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Cloning, characterization and regulation of human glutamine:fructose-6-phosphate amidotransferase.

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CLONING, CHARACTERIZATION AND REGULATION OF HUMAN GLUTAMINE:FRUCTOSE-6-PHOSPHATE AMIDOTRANSFERASE

by

JIANXIN ZHOU

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirement for the degree of Doctor of Philosophy

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ABSTRACT OF DISSERTATION
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Name of Candidate Jianxin Zhou

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Title Cloning, Characterization and Regulation of Human Glutamine:Fructose-6-Phosphate Amidotransferase

Both insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) are characterized by defective glucose-induced insulin secretion, diminished ability of insulin-stimulated glucose uptake, and long-term diabetic complication. Hyperglycemia is now recognized to be a cause of insulin resistance and a primary factor in the pathogenesis of diabetic complication. However, the mechanisms for so-called glucose toxicity are unclear.

It has been suggested that the hexosamine biosynthesis pathway mediates at least some of adverse effects of high glucose, such as insulin resistance in insulin-responsive glucose transport and stimulation of glycogen synthase. Hexosamine biosynthesis pathway is a metabolic pathway of glucose that generates precursors for glycoproteins, glycolipids, etc. Glutamine:fructose-6-phosphate amidotransferase (GFA), the first and rate-limiting enzyme in this pathway, has been shown to play a crucial role in these regulatory effects of hexosamine pathway.

DEDICATION

I would like to dedicate this to my family.

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DON	6-diazo-5-oxonorleucine
EGF	epidermal growth factor
STZ	streptozotocin
RASM	rat aortic smooth muscle
8-Br-cAMP	8-bromo-cyclic-adenosine 3', 5'-monophosphate
IBMX	3-isobutyl-1-methylxanthine
DMEM	Dulbecco's modified Eagle medium
FCS	fetal calf serum
PAGE	polyacrylamide gel electrophoresis
OPA	*o*-phthaldialdehyde
PBS	phosphate buffered saline
MODY	maturity-onset diabetes of the young
HNF-1α	hepatocyte nuclear factor-1α
HNF-4α	hepatocyte nuclear factor-4α

INTRODUCTION

Glucose is one of the principal circulating fuels in mammals. To maintain the constant energy supply and normal blood glucose level, glucose homeostasis must be achieved, primarily through the reciprocal actions of the pancreatic hormones insulin and glucagon. The major tissues involved in this regulation of glucose metabolism are the pancreas; the liver, by virtue of its unique ability to produce glucose; and the muscle and adipose tissue, due to their ability to respond to insulin and store energy in the form of glycogen or fat.

Disruption of glucose homeostasis has been shown to result in many physiological disorders and is the hallmark of diabetes mellitus. High levels of blood glucose lead to the development of the complications of diabetes, but the mechanisms by which glucose exerts these effects are not fully understood. Recently, evidence has suggested that glutamine:-fructose-6-phosphate amidotransferase (GFA), the rate-limiting enzyme in the hexosamine biosynthesis pathway, functions as a glucose sensor, and mediates some deleterious effects of high glucose. The following dissertation will characterize human GFA

years and cause most of the morbidity and mortality associated with the disease.

Despite the similarities in their pathophysiologies, the etiology of these two diseases is quite different. IDDM often arises in early life and is usually due to antoimmune destruction of pancreatic β-cells resulting in partial or complete loss of β-cell function (2). The administration of insulin is essential to prevent ketosis and preserve life. NIDDM is more common in later life (over 40 years) and accounts for 80% of all diabetes. Unlike IDDM, the etiology of NIDDM is unclear. Its key characteristics are excessive hepatic glucose output, defective glucose-induced insulin secretion from β cell and diminished ability of insulin to stimulate glucose uptake into peripheral tissues (insulin resistance), all of which contribute to hyperglycemia in NIDDM(3) (Fig. 1).

It has become clear that hyperglycemia is not only a marker of poor metabolic control, but is itself a regulator of both insulin secretion and action. In diabetic animal models, hyperglycemia appears to play a central role in the pathogenesis of the β-cell defect and insulin resistance (4, 5). Consistently, hyperglycemia induces insulin resistance in non diabetic humans, while glycemic control leads to improved β-cell function and an ameliorated insulin

resistance (6-8). Considerable evidence has also linked hyperglycemia directly or indirectly to the development and progression of diabetes complications (9-11). The results of several clinical studies, most recently the Diabetes Control and Complication Trial, convincingly demonstrated that hyperglycemia is the cause of most, if not all, of the chronic complications of diabetes (12). Intensive therapy with the goal of maintaining blood glucose concentration close to the normal range can delay the onset and slow the progression of diabetic nephropathy, neuropathy, retinopathy, and vascular diseases.

Glucose homeostasis. Blood glucose levels in normal individuals are restricted within a very narrow range, despite considerable variations in glucose availability and glucose utilization. This homeostatic control is achieved by matching the rates of glucose flux into and out of the extracellular space through tightly coordinated secretion of insulin and glucagon (13, 14) (Fig. 2). During the fasting state, the constant supply of blood glucose is maintained by hepatic glucose production, mainly gluconeogenesis, driven by glucagon secreted from pancreatic α cells. After a meal, a rise in glucose concentration induces insulin secretion from pancreatic β cells (15). As a consequence, insulin

stimulates glucose uptake and glycogen synthesis in skeletal muscle and adipose tissues. Simultaneously, insulin suppresses glucagon secretion and opposes glucagon's action in liver of glycolysis and fatty acid synthesis (16). The combination of decreased hepatic glucose production and increased tissue utilization/storage of glucose prevents significant hyperglycemia (17).

Diverse signaling pathways in insulin actions. Insulin's primary role is the regulation of glucose homeostasis, and its effects on glucose metabolism are immediate, occurring within a few seconds to minutes. More intermediate and long-term effects of insulin on cellular events include regulation of ion and amino acid uptake, protein synthesis and degradation, gene transcription and mRNA turnover, and cellular growth and differentiation (18, 19).

Insulin action can be viewed as occurring at three distinct levels (Fig. 3). Level 1 actions include the initial cell-surface events: insulin binding to its receptor, activation of the insulin receptor tyrosine kinase with subsequent phosphorylation of insulin receptor substrates, and interaction of this substrate with several downstream signaling molecules. Level II actions are composed of a complex set of distinct

phosphorylation-dephosphorylation cascades that lead to activation of several key regulatory enzymes involved in cell growth and metabolism. These include Raf-1 kinase, the mitogen-activated protein kinase-kinase (MAPKK), MAPK, and 70 kDa and 90 kDa ribosomal S6 kinase. The final biologic effects of insulin makeup level III actions. These include the movement of insulin-regulated glucose transporter (GLUT4) from an intracellular pool to the plasma membrane, activation of enzymes involved in glycogen synthesis, lipid synthesis and protein synthesis, and regulation of nuclear events including DNA synthesis and transcription of specific genes.

The insulin receptor is a transmembrane glycoprotein composed of two α-subunits (135 kDa) and two β-subunits (95 kDa) covalently linked through disulfide bonds to form an $\alpha_2\beta_2$ heterotetramer. The α-subunit is entirely extracellular and contains the sites for insulin binding, whereas the β-subunit has a small extracellular portion, a transmembrane domain, and an intracellular insulin-regulated tyrosine kinase activity. The binding of insulin to the α-subunit results in a conformational change and allows activation of tyrosine kinase of the β-subunit. The fully activated kinase activity is imperative for insulin-regulated growth and metabolism (20). In addition to tyrosine phosphorylation, the insulin

receptor undergoes serine/threonine phosphorylation in the basal state and in response to stimulation of cell by phorbol esters, cAMP analogous, and insulin itself. This kind of phosphorylation has a negative effect on insulin receptor kinase activity and plays a role in regulating receptor function (21).

As seen in Fig. 3, the pathways involved in insulin signaling are complex and overlapping. Insulin signaling primarily acts through tyrosine phosphorylation of cytoplasmic protein, primarily insulin receptor substrate 1 (IRS-1). IRS-1 is phosphorylated by activated insulin receptor on multiple tyrosine residues, which in turn recognize and bind to the SH2-(Src homology 2) domains in various signal transduction pathway. For example, phosphatidylinositol 3'-kinase (PI-3' kinase) is activated when phosphorylated IRS-1 binds to the SH2 domain in its 85 kDa of α-regulatory subunit, which establishes a direct molecular connection between circulating insulin and this cellular enzyme including its function in insulin-stimulated GLUT4 translocation (22, 23). Other signalling proteins, including Shc and Grb2 that mediate stimulation of Ras-mediated pathway, interact directly with the insulin receptor. Insulin action may also involve the activation on second messenger pathways that utilize cyclic-adenosine 3', 5'-monophosphate (cAMP), cyclic-guano-

TABLE 1
The facilitative glucose transporter family

Isoform	Major Tissue Distribution	Km (mM) in *Xenopus* oocytes	Targeting	Functions	References
GLUT1	Ubiquitous. High expression in brain endothelial cells, erythrocytes	17-21	Plasma membrane: basolateral, cell body	Basal glucose transport; transport across blood brain barrier and other barrier tissues.	31, 32
GLUT2	Liver, pancreatic β-cell, small intestinal and kidney epithelial cells	42	Plasma membrane: basolateral	Low affinity transporter; Glucose release into blood stream. Part of the glucose sensor in islet and liver.	35-37
GLUT3	Brain, placenta	11	Plasma membrane: apical, axonal	High affinity transporter. Unabated transport into central nervous system.	33, 34
GLUT4	Skeletal muscle, heart, adipocytes	2	Intracellular vesicles	Mediate insulin-regulated glucose transport; important in whole-body glucose disposal.	47-51
GLUT5	Small intestine, lesser amount in adipose, muscle, brain, and kidney tissues	ND	Plasma membrane	Fructose transporter.	64, 65
GLUT7	Liver	ND	Endoplasmic reticulum	Part of glucose-6-phosphatase complex-gluconeogenesis.	66, 67

ND: not determined

energy source, such as brain and erythrocytes (31-34). In contrast, GLUT2 is a low-affinity, high capacity transporter system, and is expressed mainly in liver and pancreas (35-37). This high-Km transporter permits rapid diffusion and equilibration of glucose in these tissues and would not be saturated at high physiological glucose concentrations. Coupled with kinetically similar high-Km glucokinase (hexokinase IV) in hepatocytes and β-cells, GLUT2 forms part of a glucose-sensing apparatus that responds to changes in blood glucose concentrations through alteration in the rate of glucose uptake into cells and the secretion of insulin, respectively (38, 39). Glucose-insulin secretion coupling depends on glucose transport into β-cell, glucose phosphorylation by glucokinase, and subsequent glucose metabolism. Although it has been agreed that glucokinase plays the predominant role in glucose sensing of β-cells (40-44), GLUT2 has been associated with diminished insulin secretary response to glucose in various diabetic animal models (37, 38, 45, 46).

GLUT4 is an insulin-sensitive isoform present in muscle and fat, the main sites of glucose disposal (47-51). The most distinguishing property of GLUT4 is its propensity to remain localized in intracellular vesicles in the absence of insulin, and then be translocated onto the plasma membrane upon insulin

stimulation under appropriately metabolic conditions (52, 53). GLUT4 in the insulin-sensitive tissues has received considerable attention because of the importance of this process in the maintenance of whole-body glucose homeostasis. For example, glucose disposals via skeletal muscle in the human account for ~20% and ~75-90% of whole-body glucose disposal under basal and insulin-stimulated condition. respectively (54). Thus, the transport step is rate-limiting for glucose uptake into muscle for glycogen synthesis under most conditions (55). The defect in insulin-resistance glucose transport in muscle and adipose tissues of NIDDM has been proposed due to a decrease in GLUT4 expression (56-59), insulin's ability to signal GLUT4 translocation (60-63), and the intrinsic activity of GLUT4 (39).

Metabolic regulation of glucose and glucose toxicity. How hyperglycemia leads to the development of insulin resistance and diabetic complications is unclear. Glucose is an important regulator of normal cell growth and metabolism, and it is likely that at least some of the adverse effects of high concentrations of glucose, referred to as glucose toxicity, may be due to dysregulation of these same processes (6, 68). It has been well established that glucose stimulates expression of a number of hepatic

enzymes involved in glycolysis, fatty acid, and triglyceride synthesis (69). In every case studied, the induction of these enzymes by glucose is due to an increase in mRNA levels through increased transcription and/or prolonged half-life of mRNA, such as phosphofructokinase (70) and L-pyruvate kinase (71, 72). In contrast, glucose reduces gene transcription rate and accelerates mRNA degradation of phosphoenolpyruvate-carboxykinase (PEPCK), the major rate-determining gluconeogenic enzyme (73). A glucose response element has been identified on L-type pyruvate kinase and S_{14} gene promoters, which consists of two CACGTTG motifs related to the consensus binding site for c-myc family of transcription factor (74).

The regulation of proteins by glucose occurs at all levels of expression. Glucose induces both insulin and insulin receptor expression by stimulating gene transcription and increasing mRNA stability (75-77). Glucose also post-translationally regulates insulin action by inducing serine phosphorylation of the insulin receptor and therefore inhibiting its tyrosine kinase activity (78). Glucose mediates both cellular protein level and catalytic activity of glucokinase in pancreatic β cells (79-81). Glucose-dependent down-regulation of glucose transporters GLUT1 and GLUT4 has been described in cultured human muscle cells (82, 83), primary rat adipocytes (84-86), and isolated rat

cataract (105, 106), and reducing motor nerve conduction velocity (107, 108).

High glucose stimulates gene transcription of growth factors and cytokines, including transforming growth factor α (TGFα) and basic fibroblast growth factor (bFGF) in vascular smooth muscle cells (109), and tumor necrosis factor α (TNFα) in human peripheral blood monocytes, which is involved in the development of diabetic atherosclerosis (110). High glucose is a potent inducer of transforming growth factor β (TGFβ) mRNA and protein in cultured proximal tubule cells and glomerular mesangial cells (101, 111, 112). In turn, TGFβ has been implicated as a key mediator in the pathogenesis of diabetic renal disease, supported by the fact that administration of anti-TGFβ antibodies in a diabetic animal model results in attenuation of whole kidney and glomerular hypertrophy and overexpression of matrix proteins (113). High glucose also causes an increased expression of basement membrane components/extracellular matrix proteins including type IV collagen, fibronectin, laminin and integrin in endothelial (114-116), glomerular epithelial (117, 118) and mesangial cells (119, 120), which in turn results in increased thickness of basement membrane or matrix lesions.

Little is known about the signaling pathway for these glucose-induced effects, although multiple

signal transduction pathways, such as PKC activation and cAMP generation, may be involved. Insulin release from islets is dependent at least partially on glucose-mediated membrane depolarization and the opening of voltage-gated Ca^{2+} channels (121). The subsequent rise in cytoplasmic free Ca^{2+} triggers insulin secretion. Glucose also stimulates insulin release by direct interacting with insulin exocytotic machinery, in which cAMP generation (122, 123), Ca^{2+}-dependent PKC translocation to plasma membrane (124), and activation of a 44-kDa mitogen-activated protein kinase (125) are involved. Glucose modulates insulin receptor signals through activation of PKC, resulting in blockage of insulin receptor-dependent tyrosine phosphorylation (126, 78). Elevated glucose concentration also depresses DNA synthesis in cultured hepatocytes, and this effect is associated with decreased expression of *c-myc* gene and is counteracted by cAMP (127).

Involvement of cAMP-dependent pathway in glucose homeostasis. The cAMP signal transduction pathway is one of several second messenger-dependent pathways that generates intracellular responses to extracellular signals (128). cAMP is synthesized from ATP by the plasma-membrane-bound enzyme adenylate cyclase, and it is rapidly and continuously hydrolyzed

to 5'-AMP by cAMP phosphodiesterase. Thus, cAMP analogues such as 8-bromo-cAMP, forskolin, an activator of adenylate cyclase, and 3-isobutyl-1-methylxanthine (IBMX), an inhibitors of cAMP phosphodiesterase, are all able to stimulate cAMP action by elevating the level of intracellular cAMP.

The primary element in cAMP signaling cascade is the cAMP-dependent protein kinase (PKA) that mediates cAMP action by phosphorylation of target proteins. The inactive form of PKA is a tetramer consisting of two regulatory subunits and two catalytic subunits. The binding of cAMP to the regulatory subunits releases the catalytic subunits, which can then phosphorylate the downstream substrates. At present, four regulatory (R) subunit genes (RIα, RIβ, RIIα, and RIIβ) and three catalytic subunit gene (Cα, Cβ, and Cγ) have been identified (129, 130). Many PKA isoforms with different localization, regulatory, and kinase properties permit the effects of cAMP to vary, depending on the target cells.

cAMP-dependent pathways have been shown to play an important role in insulin action and glucose homeostasis (131, 132). The best example is cAMP-mediated glycogen metabolism in liver and muscle cells (133, 134). As summarized in Fig. 4, the binding of glucagon to its receptor on the cell membrane increases the intracellular cAMP level by activating

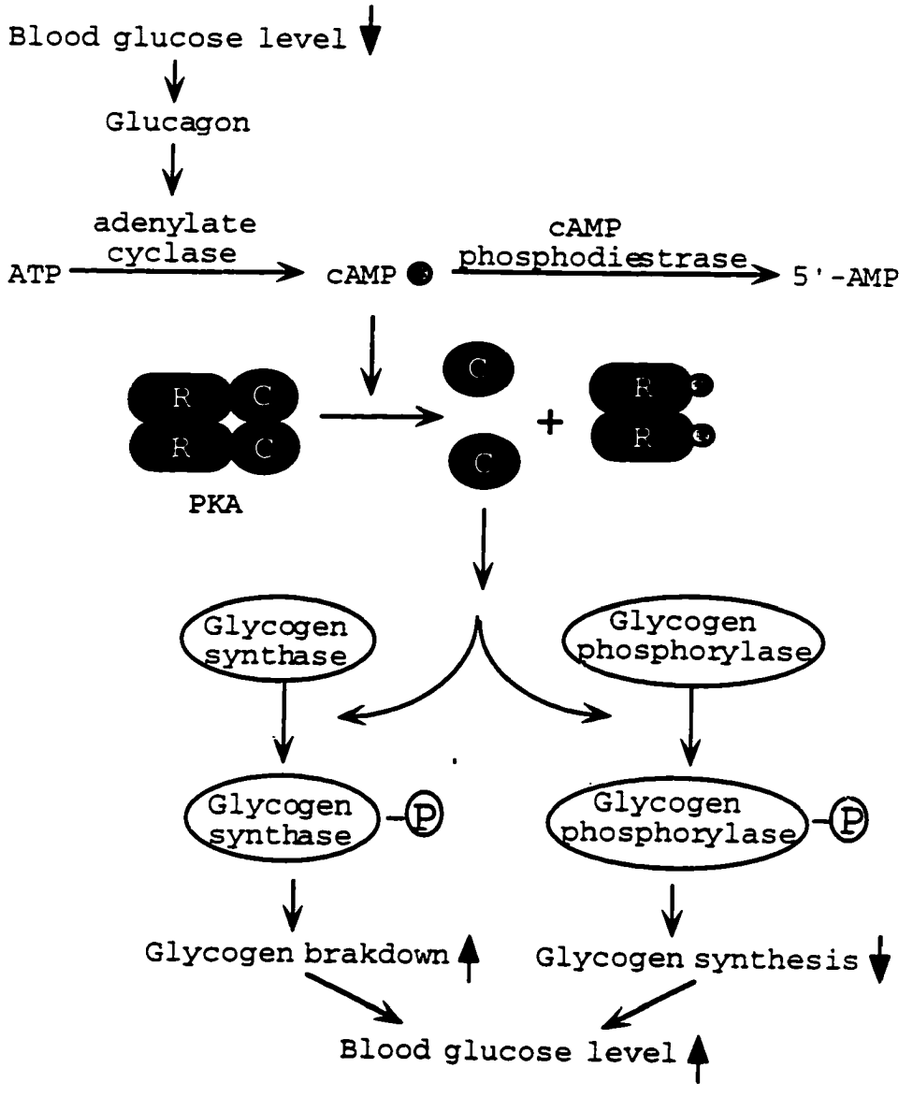

FIG. 4. The control of glycogen metabolism by cAMP. An increase in cAMP levels activates protein kinase A (PKA), which in turn phosphorylates glycogen synthase and glycogen phosphorylase. Inactivated glycogen synthase inhibits glycogen synthesis while activated glycogen phosphorylase stimulates glycogen breakdown. As a result, blood glucose level is upregulated.

adenyl cyclase. Active PKA inhibits glycogen synthase and also activates glycogen phsphorylase by phosphorylation of these two enzymes. As a result, an increase in cAMP levels both stimulates glycogen breakdown and decreases glycogen synthesis, thus maximizing the amount of glucose available to the cells.

Hexosamine biosynthesis pathway in NIDDM. Recently, it has been shown that at least some of the regulatory effects of glucose are mediated by the hexosamine biosynthesis pathway (135, 136). The first and rate limiting step for glucose entry into this pathway is catalyzed by glutamine:fructose-6-phosphate amidotransferase (GFA) (Fig. 5). GFA utilizes the amido group of glutamine to convert fructose-6-phosphate (F6P) into glucosamine-6-phosphate (GlcN-6-P). The final product of the pathway is uridine diphosphate-N-acetyl-glucosamine (UDP-GlcNAc), the major intermediate in biosynthesis of all amino sugar-containing macromolecules both in prokaryotic and in eukaryotic cells, such as glycoproteins, glycolipids etc.

Marshall et al. were the first to implicate the hexosamine pathway in cellular regulation of glucose transport in cultured adipocytes exposed to high concentrations of glucose (137). Earlier studies

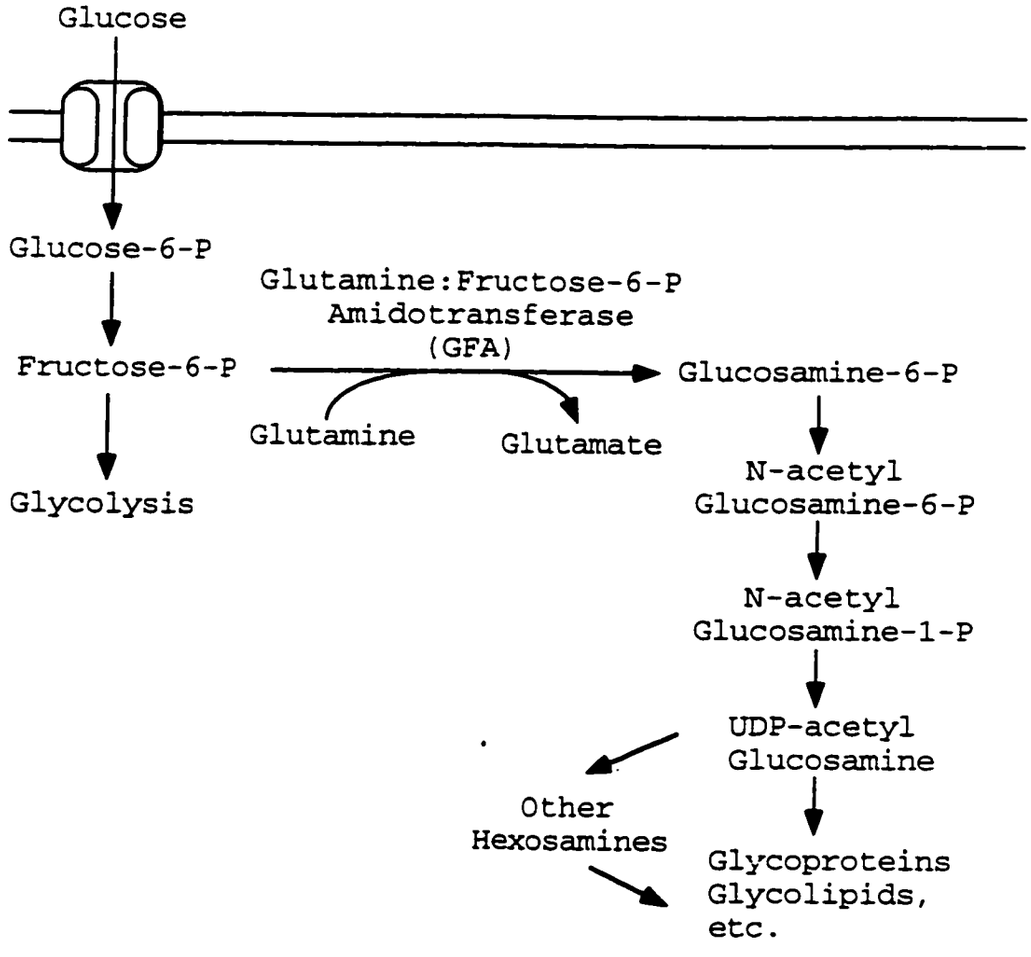

FIG. 5. Hexosamine biosynthesis pathway. Hexosamine biosynthesis pathway is a minor glucose metabolic pathway. The first step of the pathway is the formation of glucosamine-6-phosphate (GlcN-6-P) from fructose-6-phosphate and glutamine, a reaction catalyzed by glutamine:fructose-6-phosphate amidotransferase (GFA). Once formed, GlcN-6-P undergoes acetylation to acetyl-GlcN-6-P, and subsequent two reactions to form final product UDP-N-acetylglucosamine, a precursor for O- and N-linked glycosylated molecules. GFA is a rate-limiting enzyme in the pathway, and thus controls the flux of glucose into the hexosamine pathway.

showed that insulin-stimulated GLUT4 translocation could be inhibited by treating cells with high (20 mM) glucose (84). Glutamine was later found to be a required cofactor for glucose desensitization of the insulin-stimulated glucose transport system (138, 139). The ability of glucose to induce insulin resistance could be blocked by glutamine analogues, such as azaserine or diazo-oxo-norleucin (DON). Furthermore, glucosamine was many times more potent than glucose in inducing insulin resistance and was able to bypass azaserine-induced GFA inhibition. It was therefore hypothesized that hexosamine metabolism may be the pathway by which cells sense and respond to the ambient glucose levels, and moreover, excessive glucose flux through this pathway may result in down-regulation of glucose transport and insulin resistance.

Since this initial study, the glucosamine and hexosamine biosynthetic pathway has been found to mediate many of adverse effects of hyperglycemia and play a possible role in the development of diabetes and its complications. Robinson et al. showed that preexposure to glucosamine induced insulin resistance in skeletal muscle, the tissue responsible for the majority of insulin-dependent glucose utilization (140). Incubating rat hemidiaphragms in 5-22 mM glucosamine resulted in a significant reduction in

basal glucose transport and, to greater extent, in insulin stimulated increase of glucose transport. Moreover, preexposure to glucosamine abolished the ability of insulin to stimulate glycogen synthesis without affecting insulin receptor number or activation.

The effects of excessive hexosamines flux have also been reported in intact animals using euglycemic hyperinsulinaemic clamp technique. Rossetti el al. have shown that glucosamine infusion led to a 31% decrease in glucose disposal rate in normal animal, but no further impairment in insulin-mediated glucose uptake in diabetic rats which were maintained hyperglycemic for approximately 3 weeks (141). The lack of additive effect of glucosamine and chronic hyperglycemia suggests that their effects probably operate through the same pathway. Similarly, Baron et al. found that glucosamine infusion impaired translocation of insulin-stimulated glucose transporter GLUT4 in rats, similarly to what is observed in human insulin-resistant states (61). In addition, glucosamine also inhibited β-cell glucokinase activity in rat islet homogenate, and therefore diminished glucose-induced insulin secretion in both isolated islet and intact rat (142, 143).

Effects of transgenic overexpression of glutamine:fructose-6-phosphate amidotransferase. Glutamine:fructose-6-phosphate amidotransferase is the first and rate-limiting enzyme in the hexosamine biosynthetic pathway. It controls the flux of glucose into this pathway, and thus the formation of hexosamine products. To avoid the possible side effects on cells treated with high concentrations of glucosamine and to get more direct evidence of the hexosamine pathway's involvement in glucose homeostasis, a transgenic approach has been taken in our laboratory in modifying intracellular hexosamine biosynthesis by overexpressing GFA in cultured cells or in mice. Crook et al. initially showed that by transiently transfecting yeast GFA into rat-1 fibroblasts, the overactivity of GFA could blunt insulin sensitivity as reflected by a rightward shift in the dose-response curve for insulin-stimulated glycogen synthase activity (89). Subsequently, the human GFA cDNA was stably overexpressed in rat-1 fibroblasts to facilitate further studies of how hexosamine metabolism regulates glycogen synthesis (129). Both basal glycogen synthase activity and insulin sensitivity were decreased by treatment of the cells with high glucose (10-20 mM), and this decrease was observed at lower glucose concentration in GFA-transfected cells. Hebert et al. targeted GFA using

in the pathogenesis of diabetic nephropathy and atherosclerosis (101, 109).

Taken together, these studies indicate that the hexosamine biosynthesis pathway is an important regulator of glucose utilization by functioning as a glucose sensor, and some deleterious effects of high glucose could be mediated by increased glucose flux through the hexosamine pathway (Fig. 6). Hexosamine biosynthesis pathway is ubiquitously expressed and accounts for approximately 2-3% of cellular glucose metabolism. Thus, small fluctuation in hexosamine metabolism could occur without affecting overall glucose flux. The rate-limiting enzyme GFA in this pathway plays a key role in these regulatory processes.

Enzyme activity and regulation of GFA. As a principal enzyme responsible for the *de novo* synthesis of amino sugar in various mammalian tissues, the biochemistry and enzymology of mammalian GFA was extensively investigated in 1960s and 1970s (149-152). However, there have been relatively few follow-up studies, probably due to the extreme instability of the enzyme *in vitro*. Recently, it has been show that GFA in *E. coli* is encoded by the gene glmS (153, 154). In *S. cerevisiae*, the gene was cloned by complementing the *gcn1* mutation, which encodes for a protein of 80

kDa (155, 156). Later, cloning of human cDNA (157, 158) was accomplished by screening a cDNA library with a GFA probe generated from polymerase chain reaction(PCR), based on high homology of amino acid sequences between bacterial and yeast GFA.

GFA is actively regulated by a variety of mechanisms. In *E. coli*, the enzyme is repressed about 3-fold by growing on amino sugars D-glucosamine (GlcN) and N-acetylglucosamine (GlcNAc), and this regulation occurs at the level of glmS transcription (159). In mating type *a* cells of *S. cerevisiae*, the enzyme activity and GFA mRNA level was increased 1.7- and 2.3-fold, respectively, after addition of α factor, consistent with the observation that mating factors increase endogenous GlcNAc synthesis (156, 160).

More significantly, it has been well characterized that, in contrast to *E. coli* enzyme, GFA in mammalian and lower eukaryotes is allosterically inhibited by the end product of hexosamine biosynthesis pathway, UDP-GlcNAc (149). A more detailed analysis into the nature of this mechanism has been made in fungi *B. emersonii*. Recently, Selitrennikoff et al. demonstrated that the sensitivity of *B. emersonii* GFA to UDP-GlcNAc inhibition was not constant throughout the life cycle, but developmentally regulated (161, 162). Subsequent studies showed that the alterations in this sensitivity can be mimicked by *in vitro* dephospho-

(146). In rats, acute (1-2 h) hyperglycemia did not change GFA activity, while in chronic hyperglycemia (streptozotocin diabetic animals), GFA activity decreased 30-50% in muscle, liver, and epididymal fat pad (167). This decrease could be reversed by insulin treatment without significant change in GFA mRNA expression. The reasons for the partial discordance between rat and human data are not clear.

Hypotheses and specific aims. As summarized above, we hypothesize that GFA, the rate-limiting enzyme in hexosamine pathway, serves a major role in cellular glucose sensing and adaptation to changes in fuel availability. Overactivity of GFA mediates the effects of high glucose in down-regulation of insulin-stimulated glucose transport, glycogen synthesis and overexpression of growth factors. However, the mechanism by which GFA exerts its regulatory effects in these processes remains unknown. The following body of work is an attempt to begin to study the ramification of hexosamine flux on glucose homeostasis. In order to pursue my studies on regulation of GFA as well as to create the tools needed for molecular investigations and manipulation of GFA activity, it was first necessary to clone the GFA cDNA. Once in hand, this tool would allow us to examine GFA regulation, for example answering the

questions of whether GFA is itself regulated in response to the change in glucose level and whether the mechanisms of GFA regulation operate at transcriptional and/or post-translational levels.

Molecular cloning of human GFA cDNA. In the first manuscript of the Results section, "Cloning of human glutamine:fructose-6-phosphate amidotransferase (GFA) and study of glucose-mediated regulation of GFA enzymatic activity," human GFA cDNA was cloned by screening a human hepatoma cDNA library. The partial DNA fragment of human GFA was first obtained by amplifying same library using degenerate oligonucleotides derived from homologous amino acid sequences between bacterial and yeast GFA. All four of 3.5 kb clones contained a 2.0 kb open reading frame predicted to encode a 77 kDa protein. Human GFA shares high homology with other forms of GFA, with 55% amino acid identity with yeast GFA and 35% with *E. coli* GFA. The coding sequence of human GFA was ligated into a bacterial expression vector and expressed by IPTG induction. GFA protein was then purified through a Ni-agarose column, and injected into chicken for the production of anti-GFA antibody.

glucose-mediated GFA inhibition is not due to the feedback by UDP-GlcNAc.

To further examine the mechanism of this glucose-mediated inhibition of GFA activity, Western immunoblot analysis was performed. The same abundance of GFA was detected in RASM cell treated with either low (1 mM) or high (25 mM) glucose, strongly suggesting that glucose regulates GFA activity through post-translational modifications.

GFA gene is localized on chromosome 2p13 and ubiquitously expressed. In the second manuscript of the Results section, "Glutamine:fructose-6-phosphate amidotransferase: Characterization of mRNA and chromosomal assignment to 2p13," fluorescence *in situ* hybridization was performed to human metaphase chromosomes, and the GFA gene assigned to chromosome 2, band 13 of the short arm. To study tissue distribution of GFA gene expression, total RNA from a variety of rat tissue including heart, spleen, brain, smooth muscle, skeletal muscle, liver, testis, fat, and kidney were hybridized to a GFA cDNA probe. An 8 kb mRNA was ubiquitously expressed in the tissue tested. Interestingly, another 3 kb species of GFA mRNA was specifically detected in testis with approximately the same abundance as 8 kb species. The

physiological and biochemical significance of these two mRNA signals is being investigated.

GFA activity is stimulated by cAMP-dependent phosphorylation. In the third manuscript of the Results section, "Regulation of glutamine:fructose-6-phosphate amidotransferase by cAMP-dependent pathway," we studied another possible mechanisms involved in GFA regulation. We have observed that human GFA has two consensus sites for protein kinase A (PKA) phosphorylation, both located at the least conserved region. One of these sites is conserved in yeast GFA, whereas bacterial GFA has none. Prokaryotic and eukaryotic GFA have been shown to be regulated differentially at the level of GFA protein multimerization and UDP-GlcNAc feedback inhibition. We therefore tested the hypothesis that GFA is regulated by cAMP-dependent pathways. At first, we were able to show that recombinant human GFA could be phosphorylated by PKA *in vitro*. Next, we examined the effects of cAMP activators on GFA activity in RASM cells. Ten micromolar forskolin, 1 mM 8-Br-cAMP and 1 mM IBMX all stimulated GFA activity by 2-2.4-fold. In contrast, although human GFA contains several potential phosphorylation sites for protein kinase C (PKC), a potent activator of some protein kinase C isoforms, PMA, failed to induce any changes in GFA activity.

The mechanism of cAMP-stimulation of GFA activity was further investigated. Cycloheximide, an inhibitor of protein synthesis, did not abolish the forskolin effect, suggesting that post-translational modification of GFA protein is involved in this regulation process. This hypothesis was further supported by the fact that prior to assay for GFA activity, preincubation of cell extracts dramatically reduced GFA activity by up to 80%, and the loss of activity could be partially inhibited by phosphatase inhibitors, such as NaF, EDTA, and sodium vanadate.

Thus, this dissertation presents work on the cloning of human glutamine:fructose-6-phosphate amidotransferase cDNA, its chromosomal localization, mRNA characterization, and the regulation in transcription and enzyme activity of the human enzyme. These studies provide the tools to reveal the possible relationship between GFA and NIDDM, and implicate post-translational mechanisms for the regulation of GFA.

ABSTRACT

It has been previously shown that some toxic effects of high concentrations of glucose are mediated by products of the hexosamine biosynthesis pathway. For example, overactivity of glutamine:fructose-6-phosphate amidotransferase (GFA), the rate-limiting enzyme in this pathway, leads to changes in growth factor gene expression as well as impaired insulin responsiveness of glucose transport and glycogen synthesis. In order to study the molecular regulation of GFA and its relation to glucose homeostasis, we have cloned the human GFA cDNA based on the homology between yeast and bacterial GFA. The 3.5 kb cDNA contained a 2.0 kb coding region for a 77 kD protein. Regulation of GFA was then investigated at both the transcriptional and enzymatic levels. No regulation of GFA mRNA expression was observed in rat aortic smooth muscle cells and rat primary hepatocytes treated with glucose, glucosamine, or insulin. GFA enzymatic activity on the other hand, was inhibited by glucose (61% at 2 mM glucose and 90% at 25 mM glucose). The time course of this effect of glucose was rapid but not immediate; GFA enzyme activity decreased by nearly 50% after 6 h of high glucose treatment and 90% after 24 h. Glucose-mediated inhibition of GFA activity remained in Sephadex G-25 filtrates of cell extracts, indicating that this

glucose effect is not due to feedback inhibition by UDP-GlcNAc, the end product of the hexosamine pathway. Furthermore, protein expresssion of GFA was unaffected by changes in glucose concentration, determined by immunoblotting with anti-GFA antibodies. We conclude that GFA activity is possibly regulated post-translationally by glucose.

INTRODUCTION

Glucose plays an important regulatory role in normal physiology; high concentrations of glucose have multiple deleterious effects on cell metabolism and growth. For example, hyperglycemia has been shown to worsen metabolic control in diabetes mellitus by impairing insulin secretion and action (1, 2). Hyperglycemia also appears to be the primary factor associated with the development of diabetic complications (3). The mechanisms by which high concentrations of glucose exert such adverse effects are beginning to be elucidated. For example, production of toxic molecules such as advanced glycation end products may impair the structure and function of proteins (4). Other adverse effects of excessive glucose may be related to the important regulatory role that glucose plays in normal physiology. In this regard, recent studies of the hexosamine biosynthesis

activation of glycogen synthase (10, 11). Similarly, Robinson et al. have shown that pre-exposure to glucosamine markedly inhibited glycogen synthesis in isolated rat diaphragms and in fibroblasts over-expressing the human insulin receptor (12). Furthermore transgenic mice with 2.4-fold-increase in GFA activity in skeletal muscle exhibit elevated insulin-to-glucose ratio in fed state, and this hyperinsulinemia is age- and weight-dependent (13).

Taken together, these data support the hypothesis that some effects of glucose involved in metabolic homeostasis and diabetic complications could be mediated by the hexosamine biosynthetic pathway. Thus the rate-limiting enzyme GFA may play a key role in these regulatory processes. In order to study the molecular regulation of this enzyme and its relation to cellular glucose sensing mechanisms, we have cloned the human cDNA for GFA based on the homology between yeast and bacterial GFA sequences, and have examined its regulation by glucose at the levels of transcription and enzymatic activity. Our results suggest that most of the regulation of GFA in cells occurs at the post-translational level.

RESEARCH DESIGN AND METHODS

Materials. A HepG2 cDNA library in pAB23BXN was obtained from Chiron (Berkeley, CA). DNA sequencing was done with a Sequenase kit (USB, Cleveland, OH). Amplitaq DNA polymerase was from Perkin-Elmer Cetus Instruments. The plasmid PCR^{1000} used for subcloning PCR products was from InVitrogen (San Diego, CA). Restriction enzymes were from Boehringer Mannheim and USB. [α-^{32}P]-dCTP (~6000 Ci/mmol) and [^{14}C]-fructose (248 mCi/mmol) were from Amersham (Arlington Heights, IL). Cell culture media and supplies were from GIBCO/BRL (Grand Island, NY). Routine reagents were from Sigma (St. Louis, MO) unless otherwise noted.

Cell sources. Early passages of rat aortic smooth muscle (RASM) cells were prepared as described in a previous paper (9). The cells were cultured routinely in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS). Rat primary hepatocytes were prepared by the collagenase perfusion procedure as described (14). After incubation in William's medium E overnight, the cells were maintained in DMEM containing 1 mM or 5 mM glucose with 10% FCS.

Cloning of human GFA cDNA. A HepG2 cDNA library was used for GFA cDNA cloning. Based on the

sequence homology between yeast (15) and bacterial GFA (16, 17), we synthesized two degenerate oligonucleotides corresponding to amino acid sequences MQKEIYE (antisense) and VVVHNGI (sense) in yeast and bacterial GFA. Sixty ug of CsCl-purified DNA from the HepG2 cDNA library were amplified with polymerase chain reaction (PCR) for 25 cycles (9°C, 1 min; 42°C, 1 min; 72°C, 2 min) in a Perkin-Elmer Cetus DNA thermal cycler. PCR products were analyzed by 1% agarose gel electrophoresis. A 630 bp fragment was obtained. The PCR product was directly subcloned into plasmid PCR^{1000}, and sequenced. To clone the entire cDNA for GFA, approximately 2x10^5 colonies of a HepG2 cDNA library in the *E. coli* strain MC1061 were screened using this 630 bp PCR fragment. The replicated nylon filters were hybridized in 6x SSC, 5x Denhardt's, 0.5% SDS, 50% formamide, and 100 mg/ml of herring sperm DNA at 42°C overnight, and washed stringently (1x SSC, 0.1% SDS at 65°C). Four positive colonies were selected and restriction analysis confirmed that all contained a 3.5 kb cDNA insertion. One clone was used for complete sequencing by the dideoxynucleotide chain termination method.

Northern blot analysis. Total RNA from the cell lines was prepared by the guanidinum isothiocyanate-acid-phenol method (18). Twenty

micrograms of total RNA were electrophoresed in a 1% agarose-formaldehyde gel, transferred to a nylon filter, and UV cross-linked. The human GFA cDNA of the coding region was labeled with random-priming and used to probe the filters in 50% formamide, 5x Denhardt's, 0.5% SDS at 42°C overnight. Filters were given final washes at 65°C in 1x SSC, 0.1% SDS. The blots were subsequently stripped and reprobed with a β-actin cDNA (19) for the purpose of data normalization.

Cell extracts preparation. RASM cells were cultured in DMEM-10% FCS containing various concentrations of glucose as indicated in text. For cell extracts, 10 cm culture dishes of RASM cells (approximately 80% confluence) were rinsed with 2 ml Krebs-Ringer phosphate buffer-10 mM HEPES, pH 7.6 (KRP-HEPES). The cells were scraped with a rubber policeman in the same buffer and collected. The cells were then pelleted by centrifugation and resuspended in 1 ml extract buffer containing 100 mM KCl, 1 mM EDTA and 50 mM NaH_2PO_4 (pH 7.5).

Cell-free extracts were prepared by sonication on ice at output 2, 50% duty cycle for 10 bursts with a Branson 250 Sonifier. The sonicated extract was then centrifuged at 4°C, 16,000 x g for 15 min, and the supernatant used to assay GFA enzyme activity.

Aliquots of this crude cytosolic extract could be frozen using liquid nitrogen and stored at -80°C to be used later with no loss in GFA activity.

$(NH_4)_2SO_4$ precipitation of the GFA enzyme was done as previously described (20). The crude cytosolic extract from 15 dishes of treated RASM cells was brought to 40% saturation with saturated $(NH_4)_2SO_4$ (pH 7.5) at 4°C with constant stirring. After centrifugation at 10,000 x g, the precipitate was discarded and the supernatant was brought to 54% saturation by the addition of saturated $(NH_4)_2SO_4$ (pH 7.0). After centrifugation, this precipitate was dissolved in buffer containing 50 mM NaH_2PO_4 (pH 7.5), 1 mM EDTA, and 5 mM glucose-6-phosphate, and desalted using a Sephadex G-15 column equilibrated in the same buffer.

GFA enzyme activity assay and GlcN-6-P determination. To synthesize labeled F-6-P, 250 uCi of [^{14}C]-fructose were reacted in a final reaction volume of 250 ml containing 19.2 mM triethanolamine (pH 7.6), 7.6 mM $MgCl_2$, 100 units yeast hexokinase (USB), and 0.73 mM ATP for 30 min at room temperature. F-6-P was separated from unphosphorylated fructose by anion-exchange chromatography (19). The reaction mix was brought to 1 ml with dH_2O and applied to an anion-exchange column (Bio-Rad AG1-X4, formate form, 2.7 ml bed volume). The column was washed with 15 ml of 0.1

N NH_3COOH, 15 ml H_2O, and the [^{14}C]-F-6-P eluted with 0.4 M HCl. One microliter fractions were collected and neutralized with 40 ml of 10 N NaOH and counted for radioactivity. The peak fractions were pooled and stored at -20°C.

Cell extracts (30-90 mg extract proteins) were assayed for GFA activity at 37°C for 45 min in a tube containing 10 mM [^{14}C]-F-6-P, 10 mM glutamine, 40 mM NaH_2PO_4 (pH 7.5), 1 mM EDTA and 1 mM DTT in a reaction volume of 50 ml. Reactions were stopped by boiling for 3 min, and proteins were pelleted by centrifugation for 10 min 16,000 x g at 0°C. The supernatant was analyzed for GlcN-6-P content as described below. Control reactions run in parallel for all samples were preincubated for 30 min on ice with 100 mM DON and no glutamine. Protein concentrations were determined before each assay and all samples in an experiment were normalized for protein before assaying for GFA activity. Protein estimations were done using Bradford's reagent (BioRad, Hercules, CA) with bovine serum albumin used as a standard.

The amount of GlcN-6-P generated was determined using a paper electrophoresis method previously described by Callahan et al (21). Ten ml of reaction mix were spotted onto Whatman 3MM paper (15 cm x 33 cm) and electrophoresed in a BioRad DNA SUB CELL

agarose column, and finally, renatured through graduate dialysis. Polyclonal anti-GFA antisera were raised by immunizing chickens with this purified and renatured His-tagged GFA.

Western blotting analysis. RASM cells were cultured in DMEM-10% FCS containing 1 mM or 25 mM glucose for 24 hr. Cell-free extracts were prepared as described in "Cell Extracts Preparation," and protein concentrations were determined by Brodford's assay. One microgram of total cellular proteins were separated on 7.5% SDS-PAGE, and then electrophoretically transferred onto nitrocellulose filters. Western blotting analysis were performed by incubating the filters with the antibodies against human GFA (1:500), and detected with rabbit anti-chicken IgG-alkaline phosphatase-BCIP/NBT substrate method.

RESULTS

Cloning of the human glutamine:fructose-6-phosphate amidotransferase cDNA. The complete human GFA cDNA was cloned by screening HepG2 human cDNA library with the probe of a 640 bp fragment, which was generated by PCR using the degenerate oligonucleotides as described in "Research Design and Method." The 3.5 kb-cDNA clone contained a 2.0 kb open reading frame encoding 681 amino acids. The

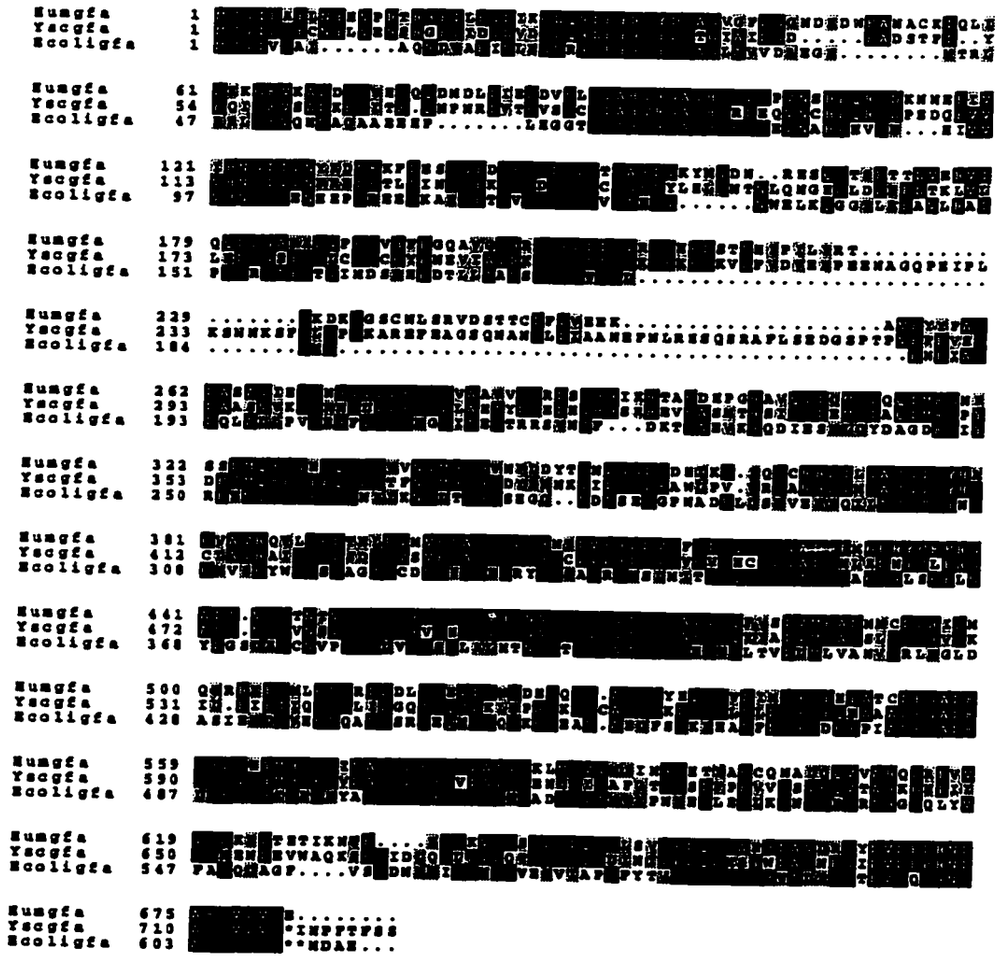

FIG. 1. Sequence alignment of human (Humgfa), yeast (Yscgfa), and bacterial (Ecoligfa) GFA. The poorly-conserved hinge region is located at amino acid 218-254 of human GFA.

Modulation of GFA activity by glucose. To determine if post-transcriptional modulation of GFA activity could occur in response to changes in glucose concentrations in cell culture, we measured GFA activity in cellular extracts from cells cultured in various concentrations of glucose. RASM cells were treated for 24 h with varying concentrations of glucose, and GFA enzyme activity was assayed in cellular extracts. As shown in Fig. 3 increasing concentrations of glucose had an inhibitory effect upon GFA activity. Glucose concentrations as low as 2 mM inhibited GFA activity by 61% (2.99 units/mg at 1 mM vs 1.18 units/mg at 2 mM), and higher concentrations of glucose (25 mM) inhibited GFA activity by 90% (0.31 units/mg). The difference in GFA activity in cells treated with 1 mM vs. 25 mM glucose was statistically significant by t-test ($p<0.050$, $n=5$).

To determine the time course of glucose inhibition on GFA enzyme activity, RASM cultures in 1 mM glucose were treated for 0, 2, 6, and 24 h with 25 mM glucose and extracts assayed for GFA activity. As shown in Fig. 4, and consistent with the data above, GFA activity was again inhibited in cells treated with high glucose. GFA enzyme activity was decreased by approximately 50% 6 h after glucose treatment (1.82 units/mg vs. 3.50 units/mg), and 24 h of high glucose

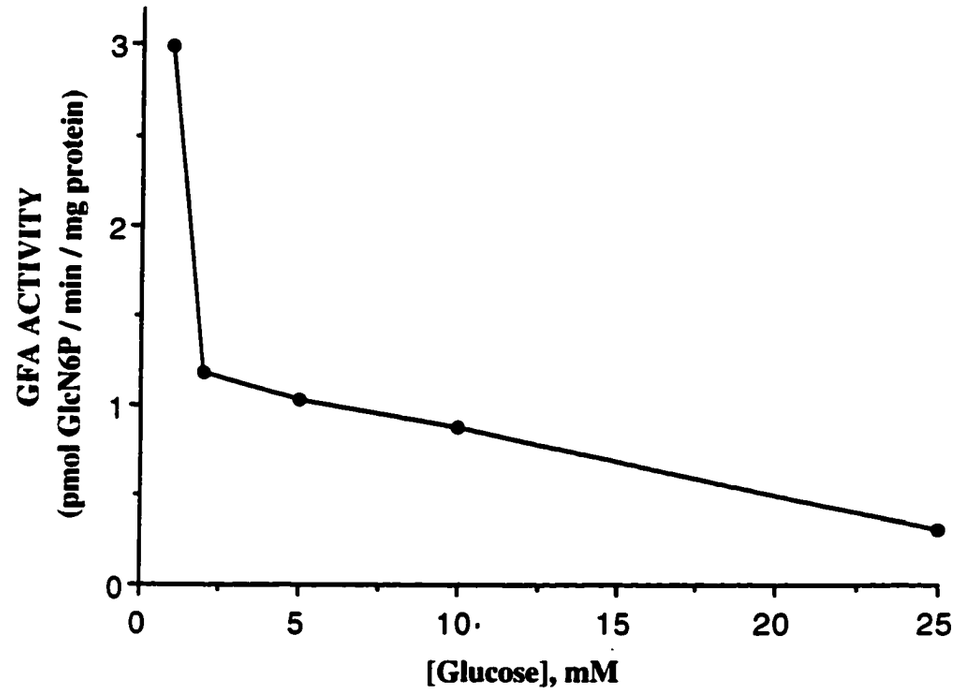

FIG. 3. Effect of glucose on GFA activity. RASM cells were passed in 1 mM glucose and grown for 48 h. Cells were then treated for 24h with various concentrations of glucose (1-25 mM). Extracts were made as described and GFA activity measured for each condition. The results represent the means of assays performed on two independently derived sets of extracts.

treatment saw a greater than 90% inhibition in GFA specific activity (0.31 units/mg vs. 3.50 units/mg).

GFA activity is inhibited by UDP-GlcNAc, the end-product of the hexosamine biosynthesis pathway. Thus, glucose could lead to the accumulation of UDP-GlcNAc in sufficient concentrations to carry over into our *in vitro* assay and cause the observed inhibition of GFA activity. To test this possibility, crude extract preparations from glucose-treated cells were subject to ammonium sulfate precipitation and G-15 Sephadex chromatography to partially purify GFA while depleting the extract of UDP-GlcNAc and other low molecular weight of compounds. As shown in Fig. 5, 25 mM glucose treatment of cells caused a 77% decrease in the partially purified GFA enzyme activity compared to the activity in cells treated with 1 mM glucose (0.71±0.72 units/mg at 25 mM vs. 3.04±1.56 units/mg at 1 mM). Inhibition by high glucose was found to be statistically significant by t-test ($p<0.025$).

To further investigate the mechanism of glucose-induced inhibition on GFA enzyme activity, we performed Western blotting analysis on protein expression of GFA in RASM cells (Fig. 6). Antibodies against His-tagged recombinant human GFA identified a band of 77 kDa with approximately the same intensity on immunoblotting of either 1 mM or 25 mM glucose-treated cell extracts. This data suggested that GFA

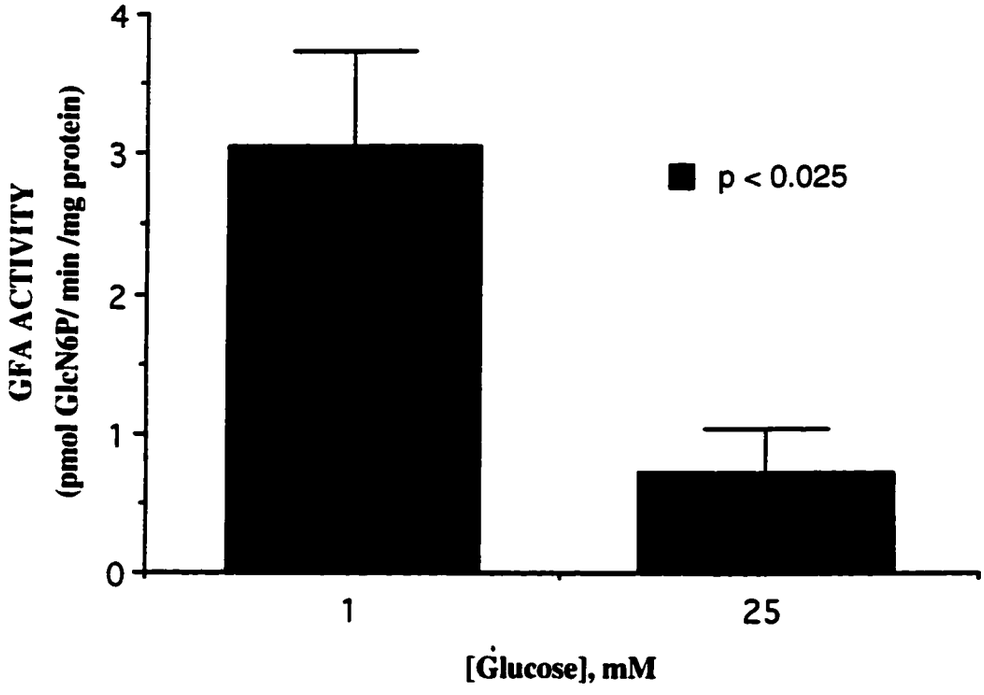

FIG. 5. Effect of high glucose on partially-purified GFA activity. RASM cells cultured in 1 mM glucose for 48 h were either left in 1 mM glucose or put in 25 mM glucose for an additional 24 h before extraction. Extracts were then subjected to ammonium sulfate precipitation as described and the resultant partially purified extracts assayed for GFA activity. Results shown are the means and SEM of five assays performed on three independently derived sets of extracts. Inhibition by high glucose was found to be statistically significant by t-test ($p<0.025$).

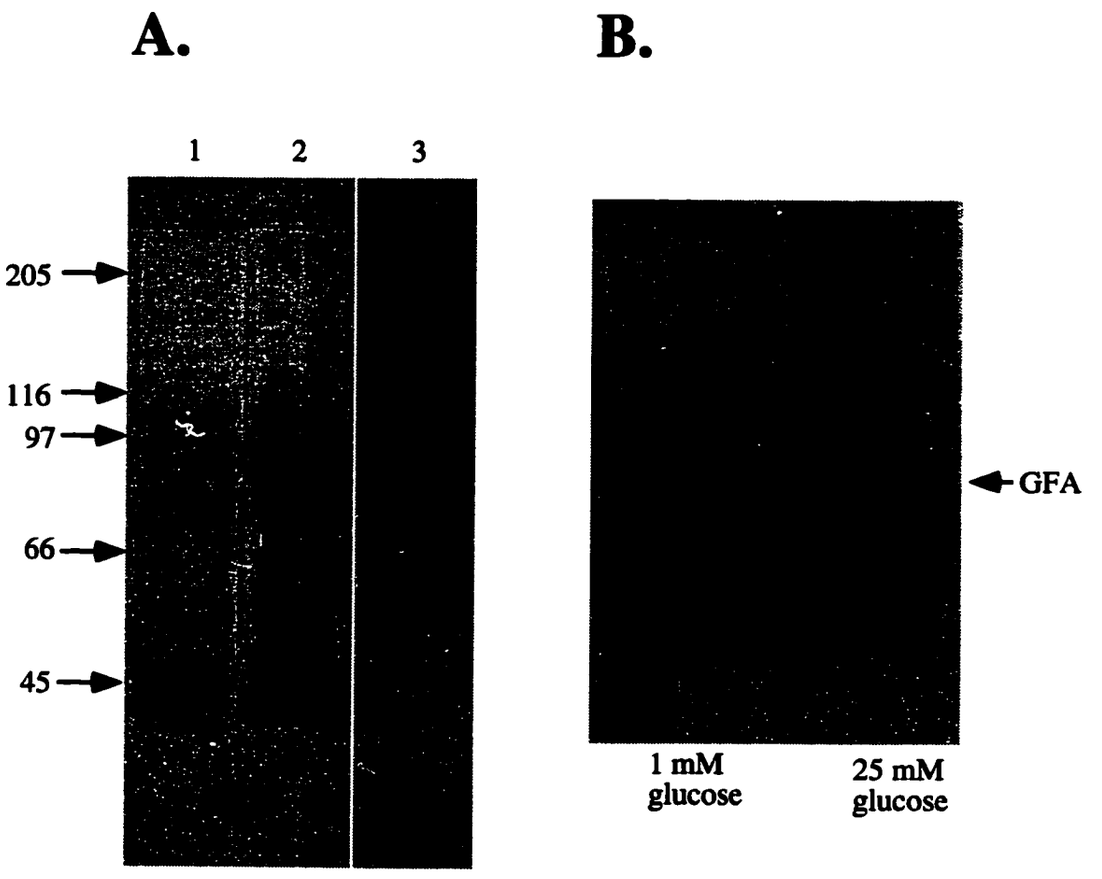

FIG. 6. GFA protein lavels in glucose-treated RASM cell. A. Staining gel of recombinant His-tagged human GFA expressed in *E. coli*. Uninduced (lane 1) or IPTG-induced (lane 2) bacterial pellets were solublized in SDS sample buffer, and applied onto 7.5% SDS-PAGE. The gel was then stained with Coomassie blue. GFA fraction purified from Ni-agarose column (lane 3) was used to raise polyclonal anti-GFA antibodies as described in "Research Design and Methods." B. Western blot of GFA protein expression in RASM cells. RASM cells were cultured in DMEM containing 1 mM glucose for 48 hr, and then treated with 25 mM glucose for 24 hr. Cell extracts were prepared as described in "Research Design and Methods." In each lane, 1 mg of total cytoplasmic proteins were loaded.

protein expression in cells was not affected by the change in glucose concentration.

DISCUSSION

We cloned and sequenced the cDNA for human glutamine:fructose-6-phosphate amidotransferase. The sequence encodes a 77 kDa protein that has been highly conserved during evolution: the overall amino acid identity is 55% with yeast GFA, and 35% with bacterial GFA (or glucosamine synthase). Structure-function studies on bacterial GFA have revealed that this enzyme can be proteolytically cleaved between Tyr^{240} and Asp^{241} to give two non-overlapping domains, an N-terminal glutamine-binding domain with amidotransferase activity, and a C-terminal F-6-P-binding domain with ketose/aldose isomerase activity (22). Sequence analysis among the three species of GFA and other amidotransferases (23, 24) confirms the conservation of these functional domains. A poorly conserved region (amino acid residue 218-254 in human GFA) connects these F-6-P- and glutamine-binding domains. Compared to bacterial GFA, both human and yeast GFA include a large insertion in this "hinge" region. Previous studies have shown that native mammalian GFA has a molecular weight of 340 kDa and probably exists as a tetramer, while the bacterial enzyme exists as a monomer (20, 25). This

oligomerization is correlated with the fact that the eukaryotic but not prokaryotic forms of GFA are sensitive to feedback inhibition by UDP-GlcNAc, the final product of hexosamine pathway. The difference in the "hinge" region between bacterial GFA and eukaryotic GFA may therefore be involved in the latter's multimerization and sensitivity to UDP-GlcNAc.

This "hinge" region of human GFA contains two consensus sequences for potential phosphorylation by cAMP-dependent protein kinase, ^{202}RRGS and ^{232}KKGS. It has been shown that GFA activity is developmentally regulated in the fungi *B. emersonii* (26). During vegetative growth the enzyme becomes constitutively active and no longer sensitive to feedback inhibition by UDP-GlcNAc. A 76 kDa-protein (consistent with the predicted size of GFA) that copurified with GFA activity could be phosphorylated by cAMP-dependent protein kinase *in vitro* and the extent of this phosphorylation paralleled the sensitization of GFA to UDP-GlcNAc inhibition. Thus, phosphorylation of this region may also operate in the human enzyme to regulate activity through sensitivity to feedback inhibition and/or multimerization.

The regulation of GFA activity in whole cells is complex. Our data suggest that regulation occurs mainly at the post-translational level. We observed

no changes in GFA mRNA levels in response to glucose, glucosamine, or insulin in different cell lines including RASM, rat primary hepatocytes, and Rat-1 fibroblasts expressing the human insulin receptor (data not shown for the latter). We also could not detect significant differences in mRNA levels between normal and STZ-treated rats in several tissues, including muscle and fat (data not shown). Marshall et al. had suggested that glucose regulation of GFA enzyme activity might occur at the level of mRNA synthesis (7), but these pharmacological studies were done before the cDNA probe was available and mRNA levels could not be directly assessed. The relatively slow changes in GFA activity we observed after altering glucose levels, combined with the previous studies, do suggest that the regulation of GFA may be indirect, and may involve transcriptional regulation of other gene products that might in turn modify GFA activity. Consistent with our data, Robinson et al. have observed that GFA activity was not affected by acute hyperglycemia (1-2 hr) in muscle and liver of control rat, but decreased 30-50% by chronic hyperglycemia in muscle, liver and epididymal fat of STZ-diabetic rats. Furthermore, they were able to show that the decrease in GFA activity was not due to change in GFA mRNA expression (27).

activity due to post-translational modification as we have shown. The end result may be a complex integrative web of regulation involving several small molecule effectors, substrate levels, and interactions between insulin-stimulated (glucose transport) and insulin-counter regulatory (cAMP-dependent kinase) pathways.

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GLUTAMINE:FRUCTOSE-6-PHOSPHATE AMIDOTRANSFERASE: CHARACTERIZATION OF mRNA AND CHROMOSOMAL ASSIGNMENT TO 2P13

by

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Human Genetics 96(1):99-101, 1995

ABSTRACT

It has been previously shown that some toxic effects of high concentrations of glucose are mediated by the hexosamine biosynthesis pathway and its rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFA). We have used the cloned human GFA cDNA to study the chromosomal localization of the gene and tissue distribution of mRNA. The human GFA gene is on chromosome 2, band p13 as determined by fluorescence *in situ* hybridization. An 8 kb species of GFA mRNA was detected in all rat tissues tested with relatively high expression in testis and smooth muscle; a unique 3 kb mRNA species was found only in testis.

INTRODUCTION

Glucose at high concentrations has multiple deleterious effects on cell metabolism and appears to be the primary factor associated with the development of diabetic complications (1). The mechanisms by which high glucose exerts such adverse effects are unknown, but recent studies of hexosamine biosynthesis have provided insights into how glucose might exert both its regulatory and pathophysiologic effects.

It has been shown that glucose-induced desensitization of the insulin-responsive glucose transport system is mediated by products of the

hexosamine pathway (2). The rate-limiting step in this pathway is the amination of fructose-6-phosphate to glucosamine-6-phosphate, catalyzed by the enzyme glutamine:fructose-6-phosphate amidotransferase (GFA). We have shown that overexpressing GFA mediates the regulation of transforming growth factor a by glucose (3, 4), and causes insulin resistance of the stimulation of glycogen synthase (5). These data support the hypothesis that some effects of glucose involved in metabolic homeostasis and diabetic complications could be mediated by GFA and the hexosamine biosynthesis pathway. We have, therefore, cloned the human GFA cDNA and report here the chromosomal localization of human GFA gene and distribution of GFA mRNA in rat tissues.

RESEARCH DESIGN AND METHODS

Isolation of a human genomic GFA fragment and fluorescence *in situ* chromosome hybridization. The 3.5 kb human GFA cDNA was cloned by screening a HepG2 cDNA library with a PCR-derived probe. Sequences of the coding region were identical to that published by McKnight et al. (6) while this work in progress. A human placental genomic DNA library in phage 1 (Stratagene, La Jolla, CA) was screened with a ^{32}P-labeled human GFA cDNA probe. A clone containing a 13 kb insert was identified and

found by sequencing to include five exons encoding base pairs 658 to 1149 of cDNA. Phage was purified by CsCl gradient and DNA extracted as described (7) and used for the chromosome hybridization studies.

Human metaphase cells were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes. Fluorescence *in situ* hybridization was performed as described (8). A biotin-labeled GFA gene fragment was prepared by nick-translation using Bio-11-DUTP (Enzo Diagnostics, New York, NY). Hybridization was detected with fluorescein-conjugated avidin (Vector Laboratories, Burlingame, CA), and chromosomes were identified by staining with 4,6-diamidino-2-phenylindole-dihydrochloride.

Northern blot analysis. Rat tissues were surgically excised and rapidly frozen with liquid nitrogen. Total RNA from the tissues was prepared by the guanidinum isothiocyanate-acid-phenol method. A 20 ug sample of total RNA was subjected to electrophoresis in a 1% agarose-formaldehyde gel, transferred to a nylon filter, and hybridized with a probe of human GFA cDNA in 50% formamide, 5x Denhardt's, 0.5% SDS at 42°C overnight. Filters were given final washes at 65°C in 1x SSC, 0.1% SDS. The blot was subsequently stripped and reprobed with a β-actin cDNA.

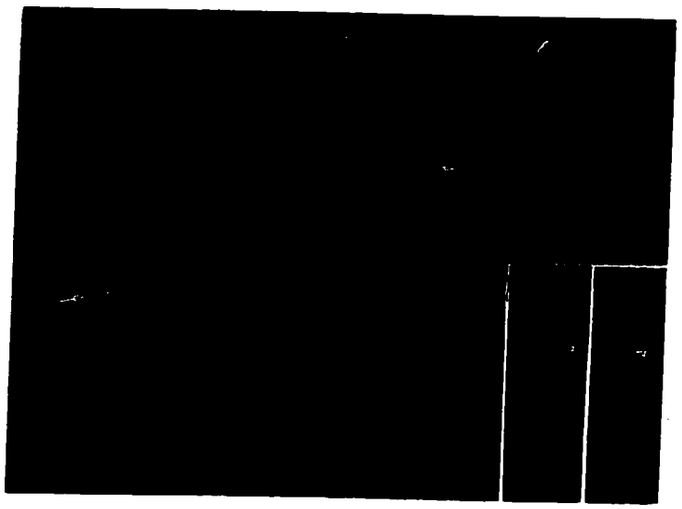

FIG. 1. *In situ* hybridization of a biotin-labeled GFA probe to human metaphase chromosomes. The chromosome-2 homologues are identified with *arrows* in the partial metaphase cell; specific labeling was observed at 2p13. The *inset* illustrates partialkaryotypes of two chromosome-2 homologues illustrating specific labeling at 2p13 (*arrowheads*).

amounts of GFA mRNA were found in all rat tissues tested. The Northern blot in Fig. 2, normalized for loading of total RNA, showed the highest relative GFA mRNA levels in smooth muscle and testis; lower relative levels were found in liver and heart. When the blot was reprobed for β-actin mRNA, however, all non-muscle tissues had similar ratios of GFA to β-actin mRNA. Two mRNA species of GFA were detected, an 8 kb mRNA in all the tissues and a 3 kb mRNA only in testis in approximately the same abundance as the 8 kb species.

The molecular basis for the size difference between these mRNAs is unknown, as is whether the transcripts or their proteins differ functionally. Seminal fluid is fructose-rich, and GLUT5, the fructose transporter, is highly expressed in human testis and sperm (9). Because F-6-P is the direct substrate of GFA, it could be speculated that the high level of GFA mRNA and functional differences exhibited by the product of the smaller mRNA in testis might be related to the high levels of fructose in this tissue.

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REGULATION OF GLUTAMINE:FRUCTOSE-6-PHOSPHATE AMIDOTRANSFERASE BY cAMP-DEPENDENT PATHWAY

by

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Prepared for *Diabetes*

ABSTRACT

Glutamine:fructose-6-phosphate amidotransferase (GFA) is the rate-limiting enzyme in the hexosamine biosynthesis pathway and has been shown to play a key role in regulating glucose metabolism. Human GFA has two potential protein kinase A (PKA)-phosphorylation sites, both located in the least evolutionarily conserved "hinge" region; one of these consensus sites is conserved in yeast GFA whereas bacterial GFA has neither. To test whether GFA activity is regulated by cAMP-dependent phosphorylation, rat aortic smooth muscle (RASM) cells were treated *in vivo* with cAMP-elevating agents, 10 uM forskolin, 1 mM 8-Br-cAMP or 3-isobutyl-1-methylxanthine (IBMX). The cytosolic extracts were then prepared and assayed for GFA activity. All treatments resulted in significant increases (2-2.4-fold) in GFA activity. Stimulation by forskolin was dose- and time-dependent, with maximal effects observed at 10 uM forskolin and after 60 min treatment. Preincubation of cells with cycloheximide did not abolish the effect of forskolin. Incubation of RASM cytosolic extracts at 37°C for 10 min without phosphatase inhibitors led to a 79% decrease of GFA activity. This loss of activity was partially inhibited by the addition of phosphatase inhibitors (5 mM sodium orthovanadate, 50 mM sodium fluoride or 5 mM EDTA, but not 100 nM okadaic acid),

synthase in rat-1 fibroblasts (10, 11). These data suggested that GFA plays an important role in the regulation of glucose homeostasis and glucose-dependent pathway.

Bacterial (12), yeast (13), and human (14, 15) GFA have been cloned and GFA is highly evolutionarily-conserved. In a fungi *B. emersonii* GFA enzyme activity is developmentally regulated (16); GFA remains dephosphorylated and constitutively active during vegetative growth, but during sporulation GFA is phosphorylated and is able to feedback-inhibited by the end product of the pathway, UDP-N-acetyl-glucosamine. Furthermore, an *in vitro* phosphorylation assay suggested that partially purified GFA could be desensitized by cAMP-dependent protein kinase (PKA).

Amino acid sequence analysis revealed that human GFA has two potential PKA-phosphorylation sites (^{202}RRGS and ^{232}KKGS); one of these consensus sites is conserved in yeast GFA whereas bacterial GFA has neither (Fig. 1). In the present work, we have studied the effects of cAMP-elevating agents and phosphatase inhibitors on GFA activity in order to characterize the pathway involved in the regulation of GFA in mammals.

RESEARCH DESIGN AND METHODS

Materials and cell culture. Forskolin, 8-bromo-cAMP, IBMX, sodium fluoride and sodium orthovanadate were obtained from Sigma. Okadaic acid was from Calbiochem(La Jolla, CA), and microcystin-LR from Life Technologies (Gaithersburg, MD).

Rat aortic smooth muscle (RASM) cells were prepared as described previously (17). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fatal calf serum (FCS) and routinely passed every 4 days at confluence. Cells were equilibrated in DMEM-0.1% FCS for 1 hr prior to treatment *in vivo*.

Phosphorylation of recombinant GFA with protein kinase A (PKA) *in vitro*. A 2.0 kb open reading frame of human GFA cDNA was subcloned into a bacterial expression vector (pET-His•tag vector, Novagen) under control of T7 promoter, and expressed by IPTG (1 mM) induction for 3 hr. Soluble GFA protein in crude bacterial extracts was purified through Ni-agarose column. For phosphorylation reactions, 30-50 ug of total proteins were incubated with the buffer containing 20 mM Tris/HCl, pH 7.4, 10 mM MgCl2, 1 mM PMSF, 1 mM DTT, 50 uM ATP, 10 uCi [r-^{32}P]ATP (Amersham) and 100 ng of the catalytic subunit of PKA (Promega) for 30 min at 37 °C. The proteins

were then precipitated by acetone, separated on 7.5% polyacrylamide gel, and autographed.

Preparation of cytosolic extracts and GFA activity assay. GFA activity was assayed using the method published previously (11). Briefly, at the end of treatment, cells were rinsed twice with ice-cold Krebs-Ringer phosphate buffer-10 mM HEPES, pH 7.6, and harvested with a rubber policeman. Cells were pelleted, and sonicated in extraction buffer containing 100 mM KCl, 1 mM EDTA, and 50 mM sodium phosphate, pH 7.5. Cytosolic extracts were obtained by centrifugation at 16,000 x g for 10 min at 4°C. The extracts (25-30 ug) in 50 ul volume were then incubated with 50 ul of substrate mix (final concentration: 6 mM F6P, 12 mM glutamine, 40 mM sodium phosphate, pH 7.4, 1 mM EDTA and 1 mM dithiothreitol) at 37°C for 45 min. GFA enzyme activity were measured by the generation of GlcN6P, quantified by fluorescence after derivatization by o-phthaldi-aldehyde and fractionation by reverse-phase HPLC. GFA activity was expressed as 1 U/mg protein where 1 U represents the generation of 1 pmol of GlcN6P/min.

***In vitro* dephosphorylation of GFA.** Cells were cultured and harvested as described above, except cytosolic extracts were prepared by sonicating cells

in the buffer 25 mM Tris (pH 7.4), 1 mM EDTA, 0.5 mM EGTA, 200 uM PMSF, 10 ug/ml leupeptin, and 20 ug/ml aporotinin. Twenty to thirty of cytosolic extracts were preincubated at 37°C for 10 min in the presence or absence of phosphatase inhibitor, cooled on ice for 5 min, and then assayed GFA activity by adding substrate mix.

RESULTS

Recombinant GFA is phosphorylated by PKA *in vitro*. As shown in Fig. 1, human GFA has two consensus sites for PKA phosphorylation, ^{202}RRGS and ^{232}KKGS, both located in the least conserved region. To test whether these sites could be phosphorylated by PKA, *in vitro* phosphorylation assay was performed using His-tagged recombinant human GFA purified through Ni-agarose column. In the autorad of Fig. 2, ^{32}p-labeled 77 kDa of GFA protein was only detected in GFA-expressed bacterial extracts with PKA added in phosphorylation reaction. Since only tiny amount of GFA is in soluble fraction of bacterial extracts, we failed to detect GFA enzyme activity on such recombinant GFA.

GFA activity is increased by treatment of cells with cAMP-elevating agents. To further investigate whether cAMP-dependent pathway plays a

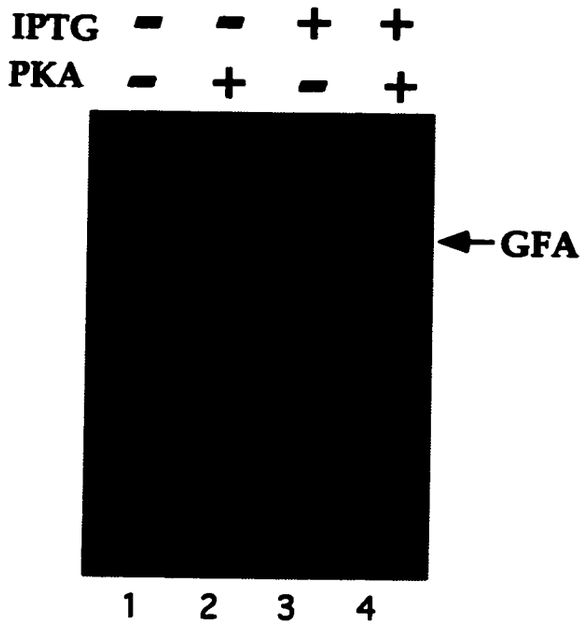

FIG. 2. Phosphorylation of recombinant human GFA by PKA *in vitro*. His-tagged human GFA was expressed in *E. coli* following IPTG (1 mM) induction for 3 hours (lane 3 & 4). Soluble GFA protein purified through Ni-agarose column was incubated with [r-^{32}P]-ATP and the catalytic subunit of PKA in phosphorylation buffer at 37 °C for 30 min. The proteins were then applied to SDS-PAGE, and autographed. Lane 1 & 2, proteins purified from bacterial extracs without IPTG induction.

role in the regulation of GFA enzyme activity, RASM cells were treated for 30 min with 10 uM forskolin, an activator of adenylate cyclase. Cytosolic extracts were then prepared and assayed for GFA activity. As shown in Fig.3, GFA enzyme activity in forskolin-treated cells was increased 2.2-fold (902.9 vs. 410.4 U/mg, $p<0.001$) compared to activity in control cells. Preincubation of cells with 2 ug/ml cycloheximide, a general and potent inhibitor for protein synthesis, for 2 hours did not abolish the effect of forskolin. This data strongly suggests that forskolin-mediated stimulation of GFA activity occurs through post-translational modification of GFA without affecting transcription or translation of GFA enzyme. Other intracellular cAMP-elevating agents such as 8-Br-cAMP (1mM) and IBMX (1 mM), an inhibitor of phospho-diesterase, also led to respectively 2-fold (827.7 U/mg) and 2.4-fold (1010.0 U/mg) increase of GFA activity at the end of 30 min incubation period ($p<0.001$).

The effect of cAMP-elevating agents on GFA activity is time- and dose-dependent. RASM cells treated with 10 uM forskolin exhibited an increase (2-fold) in GFA activity as soon as 15 min, with maximal stimulation observed at 60 min (2.3-fold, Fig. 4A). GFA activity was maximally stimulated by 10 uM

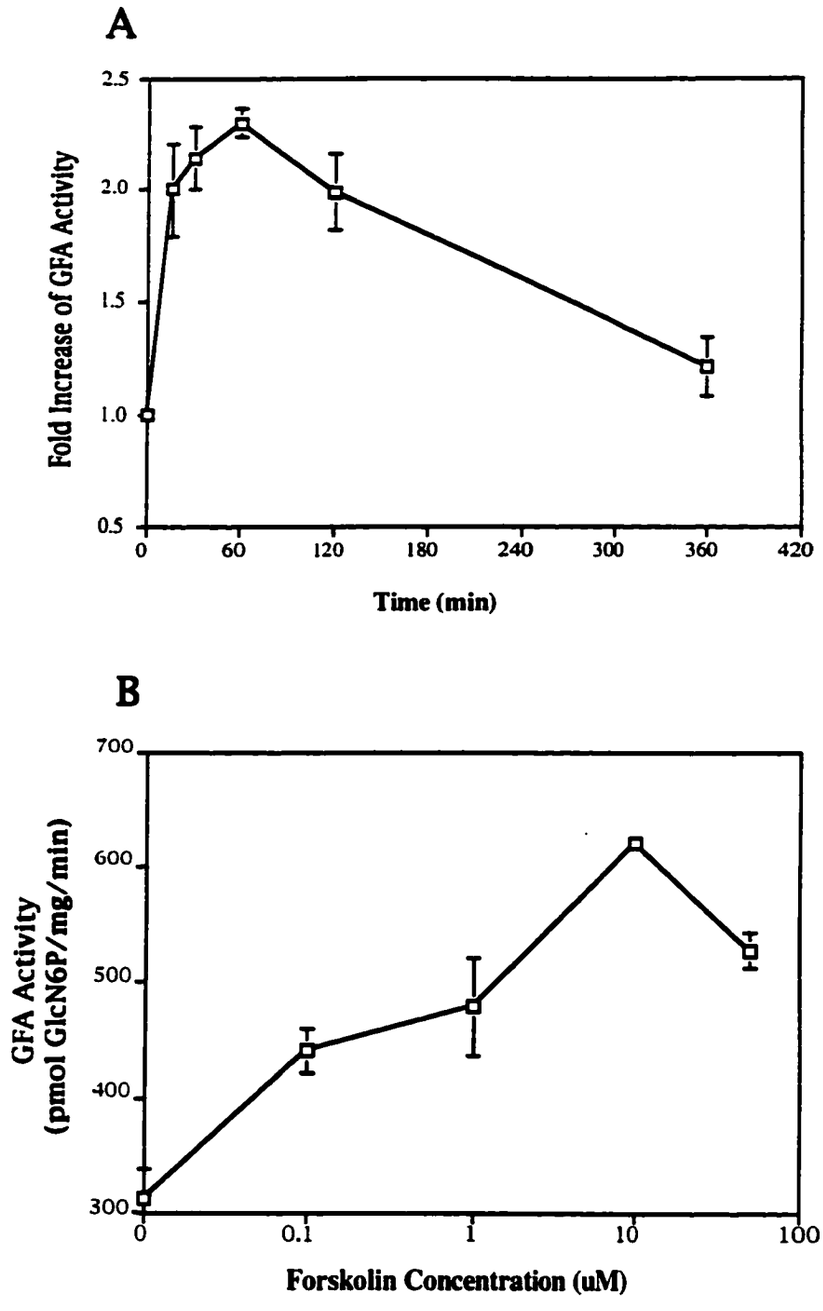

FIG. 4. Time- and dose-dependent effect of forskolin on GFA enzyme activity. A, RASM cells were treated with 10 uM forskolin for 0, 15, 30, 60, 120 or 360 min. B, Cells were incubated for 30 min with 0, 0.1, 1, 10 or 50 uM forskolin before harvest. Each point represents the average of three (A) or two (B) independent experiments.

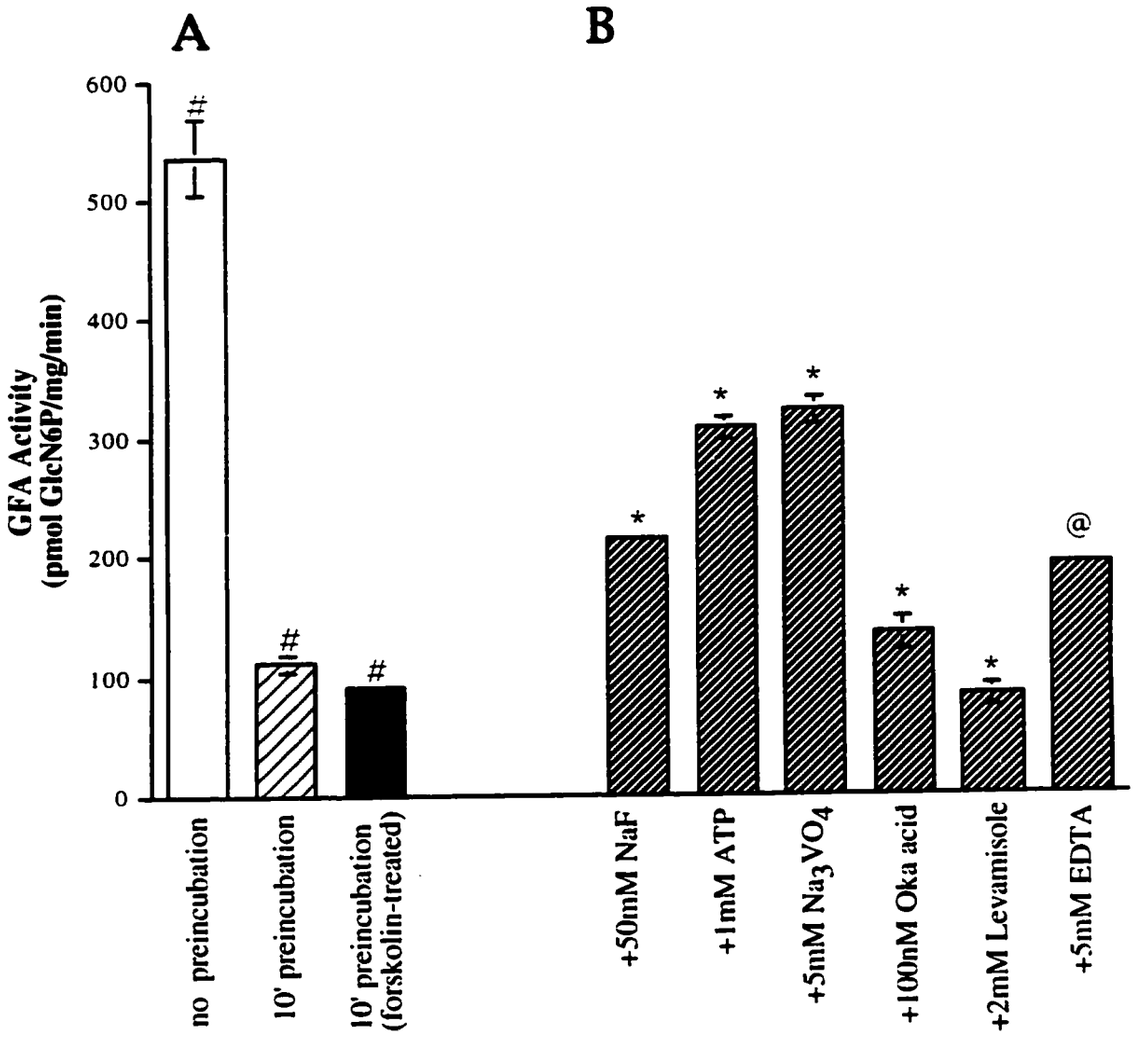

FIG. 5. Effects of endogeneous phosphatases and phosphatase inhibitors on GFA activity. RASM cytosolic extracts were prepared in the extraction buffer containing 25 mM Tris (pH 7.4), 1 mM EDTA, 0.5 mM EGTA, 200 uM PMSF, 10 ug/ml leupeptin and 20 ug/ml aporotinin. GFA activities were then assayed with or without preincubation at 37 ℃ for 10 min. Phosphatase inhibitors were added prior to preincubation. All the data were obtained from cells without forskolin treatment unless specifically noted. Each column represents the average of six (#), four (*), or two (@) independent experiments.

effects of several specific phosphatase inhibitors. Sodium orthovanadate was the most effective agent in preventing the loss of GFA activity (49%, Fig. 5B). This agent is known to inhibit serine/threonine phosphatases and more potently, at micromolar concentration, inhibit tyrosine phospha-tases (18, 19). The facts that the effects of vanadate were only seen at higher concentrations and that an monoclonal anti-phosphotyrosine antibody failed to immuno-precipitate GFA activity (data not shown) suggest that vanadate effect is more likely due to inhibition of serine/threonine phosphatases.

The addition of up to 100 nM okadaic acid (Fig. 5B) had very little effect on the decrease of GFA activity, indicating the okadaic acid-sensitive serine/threonine phosphatases, PP1 and PP2A (20), are not involved in this regulatory process. Consistent with this conclusion, microcystin-LR, a cyclic heptapeptide inhibitor of PP1 and PP2A but not PP2C (21), did not protect GFA from deactivation during the 37°C incubation (data not shown). Two millimolar levamisole (22), a specific inhibitor of alkaline phosphatase, had no effect for the recovery of GFA activity. On the other hand, 5 mM EDTA inhibited 18% of the decrease.

uM phorbol-12-myristate, 13-acetate for 15-60 min *in vivo* (data not shown).

The cAMP-dependent effect of GFA activity is probably post-translational. First, the increase of GFA activity by forskolin was very rapid, being near maximal 15 min after *in vivo* treatment (Fig. 4). Second, pretreatment by cycloheximide did not affect the forskolin-induced increase of GFA activity. Finally, GFA activity from control extracts was rapidly reduced by simply incubating cytosolic extracts at 37°C for 10 min. More importantly, the added increment of GFA activity seen in forskolin-treated cells was abolished by the same incubation of the cell extract at 37°C. This decrease in GFA activity could be inhibited by the addition of the phosphatase inhibitors sodium orthovanadate, NaF, or EDTA.

The endogenous protein phosphatase(s) responsible for down-regulation of GFA activity remain unidentified. Our data (Fig. 5) suggest that GFA is dephosphorylated by serine/threonine phosphatases, consistent with up-regulation of GFA activity by cAMP-dependent phosphorylation. Etchebehere et al. have shown that GFA in fungi *B. emersonii* was developmentally regulated by PP2C and, to a lesser extent, PP2A (26). The activity of endogenous phosphatase in RASM cytosolic extracts was inhibited

by 5 mM EDTA but not by okadaic acid. This suggests that PP2C, which requires free Mg^{2+} for its activity (27), may also be partially responsible for GFA regulation in mammals.

Regulation of GFA activity is only beginning to be examined but may be complex. For example, GFA activity increases significantly with high glucose or insulin treatment of cultured human skeletal muscle cells (28, 29), but is reduced by glucose and glucosamine in adipocytes (30). In hyperglycemic and hypoinsulinemic streptozotocin-treated rats, GFA activity was decreased (31). The sensitivity of GFA to the feedback inhibitor UDP-N-acetylglucosamine is developmentally regulated by phosphorylation in the fungi *B. emersonii* (16). In the present work, we have shown that GFA activity is stimulated by a cAMP-dependent pathway in RASM cells. These data support the hypothesis that GFA is an actively regulated molecule, consistent with its important role in mediating glucose uptake and cellular utilization of glucose (4, 10). To further elucidate the mechanism and significance of GFA regulation by cAMP-dependent pathway, the mutagenesis of PKA-consensus sites and additional GFA enzymatic studies are suggested.

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SUMMARY AND DISCUSSION

Hyperglycemia is now recognized to be a cause of insulin resistance in both insulin-dependent diabetes and non-insulin-dependent diabetes by leading to decreased glucose uptake and glucose utilization (6, 68). Hyperglycemia is also the primary factor in the pathogenesis of diabetic microvascular complications (13). The mechanisms for so-called glucose toxicity are unclear. Recent evidence suggests the hexosamine biosynthesis pathway of glucose metabolism mediates at least some of these adverse effects of glucose (135, 136).

The enzyme-controlled rate-limiting glucose flux through the hexosamine pathway is mediated via glutamine:fructose-6-phosphate amidotransferase (GFA). GFA is a necessary factor in desensitizing insulin-responsive glucose transport system in adipocytes (137,138). GFA also regulates growth factor expression in fibroblasts (109) and causes insulin resistance in stimulation of glycogen synthase in fibroblasts (89, 144) and isolated rat diaphragms (140). Transgenic mice that overexpress GFA in muscle and adipose tissues develop insulin resistance and a decreased glucose disposal rate. Hyperinsulinemia

observed in these random-fed transgenic mice is both age- and weight-dependent (145). Consistently, GFA activity in human skeletal muscle biopsies (146) and primary cultures of muscle cells (147) is positively correlated with glucose disposal in NIDDM patients. The abnormalities induced by glucose flux into the hexosamine pathway are targeted and specific. For instance, Robinson et al. have recently found that glucosamine-induced inhibition in insulin signal transduction and stimulation of glucose transport appears to be restricted in GLUT4-expressing cells, e.g., skeletal muscle cells and adipocytes, but not in rat-1 fibroblasts that exclusively express GLUT1 (140). Taken together, these data support the hypothesis that GFA plays a key role in glucose homeostasis and the development of diabetic complications.

How hexosamine flux is itself regulated is unknown. It is estimated that hexosamine metabolism normally accounts for only 2-3% of cellular glucose utilization (137). Maximal overexpression of GFA in either transfected cells (89, 144) or transgenic mice (145) could only be achieved to 2-3-fold levels, suggesting that cells are intolerant of major alterations in hexosamine flux.

The molecular basis of GFA mediated effects could involve alterations in gene transcription or post-

translational modification of critical proteins. For example, GFA stimulates gene expression of TGFα in fibroblasts (109) and pyruvate kinase in rat adipocytes (168). GFA also modulates glycogen synthase by modifying its phosphorylation state possibly through a transcriptional mechanism (169). The metabolic end product of this pathway, UDP-N-acetyl-glucosamine, is a critical precursor for both *N*-linked and *O*-linked glycosylation. As could be predicted, blockage of GFA expression or inhibition of GFA activity significantly decrease O-linked GlcNAc content on intracellular proteins (170). Thus, modulation of the activity of cellular proteins by post-translational glycosylation is a possible mechanism for the regulatory effects of glucose and the hexosamine pathway. For instance, the subcellular distribution and N-linked glycosylation of GLUT1 and GLUT4 are developmentally regulated in L6 myoblasts (171), and this post-translational modification of proteins is important for transporter function (172). Purrello et al. have shown that high-glucose induced desensitization in insulin secretion could be prevented by adding N-glycosylation inhibitors such as tunicamycin or Swainsonine in isolated rat islets and purified β-cell (173). Similarly, Daniels et al. have found that tunicamycin could abolish the effects of high glucose and glucosamine on TGFα gene expression

(148). Therefore, hexosamine-induced insulin resistance of glucose transport in adipocytes (138) and in skeletal muscle (140) may reflect altered glycosylation of GLUT4 or vesicle-associated proteins, which may be involved in GLUT4 targeting and/or trafficking as suggested by Baron et al (61).

Recently, a new group of glycoproteins has been identified that are O-glycosylated by addition of single GlcNAc moieties to serine or threonine residues (174). This O-glycosylation is a widespread event that occurs on numerous nuclear and cytoplasmic proteins and is highly dynamic and regulated (175, 176). Several transcriptional factors, including Sp1, can be rapidly modified by O-linked glycosylation, possibly affecting binding affinity at consensus sites in gene promoter regions. (177, 178). Interestingly, pyruvate kinase (74) and TGFα genes possess Sp1 binding sites in 5'-flanking DNA (109).

Given the fact that GFA plays a key role in glucose homeostasis and diabetic complications, the goal of this dissertation was to clone and characterize human GFA in order to eventually elucidate the mechanism by which GFA exerts its regulatory roles. As described in the first manuscript, human GFA was cloned by screening a human cDNA library with a 640 bp DNA fragment, which was generated from polymerase chain reaction (PCR) using

degenerated oligonucleotides derived from the highly homologous amino acid sequences between yeast and bacterial GFA. The 3.5 kb cDNA clones containing a 2.0 kb open reading frame were sequenced. His-tagged human GFA was expressed in *E. Coli* as a 77 kD protein, and purified through Ni-agarose. Polyclonal antibodies were then raised against this recombinant GFA in chicken. Subsequently, the regulation of GFA gene expression and enzyme activity was investigated by Northern and Western blot analysis. In cultured rat aortic smooth muscle cells and isolated primary hepatocytes, no significant changes in GFA mRNA levels were detected after treatment with 25 mM glucose, 7.5 mM glucosamine or 170 nM insulin for 2-24 hr. GFA enzyme activity in cytoplasmic extracts on the other hand, was inhibited by high concentration of glucose (61 % at 2 mM, and 90% at 25 mM glucose). This glucose-mediated reduction of GFA activity remained the same in Sephadex G-25 filtrates of cell extracts after removal of small molecules, suggesting that the glucose effect on GFA was not due to the feedback inhibition of UDP-GlcNAc, the end product of the hexosamine pathway. Furthermore, immunoblotting a-nalysis showed that GFA protein expression was unaffected by glucose. We therefore conclude that high concentrations of glucose may posttranslationally regulate GFA enzymatic activity.

Glucose-mediated inhibition of GFA activity is a graduate process ($t_{1/2}$=6 hr, Fig. 4 in the first manuscript). It is therefore possible that this glucose effect is indirectly modulated by a upstream mediator in glucose signaling pathway, which in turn causes post-translational modification of downstream GFA protein. The mechanism for this posttranslational modification of GFA remains unknown. The fact that GFA in RASM cells extracts failed to bind to Wheat Germ Agglutinin (WGA) suggests that GFA itself is not glycosylated (Daniels MC, unpublished data). As I have discussed in the third manuscript, GFA activity is stimulated by cAMP-dependent phosphorylation. It is intriguing to know whether glucose signaling pathway would cross-talk with cAMP-dependent phosphorylation in regulation of GFA activity. For this purpose, GFA protein in cells treated with different concentrated glucose could be immunoprecipaitated with anti-GFA antibody, and then examined for changes in the phosphorylation status of GFA. Alternatively, HA- or *c-myc*-tagged GFA in a mammalian expression vector could be expressed in RASM cells by transiently transfection. Monoclonal anti-HA- or anti-*c-myc*-antibodies would then be used to pellet recombinant GFA for further investigation. Unfortunately, GFA tagged with *c-myc* at C-terminal completely abolished its enzymatic activity when expressed in cells, while

for human GFA cDNA isolated from human HepG2 cDNA library is 3.5 kb containing 2.0 kb open reading frame and about 1.3 kb of 5'-untranslated region (5'-UTR). The significant difference in size between GFA coding region (2.0 kb) and the major GFA mRNA (8 kb) suggests that a large 5'-UTR exists at GFA mRNA. The 3 kb of GFA mRNA is thus more likely due to truncated 5'-UTR. Further studies will be performed to isolate these two mRNAs and analyze their RT-PCR products by sequencing. Furthermore, the stability of two mRNAs could be measured and compared by addition of actinomycin D, a inhibitor of mRNA synthesis. The result will reveal whether two mRNA are differentially regulated at transcriptional and translational levels.

The availability of the human GFA cDNA and anti-GFA antibodies has allowed determination of GFA mRNA and protein levels in various insulin-resistant states such as NIDDM and obesity, as well as in insulin-targeted tissues of normal and diabetic individuals. These studies will help to determine the role of the hexosamine pathway in diabetes mellitus. For example, this cloned human GFA cDNA has already been used in investigations using transfected cells and transgenic mice (144, 145).

Cloning of human cDNA and chromosomal assignment of this gene to chromosome 2p13 also allows further studies of potential genetic relationships between GFA

contribution of these genes to NIDDM is not significant. GFA is a key mediator in the hexosamine biosynthesis pathway of glucose metabolism. The low affinity of the enzyme for its substrates (Kms in the near mM range for both F6P and glutamine) (146) would give it the ability to serve a sensing function for the concentration of cellular glucose and amino acid pools and allow the modulation of metabolic pathway in response to the cell's nutrient need. In fact, it has been shown that GFA acts as a glucose sensor for regulation of insulin-stimulated glucose uptake and glycogen synthesis (89, 144, 145). Moreover, GFA and its enzyme activity are under the regulation of both insulin and glucose (138, 147, 167). GFA is therefore a potential candidate for the defect in insulin resistance in NIDDM.

GFA is an evolutionarily conserved protein; human GFA shares overall 55% amino acid identity with *S. cerevisae* GFA and 35% with *E. coli* GFA. We have observed that a much less homologous "hinge" region exists between two well-conserved substrate binding domains, the N-terminal glutamine- and C-terminal F6P-binding domains. More interestingly, all the consensus sites for PKA-phosphorylation are found in this region; there are two in human GFA (^{202}RRGS and ^{232}KKGS) and one in yeast GFA (^{202}RKGS) whereas *E. coli* has none. The third manuscript has shown that this poorly

conserved hinge region may serve a regulatory function for GFA activity through cAMP-dependent pathway. cAMP-elevating reagents such as forskolin, 8-Br-cAMP, and IBMX all stimulate GFA enzyme activity 2-2.4-fold in RASM cells treated for 24 hr. This effect on GFA is probably post-translational, supported by the facts that cycloheximide did not affect the forskolin-induced increase in GFA activity, and that phosphatase inhibitors could partially prevent the loss of GFA activity during preincubation of cell extracts. The correlation of GFA sensitivity to UDP-GlcNAc and cAMP-dependent phosphorylation of GFA, which are both specific in eukaryotes, suggested a mechanism for GFA regulation. Future studies will investigate the significance of GFA regulation by cAMP-dependent phosphorylation *in vivo* and *in vitro*. Analysis of baculovirus-expressed recombinant GFA, which is enriched in the soluble form of the protein, is underway to assess the effects of *in vitro* phosphorylation with PKA on GFA activity. In parallel with wild type GFA, GFA with mutated PKA-consensus sites could be analyzed in the same system to address which site is responsible for the observed changes in GFA activity. When a precipitating anti-GFA antibody is available, *in vivo* labeling of RASM cells with [r-^{32}P]-ATP, and then immunoprecipitating GFA protein with anti-GFA antibodies could be performed. Such experi-

ments will directly examine GFA phosphorylation *in vivo*, and phosphoamino acid(s) of GFA could also be identified following 2-dimensional electrophoresis of GFA peptide.

In summary, the results of the work described in this dissertation have provided the availability of the cDNA clone and anti-GFA antibody for further study of the involvement of GFA in glucose homeostasis and the development of NIDDM. Glucose-mediated regulation of GFA activity supports the hypothesis that GFA functions as a glucose sensor by changing its activity rapidly up and down in response to glucose signal. The regulation of GFA activity by cAMP-dependent phosphorylation suggests a possible mechanism by which GFA's regulatory effects might be modulated.

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I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this students are such that she may be recommended for the degree of Doctor of Philosophy.

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