutant forms of GFP-HMGS bound to glyconogen was also an indication that the glyconogen-binding site of the enzyme was not significantly disturbed by the single point mutations. Additionally, Lys-38 of the rabbit muscle GS has been implicated in UMP-glucose binding, suggesting that this substrate binds to the N-terminal half of the enzyme (18). It is therefore reasonable to assume that Glu-510 and Glu-518 are part of the HMGS active site machinery, and by analogy, the corresponding residues of other eukaryotic GSs play an identical role. The same may be true for the glycosyl transferases from families 4, 5, and 15 of Campbell’s classification, although in these cases, experimental confirmation would be required. This type of evidence has been obtained for Ace-A (35, 46), an α-mannosyltransferase that belongs to family 4. Geremia et al. (42) found an E-X7-E motif similar to that described here in a group of prokaryotic α-mannosyltransferases and proposed that both conserved Glu residues were important for catalysis. The replacement by Ala residues of Glu-287 or Glu-295 in Ace-A (equivalent to Glu-510 and Glu-518 in HMGS, respectively) led to the same changes in enzymatic activity as those observed in HMGS. The E827A variant was inactive, whereas Ace-A (E295A) showed very little activity. Very recently, Nichols et al. (37) have shown that Glu-391 of maize starch synthase IIB-2, a glycosyltransferase from family 5, is essential for activity. According to our alignments, this residue corresponds to the indispensable Glu-510 in HMGS.

Enzymatic reactions that involve the substitution of a group at an asymmetric carbon atom and yield a product with the same configuration as the substrate generally operate by two successive displacements on the asymmetric carbon (10). In retaining glycosidases, the first step involves the formation of an inverted substrate-enzyme intermediate through the coordinated attack of a nucleophile at the sugar anomeric center and the protonation of the glycosidic oxygen by a residue acting as a general acid catalyst. In the second step, the latter provides general base catalytic assistance and deprotonates a water molecule, which in turn attacks the anomeric carbon once again, thus yielding the final product. Through site-directed mutagenesis and kinetic analysis of the mutants, the catalytic residues of several retaining glycosidases, always Asp or Glu residues, have been identified and their respective roles assigned (39). Mutant enzymes in which the nucleophile has been replaced by an Ala residue are essentially inactive. When the acid/base catalytic residue is eliminated, the resulting protein retains some activity with very good substrates, i.e., those bearing good leaving groups. In this situation, protonation of the leaving group is not crucial for catalysis (39).

Kapitonov and Yu described a domain (NRD1α), present in several members of the glycosyltransferases of family 4 different from the α-mannosyltransferases analyzed by Geremia et al. (42), which also contained an E-X7-E segment. The authors arbitrarily proposed, by analogy with the mechanism of retaining glycosidases, that the first conserved Glu residue was the general acid/base catalyst, while the second one acted as the nucleophile (43). However, these assumptions were not supported experimentally.

Our results argue against the roles assigned to the two conserved Glu residues by Kapitonov and Yu. First, the sequence comparisons with selected glycosyltransferases show that, although the first Glu residue of the motif is invariant, the second Glu is more variable and therefore better fits the secondary role of the acid/base catalyst. It has to be noted that, in all the enzymes analyzed in this study, the second residue is always an amino acid whose lateral chain can putatively act as a proton donor/acceptor. Second, the E510A mutation in HMGS completely inactivates the enzyme, whereas the E518A mutant maintains some residual activity. The glycosyl donor in the synthesis of glycogen is UDP-glucose. The chemical nature of UDP dictates that this moiety can act as a good leaving group even when it is not protonated and thus the glycosidic reaction might still proceed at a measurable rate in the absence of an acid catalyst. Our results are consistent with Glu-510 being the
fundamental nucleophile and with Glu-518 providing important but not essential catalytic assistance, possibly as the general acid/base catalyst. Further experiments are in progress to determine the exact roles of both conserved Glu residues of the E-X-E motif in the catalysis by GS.

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