Identification of a Novel Serine Phosphorylation Site in Human Glutamine:Fructose-6-phosphate Amidotransferase Isoform 1[†]

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ABSTRACT: Glutamine:fructose-6-phosphate amidotransferase (Gfat) catalyzes the first and rate-limiting step in the hexosamine biosynthetic pathway. The increasing amount of evidence that links excess hexosamine biosynthesis with pathogenic complications of type II diabetes highlights the need to understand the regulation of Gfat. Previous studies showed that eukaryotic Gfat is subjected to feedback inhibition by UDP-*N*-acetyl-D-glucosamine (UDP-GlcNAc) and to phosphorylation by cAMP-activated protein kinase A (PKA). In this study, overexpression of human Gfat isoform 1 (hGfat1) in insect cells revealed that hGfat1 is phosphorylated *in vivo*. Using matrix-assisted laser desorption/ionization and electrospray tandem mass spectrometry, we have identified Ser243 as a novel phosphorylation site. Biochemical properties of the wild type and the Ser243Glu mutant of hGfat1 overexpressed in *Escherichia coli* were compared. Our results provide evidence that phosphorylation at Ser243 stimulates glucosamine 6-phosphate-synthesizing activity, lowers amidohydrolyzing activity in the absence of fructose 6-phosphate (F6P) (glutaminase activity), and lowers K_m(F6P) 2-fold, but has no effect on UDP-GlcNAc inhibition. On the basis of the sequence consensus, AMP-activated protein kinase and calcium/calmodulin-dependent kinase II were identified to phosphorylate specifically Ser243 *in vitro*. Phosphorylation by these two kinases results in an increase of enzymatic activity by 1.4-fold. These findings suggest for the first time that hGfat1 may be regulated by kinases other than PKA.

L-Glutamine:D-fructose-6-phosphate (F6P)[1] amidotransferase (Gfat) catalyzes the conversion of F6P to glucosamine 6-phosphate (GlcN6P) in conjunction with the hydrolysis of glutamine to glutamate. This reaction is the first and rate-limiting step in the hexosamine biosynthetic pathway (*1*). Subsequent steps metabolize GlcN6P to the major end product UDP-*N*-acetylglucosamine (UDP-GlcNAc), which is the essential precursor of glycoproteins and glycolipids in the endoplasmic reticulum and Golgi apparatus. Moreover, UDP-GlcNAc is the direct donor for O-linked β-*N*-acetylglucosamine modification (*O*-GlcNAc) on serine or threonine residues of numerous nucleocytoplasmic proteins (*2*). The hexosamine pathway was proposed to function as a nutrient-sensor controlling glucose metabolism and fuel storage (*3*, *4*). Overexpression of Gfat in liver cells (*5*), fat cells (*6*), or β-cells (*7*), which increases glucose flux through the hexosamine pathway, causes insulin resistance, a hallmark of type II diabetes. The mechanism of nutrient-sensing is likely mediated by the cellular level of *O*-GlcNAc modification, e.g., on regulatory proteins such as transcription factor Sp1 that regulates expression of related genes in response to the nutrient signals (*8*). Convincing evidence has further established the link between elevated *O*-GlcNAc modification and insulin resistance (*9*). The role that the hexosamine pathway may play in the pathogenic complications of type II diabetes therefore underlines the need to understand the basic regulation of Gfat by post-translational modifications.

The enzymatic properties were studied extensively for GlmS, the Gfat homologue from *Escherichia coli*. GlmS belongs to the N-terminal nucleophile subclass of amidotransferases which invariably utilize the Cys1 residue for nucleophilic attack during L-Gln hydrolysis (*10*). It is a dimeric and modular enzyme in which each monomer is composed of two domains: the glutaminase domain where L-Gln is hydrolyzed and the sugar isomerase domain where amination of F6P and isomerization occur. Crystal structures of separate domains and the entire protein have revealed the mechanism of interdomain communication (*11*). Bacterial Gfat is regulated at the gene expression level by the *glmS* ribozyme, which responds to changes of GlcN6P concentration (*12*).

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[1] Abbreviations: APAD, 3-acetylpyridine adenine dinucleotide; AMPK, AMP-activated protein kinase; BSA, bovine serum albumin; CaMKII, calcium/calmodulin-dependent kinase II; CIAP, calf intestinal alkaline phosphatase; DTT, dithiothreitol; F6P, D-fructose 6-phosphate; GDH, glutamate dehydrogenase; Gfat, glutamine:fructose-6-phosphate amidotransferase; GlcN6P, D-glucosamine 6-phosphate; GlmS, bacterial glucosamine-6-phosphate synthase; GluPA, L-γ-glutamyl-*p*-nitroanilide; hGfat1, human glutamine:fructose-6-phosphate amidotransferase isoform 1; IMAC, immobilized metal affinity column; MALDI, matrix-assisted laser desorption ionization; ESI-MS, electrospray ionization mass spectrometry; *O*-GlcNAc, O-linked β-*N*-acetylglucosamine; PKA, cAMP-dependent protein kinase A; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; UDP-GlcNAc, UDP-*N*-acetyl-D-glucosamine; WT, wild type.

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Gfat from several eukaryotic sources, e.g., human (*13*), rat (*14*), mouse (*15*), and yeast (*16*), have been isolated or cloned. Sharing 35−55% sequence identity with their bacterial homologues, they conserve all the active-site residues, which suggests a similar catalytic mechanism. The major difference at the primary structure level is that eukaryotic Gfat possesses an extra insert region of 50−70 amino acids at the end of the glutaminase domain. UDP-GlcNAc, the end product of hexosamine biosynthesis, is a competitive inhibitor of the F6P site of eukaryotic Gfat (*17*). This regulation by feedback inhibition agrees with the important role that Gfat plays in the glucose metabolism. A recent kinetic study on human Gfat isoform 1 (hGfat1) using a radioactive assay revealed that GlcN6P is also a potent inhibitor (*18*). Inhibition by GlcN6P has not been reported yet for Gfat from other eukaryotic sources. Gfat is subjected to phosphorylation by cAMP-dependent protein kinase A (PKA). PKA upregulated the activity of Gfat from rat liver (*19*), *Drosophila melanogaster* (*20*), and *Candida albicans* (*21*). In contrast, *in vitro* phosphorylation of recombinant hGfat1 by PKA reduced enzyme activity (*22*). The authors argued that PKA regulation might be isoform-dependent. Two putative PKA sites (Ser205 and Ser235) are present in hGfat1, while only one is present in Gfat isoform 2 (Ser205). Indeed mouse Gfat isoform 2 was stimulated by PKA *in vitro* (*15*). At the gene expression level, Gfat is strictly regulated and tissue-dependent in human (*23*). A splice variant of hGfat1 which has an insert of 18 amino acids at position 229 was found to be selectively expressed in striated muscle. It showed higher sensitivity to UDP-GlcNAc inhibition (*24*). Taken altogether, the regulation of eukaryotic Gfat is complex and has just started to be understood.

Purification of eukaryotic Gfat has been a challenge due to the low expression level and instability of the enzyme. Addition of tags at either the N- or the C-terminus to facilitate purification failed since both ends are essential for catalytic activity (*25*). Overexpression of hGfat1 in insect cells was recently reported. However, it required a multistep purification, and the yield was low (*18*). To advance our understanding of biochemical and structural properties of eukaryotic Gfat, the availability of active recombinant enzyme by a simple procedure seems to be the prerequisite. In a project initiated for inhibitor screenings, we have previously overexpressed hGfat1 with an internal His_6 tag in SF9 insect cells (*26*). Here we report identification of a novel phosphorylation site of hGfat1 and the corresponding kinases *in vitro*. A convenient bacterial expression system to produce active His_6-tagged hGfat1 is also presented.

EXPERIMENTAL PROCEDURES

General Materials. SF9 insect cells expressing His_6-tagged hGfat1 from pMAD1 were from GTP Biotechnology (France) (*26*). PrimeSTARHS DNA polymerase (TaKaRa Bio Inc.) was used for PCR amplification; AMP-activated protein kinase (AMPK) was purchased from Upstate. Calcium/calmodulin-dependent protein kinase II (CaMKII) was from New England Biolabs. Calf intestinal alkaline phosphatase (CIAP) was from Promega. Anti-pSer antibody was purchased from Qiagen; anti-pThr and anti-Tyr were from Cell Signaling Inc.

Cloning of Human gfat1 for Expression in E. coli. The gene *hgfat1* was amplified by PCR from donor plasmid pMAD1 for baculovirus expression using the following primers: forward 5′-CCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATAATGTGTGGTATATTTGCTTAC-3′ and reverse 5′-GCAAAAGCTTATTCGGCTTACGGATCCTCA-3′ (restriction sites are underlined). The purified 2.1 kb PCR product was digested and inserted between the *Xba*I and *Hin*dIII sites of pET-28a(+) (Invitrogen), yielding the construct PYLWT, which encoded the full-length hGfat1 with a His_6 tag inserted at position 298. *E. coli* DH5α strain (Invitrogen) was used for plasmid propagation. Site-directed mutagenesis was performed according to the manufacturer's protocol (GeneTaylor, Invitrogen) using pYLWT as the template. The primers used were forward 5′- GTTGGCACAAGGCGAGGGAACCCTCTGTT-3′ for Ser205Glu, forward 5′-CAAAGACAAGAAAGGGAACTGCAATCT-3′ for Ser235Glu, forward 5′-GCAATCTCTCTCGTGTGGACGCCACAACCTGCCTT-3′ for Ser243Ala, forward 5′- GCAATCTCTCTCGTGTGGACGAAACAACCTGCCTT-3′ for Ser243Glu (mutation sites are underlined), and reverse 5′-GTCCACACGAGAGAGATTGCAGCTTCCTTTC-3′.

Overexpression and Purification of Recombinant Internal His_6-Tagged hGfat1. Two liters of cultures of *E. coli* Rosetta (DE3) (Novagen) harboring pYLWT in LB medium supplemented with kanamycin (30 μg/mL) was grown at 37 °C with agitation until OD_{600} reached 0.5−1.0. Protein overexpression was induced by addition of 0.5 mM isopropyl β-D-thiogalactopyranoside. Cells were grown for an additional 4 h at 37 °C before being harvested. The cell pellets were resuspended in the lysis buffer (50 mM Tris−HCl, pH 7.8, 200 mM NaCl, 10 mM imidazole, 1 mM F6P, 2 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 10% glycerol, Roche EDTA-free protease inhibitor cocktail) and disrupted by sonication. The recombinant protein was purified to homogeneity in one step on a 5 mL HiTrap Ni−NTA column (GE Healthcare) connected to an AKTAExplorer system. The purified hGfat1 was stored in the elution buffer (50 mM Tris−HCl, pH 7.8, 200 mM NaCl, 1 mM F6P, 2 mM TCEP, 200 mM imidazole, 10% glycerol) at −80 °C and was stable for up to 12 months.

The recombinant hGfat1 expressed in insect cells was purified as above with some modifications. The cells were lysed using glass beads on a DynoMill system. After nickel affinity chromatography, hGfat1 was further purified by gel filtration on a Superdex 200 HR 26/60 column (GE Healthcare) and stored at − 80 °C in the following buffer: 50 mM Tris−HCl, pH 7.5, 2 mM TCEP, 1 mM F6P, 10% glycerol.

Phosphoprotein Detection by Gel Staining and Western Immunoblotting. The purified hGfat1 from baculovirus-infected insect cells was separated by SDS−PAGE and stained with Pro-Q Diamond phosphoprotein dye following the manufacturer's protocol (Molecular Probes). The total quantity of protein was analyzed by staining with SYPRO Ruby gel dye (Molecular Probes). The gel was visualized using a UV transilluminator of the BioRad ChemDoc XRS system. Western immunoblotting analysis was performed using standard protocols.

In Vitro Phosphorylation by Protein Kinases. For a standard 20 μL kinase assay, hGfat1 purified from *E. coli* (2 μg) was incubated with different quantities of AMPK (0 to 0.3 units) or activated CaMKII (0−100 units) in the presence of 0.2 mM ATP in 1× kinase buffer (50 mM Tris−

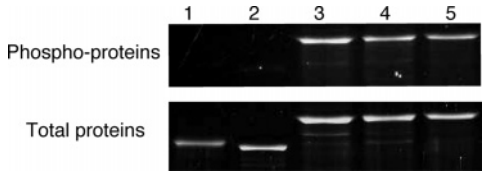

FIGURE 1: Determination of the phosphorylation states of recombinant hGfat1 expressed in insect cells. Proteins (500 ng each) were separated on 8% SDS−PAGE. The gel was stained first with phosphoprotein dye and then with SYPRO Ruby dye to determine the total protein level. Key: lane 1, BSA; lane 2, GlmS; lane 3, hGfat1 WT; lane 4, Ser205Glu; lane 5, Ser235Glu.

HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 20 μM F6P) at 37 °C for 20 min. In the case of AMPK assay, 0.1 mM AMP was included in the reaction. CaMKII was activated by 1.2 μM calmodulin, 2 mM $CaCl_2$, and 0.1 mM ATP in 1× kinase buffer at 30 °C for 10 min before use.

Determination of the Phosphorylation Site by Mass Spectrometry. In-gel digestion of recombinant hGfat1 was performed according to the established procedure (*27*). hGfat1 was reduced, alkylated by iodoacetamide, and then digested by trypsin (Promega, MS grade) at 37 °C for 8 h. The subsequent peptides were extracted by 5% formic acid/50% acetonitrile. Phosphopeptides were enriched using a ZipTipMC column (Millipore). MALDI experiments were performed on a Voyager DE-STR (Applied Biosystems), and the mass spectra were acquired on the reflectron in positive ion mode. ESI tandem MS was performed on a hybrid quadrupole-TOF mass spectrometer (API Qstar Pulsar *i*, Applied Biosystems) equipped with a nano-ESI capillary (Proxeon, Denmark). Collision-induced dissociation of the phosphorylated peptide was carried out by selecting the doubly charged species at *m*/*z* 802.8 as the precursor ion.

Enzymatic Activity Assays. Enzyme activity was measured following established protocols (*28*). One enzyme unit is defined as 1 μmol of Glu or GlcN6P produced per min. GlcN6P-synthesizing activity was measured by the modified colorimetric method of Morgan−Elson. The reaction mixture was incubated at 37 °C for 20 min before GlcN6P detection. For kinetic studies, the concentration of one substrate was varied while the other one was kept at saturation concentration (6 mM for F6P and 10 mM for Gln). The data were analyzed by the Igor Pro program (Oregon). The production of Glu was coupled to glutamate dehydrogenase (GDH) and followed spectrometrically at 37 °C by the appearance of reduced 3-acetylpyridine adenine dinucleotide (APADH) at 365 nm. The assay mixture contained 50 mM potassium phosphate, pH 7.5, 1 mM EDTA, 50 mM KCl, 1 mM APAD, 13 units of GDH, 6 mM F6P, 10 mM Gln, and hGfat1 (0.5−1 μg) in a total volume of 1 mL. The amidohydrolyzing activity in the absence of F6P (glutaminase activity) was determined using a glutamine analogue, L-γ-glutamyl-*p*-nitroanilide (GluPA), in a 96-well microplate format as previously described (*18*).

RESULTS

Gfat Is a Phosphoprotein in Vivo. Considering that the eukaryotic expression system is suitable for producing recombinant proteins with correct post-translational modifications, hGfat1 was expressed in baculovirus-transfected SF9 insect cells. The recombinant protein carries an insert of a His_6 tag at position 298 which facilitates purification by nickel affinity chromatography and does not affect activity. The phosphorylation state of the purified hGfat1 was determined by phosphoprotein gel staining (*29*). As shown in Figure 1, hGfat1 exhibited strong fluorescence, whereas the negative control, GlmS from *E. coli*, remained

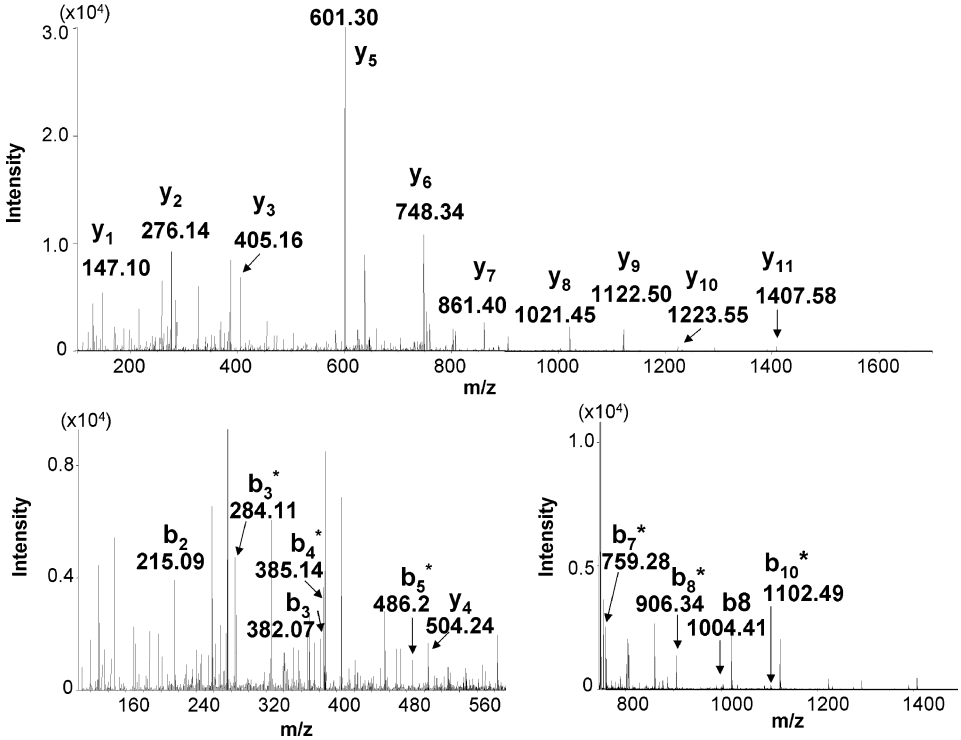

FIGURE 2: MS/MS spectrum of the phosphopeptide ^{241}VDpSTTCLFPVEEK^{253}. The doubly charged species at *m*/*z* 802.8 was selected as the precursor ion for tandem MS. The upper panel shows the full MS/MS spectrum and assignment of the y series ion. The lower panel shows spectra which zoom in to display the b series ion. b* indicates ion species on which the phosphate is lost.

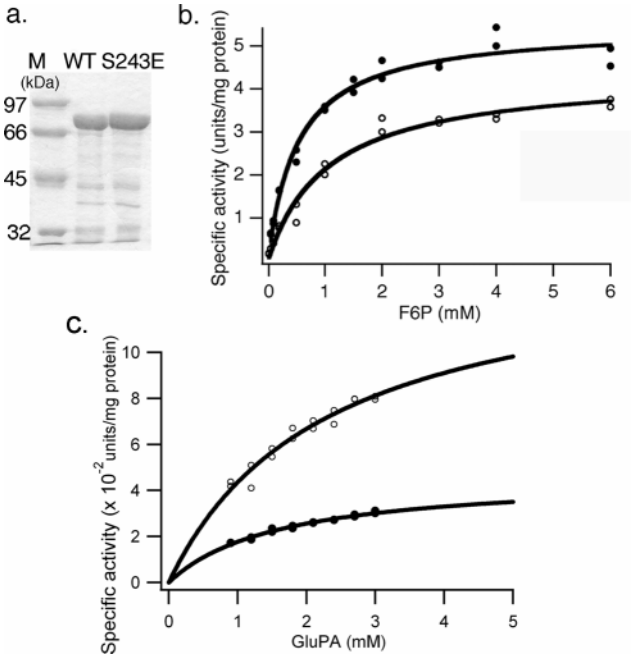

FIGURE 3: Kinetic studies of the WT and the Ser243Glu mutant. (a) SDS−PAGE of purified hGfat1 WT and Ser243Glu (2 μg each). (b) Determination of K_m(F6P) by a Michaelis−Menten plot (hGfat1 WT, ○; Ser243Glu, ●). GlcN6P-synthesizing activity (0.8 μg of enzyme per assay) was measured by the modified Morgan−Elson method under various F6P concentrations while L-Gln was kept at 10 mM. WT: K_m(F6P) = 1.04 ± 0.09 mM, V_{max} = 4.4 ± 0.2 units/mg of protein. Ser243Glu mutant: K_m(F6P) = 0.52 ± 0.07 mM, V_{max} = 5.5 ± 0.2 units/mg of protein. (c) Determination of glutaminase activity (hGfat1 WT, ○; Ser243Glu, ●). V_{max}(glutaminase) is 0.14 ± 0.01 and 0.046 ± 0.002 units/mg of protein for the WT and the Ser243Glu mutant, respectively. K_m(GluPA) is 1.8 ± 0.2 mM for both enzymes.

in the background as the BSA control (Figure 1). This indicates that recombinant hGfat1 is phosphorylated *in vivo*. The phosphorylation sites appear to be exclusively on serine residues as indicated by Western blot analysis using specific antibodies against phospho-Ser, Thr, or Tyr residues (data not shown).

To further determine the exact position of phosphorylation, the recombinant hGfat1 was subjected to in-gel digestion by trypsin and the subsequent peptides were analyzed by MALDI and ESI tandem mass spectrometry. After enrichment using an immobilized metal affinity column (IMAC), a phosphopeptide of 1604.55 Da was detected, corresponding to ^{241}VDSTTCLFPVEEK^{253} with an alkylated Cys residue. Loss of 80 Da in this peptide was observed after treatment of alkaline phosphatase, which is characteristic of a phosphate loss (Supporting information). Nanospray tandem MS experiments unambiguously identified that phosphorylation occurs on Ser243 (Figure 2). Nonphosphorylated peptides containing either putative PKA site (Ser205 or Ser235) have been observed, while the corresponding phosphopeptides were not detected. We have also overexpressed the mutants Ser205Glu and Ser235Glu, which mimic the phosphorylation states regulated by PKA. The two mutants were also found to be phosphorylated on Ser243 by MS analysis. These results clearly indicate Ser243 is the major modification site of hGfat1 and the PKA sites are not phosphorylated under the same growth conditions. However, we cannot rule out the presence of other minor phosphoserine residues below the detection limits of MS.

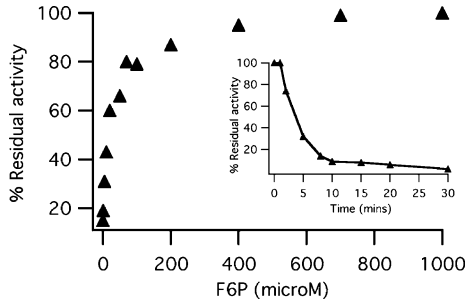

FIGURE 4: Substrate protection of hGfat1. The WT hGfat1 (1.6 μg) was incubated in 50 mM Tris−HCl (pH 7.5) and 2 mM DTT with various concentrations of F6P in a total volume of 10 μL at 37 °C for 10 min. The activity was determined by GDH-coupled assay. The inlet graph shows the time course of hGfat1 inactivation at 37 °C without F6P. The Ser243Glu mutant has a curve of substrate protection identical to that of the WT.

Overexpression of hGfat1 in E. coli. Understanding the effect of phosphorylation requires the full biochemical characterization of hGfat1 in different phosphorylation states. Initial attempts were made to dephosphorylate purified hGfat1 by CIAP followed by activity measurement. However, spontaneous CIAP-induced dephosphorylation of F6P, which was present in the reaction mixture, resulted in the loss of substrate protection and therefore in a dramatic decrease of enzyme activity (10-fold). Substrate protection by F6P of human Gfat against thermal inactivation was previously observed (*14*). Therefore, it was impossible to draw any conclusion from the CIAP dephosphorylation assays, but they did underline the need to switch to a bacterial expression system which generates recombinant proteins without post-translational modifications.

The gene *hgfat1* was then amplified from pMAD1 plasmid for insect cell expression and cloned into pET-28a(+) under control of the T7 promoter. The hGfat1 with an internal His_6 tag was reasonably expressed in the *E. coli* Rosetta (DE3) strain. hGfat1 was purified to homogeneity with >90% purity after one-step purification by metal affinity chromatography (Figure 3a). A mass of 0.5−1 mg of protein/g of cell mass was routinely obtained. Consistent with previous studies, addition of F6P and reducing agent such as TCEP was found essential for protein stabilization throughout the purification process (*26*). The purified protein had a V_{max} in the range of 3−6 units/mg depending on the batch used. Mutant Ser243Glu was generated to mimic the fully phosphorylated form of hGfat1. For the sake of clarity, the following experiments were all carried out with recombinant hGfat1 expressed in *E. coli*.

Biochemical Properties of the Wild-Type and Ser243Glu Mutant of hGfat1. Both the wild-type (WT) and Ser243Glu mutant existed as homotetramers in solution as measured by gel filtration chromatography, indicating Ser243 phosphorylation does not affect the oligomeric state (data not shown). The GlcN6P-synthesizing activity was measured for all kinetic studies by the Morgan−Elson method, and the amidohydrolyzing activity in the absence of F6P (glutaminase activity) was measured using a GluPA assay as described in the Experimental Procedures. Under the same conditions of enzyme preparation and with the same purity as determined by densitometric analysis, hGfat1 WT and mutant Ser243Glu exhibited comparable and similar kinetic properties. Both enzymes followed Michaelis−Menten kinetics toward both

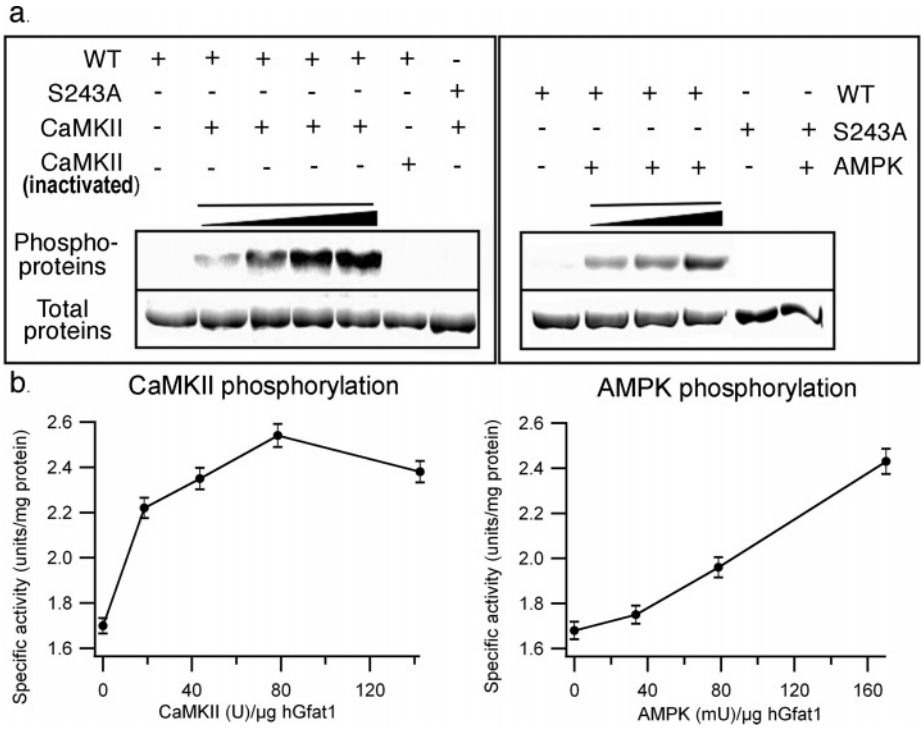

FIGURE 5: *In vitro* phosphorylation by AMPK and CaMKII. A 20 μL kinase assay included 50 mM Tris−HCl (pH7.5), 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 0.2 mM ATP, hGfat1 (2 μg), and increasing amounts of kinases (0.05−0.3 units of AMPK or 50−500 units of CaMKII). In the AMPK assay 0.1 mM AMP was included. The reaction was incubated at 37 °C for 20 min. (a) Gel staining of hGfat1 after kinase reactions. The upper panel shows phosphoproteins, and the lower panel shows the total protein level. The gel is presented in gray scale. (b) S243 phosphorylation stimulates hGfat1 activity in a kinase-dose-dependent manner.

substrates. The V_{max} values for WT and Ser243Glu were 4.4 ± 0.2 and 5.5 ± 0.2 units/mg of protein, respectively, showing Ser243Glu is more active (Figure 3b). Ser243Glu had an apparent K_m for F6P 2-fold lower than that of the WT (0.52 ± 0.07 vs 1.04 ± 0.09 mM), whereas the K_m values for Gln are similar (0.72 ± 0.10 vs 0.80 ± 0.07 mM). Interestingly, Ser243Glu exhibited a 3-fold lower glutaminase activity than the WT (0.14 ± 0.01 vs 0.046 ± 0.002 units/mg of protein), while K_m for GluPA remained unchanged (1.8 ± 0.2 mM) (Figure 3c). Both were equally sensitive to UDP-GlcNAc inhibition, with an IC_{50} of 0.12 mM corresponding to about 90% inhibition at 1 mM inhibitor under the assay conditions. Worthy of note, both enzymes lost 80−90% activity when preincubated without F6P under mild conditions such as at 37 °C for 10 min (Figure 4). Both GlcN6P-synthesizing and amidohydrolyzing activities were affected to the same degree as measured with the Morgan−Elson and the GDH-coupled assay, respectively. The binding curve of F6P was drawn from the protection against thermal inactivation, as described for phosphofructokinase (*30*). Both the WT and the SerS243Glu mutant had identical curves with a dissociation constant of 15 μM for F6P. This value is in the range of the one previously determined by the activation curve of the glutaminase domain (2 μM) (*18*).

To confirm that the increase of activity of the Ser243Glu mutant was an intrinsic property and did not result from experimental errors, the experimental conditions to phosphorylate hGfat1 *in vitro* were then pursued.

Determination of Responsible Kinases in Vitro. Using the group-based phosphorylation scoring method, Ser243 was predicted to be a potential recognition site of CaMKII (*31*). Ser243 is located in the consensus ØXRXXS/TXXXØ (Ø represents a hydrophobic residue). CaMKII has been reported to have a substrate recognition motif similar to that of AMPK except that AMPK tolerates other residues at position −3 (*32*). Therefore, we predicted that AMPK could also be a potential kinase responsible for Ser243 phosphorylation. Indeed, *in vitro* assays showed that CaMKII and AMPK could phosphorylate hGfat1 in a dose-dependent manner as analyzed by phosphoprotein gel staining (Figure 5a). No fluorescent signals were detected when the Ser243Ala mutant was used in the kinase assay. MS analysis of the tryptic peptides of phosphorylated hGfat1 further confirmed Ser243 was phosphorylated specifically. The activity of hGfat1 was stimulated a maximum 1.4-fold by Ser243 modification in a kinase dose-dependent manner (Figure 5b). When using CaMKII which was not preactivated by Ca^{2+} and calmodulin, activation of hGfat1 was not observed. When AMP was absent in the AMPK assay, only a slight increase (~1.1-fold) in activity was observed. In all *in vitro* assays, 20 μM F6P was added to protect enzyme activity.

DISCUSSION

In this study Ser243 was identified as a novel phosphorylation site of hGfat1 using an insect cell expression system. Two recent studies of the phosphoproteome in HeLa cells by MS independently identified hGfat1 phosphorylated at Ser243, which unequivocally confirms our speculation that Ser243 is a basal phosphorylation site *in vivo* (*33*, *34*). Sequence analysis shows that Ser243 is conserved in eukaryotic Gfat from different sources (Figure 6), suggesting regulation at this site may be a general feature. Particularly Ser243 is strictly conserved in two Gfat isoforms from human and mouse. This is different from PKA regulation that may be isoform-dependent. Furthermore, we demonstrate for the first time that hGfat1 can be regulated by kinases other than

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Gfat1_Human              226 RTARTQIGSKFTRWGSQGERGKDKKGSCN-----------------LSRVDSTTCLFPVEE 270
Gfat1_Mouse              227 RTG-----------------KDKKGSCG-----------------LSRVDSTTCLFPVEE 252
Gfat2_Human              224 RTCT----------------LENVKNICKTR---------------MKRLDSSACLHAVGD 253
Gfat2_Mouse              224 PTCN----------------IENVKNICKTR---------------MKRLDSSTCLHAVGD 253
Gfat1_D. melanogaster    218 GKDDKK---LCTDQDADSGKPQDIRPHGQSRE-----------LPVLPRSESTSEFMPLEE 265
Gfat1_C. albicans        230 EAQQQHRPQQPQINHN-----GATSAAELG------FIPVAPGEQNLRTSQSRAFLSEDDL 279
Gfat1_Yeast              227 EENAGQPEIPLKSNNKSFGLGPKKAREFEAGSQNANLLPIAANEFNLRHSQSRAFLSEDGS 281
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FIGURE 6: Sequence alignment of the insert region of eukaryotic Gfat. Alignment was done with the ClustalW program. The conserved Ser243 (hGfat1 numbering) is highlighted. The hydrophobic residues at the −6 and +4 positions (shadowed) are in the consensus of AMPK/CaMKII recognition. The R residue at the −3 position (shadowed) is essential for CaMKII function.

PKA. AMPK and CaMKII are two kinases that specifically phosphorylate Ser243 *in vitro*. Although the physiological relevance of this finding awaits further investigations, it is of particular interest since it indicates a potential direct link between hexosamine biosynthesis and the AMPK signaling pathway via Gfat regulation. AMPK is proposed to be a metabolic switch, whose downstream substrates include key enzymes involved in lipid metabolism, lipolysis, fatty acid oxidation, and glucose transport (*3*). A recent study showing that AMPK activity is regulated by hexosamine biosynthesis in adipocytes constitutes the first report to confirm the functional link of the two pathways (*35*). Our findings may open a new direction to study Gfat regulation in physiological conditions. *In vivo* experiments to study the effect of Ser243 phosphorylation are currently ongoing in the laboratory. The relationship between Ser243 phosphorylation and PKA regulation remains another interesting issue to investigate. It is not uncommon for a single enzyme to be regulated by different kinases. For example, in the case of hormone-sensitive lipase, phosphorylations at Ser565 by AMPK/CaMKII and at Ser563 by PKA are mutually exclusive. The antilipolytic effect of AMPK regulation and subsequent activation of the enzyme is mediated by preventing PKA phosphorylation at Ser563 (*36*). Preliminary experiments expressing the Ser205Glu or Ser235Glu mutant of hGfat1 in SF9 cells showed that PKA phosphorylation has no apparent inhibition effect on Ser243 phosphorylation.

Our results show that hGfat1 phosphorylation at S243 lowers glutaminase activity while it increases GlcN6P-synthesizing activity. A tight control of glutaminase activity, which avoids unnecessary breakdown of L-glutamine in the absence of F6P, may be advantageous in physiological conditions. This might explain that Ser243 is a basal phosphorylation site *in vivo*. Ser243 is located in the insert region of hGfat1 (residues 211−254). Previous studies on Gfat from *C. albicans* showed that the insert fragment is located at the C-terminal part of the glutaminase domain. Deletion of the insert region completely abolished GlcN6P-synthesizing activity, while the glutaminase and sugar isomerase activities remained intact (*37*), suggesting that this fragment plays a role in interdomain communication, probably by keeping the right orientation of the glutaminase domain. hGfat1 is highly likely to function in the same way. In contrast to the Ser243 phosphorylation, phosphorylation by PKA on Ser208 in fungal Gfat (equivalent to Ser205 in hGfat1) increased GlcN6P-synthesizing activity by increasing glutaminase activity (*37*). The authors reasoned from the crystal structure of bacterial Gfat that Ser208 was located close to the catalytic Cys1 and phosphorylation on Ser208 might help keep Cys1 in an active conformation. Understanding the molecular mechanism of Ser243 phosphorylation awaits detailed structural information on hGfat1. However, one may speculate that phosphorylation on the insert fragment could provide diverse additional controls over subtle conformational change, which may mediate the cross talk between the glutaminase and isomerase domain.

Previous studies of human Gfat phosphorylation used N-terminal-tagged recombinant enzymes (*15*), and the effect of substrate protection was not taken into account in *in vitro* assays. Hence, the enzyme activity in these studies was too low to have a reliable interpretation. The present work is the first report of bacterial expression of a eukaryotic Gfat with an internal His_6 tag. This protocol allows rapid purification and affords active hGfat1 that is suitable for *in vitro* phosphorylation studies. Furthermore, it will greatly facilitate detailed mechanistic and structural investigations of hGfat1.

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SUPPORTING INFORMATION AVAILABLE

MS spectra of the phosphopeptide before and after AP treatment. This material is available free of charge via the Internet at http://pubs.acs.org.

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