Histidine 167 Is the Phosphate Acceptor in Glucose-6-phosphatase-β Forming a Phosphohistidine Enzyme Intermediate during Catalysis

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The glucose-6-phosphatase (Glc-6-Pase) family comprises two active endoplasmic reticulum (ER)-associated isozymes: the liver/kidney/intestine Glc-6-Pase-α and the ubiquitous Glc-6-Pase-β. Both share similar kinetic properties. Sequence alignments predict the two proteins are structurally similar. During glucose 6-phosphate (Glc-6-P) hydrolysis, Glc-6-Pase-α, a nine-transmembrane domain protein, forms a covalently bound phosphoryl enzyme intermediate through His176, which lies on the luminal side of the ER membrane. We showed that Glc-6-Pase-β is also a nine-transmembrane domain protein that forms a covalently bound phosphoryl enzyme intermediate during Glc-6-P hydrolysis. However, the intermediate was not detectable in Glc-6-Pase-β active site mutants R79A, H114A, and H167A. Using [32P]Glc-6-P coupled with cyanogen bromide mapping, we demonstrated that the phosphate acceptor in Glc-6-Pase-β is His167 and that it lies inside the ER lumen with the active site residues, Arg79 and His114. Therefore Glc-6-Pase-α and Glc-6-Pase-β share a similar active site structure, topology, and mechanism of action.

The glucose-6-phosphatase (Glc-6-Pase) family is composed of three proteins: Glc-6-Pase-α (1–4), Glc-6-Pase-β (5–7) (previously known as UGRP ubiquitously expressed Glc-6-Pase related protein), and islet-specific Glc-6-Pase-related protein (8, 9). Whereas Glc-6-Pase-α and Glc-6-Pase-β are functional phosphohydrolases, the islet-specific Glc-6-Pase-related protein lacks enzymatic activity.

The prototype of the family, Glc-6-Pase-α, is a 357-amino acid, nine-transmembrane domain, endoplasmic reticulum (ER)-associated protein (10, 11), which is expressed primarily in the liver, kidney, and intestine (12, 13). Glc-6-Pase-α catalyzes the hydrolysis of glucose 6-phosphate (Glc-6-P) to glucose in the terminal step of gluconeogenesis and glycogenolysis (13). Between meals, the resulting release of glucose to the blood maintains glucose homeostasis. Naturally occurring loss of function mutations in Glc-6-Pase-α cause glycogen storage disease type Ia, a disorder that is characterized by loss of blood glucose homeostasis and disorders of glycogen and lipid metabolism (reviewed in Refs. 14 and 15).

Glc-6-Pase-β is a ubiquitously expressed, 346-amino acid membrane protein that shares a 36% sequence identity to Glc-6-Pase-α (5–7). Despite the absence of any apparent ER retention motif, Glc-6-Pase-β is also localized in the ER membrane (6), although its orientation in the membrane is not known. The subcellular localization of the Glc-6-Pase-β active site is not known, although it is assumed to be similar to Glc-6-Pase-α. Both Glc-6-Pase-β and Glc-6-Pase-α couple with the Glc-6-P transporter to form an active Glc-6-Pase complex, and both share similar kinetic properties with respect to Glc-6-P hydrolysis (6).

The active site of Glc-6-Pase-α was originally identified by the presence of a conserved phosphatase signature motif found in lipid phosphatases, acid phosphatases, and vanadium halo-peroxidases (16, 17). This motif was shown to contribute to the active site of a vanadium chloroperoxidase by both x-ray crystal structure analysis (18) and mutational studies (19, 20) and has since been shown to have a similar role in Glc-6-Pase-α (21, 22). Hydrolysis of Glc-6-P to glucose and phosphate via a covalent phosphohistidine-Glc-6-Pase-α intermediate was first proposed by Nordlie and Lygre (23) based on pH kinetic studies of Glc-6-Pase-α catalysis, which was confirmed by the identification of an enzyme-bound 32P-labeled histidine after incubating rat liver microsomes with [32P]Glc-6-P (24–26). Critical residues for Glc-6-Pase-α catalysis in the active site motif include: Arg83, which donates hydrogen ions to the phosphate and stabilizes the transition state; His114, which provides a proton to liberate the glucose moiety; and His176, which undertakes a nucleophilic attack on the phosphate to form a covalently bound phosphoryl enzyme intermediate (21, 27). In Glc-6-Pase-α, all of these residues lie together on the luminal side of the ER membrane (10, 11).

Given the sequence similarity between Glc-6-Pase-α and Glc-6-Pase-β, a similar active site structure would be anticipated. Sequence alignments predict that His167 is the residue that forms a covalent bond with phosphate to create a phosphoryl-Glc-6-Pase-β intermediate, whereas Arg79 and His114 are the hydrogen donors. These alignments also suggest that Glc-6-Pase-β contains nine putative transmembrane helices and that Arg79, His114, and His167 lie inside the ER lumen.

In an effort to understand the role of this ubiquitous Glc-6-Pase activity, we have undertaken a study of the active site of Glc-6-Pase-β and confirmed that it is similar to Glc-6-Pase-α. This information can now be used to design experiments that disrupt the activity of Glc-6-Pase-β and investigate the biological importance of a Glc-6-Pase activity that is not limited in expression to the liver, kidney, and intestine, the organs pre-
EXPERIMENTAL PROCEDURES

Construction of Glc-6-Pase-β Mutants—Mutants were constructed using the same general strategy used for Glc-6-Pase-α (22). Briefly, the human Glc-6-Pase-β cDNA was engineered to remove the internal SfiI site (6) and then used as a template for PCR-based N-terminal epitope tagging using either the FLAG (DYKDDDDK) or His6, epitopes. The resulting PCR products were cloned directionally into the pSVL vector to generate pSVL-Glc-6-Pase-β-5FLAG and pSVL-Glc-6-Pase-β-His10. The human pSVL-Glc-6-Pase-β-3FLAG construct has been described by Shieh, et al. (6).

For the protease protection assays, Glc-6-Pase-β constructs incorporating two cleavable factor Xa tetrapeptide (IEGR) (28) recognition motifs were constructed by PCR and cloned into the pSVL vector. To locate the factor Xa tetrapeptide recognition motifs between the N-terminal FLAG epitope and the Glc-6-Pase-β coding sequence, a 5′ primer containing an ATG initiation codon followed by the 24-bp FLAG coding sequence, two 5′a recognition sequences (back to back) (ATCGAGGGTAGAATCGAGGGTAGA), and nucleotides 217–237 of Glc-6-Pase-β (GenBank™ accession number XM_045901) was used. The 3′ primer represented nucleotides 1237–1257 of human Glc-6-Pase-β. The region was then named Glc-6-Pase-β-5XaFLAG. To locate the factor Xa tetrapeptide recognition motifs between the Glc-6-Pase-β coding sequence and the C-terminal FLAG epitope, the 5′ primer represented nucleotides 217–237 of human Glc-6-Pase-β, whereas the 3′ primer contained human Glc-6-Pase-β nucleotides 1237–1257 followed by the 24-bp 5′a recognition sequence, the 24-bp FLAG coding sequence, and a termination codon. This construct was named Glc-6-Pase-β-3XaFLAG. All constructs were verified by DNA sequencing.

Construction of Recombinant Adenoviral Glc-6-Pase-β Mutants—The recombinant adenovirus containing Glc-6-Pase-β-His10 was generated by the Cre-lox recombination system described by Hardy, et al. (29). The Ad-Glc-6-Pase-β-His10 virus was plaque-purified and amplified to produce large titers of titers of 5–1010 plaque-forming units/ml. The C-terminal FLAG-tagged constructs, Ad-Glc-6-Pase-β, Ad-Glc-6-Pase-β-R79A, Ad-Glc-6-Pase-β-H1114A, and Ad-Glc-6-Pase-β-H167A, were described previously (6).

Protease Protection Assays and Western Blot Analysis—COS-1 cells in 25-cm2 flasks were transfected with 10 μg of Glc-6-Pase-β-5FLAG, Glc-6-Pase-β-3FLAG, Glc-6-Pase-β-3XaFLAG, or Glc-6-Pase-β-3XaFLAG transfected cells were then treated with trypsin or factor Xa treatment, were used as controls. The 32P-labeled phosphoryl microsomal proteins from Ad-Glc-6-Pase-β-His10-infected cells were precipitated by trichloroacetic acid as described above. The pellet was washed twice with 100 μl of ice-cold 0.5 M Tris-HCl, pH 8.0, and once with 100 μl of cold water and finally dissolved in 400 μl of Solution A (25 mM Tris-HCl, pH 8.0, 1% Triton X-100, 150 mM NaCl) containing the complete EDTA-free protease inhibitor mixture. The resulting proteins were fractionated by electrophoresis through a 12% SDS-polyacrylamide gel and the 32P-labeled polypeptide band was visualized by autoradiography.

Cyanogen Bromide Cleavage of 32P-Phosphoryl-Glc-6-Pase-β Peptides by Gel Electrophoresis and Isoelectric Focusing—The 32P-phosphoryl-Glc-6-Pase-β-His10 intermediate was resuspended in 400 μl of a solution containing 32 mg of cyanogen bromide/ml of 70% formic acid (30, 31) and digested overnight in the dark at room temperature. Following digestion, the products were dialyzed against water at 4 °C using Slide-A-Lyzer 3.5K Dialysis Cassettes (Pierce), lyophilized, dissolved in SDS sample buffer, resolved by electrophoresis through a 10% NuPAGE Bis-Tris gel, and visualized by autoradiography.

Membrane Topography of Glc-6-Pase-β—Glc-6-Pase-β-a is embedded in the ER membrane by nine-transmembrane domains (10, 11) oriented with the N terminus in the ER lumen and the C terminus in the cytoplasm. Hydrophy analysis of the Glc-6-Pase-β amino acid sequence using the TMpred program (32) predicts that Glc-6-Pase-β is also anchored in the ER by nine putative transmembrane helices (Fig. 1). If this is correct, the N and C termini of the protein will be on opposite sides of the ER membrane. To determine the subcellular localization of the termini of Glc-6-Pase-β, we undertook a protease protection assay using the N- and C-terminal FLAG-tagged Glc-6-Pase-β constructs. Intact microsomes from COS-1 cells transfected with Glc-6-Pase-β-5FLAG or Glc-6-Pase-β-3FLAG were digested with trypsin, and the trypsin fragments were analyzed by Western blot analysis (Fig. 2A). Microsomes expressing Glc-6-Pase-β-3FLAG yielded no FLAG-tagged peptides upon digestion consistent with an exposed cytoplasmic C terminus. In contrast, microsomes expressing Glc-6-Pase-β-5FLAG were more resistant to digestion. However, the trypsin-resistant
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Identification of the Phosphoryl-Glc-6-Pase-β Intermediate—We have shown previously that during catalysis, His^{176} in Glc-6-Pase-α acts as a phosphate acceptor forming a covalently bound phosphoryl enzyme intermediate (21). The kinetic and topographical similarities between Glc-6-Pase-β and Glc-6-Pase-α suggest that a phosphoryl enzyme intermediate is also formed during Glc-6-Pase-β catalysis. Microsomes isolated from C-terminal FLAG-tagged Ad-Glc-6-Pase-β-, Ad-Glc-6-Pase-β-R79A-, Ad-Glc-6-Pase-β-H114A-, or Ad-Glc-6-Pase-β-H167A-infected COS-1 cells were incubated with ^{32}P[Glc-6-P, and the resulting ^{32}P[phosphoryl-Glc-6-Pase-β intermediate was isolated by immunoprecipitation and analyzed by gel electrophoresis and autoradiography. As expected, a 35-kDa ^{32}P[phosphoryl enzyme intermediate was identified in wild-type Glc-6-Pase-β-infected cells but not in R79A, H114A, or H167A active site mutant-infected cells (Fig. 3).

His^{167} Is the Phosphate Acceptor in Glc-6-Pase-β—Based on the nine helical domain topography of Glc-6-Pase-β (Fig. 1), the active site residues, Arg^{124}, His^{114}, and His^{167}, are predicted to lie inside the lumen of the ER. Sequence alignment predicts His^{167} is the phosphate acceptor in Glc-6-Pase-β. Human Glc-6-Pase-β contains nine methionine residues at positions, 1, 116, 126, 145, 186, 192, 207, 291, and 334 (Fig. 1), which can be cleaved by cyanogen bromide (30, 31). After cleavage, the His^{167} is predicted to lie within a 4.4-kDa peptide (residues 146–186) with a theoretical isoelectric point of 8.6.

To purify the ^{32}P[phosphoryl-Glc-6-Pase-β intermediate, we generated a Glc-6-Pase-β construct carrying a His^{147} tag at the N terminus (Ad-Glc-6-Pase-β-His^{147}). Microsomes from Ad-Glc-6-Pase-β-His^{147}-infected COS-1 cells were labeled with ^{32}P[Glc-6-P, and the ^{32}P[phosphoryl-Glc-6-Pase-β intermediate was enriched by affinity chromatography and further purified by electrophoresis through a SDS-polyacrylamide gel. The gel-purified ^{32}P[phosphoryl-Glc-6-Pase-β intermediate was then cleaved by cyanogen bromide, and the cleaved products were
fractionated through a NuPAGE Bis-Tris gel. A major band with an apparent molecular mass of 4.4 kDa and minor bands with apparent molecular masses of 18, 16, and 10 kDa were identified (Fig. 4A). The 4.4-kDa peptide corresponds to the expected molecular weight of the His\(^{167}\)-containing peptide of 41 amino acids (residues 146–186).

To further demonstrate that the His\(^{167}\)-containing peptide of 4.4-kDa carries the \(^{32}\)P-labeled phosphate moiety, this 4.4-kDa band was eluted from the gel and analyzed by gel electrophoresis and isoelectric focusing. The purified \(^{32}\)P-labeled peptide migrated as a 4.4-kDa band through a 10% NuPAGE Bis-Tris gel (Fig. 4B) with an isoelectric point above 8.3 (Fig. 4C). The results firmly established that in Glc-6-Pase-β, His\(^{167}\) is the amino acid that covalently binds the phosphoryl moiety during Glc-6-Pase-β catalysis.

**DISCUSSION**

Blood glucose homeostasis between meals depends on gluconeogenesis and glycogenolysis. In the terminal step common to both pathways, Glc-6-P is hydrolyzed to glucose by the Glc-6-Pase complex, which is composed of two functionally coupled proteins, the Glc-6-P transporter and a Glc-6-P phosphohydrolase (reviewed in Ref. 14). Until recently, only one functional Glc-6-Pase had been reported. This enzyme, previously called Glc-6-Pase, is expressed in the liver/kidney/intestine consistent with its role in glucose homeostasis (reviewed in Ref. 13). More recently, a ubiquitously expressed Glc-6-Pase-related protein, which was previously called UGRP and thought to be inactive (5), was demonstrated to be catalytically active (6, 7). As a result, the proteins have been renamed Glc-6-Pase-α (liver/kidney/intestine Glc-6-Pase) and Glc-6-Pase-β (UGRP). Glc-6-Pase-α is well characterized, but little beyond the kinetic activity (6) and ER localization (6) of Glc-6-Pase-β is known. As a first step to characterizing the biological relevance of Glc-6-Pase-β, we have sought to examine whether it is structurally similar to Glc-6-Pase-α, a hydrophobic protein embedded in the ER by nine-transmembrane helical domains (10, 11). The key active site residues in Glc-6-Pase-α have been characterized (21, 22) and shown to lie facing into the lumen of the ER. During catalysis, Glc-6-Pase-α forms a phosphoryl enzyme intermediate (23–26) by the formation of a covalent bond between the phosphoryl group of Glc-6-P and His\(^{170}\) in Glc-6-Pase-α (21).

Glc-6-Pase-β lacks any apparent ER transmembrane protein retention motif, but double immunofluorescence microscopy studies have shown that it is localized in the ER (6). Theoretical modeling of the topography of Glc-6-Pase-β using the TMpred program (32) predicts that Glc-6-Pase-β is anchored in the membrane by nine helical domains. Using epitope-tagged Glc-6-Pase-β constructs and a protease protection assay by limited trypsin digestion, we found that the C terminus of Glc-6-Pase-β is exposed to the cellular cytoplasm but that the N terminus appears protected. Cleavage of the N-terminally FLAG-tagged protein yielded a 14-kDa peptide fragment that is consistent with a nine-transmembrane domain topology. A prediction of tryptic cleavage sites in the FLAG-tagged Glc-6-Pase-β by the Ensembl (www.ensembl.org) Peptide Cutter server reveals 20 potential cleavage sites (Fig. 1). Cleavage between residues 136 and 137 (...VAT(R/A)RSR....) would yield the closest theoretical fragment (15.5 kDa) to that observed. Given the increased electrophoretic mobility expected from the negatively charged FLAG epitope, this suggests that the FLAG epitope lies on a fragment representing the first 136 residues of Glc-6-Pase-β. Sequence alignment to the nine-transmembrane domain model of Glc-6-Pase-α (10, 11) corresponds to the first cytoplasmically exposed cleavage site that lies in the putative second cytoplasmic loop (Fig. 1). There are five other trypsin sites between residues 1 and 136 in Glc-6-Pase-β, but by this model the first site is predicted to lie within the first transmembrane helix, the second site lies at the junction between cytoplasmic loop 1 and transmembrane helix 2, the next two sites lie within the second transmembrane helix, and the fifth site lies at the beginning of the first luminal loop. When the protease protection assays were repeated using constructs engineered to contain a unique protease cleavage site the results were consistent with a cytoplasmic C terminus and luminal N terminus. Taken together, Glc-6-Pase-β has been shown to have an odd number of transmembrane domains, and proteolytic digests are consistent with the nine-transmembrane topology predicted both by hydropathy analysis (32) and sequence alignment to Glc-6-Pase-α. In an earlier study, we showed that Glc-6-Pase-β couples with the Glc-6-P transporter to form an active Glc-6-Pase complex (6). This is also consistent with the nine-transmembrane domain model that puts the

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**Fig. 3. Identification of the phosphoryl-Glc-6-Pase-β intermediate by immunoprecipitation.** COS-1 microsomes expressing wild-type, R79A, H114A, or H167A C-terminally FLAG-tagged Glc-6-Pase-β were incubated with \(^{32}\)P[Glc-6-P. The resultant \(^{32}\)P phosphoryl enzyme intermediate was isolated by immunoprecipitation using a monoclonal anti-FLAG antibody and identified following fractionation through a 12% SDS-polyacrylamide gel by autoradiography as described under "Experimental Procedures.”

**Fig. 4. Cyanogen bromide mapping of the phosphoryl-Glc-6-Pase-β intermediate.** COS-1 microsomes expressing Glc-6-Pase-β, His\(^{10}\), were incubated with \(^{32}\)P[Glc-6-P to form the \(^{32}\)P phosphoryl enzyme intermediate, which was then enriched on a nickel chelate column and further purified by gel electrophoresis as described under “Experimental Procedures.” A, identification of the cyanogen bromide cleaved phosphoryl-Glc-6-Pase-β peptides by SDS-polyacrylamide gel electrophoresis and autoradiography. B, enrichment of the 4.4-kDa \(^{32}\)P phosphoryl-Glc-6-Pase-β peptide by gel electrophoresis. The 4.4-kDa \(^{32}\)P-labeled phosphoryl-cyogon bromide peptide was eluted from the SDS-polyacrylamide gel and its identity confirmed by NuPAGE Bis-Tris gel electrophoresis and autoradiography. C, identification of the 4.4-kDa \(^{32}\)P phosphoryl-Glc-6-Pase-β peptide by isoelectric focusing. The 4.4-kDa \(^{32}\)P-labeled phosphoryl-cyogon bromide peptide was eluted from the SDS-polyacrylamide gel was characterized by isoelectric focusing through a Novex Pre-Cast isoelectric focusing gel followed by autoradiography as described under “Experimental Procedures.” Numbers on the left of the figures are molecular weight markers, and numbers on the right are pH markers.
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active site residues Arg29, His114, and His167 on the luminal side of the membrane.

The kinetic and structural similarity between Glc-6-Pase-α and Glc-6-Pase-β catalysis suggests that the mechanism of action of the active site is also similar. In the hydrolysis of Glc-6-P, Glc-6-Pase-α forms a covalently bound intermediate between His126 and the phosphate moiety (21). The analogous residue in Glc-6-Pase-β is His167. Using 32P-labeled Glc-6-P we detected the [32P]phosphoryl-Glc-6-Pase-β intermediate from microsomes expressing Glc-6-Pase-β. However the intermediate is not detectable in microsomes expressing the Glc-6-Pase-α active site mutants R79A, H114A, or H167A.

To further identify His167 as the phosphate acceptor, we purified the [32P]phosphoryl-Glc-6-Pase-β intermediate by affinity chromatography and gel electrophoresis. Complete cleavage of the intermediate with cyanogen bromide is predicted to create 10 peptide fragments. The His167 is predicted to lie on a 4.4-kDa peptide with a theoretical pI of 8.6. When cleaved, the intermediate yielded a predominant fragment of 4.4 kDa having a pI above 8.3, which is consistent with His167 as the phosphate acceptor. In addition, several minor bands of 10, 16, and 18 kDa were also detected bound to the [32P]phosphoryl moiety. Complete cyanogen bromide digestion yielded no fragment greater than 13.2 kDa, whereas the next largest peptides were 9.2 and 5.0 kDa. The minor bands are, however, consistent with partial digests that contain His167. The 18-kDa band is consistent with the peptide containing amino acids 127–291, the 16-kDa band is consistent with the peptide containing amino acids 146–291, and the 10-kDa band is consistent with the peptide containing amino acids 127–192. Our results firmly established that His167 acts as the nucleophile forming the phosphohistidine enzyme intermediate during Glc-6-Pase-β catalysis.

In summary, we showed that the ubiquitous Glc-6-Pase-β is anchored in the ER by nine-transmembrane helices oriented with its active site inside the lumen, like the liver/kidney/intestine Glc-6-Pase-α. The similarity between Glc-6-Pase-β and Glc-6-Pase-α also extended to the nature of the active site residues Arg29, His114, and His167 and the formation of a covalently bound phosphoryl enzyme intermediate during catalysis. We are now in a position to study the effect of inactivation of Glc-6-Pase-β and assess its importance to blood glucose homeostasis and metabolism in general.

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