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Blockage of O-GlcNAc metabolism by overexpressing a dominant-negative splice variant of NCOAT results in cell apoptosis.

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BLOCKAGE OF *O*-GlcNAc METABOLISM BY OVEREXPRESSING A DOMINANT-NEGATIVE SPLICE VARIANT OF NCOAT RESULTS IN CELL APOPTOSIS

by

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ABSTRACT OF DISSERTATION
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Title Blockage of *O*-GlcNAc Metabolism by Overexpressing a Dominant-Negative Splice Variant of NCOAT results in Cell Apoptosis

The addition of *O*-linked ß-*N*-acetylglucosamine (*O*-GlcNAc) to serine and threonine residues is a post-translational modification of nucleocytoplasmic proteins. This modification plays important roles in regulating gene transcription and proteasome activity. *O*-GlcNAc transferase (OGT) and nuclear cytoplasmic *O*-GlcNAcase and acetyltransferase (NCOAT) are responsible for addition and removal of this modification to and from proteins. A splice variant of NCOAT was cloned from the Goto-Kakazaki (GK) rat, a model animal for diabetes. This variant deletes amino acids 250 to 345, resulting in an approximately 90 kDa protein with a removal of exon 8 from the wild type form. *In vitro* experiments show that this splice variant (GK-NCOAT) loses the *O*-GlcNAcase activity but retains the histone acytltansferase (HAT) activity. In the study presented in this dissertation, the *in vivo* role of GK-NCOAT was analyzed by transfecting it into mammalian cells. It has been found that overexpression of GK-NCOAT results in an increased cellular *O*-GlcNAc modification level, decreased proteasome activity, and cell apoptosis. These results suggest that GK-NCOAT may act as a dominant negative of wild type NCOAT *O*-GlcNAcase, blocking its function. Since the *O*-GlcNAcase is required to offset the action of OGT that also mediates the inhibition of the proteasome, cell death may result from the blockage of *O*-GlcNAc metabolism and proteasome inactivation. These results are consis-

tent with our previous observations that blockage of *O*-GlcNAc removal with the chemical streptozocin (STZ) results in pancreas ß cell and neuron cell apoptosis.

In the other part of the research presented in this dissertation, the amidotransferase activity of glutamine: fructose-6-phosphate amidotransferase 2 (GFAT2) has been proved for the first time. The kinetic characters of GFAT2 and its regulation by phosphorylation and UDP-GlcNAc were also measured.

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LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
cAMP	cyclic adenosine monophosphate
EDTA	ethylenediaminetetraacetic acid
FFA	free fatty acid
GalNAc	*N*-acetylgalacosamine
GFAT	glutamine- fructose-6-phosphate amidotransferase
GlcNAc	*N*-acetylglucosamine
GST	glutathione *S*-transferase
HAT	histone acetyltransferase
HSP	hexosamine biosynthesis pathway
NCOAT	nuclear cytoplasmic beta-*N*-aceylglucosaminidase and acetyltransferase
O-GlcNAcase	beta-*N*-acetylglucosaminidase
O-GlcNAc	*O*-linked beta-*N*-acetylglucosamine
OGN	O-GlcNAcase
OGT	O-GlcNAc transferase
PBS	phosphate-buffered saline
PKA	cyclic adenosine monophosphate -dependent protein kinase
SDS	sodium dodecyl sulfate
STZ	streptozotocin
Tris	tris(hydroxymethyl) aminomethane

UDP	uridine diphosphate
UDP-GlcNAc	UDP-*N*-acetylglucosamine

INTRODUCTION

The Hexosamine Biosynthetic Pathway (HSP)

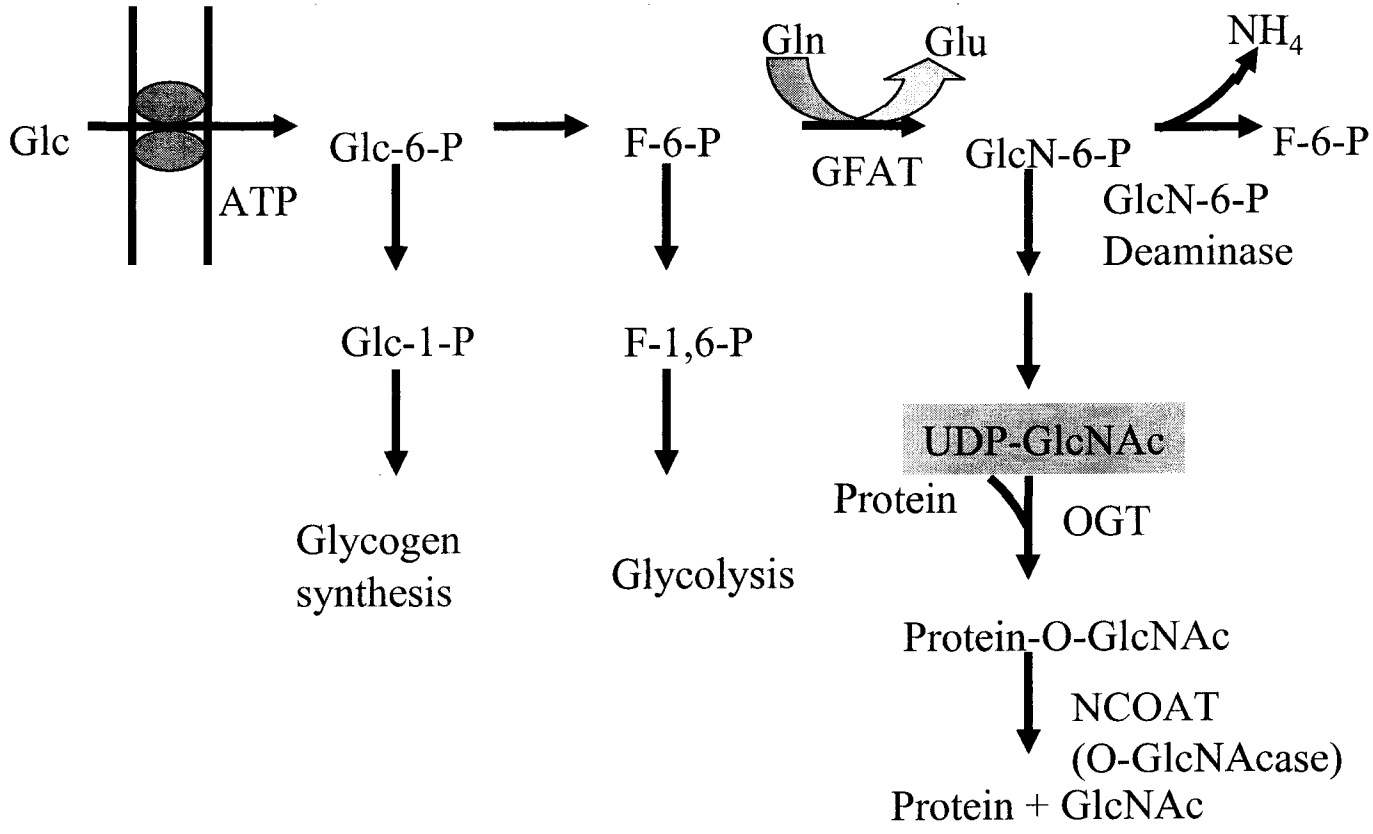
Glucose, a major metabolic fuel source, is mainly degraded via glycolysis to produce ATP, or it is stored as glycogen in animals. When glucose enters a cell, it is phosphorylated, isomerized, and converted to fructose-6-phosphate (Fru-6-P). Most of the fructose-6-phosphate is further phosphorylated and lysed to produce ATP for organism energy requirements; however, there is still 1-3% of fructose-6-phosphate that enters another pathway, HSP (Fig. 1).

The HSP, a universally conserved pathway in all life forms, metabolizes fructose-6-phosphate in conjunction with glutamine and acetate to make UDP-*N*-acetylhexosamines (UDP-HexNAcs) as the main end products (Fig.1). UDP-HexNAcs include UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetylgalacosamine (UDP-GalNAc). These UDP-HexNAcs are used mainly for the synthesis of exported proteins that are modified by complex carbohydrate side chains prior to their exit from the interior of the cell. In addition, UDP-HexNAcs also serve as essential substrates for the synthesis of glycol side chains of glycolipids, and UDP-GlcNAc is also an active precursor of chitin, an essential component of bacterial and fungal cell wall and insect cuticle.

The HSP also has been proposed to be a nutrient sensor of energy availability (1) and has been implicated in insulin resistance (2).

Figure 1. The disposal of glucose, the hexosamine biosynthesis pathway and O-GlcNAc. When glucose (Glc) enter the cell, it is phosphorylated by hexokinase to glucose-6-phosphate (Glc-6-P). Glc-6-P is converted to glucose-1-phosphate (Glc-1-P) or fructose-6-phosphate (Fru-6-P). Glc-1-P can be used for storage as glycogen; Fru-6-P is glycolyzed to generate energy or enter the hexosamine biosynthesis pathway (HSP) to produce UDP-GlcNAc.The first step of the HSP is the catalysis by glutamine:fructose-6-phosphate amidotransferase (GFAT) to produce glucosamine-6-phosphate (GlcN-6-P). UDP-GlcNAc is the substrate for O-GlcNAc modification on proteins. O-GlcNAc transferase (OGT) and nuclear and cytoplasmic O-GlcNAcase and histon acetyltransferase (NCOAT) are the enzymes that add or remove this modification to/from the proteins.

Hexosamine Biosynthesis Pathway and Protein O-GlcNAcylation



The fructose-6- phosphate pool in the cell is very important for the flux through the pathway. If the availability of the fructose-6-phosphate is increased, the flux through the hexosamine pathway will be increased. An expanded pool of fructose-6-phosphate, whether due to increased phosphorylation of glucose (e.g. hyperglycemia) or due to decreased utilization of fructose-6-phosphate (e.g. increased availability of free fatty acid), would increase the carbon flux through the hexosamine pathway and its end product, UDP-GlcNAc (1). There are some evidences supporting this idea: Prolonged exposure of muscle cells to high concentrations of free fatty acid increases flux into the hexosamine pathway and can induce insulin resistance (3). An increase of the hexosamine flux by overexpressing the rate-limiting enzyme for hexosamine synthesis, glutamine: fructose-6-phosphate amidotransferase (GFAT) in transgenic animals results in many effects that mimic type 2 diabetes (muscles becomes insulin resistant, the liver synthesizes excess fatty acid, and the ß cell secretes excess insulin, leading to hyperinsulinemia) (4). Increased activity of GFAT has been demonstrated in ob/ob mice (5) and in patients with type 2 diabetes (6). However, how the HSP senses the nutrition state of an organism is not completely clear.

The end product of the hexosamine pathways, UDP-GlcNAc, is the donor substrate for modification of nucleocytoplasmic proteins at serine and threonine residues with *O*-linked *N*-acetylglucosamine (*O*-GlcNAc). Theoretically, by this post-translational modification, the HSP serves as the nutrient sensor as proposed (7). There are some evidences supporting this idea as well: Overexpressing *O*-linked *N*-acetylglucosamine transferase (OGT) impacts the *O*-GlcNAc modification of proteins and results in insulin resistance in adipocytes (8, 9).

The Rate-Limiting Enzyme of the Hexosamine Pathway—GFAT

As mentioned above, the hexosamine biosynthesis pathway has been proposed to act as a nutrient sensor, and evidence suggest that it is implicated in insulin resistance, which is the important characteristic of type II diabetes. For example, the transgenic mice that overexpress GFAT develop the insulin resistance in muscle and adipose tissue (10). Since GFAT is the rate-limiting enzyme of the hexosamine pathway, its characteristics and functions have been widely studied.

GFAT catalyzes the first step of the HSP: the synthesis of Glutamine-6-phosphate and glutamate from fructose-6-phosphate and glutamine (Fig. 2). GFAT is a cytoplasmic enzyme, and its activity has been detected in almost every organism and tissue. The enzyme is composed of two domains: a glutaminase domain, which catalyzes the hydrolysis of glutamine to glutamate and ammonia, and a synthase domain, which catalyzes amination of fructose-6-phosphate (11). The *Escherichia coli* GFAT has been crystallized, and the structure of the enzyme has been elucidated (12). UDP-HexNAcs also serve as essential substrates for the synthesis of glycosyl side chain and lipids (13), and UDP-GlcNAc is an active precursor of chitin, which is the essential component of fungi and insect cuticle. On the base of its physiologic role in the hexosamine pathway, GFAT has been one of the targets for antifungal drug design (14), and GFAT was identified as a primary target of methylmercury toxicity in yeast (15).

Fru-6-P is the initial substrate of the hexosamine pathway. It is also a key intermediary metabolite of glucose as it can be metabolized through glycolysis to create ATP, and/or it can be metabolized to glucosamine for use in glycoprotein synthesis by the enzyme GFAT (Fig. 1). In liver and kidney, Fru-6-P can also be utilized for gluconeogene

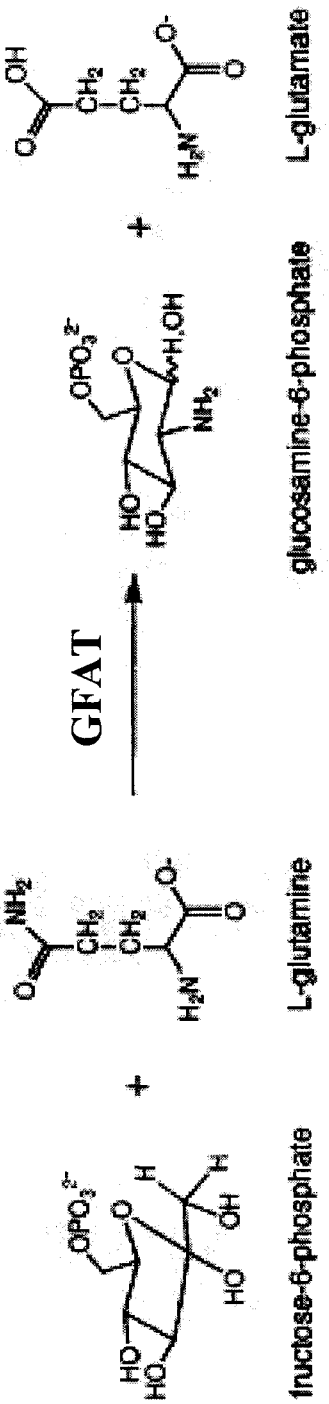


Figure 2. The reaction catalyzed by glutamine:fructose-6-phosphate amidotransferase (GFAT)

sis. Fru-6-P metabolism is tightly regulated allosterically and by hormones in concert with the nutritional status of the intact organism. During starvation or stress, glucagon and epinephrine signal the accumulation of cAMP, which directs the flux of Fru-6-P into gluconeogenesis in the liver and glycolysis in the heart. Thus, the net effect is for the liver to release glucose to provide for the energy requirements of muscle, heart and brain.

Our laboratory has found that phosphorylation of human GFAT1 at serine 205 by protein kinase A (PKA) can block the enzyme's activity (16). This implicates that the hexosamine pathway is also regulated by hormones like glucagon and epinephrine, which accumulate cAMP and activate PKA, resulting in a shutting down of the macromolecular synthesis during the starvation, which makes more Fru-6-P available for yielding energy. It has been shown that the end product of the hexosamine pathway can inhibit the activity of human GFAT1 (17, 18). This feedback inhibition of GFAT by the end product of the hexosamine pathway, UDP-GlcNAc, may help to prevent excess glucose flux into this pathway.

In 1999, a gene encoding a protein with 75% homology to GFAT was cloned. The new gene was named GFAT2, and the previous gene was designated GFAT1. Both the human and the mouse GFAT2 proteins are composed of 682 amino acids and are approximately 77.0 kDaa. At the amino acid level, homologies between the human GFAT1 and GFAT2, between the mouse GFAT1 and GFAT2, and between the human GFAT2 and the mouse GFAT2 are 75.6, 74.7, and 97.2%, respectively. Analysis also revealed different tissue distribution between GFAT1 and GFAT2: GFAT1 was more highly expressed in the placenta, pancreas, and testis than GFAT2, and GFAT2 was expressed throughout the central nervous system, especially in the spinal cord, while GFAT1 ex-

pression was weak. The gene locus was mapped to human chromosome 5q and mouse chromosome 11 (19).

In contrast to GFAT1, little was known about GFAT2. It is named GFAT2 based solely on the sequence homology. Since these two isoforms of GFAT have different gene locations and quite different tissue distribution, we wanted to characterize this new enzyme to provide more information about the hexosamine pathway.

O-GlcNAc

The end product of the hexosamine synthesis pathway, UDP-GlcNAc is the donor substrate for O-GlcNAc modification of proteins. O-GlcNAc modification is a ubiquitous post-translational modification consisting of the monosaccharide *N*-acetylglucosamine linked to the side chain hydroxyl of either serine or threonine residues of the protein. The O-GlcNAcylation is a cyclic and highly dynamic process, with the addition and removal of O-GlcNAc from protein carried out by two enzymes OGT and Nuclear Cytoplasmic O-GlcNAcase and AcetylTransferase (NCOAT).

The O-GlcNAc transferase (OGT) transfers *N*-acetylglucosamine from UDP-GlcNAc to the hydroxyl oxygen of serine and threonine in a ß confirmation. The OGT gene is on the X-chromosome, and a knockout of the gene is lethal at the embryonic stem cell stage (20). This 110-kDaa polypeptide has two domains: an N terminus with 11.5 tetratricopeptide repeats and a putative catalytic C terminus. TPRs are known protein–protein association domains. The enzyme functions as a trimer with the polypeptide chains interacting via the tetratricopeptide repeats. OGT is localized to the cytosol, and nucleus and is modified by tyrosine phosphorylation as well as being modified by O-

GlcNAc itself (21-23). mSin3A can recruit O-GlcNAc transferase (OGT) along with histone deacetylase into co-repressor complexes to regulate gene transcription (24).

NCOAT is a 103-kDaa protein with an acidic isoelectric point. The cloned cDNA encodes a polypeptide of 916 amino acids with a predicted molecular mass of 103 kDaa and a pI value of 4.63, but the protein migrates as a 130-kDaa band on SDS-polyacrylamide gel electrophoresis. The cloned enzyme cleaves GlcNAc, but not GalNAc, from glycopeptides. Cell fractionation suggests that the overexpressed protein is mostly localized in the cytosol. Northern blots show that the transcript is expressed in every human tissue examined, but the highest expression is in the brain, placenta, and pancreas (25). NCOAT is a substrate for the executioner apoptotic caspase-3 and is cleaved during the induction of apoptosis in cells by treatment with cytotoxic lymphocyte granules. Surprisingly, cleavage of the full-length 130 kDaa NCOAT into two approximately 65 kDa fragments has no effect on *in vitro* NCOAT activity (26). This protein had previously been named *O*-GlcNAcase (OGN), however, it has been renamed Nuclear Cytoplasmic *O*-GlcNAcase and AcetylTransferase (NCOAT) since it has been discovered to possess intrinsic histone acetyltransferase (HAT) activity in addition to its *O*-GlcNAcase activity. The domains for HAT activity and *O*-GlcNAcase activity reside separately in the C terminus and N terminus of the protein (27). A recent study in our laboratory showed that NCOAT can be found in the co-repression complexes with OGT, and the complexes sit on the DNA to regulate transcription. This result is quite interesting: the transcriptional activation capability of many transcriptional factors like Sp1 and CREB can be inhibited when they are *O*-GlcNAcylated. The *O*-GlcNAcase of NCOAT can cleave this modification from those factors and up-regulate their activity; at the same time the his-

tone acetyltransferase activity of NCOAT can add the acetyl group to histones, which helps to loose DNA from histones. When NCOAT and OGT form the complex and sit on DNA, both enzyme activities of NCOAT will help the gene to be actively transcribed. However, further experiments need be done to test this idea.

It has been reported that there are at least two variants of this protein, each having the same N terminus; however, one of them is lacking the C terminal 240 amino acids. This short form still has the *O*-GlcNAcase activity, which means the active site for the enzyme is in the N terminus of the protein (28). Our laboratory's recent study on NCOAT shows that there are two other isoforms of the enzyme that are both splice variants: the first one results in a protein of ~90 kDa, due to the removal of exon 8 (missing amino amino acids 250 to 345, named as GK-NCOAT, Figure 3), and the second is ~84 kDa resulting from a variant that is missing exons 8 and 9 (lacking amino acids 250 to 398, named as SD-NCOAT) (Fig. 3). It has also been shown that both of GK-NCOAT (~90kDa protein) and SD-NCOAT (~84 kDa protein) lose the O-GlcNAcase activity and still retain complete histone acetyltransferase activity (27). Interestingly, the Goto-Kakizaki (GK) rat, a model animal for diabetes, has been shown to express two forms of NCOAT, the full-length form and the 90 kDa form mentioned above. The Sprague-Dawley rat, which does not get diabetes even under high glucose diets, only expresses the 84 kDa form of NCOAT. The potential significance of these findings is unknown.

In vitro experiments show that GK-NCOAT loses *O*-GlcNAcase activity and still retains complete HAT activity, and the GK-NCOAT can also form a complex with OGT. Can GK-NCOAT be a dominant negative *in vivo* to block the wild type NCOAT's function? If it can, we may use this dominant negative form as a tool to study the physiologi

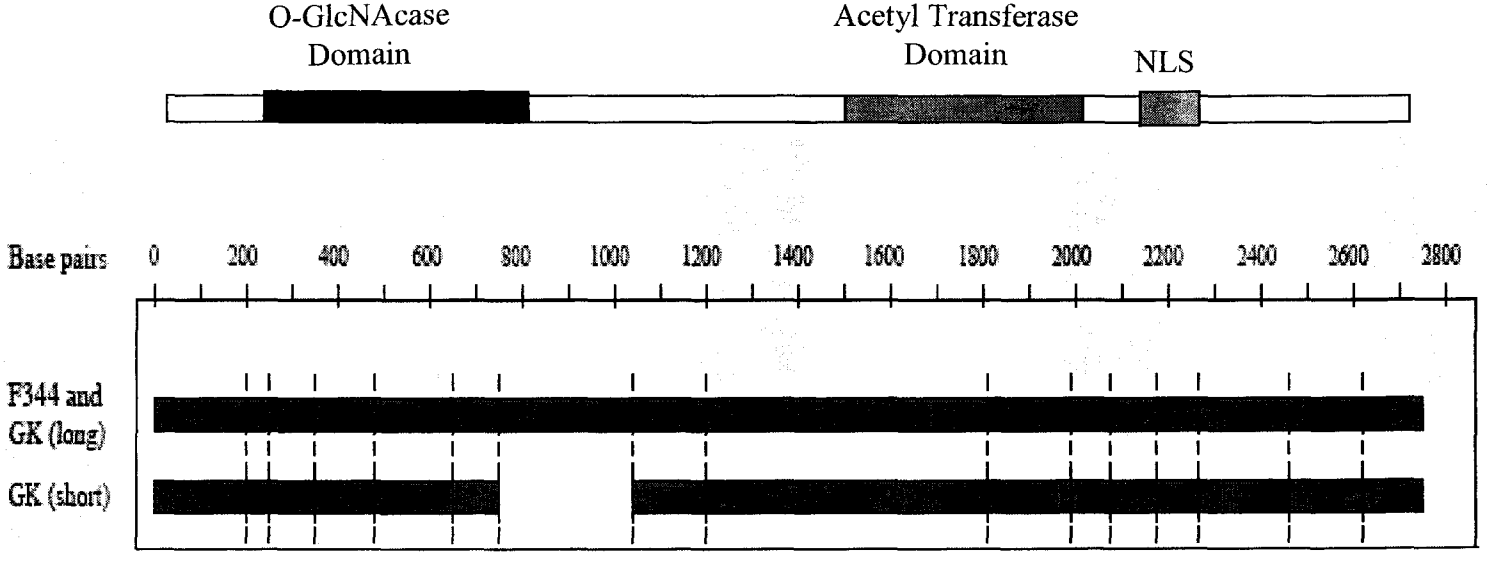


Figure 3. Diagram of NCOAT and its splice variant GK-NCOAT. The domains for the O-GlcNAcase and histon acetyltransferase are in the N-terminus and C-terminus of NCOAT separately. In the C-terminus of NCOAT, there is a nuclear localization sequence (NLS). GK-NCOAT lacks the exon 8, which overlaps in the O-GlcNAcase domain.

cal phenomenon caused by disrupting *O*-GlcNAc metabolism, which will give a base to further study on the relationship between *O*-GlcNAc modifications and some human diseases like diabetes and neurodegeneration diseases.

The Roles of *O*-GlcNAc Modification

Currently, there are over 100 cytoplasmic or nuclear proteins that have been identified as carrying the *O*-GlcNAc modification, including transcription factors, cytoskeletal components, chaperones, metabolic enzymes, and signaling components. However, these proteins are surely just a small part of all the *O*-GlcNAc modified proteins in a cell (29,30). *O*-GlcNAc modification has wide spread physiological roles in a cell, and it has a role in protein transport, gene transcription, translation, and cell signaling.

The *O*-GlcNAc modification appears to modulate transcriptional and signal transduction events. So far, most of the proteins with mapped *O*-GlcNAc sites have been transcription factors (31-33). Indeed, the first transcription factor shown to contain *O*-GlcNAc was Sp1 (34). However, the serum response factor (35), c-myc (36), estrogen receptors (37-39), and RNA polymerase II itself (40) are now among the growing list of transcription factors known to contain this modification (32). Those transcriptional factors are modified by *O*-GlcNAc, and the modification often occurs in the transcriptional activation domain, which suggests that this modification may have a role in the transcriptional control function of these proteins. For example, the *O*-GlcNAc modification on CREB's Q2 domain disrupts the association of CREB and $TAF_{II}130$, thereby repressing the transcriptional activity of CREB in vitro (41). Similarly, increased glycosylation of Sp-1 inhibits its transactivation capability (24). Furthermore, it has been shown that Sp-1

is O-GlcNAc modified in a cell type-specific manner. OGT was found to associate with the mSin3A co-repressor complex, and its activity was necessary for maximal gene silencing (42). Recently, we have found that NCOAT and OGT can form a co-repression complex and sit on gene promoters to regulate gene transcription.

There is also some evidence suggesting that glycosylation plays a role in regulating translation via glycosylation of the eIF-2-associated p67 (eIF-2A) protein. *O*-GlcNAc modification of eIF-2A appears to protect the protein from degradation and promotes translation by binding eIF-2 and impeding the inhibitory phosphorylation of eIF-2. Interestingly, this system appears to be sensitive to the nutritional state of the cell, and starvation results in the loss of glycosylated eIF-2A and culminates in the inhibition of translation (43,44).

It has been shown that *O*-GlcNAc can modify the proteasome, and this modification can regulate its activity (45). The study of the role of *O*-GlcNAc on the proteasome initiated from the interest in the proteasomal degradation of the transcription factor Sp1 (45,46). Subsequent studies showed that *O*-GlcNAc modification can regulate the activity of the proteasome. The activity of the 26S proteasome can be inhibited by modification with the enzyme *O*-GlcNAc transferase (OGT). This reversible modification of the proteasome inhibits the proteolysis of the transcription factor Sp1 and a hydrophobic peptide by inhibiting the ATPase activity of the 26S proteasome. The Rpt2 ATPase in the mammalian proteasome 19S cap is modified by *O*-GlcNAc *in vitro* and *in vivo* and as modification increases, proteasome activity decreases (45). This provides a linkage between the nutrient metabolic pathway and the control of the proteasome. The O-GlcNAc modification of proteasomes may allow the organism to respond to its metabolic needs by

controlling the availability of amino acids and regulatory proteins. The reduction of proteasome activity may slow down protein degradation and result in the accumulation and aggregation of some proteins.

Besides the studies mentioned above, more physiologic roles of *O*-GlcNAc modification will be explored in the future. From current data, it seems that the misregulation of *O*-GlcNAc metabolism will impair gene transcription, translation, and proteasome function, which disrupts the normal protein synthesis and degradation and results in cell death. It has been shown that a knock-out of the glucosamine-6-phosphate acetyltransferase gene, which is required for GlcNAc synthesis, causes cells to fail to divide or withstand a number of apoptotic stimuli. Therefore, UDP-GlcNAc levels influence cell cycle progression and susceptibility to apoptotic stimuli (47).

O-GlcNAc and Apoptosis

Considerable progress has been made in the general understanding of the pathways leading to apoptosis. Apoptosis is induced through the activation of "death-receptors" on the cell surface or as a result of internal protective mechanisms such as the p53 response to DNA damage. Central to apoptosis is the mitochondrion. The apoptotic signals largely appear to converge on the mitochondrion, resulting in the release of cytochrome c and other molecules. A p53 target gene stimulates the release of cytochrome C and Bax, a member of the Bcl family. The release of cytochrome C stimulates the assembly of the "apoptosome" that cleaves procaspase-3 to activated caspase-3. The activated caspase-3 then becomes the major trigger of the multitude of events leading to the disassembly of the cell. Proapoptotic factors such as p53 and Bax are degraded by the protea-

some (48-51). Proteasome inhibitors are being used as an adjunct to cancer chemotherapy because these inhibitors promote apoptosis (52-56). Proteasome inhibition in neurons results in apoptosis (57) and protein aggregate formation (58).

O-GlcNAc modification on the Rpt2 subunit of the proteasome can inhibit its activity and causes the blockage of protein degradation, especially the clearance of those apoptotic factors that may initiate cells to undergo apoptotic death.

Streptozotocin (STZ) is chemical produced by fungi and has a molecular structure very similar to that of GlcNAc (Fig. 4). It can induce diabetes in rat. STZ is an inhibitor of NCOAT *O*-GlcNAcase activity (8,23,59-61). Kinetic studies using recombinant NCOAT indicate that STZ is a noncompetitive inhibitor of the enzyme. Following treatment with STZ, pancreatic β-cells undergo glucose-dependent apoptosis (62). Our more recent studies show that intraventricular administration of STZ, hippocampal neurons and Purkinje cells in the cerebellum, the area displaying the highest expression of OGT and NCOAT in the brain, show rapid accumulation of *O*-GlcNAc, and cerebral proteasomes are inactivated. Ubiquitin and P53 accumulate in these area, and later some hippocampal neurons undergo apoptosis (63). These data suggest that the *O*-GlcNAc system is a functional candidate for diabetes and neurodegeneration diseases.

O-GlcNAc and Diseases

The hypothesis of "glucose toxicity" has been proposed for several years; in which excessive flux of nutrients through the UDP-hexosamine biosynthetic pathway (HSP) leads to insulin resistance and ß cell death, the key features of type II diabetes. The in vivo fructose-6-phosphate pool is very important to the hexosamine pathway. As the

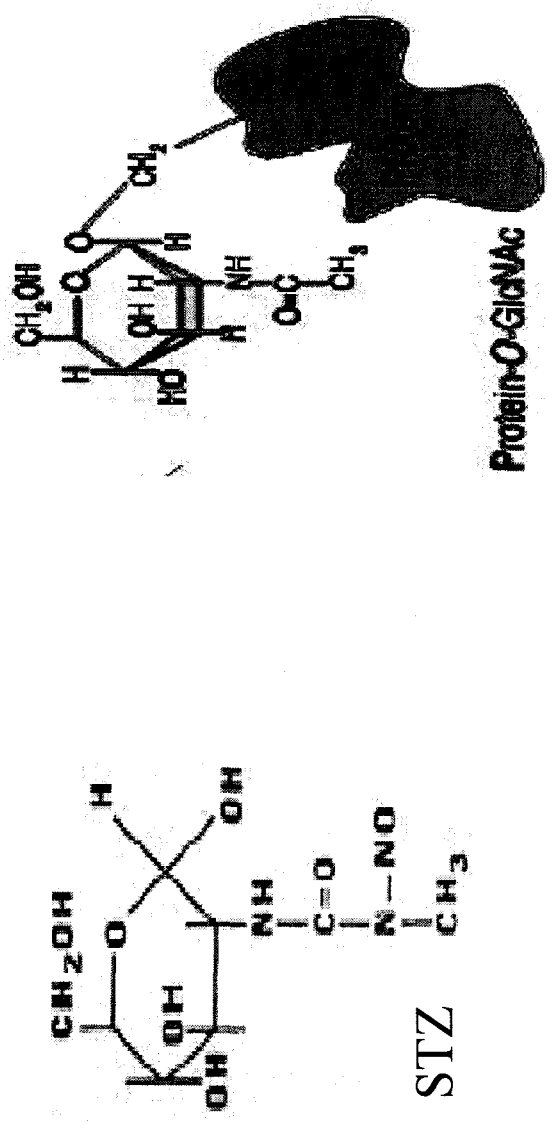


Figure 4. Structure of STZ and O-GlcNAc modified protein.

availability of fructose-6-phosphate increases, more sugar will flux into this pathway. High glucose environment or reducing the assumption of fructose-6-phosphate by providing high concentration's free fatty acids causes more glucose to go into hexosamine biosynthesis pathway (3,64). In 1991, Marshall *et al.* also showed that inhibition of the HSP, which converts fructose-6-phosphate to UDP-GlcNAc, the donor sugar nucleotide for O-GlcNAc modification, prevented hyperglycemia-induced insulin resistance in peripheral tissue (64). Transgenic mice overexpressing the rate-limiting enzyme in hexosamine synthesis GFAT in skeletal muscle or adipose tissue exhibit total body insulin resistance (10). However, how the products of the hexosamine pathway mediate these effects is not clear. Further work in 1998 by Yki-Jarvinen *et al.* demonstrated that mice with insulin resistance had elevated O-GlcNAc levels in skeletal muscle (65). Thus, a correlation between elevated O-GlcNAc levels and insulin resistance was proposed. Recently, it has been reported that transgenic mice overexpressing OGT in skeletal muscle and adipose tissue were mildly diabetic, which provides new evidence to suggest that the elevation of O-GlcNAc levels results in insulin resistance (9).

Data have also been accruing data that *O*-GlcNAc plays a role in apoptosis and neuropathology, particularly in Alzheimer's disease. Several neuronal proteins have been identified as being modified with *O*-GlcNAc; these proteins could form part of the inclusion bodies found, for example, in the most frequently observed neurological disorder (i.e., in Alzheimer's disease, the Tau protein and the ß-amyloid peptide are the well known aggregated proteins). Inclusion bodies are partly characterized by a deficiency in the ubiquitin-proteasome system, and a disruption in the degradation of aggregated proteins. From this perspective, it appears interesting that substrate proteins could be pro-

tected against proteasomal degradation by being covalently modified with single N-acetylglucosamine on serine or threonine and that the proteasome itself is modified and regulated by *O*-GlcNAc. (In this case, the turnover of neuronal proteins correlates with extracellular glucose.) Interestingly, glucose uptake and metabolism are impaired in neuronal disorders.

Lubas and co-workers have shown that the brain is second only to the pancreas in its level of OGT expression (66). *In situ* hybridization on brain slices indicated that the hippocampus and Purkinje cells in the cerebellum contain the highest levels of OGT mRNA (63). NCOAT mRNA was also abundant in these cells. The hippocampus is a very important region of the brain for the laying down of short-term memory and is a site for the development of the pathological changes of Alzheimer's disease.

Disease-specific protein aggregates are the hallmark of many of the neurodegenerative disorders. The most common of these disorders is Alzheimer's disease, in which the aggregate is composed mainly of β-amyloid in the extracellular space, and of the Tau protein in the intracellular neurofibrillary tangles. However, Parkinson's disease, polyglutamine disease, familial amyotrophic lateral sclerosis, and even prion disease (Jakob-Creutzfeld) are all characterized by their specific protein aggregates and inclusions (67). For these aggregates to develop, there must be either a defect that increases the synthesis of the protein from which the aggregate is composed or a defect in the degradation of the protein. Indeed, there are examples of both. For Alzheimer's disease, mutations in the β-amyloid precursor protein (APP) or mutations in the presenilin 1 or 2 result in a greater rate of conversion of APP to Aβ by the secretases (68,69). It is the Aβ, with its tendency to aggregate, that is thought to result in the initiation of the disease (70). On the other

hand, there are also examples of the delayed clearance of the aggregating protein that also results in disease. For example, in Parkinson's disease, one of the mutations that are associated with this condition is in a gene that encodes a ubiquitin C-terminal hydrolase (71,72). It is thought that the partial loss of catalytic activity of the mutant thiol protease leads to the accumulation of α-synuclein in the neurons of the substantial nigra. This aggregate accumulation and formation of the characteristic Lewy bodies is associated with toxicity to these neurons leading to their premature failure and early onset Parkinson's disease. The aggregates in Alzheimer's disease and the other neurodegenerative conditions contain ubiquitin (73-75). In addition, the proteins that aggregate in the lesions are cleared by the ubiquitin-proteasome system (76). Proteasomal function has been shown to decrease during aging in general (77) and in Alzheimer's disease in particular (78). Finally, inhibition of proteasomal function by the expression of abnormal ubiquitin leads to neuronal cell apoptotic death (57), and inhibition of the proteasomes with MG132 in cultured cells stably transfected with a presenilin 1 mutant formed aggresomes (58). These observations have led to the conclusion that part of the pathogenic mechanism of neurodegeneration might result from the impairment of proteasomal function. Because the protein aggregates and impaired proteasomal function are observed in well-developed neurodegenerative disorders, the question arises as to what causes what. Do the aggregates impede the function of the proteasome or do the aggregates arise because proteasome function is already impeded. The amyloid hypothesis of Alzheimer's disease (70) would suggest the former possibility. That is, in the hereditary form of neurodegenerative diseases, the increased production of the aggregating protein could swamp the ubiquitin-proteasome system (79,80) leading to the accumulation not only of the characteristic

aggregate but also of other toxic products that normally would be cleared by the proteasome. The chief problem with this idea is that most Alzheimer's disease occurs in individuals with no known genetic defect in the APP or the presenilins. Therefore, these patients should not accumulate the Aβ because of the overproduction of these peptides. Nevertheless, these patients do display the same Aβ aggregates and proteasomal impairment.

Since proteasome function is directly inhibited by *O*-GlcNAc, this alteration may affect the degradation of many vital cellular proteins involved both in cell growth and in apoptosis. In addition, since failure of adequate proteasome function appears responsible for neurodegeneration, this novel mechanism may explain how proteasomal degradation of aggregating proteins is coupled to nutrition through glucose metabolism to *O*-GlcNAc. The studies determine how the *O*-GlcNAc pathway might be functionally connected to neurodegeneration. Since nutrient deprivation is the only known means of extending life span, the connection among nutrients, proteasomes, and neurodegeneration may give insight into aging itself. Because the pathway under study is well defined, drugs may even be developed that can partially block this pathway, leading to a novel approach to the prevention of neurodegeneration.

O-GlcNAcylated proteins are also implicated in synaptosomal transport (e.g. synapsins and clathrin-assembly proteins). *NCOAT* blockade in the endocrine tissues where OGT is abundant results in a defect in vesicular trafficking (81). Since synaptosomes in the brain contain OGT and *NCOAT* (82), it remains possible that these enzymes play some role in vesicular traffic in the brain. Thus, a defect in *NCOAT* may result in a func-

tional defect in neurotransmitter release that could lead to a phenotypic response even before brain cell apoptosis supervenes.

The Transgenic Animal Model Study

It has been shown that the brain is second to the pancreas in its level of OGT expression (66). *In situ* hybridization on brain slices indicated that the hippocampus and Purkinje cells in the cerebellum contain the highest levels of OGT and NCOAT mRNA (63). The hippocampus is a very important region of the brain for the laying down of short-term memory and is a site for the development of the pathological changes of Alzheimer's disease. In the same study, Liu and his colleagues (63) observed the effect of chemical blockade of *O*-GlcNAc catabolism in the brain. After intraventricular administration of STZ, a chemical inhibitor of *O*-GlcNAcase, the hippocampal neurons and Purkinje cells display the most rapid accumulation of *O*-GlcNAc. Cerebral proteasome activity is reduced, and ubiquitin and p53 accumulate in those brain regions, with the subsequent activation of a p53-dependent transgene and the endogenous mdm2 gene. Later, some hippocampal cells, but not Purkinje cells, undergo apoptosis. These observations suggest that the *O*-GlcNAc system may participate in neurodegeneration, particularly in the hippocampus.

For several decades, streptozotocin (STZ), an analogue of GlcNAc (Fig. 4), has been used to create animal models of diabetes despite an incomplete understanding of how STZ actually causes ß-cell death (83). STZ has been shown to inhibit the enzyme *O*-GlcNAc-selective *O*-GlcNAcase, which removes *O*-GlcNAc from protein and is the final enzyme in the pathway of *O*-glycosylation in the ß-cell (59,62). Because of the linkage

between *O*-GlcNAc modification and diabetes and neurodegeneration diseases mentioned above, we propose that the physiological effects caused by STZ treatment result from the impaired *O*-GlcNAc metabolism as seen when STZ inhibits *O*-GlcNAcase activity. However, besides inhibiting the *O*-GlcNAcase activity, STZ can also act as a NO donor, which has led many investigators to postulate that NO is involved (84-86); however, the effect of STZ cannot be readily duplicated with *N*-methyl-*N*-nitrosourea (MNU, the portion of STZ that actually donates NO) (87-91). What causes the cell apoptosis when the cell is treated with STZ? Does it result from impaired *O*-GlcNAc metabolism or from stress caused by released NO or from both effects? To answer these questions, a new transgenic mouse model will be made in which genetic tools will be used to interrupt cellular *O*-GlcNAc metabolism in mouse brain or pancreatic ß cells.

Proteasome function is controlled by the post-translational modification of its cap by *O*-GlcNAc (45,46,92,93), and proteasomal inhibition induces nerve cell death (57,94,95) or degeneration (96). Some aggregates themselves could inhibit the proteasome (80,97). These phenomena raise one important question: In the process of neurodegeneration, what comes first, the proteasomal inhibition or the aggregates? However, once aggregate formation is initiated, a positive-feedback cascade could result and lead to the rapid terminus development seen in the neurodegeneration diseases. In the transgenic mouse model with impaired *O*-GlcNAc metabolism in the brain, we are going to investigate the relationship between *O*-GlcNAc modification and proteasome; the relationship between proteasome and protein aggregates could be investigated, as well.

Our laboratory has been using the tetracycline inducible system to target the conditional expression of transgenes (98,99). A schematic of this system is shown in Fig. 5

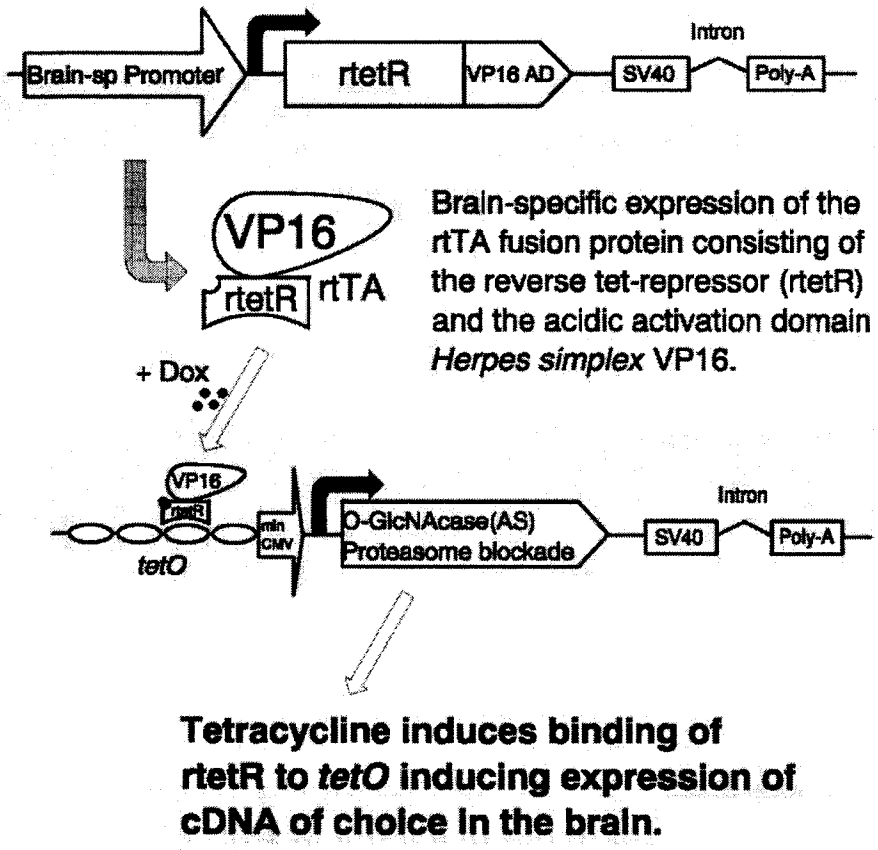


Fig. 5. The diagram of tetracycline transgene system. ***Dox* means doxcycline.**

as it applies to the brain. In this system, two lines of transgenic mice are required: The first line specifically in mouse brain expresses the transcriptional factor rtTA fusion protein that consists of the reverse tet-repressor (rtetR) and the acidic activation domain Herpes simplex VP16 with a brain-specific promoter CaMKIIα promoter ahead of the rtTA coding sequence (100). The second line contains antisense NCOAT or splice variant GK-NCOAT to block the *O*-GlcNAc metabolism. The transgene in the second line has a mini CMV promoter that can not initiate the downstream transgene's expression until the rtTA binds to the tetO operator ahead of mini CMV promoter. However, even these two transgenes reside in the same cell, the transcriptional factor rtTA can't bind to the tetO operator by itself but can do so only when the small molecule Doxycycline/ Tetracycline is used to induce the rtTA to bind with tetO. In biotransgenic mice that have both transgenes, transgene's expression can be induced by doxycycline/ tetracycline given simply in drinking water at a concentration of 2 mg/ml. By using a brain-specific promoter, and providing or withdrawing the doxycyline/tetracycline, we can control the expression of the transgene (antisense NCOAT or GK-NCOAT) temporally and spatially. This tetracycline system is therefore ideal for the study of the relationship between proteasome and protein aggregates. With this system, we can test the hypothesis that any inhibitory perturbation to the proteasome, even temporarily, should be perpetuated because of the positive feedback loop by which protein like Aβ accumulation continues to inhibit proteasomes. Since the tet-on system can be reversibly activated by the administration and then removal of doxycycline/tetracycline, the temporal requirements of the proteasome inhibition can be tested.

The antisense NCOAT will be used as a transgene to block endogenous wild type NCOAT and induce cellular *O*-GlcNAc modification accumulation. However, antisense NCOAT will block both *O*-GlcNAcase activity and histone acetyltransferase activity. Thus, the splice variant GK-NCOAT will be considered as the other transgene candidate to block endogenous *O*-GlcNAcase activity, specifically because this splice variant only losses its *O*-GlcNAcase activity by lacking of exon 8 and retains complete HAT activity. GK-NCOAT can also form a complex with OGT like wild type NCOAT does, but this complex is dysfunctional because of the lack of *O*-GlcNAcase activity (unpublished data). Before we develop the transgenic mouse model with impaired *O*-GlcNAc removal as proposed above, studies need to be done on GK-NCOAT to test this potential "dominant negative" idea.

Objective of Dissertation

The hexosamine biosynthesis pathway produces UDP-GlcNAc as the donor substrate of *O*-GlcNAc modification. GFAT is the rate-limiting enzyme of this pathway. In humans and mice, there are two isoforms of GFAT: GFAT1 and GFAT2. Many studies have been done on GFAT1. Compared with GFAT1, GFAT2 remains little understood. It has been called GFAT2 based only on their sequence homology, although it was even unknown whether it has the fructose-6-phosphate: glutamine amidotransferase activity like GFAT1 does. These two enzymes have high homology at the amino acid level, but they are still encoded by different genes located on two chromosomes and have quite different tissue distribution. In the studies presented in this dissertation, to characterize this new enzyme GFAT2, the mouse GFAT2 will be expressed in mammalian cells with the

use of recombinant vaccinia virus. To simplify the purification of expressed mGFAT2, His-tagged or GST-tagged mouse GFAT2 fusion proteins were designed. With beads purified mouse GFAT2, the enzyme activity was tested. We also found that in mouse GFAT2 there is the conserved serine (serine 202 of mouse GFAT2). This serine can be phosphorylated as seen in GFAT1, which regulates GFAT1's enzyme activity. So the regulation of mouse GFAT2 enzyme activity by phosphorylation by cAMP dependent protein kinase (PKA) was checked.

In the second part of the studies presented in this dissertation, the *in vivo* role of newly cloned splice variant GK-NCOAT was investigated. GK-NCOAT is a splice variant of NCOAT that naturally occurs in a diabetic animal model, the Goto-Kakizaki rat. Previous studies have shown that GK-NCOAT lacks the exon 8 of the full length NCOAT; which overlaps part of the *O*-GlcNAcase domain of NCOAT. In *in vitro* assay, mammalian cells expressing GK-NCOAT have no GlcNAcase activity but still retain HAT activity. In addition, GK-NCOAT can also form a complex with OGT that is just like the OGT: NCOAT co-repression complex that has been proved to play an important role in gene transcriptional regulation (unpublished data from J. E. Kudlow's lab). On the base of these observations, we want to test whether overexpressing splice variant GK-NCOAT will block the wild type *O*-GlcNAcase activity as a dominant negative. This is the pioneer study for future *O*-GlcNAc research in the transgenic animal models mentioned above. If overexpressed GK-NCOAT can perform the dominant negative role in vivo to block endogenous *O*-GlcNAcase function, it could be used as the transgene being expressed specifically in the brain (or pancreas ß cells) for the further studies of *O*-

GlcNAc in vivo. At the same time, the study on the cellular function of GK-NCOAT may help to uncover the mechanism for disease development in the Goto-Kakizaki rat.

PHOSPHORYLATION OF MOUSE GLUTAMINE-FRUCTOSE-6-PHOSPHATE AMIDOTRANSFERASE 2 (GFAT2) BY CAMP-DEPENDENT PROTEIN KINASE INCREASES THE ENZYME ACTIVITY

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Abstract

A protein encoded by a new gene with approximately 75% homology to glutamine-fructose-6-phosphate amidotransferase (GFAT) was termed GFAT2 on the basis of this similarity. The mouse GFAT2 cDNA was cloned, and the protein was expressed with either an N-terminal glutathione *S*-transferase or His tag. The purified protein expressed in mammalian cells had GFAT activity. The K_m values for the two substrates of reaction, fructose 6-phosphate and glutamine, were determined to be 0.8 mM for fructose 6-phosphate and 1.2 mM for glutamine, which are within the ranges determined for GFAT1. The protein sequence around the serine 202 of GFAT2 was conserved to the serine 205 of GFAT1, whereas the serine at 235 in GFAT1 was not present in GFAT2. Previously we showed that phosphorylation of serine 205 in GFAT1 by the catalytic subunit of cAMP-dependent protein kinase (PKA) inhibits its activity. Like GFAT1, GFAT2 was phosphorylated by PKA, but GFAT2 activity increased approximately 2.2-fold by this modification. When serine 202 of GFAT2 was mutated to an alanine, the enzyme not only became resistant to phosphorylation, but also the increase in activity in response to PKA also was blocked. These results indicated that the phosphorylation of serine 202 was necessary and sufficient for these alterations by PKA. GFAT2 was modestly inhibited (15%) by UDP-GlcNAc but not through detectable *O*-glycosylation. GFAT2 is, therefore, an isoenzyme of GFAT1, but its regulation by cAMP is the opposite, allowing differential regulation of the hexosamine pathway in specialized tissues.

Introduction

The hexosamine biosynthetic pathway, a universally conserved pathway in all life forms, metabolizes fructose 6-phosphate in conjunction with glutamine and acetate to

UDP-*N*-acetylhexosamine as its main end product. These UDP-*N*-acetylhexosamines are used mainly for the synthesis of exported proteins that are modified by complex carbohydrate side chains prior to their exit from the interior of the cell. In addition, UDP-*N*-acetylhexosamines also serve as essential substrates for the synthesis of the glycosyl side chain of glycolipids, and the ratio of UDP-*N*-acetylglucosamine (UDP-GlcNAc) to UDP-*N*-acetylgalactosamine usually is 3:1 (1, 2). UDP-GlcNAc is an active precursor of chitin that is polymerized by bacterial, fungal, and insect cells to form the major component of the wall and insect cuticle.

In higher eukaryotes, part of the UDP-GlcNAc is used by the enzyme *O*-GlcNAc transferase (OGT)[1] to modify nucleocytoplasmic proteins by the addition of the monosaccharide GlcNAc to the hydroxyl groups on serine or threonine residues of proteins. This *O*-GlcNAc modification can be removed from proteins by the enzyme *O*-GlcNAcase. Important observations have been linked to this modification of proteins by OGT. Overexpression of OGT causes insulin resistance in adipocytes (3, 4), whereas inhibition of the counteracting enzyme *O*-GlcNAcase is associated with pancreatic β cell and brain hippocampal neuron apoptosis (5, 6) and a hormone secretory defect (7). Although the modification has been found in 80 or more proteins, many of which are transcription factors, only recently has there been concrete evidence for a direct role of the *O*-GlcNAc modification in the control of protein functions (8). The enzyme OGT has been localized to gene-targeted co-repression complexes (9), and the modification of the transcription factor Sp1 has been shown to repress the transcriptional activation capacity of OGT (10). The 26 S proteasome is also regulated reversibly by this modification: OGT inhibits the function of proteasomes, whereas *O*-GlcNAcase activates the organelle (11). Because the proteasome controls the abundance of many regulatory proteins, including proapoptotic

and developmental factors, and gene co-repression similarly determines cellular outcome, the hexosamine pathway, with its ability to sample the metabolism of sugars (glucose backbone of GlcNAc), amino acids (nitrogen in GlcNAc), and lipids (acetate in GlcNAc), could provide a metabolic input into vital cell processes. Indeed, an absence of the OGT gene on the X chromosome is lethal (12).

The rate-limiting step in glucosamine synthesis is catalyzed by the enzyme glutamine-fructose-6-phosphate amidotransferase (GFAT). The flux of glucose into the hexosamine pathway can therefore be controlled by the activity of the GFAT enzyme. GFAT activity has been detected in almost every organism and tissue. The enzyme is composed of two domains: a glutaminase domain, which catalyzes the hydrolysis of glutamine to glutamate and ammonia, and a synthase domain, which catalyzes amination and isomerization of fructose 6-phosphate to glucosamine 6-phosphate (13). The *Escherichia coli* GFAT has been crystallized, and the structure of the enzyme has been elucidated (14).

Because the activity of GFAT controls the entry of glucose into the hexosamine pathway, the control of GFAT activity might have implications for these and other regulatory processes. Previously, our laboratory expressed and purified recombinant GST-GFAT in a mammalian expression system and showed that the phosphorylation of serine 205 of the GFAT protein by cAMP-dependent protein kinase (PKA) inhibited the activity of the enzyme. Such inhibition of GFAT would cause the preferential metabolism of glucose to ATP rather than glucosamine in pancreatic βcells, perhaps causing more ATP-stimulated insulin secretion (15) for a given glucose challenge. Because incretins such as Glp1, which are made in the gastrointestinal tract in response to food, increase cAMP in

the βcell (16), part of their mechanism of action might result from this shift in the metabolism of glucose.

When GFAT was purified from liver and subjected to phosphorylation by PKA, however, another investigative group (17) found that the enzyme activity was increased by approximately 1.7-fold. At approximately the same time of that study, a gene encoding a protein with approximately 75% homology to GFAT was cloned. The gene product was named GFAT2 on the basis of this homology but not on the basis of activity. Northern blot analysis revealed different tissue distribution between GFAT1 and GFAT2. GFAT1 was more highly expressed in the pancreas, placenta, and testis than GFAT2, whereas GFAT2 was expressed throughout the central nervous system, especially in the spinal cord. The GFAT2 locus was mapped to human chromosome 5q and mouse chromosome 11 (18).

To determine whether GFAT2 indeed has GFAT enzyme activity and the effect of PKA phosphorylation on the enzyme activity, GFAT2 was cloned, expressed, and purified. Our studies showed that GFAT2 has GFAT enzyme activity. It is phosphorylated at a site similar to GFAT1, but instead of inhibiting its activity on phosphorylation, the modification accentuated the enzyme activity. These isozymes of GFAT would allow different tissues under hormonal stimulation to metabolize glucose differently.

Experimental Procedures

Materials

Protein kinase A catalytic subunit, protein phosphatase catalytic subunit, fructose 6-phosphate, 5-bromo-2'-deoxyuridine, L-glutamine, glutamic acid, 3-acetylpyridine adenine dinucleotide, mycophenolic acid, thrombin, protein kinase A inhibitor, protease in-

hibitors mixture, monoclonal anti-GST tag antibody, and trifluoroacetic acid were purchased from Sigma. Monoclonal anti-His_6 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). [γ-^{32}P]ATP was purchased from PerkinElmer Life Sciences. Glutamate dehydrogenase and ATP were purchased from Roche Applied Science. Glutathione-Sepharose 4B was purchased from Amersham Biosciences.

Cell Culture

BSC40 cells were grown in Dulbecco's modified Eagle's medium with 10% newborn calf serum (Invitrogen), 100 μg/ml penicillin, and 50 μg/ml gentamicin at 37 °C in a humidified incubator with 7.5% CO_2.

Cloning and Expression of Recombinant Mouse GST-GFAT2

The 5' end 769 base pairs of the mouse GFAT2 cDNA (accession number NM_013529 [GenBank]) were amplified by PCR using oligonucleotides with the sequence 5'-CATCGGTACCGAATTCTGCGGAATCTTTGCCTAC-3' and 5'-TTCTATGATGGCACTTGC-3'. The product was inserted into the EcoRI site of pBS and sequenced. The 1280 base pairs of 3' end of mouse GFAT2 were amplified by PCR using the oligonucleotides 5'-CGCTGTGGGCGATAAAGC-3' and 5'-CGAGGCCTCGAGTCATTCCACAGTGACAGAC-3'. This portion of mGFAT2 was inserted between the XhoI and EcoRI sites of pTMGST and then sequenced. The 5' end of mGFAT2 was then inserted into the EcoRI site upstream of the remainder in the correct direction to create the intact sequence in pTMGST. This construct was used to express the N-terminal GST-tagged mGFAT. The full-length GFAT2 was also cloned into pCDNA3.0HisC (Invitrogen) between EcoRI and XhoI, and then the fusion fragment en-

coding the His tag and mGFAT2 sequence was cloned into pTM3 between the NcoI and XhoI sites. This construct was used to express the N-terminal His-tagged mGFAT2 fusion protein.

Mutagenesis Study of mGFAT2

The potential PKA phosphorylation site (serine 202) was mutated to alanine by PCR using the oligonucleotides 5'-CGAGGAGAGGCGCCCCCTTGCTCATC-3' and 5'-GATGAGCAAGGGGGCGCCTCTCCTCG-3'. The mutated segment was cloned into mGFAT2 and sequenced to confirm placement of the mutation.

Vaccinia Virus Expression and Purification of Recombinant Fusion Proteins

Procedures for the generation of recombinant GST-mGFAT2 vaccinia virus were as described using both mycophenolic acid and bromodeoxyuridine selections (19, 20). The vaccinia virus system was a kind gift from Dr. B. Moss (19). To express GST-mGFAT2, the BSC40 cells were infected with recombinant GST-mGFAT2 virus and VTF7-3 virus, which expresses T7 polymerase in infected mammalian cells; T7 polymerase can bind to the promoter of GST-mGFAT2, resulting in the overexpression of GST-mGFAT2 proteins. After 24 h of infection, the cells were collected and lysed in the extraction buffer containing 20 mM Tris (pH 7.5), 0.5 M NaCl, 0.5% Nonidet P-40, 5 mM $MgCl_2$, 5mM EDTA, 20% glycerol, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 2 mM fructose 6-phosphate, and protease inhibitor mixture. After standing on ice for 20–30 min, the supernatant was collected by centrifugation, and the GST-mGFAT2 was purified by incubation with a 50% slurry of glutathione-Sepharose beads at 4 °C for 30–60 min. The beads were collected by centrifugation and washed

three times with the extraction buffer. The fusion protein bound to the glutathione beads was eluted with 20 mM reduced glutathione. To express the His-tagged mGFAT2, the plasmid pTM3His-mGFAT2 was transfected into BSC40 cells by electroporation at 250 V and 500 microfarads. After overnight recovery, the cells were infected with recombinant vaccinia virus VTF7-3. After incubating for 24 h, the cells were lysed in the extraction buffer (without EDTA). The N-terminal His-tagged mGFAT2 was purified with His-tagged beads as directed by the manufacturer (Novagen).

Enzyme Assay of mGFAT2

The enzyme activity was determined by a spectrophotometric method (21, 22) in a 1-ml standard assay as described previously. The protein concentration was determined by SDS-PAGE. A blank calibration control consisted of the entire reaction mixture with the same volume of GST- or His-tagged bead elution buffer.

Phosphorylation of mGFAT2

The purified GST-mGFAT2 and His-mGFAT2 fusion proteins (0.1 μg) were phosphorylated *in vitro* by the catalytic subunit of PKA in phosphorylation buffer containing 50 mM Tris-Cl (pH 7.5), 100 μM ATP, 10 mM$MgCl_2$, 5 mM dithiothreitol at 30 °C for 20 min. After the phosphorylation reaction, the GFAT activity was measured. Fructose 6-phosphate and glutamine were added to final concentrations of 10 mM and incubated at 37 °C for 1 h. The reactions were stopped by boiling for 2 min. After the temperature was lowered by sitting the reaction mixture on ice for 3 min, the supernatants were collected by centrifugation at 14,000 rpm for 5 min. Glutamate dehydrogenase (20 units) and 3-acetylpyridine adenine dinucleotide at a final concentration of 0.5 mM were

added in an enzyme activity assay buffer to a final volume of 1 ml. After incubation at 30 °C for 30 min, the A_{365} was determined.

[γ-^{32}P]ATP Labeling of mGFAT2

The GST-mGFAT2 or mutant GST-mGFAT2 (0.5 μg) was incubated with or without 100 units of the catalytic subunit of PKA in the phosphorylation buffer described above. Unlabeled ATP at a final concentration of 0.1 mM and 1 μlof[γ^{32}P]ATP (10 mCi/ml) were added to the reaction. After incubation for 20 min at 30 °C, the GST beads were added into the reaction mixture and incubated at room temperature for 10 min with gentle shaking, and then the beads were spun down and washed three times with phosphorylation buffer. The sample with beads was then boiled, and the protein was separated by 8% SDS-PAGE followed by autoradiography of the dried gel.

O-GlcNAc Modification of mGFAT2

Prior to the *O*-GlcNAc modification assay, the purified GST-mGFAT2 still bound to glutathione beads was treated with protein phosphatase catalytic subunit at 30 °C for 20 min in the phosphatase reaction buffer (25 mM imidazole (pH 7.4), 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 50 mM NaCl). After the reaction, the beads were spun down and washed, and the GST-mGFAT2 protein was eluted with 20 mM reduced glutathione. With UDP-GlcNAc at a final concentration of 5 μM, the eluted GST-mGFAT2 was mixed with the purified GST-OGT, which was expressed in BSC40 cells using the same recombinant vaccinia virus expression system as mGFAT2 (11). The activity of the purified GST-OGT was confirmed as described (10). After incubation at room temperature for 1 h, the reaction mixtures were loaded onto an 8% SDS polyacrylamide gel. The

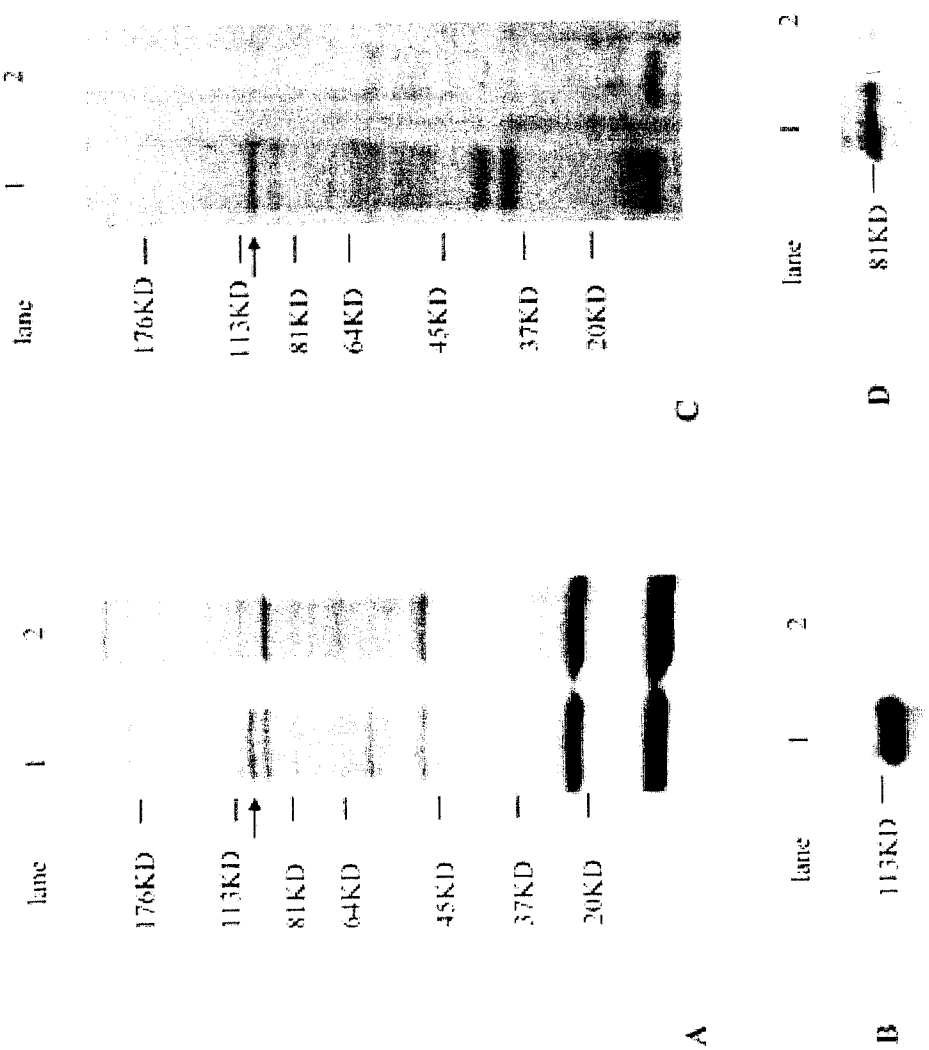
O-GlcNAc-acylated proteins were detected with RL2 antibody (23, 24) by Western blot, and the same blot was stripped and reblotted by anti-GST tag antibody to detect the GST-tagged proteins.

Results

Expression of GST-mGFAT2 and His-mGFAT2 in BSC40 Cells

The GST-mGFAT2 was expressed in BSC40 cells coinfected with VTF7-3 virus and the recombinant vaccinia virus that encodes GST-mGFAT2. The control cell lysate from the cells infected only with VTF7-3 virus showed many bands (Fig. 1*A, lane 2*). When the cells were infected with VTF7-3 virus and the GST-mGFAT2 recombinant virus, a new band appeared at approximately 110 kDa (Fig. 1*A, lane 1*). Western blot analysis of the lysates with an anti-GST tag antibody showed that the 110-kDa band of the cell lysate programmed for expression of the recombinant GST-mGFAT2 was a GST-reactive band (Fig. 1*B, lane 1*). The fusion protein was purified on glutathione beads. The eluted GST-mGFAT2, seen by Coomassie Blue staining after purification on GST beads (Fig. 1*C, lane 1*), was used in the enzyme activity and phosphorylation assays. Similar purification and elution steps were performed on the control lysate, yielding a few bands of lower intensity background (Fig. 1*C, lane 2*). The cells were also programmed to express His-mGFAT2, in which the six histidines were substituted for the GST on the N terminus of mGFAT2. Compared with the control lysate, which was from cells that were transfected with vector pTM3 and infected with VTF7-3 virus (Fig. 1*D, lane 2*), the lysate of the cells transfected with pTM3His-mGFAT2 contained a new band, which was visible on analysis by Western blot, with anti-His-tag antibody. The band was at 81 kDa (Fig. 1*D, lane 1*) as predicted from the size of the His-mGFAT2 cDNA. The nickel affinity-

Figure 1. Expression of recombinant mouse GFAT2 in mammalian cells. GST-mGFAT2 is approximately 110 kDa, and His-mGFAT2 is approximately 80 kDa. *A*, the proteins in the cell lysate were separated by 10% SDS-PAGE stained with Coomassie Blue. *Lane 1*, cell lysate proteins from cells infected with VTF7-3 virus and GST-mGFAT2 recombinant virus; *lane 2*, control cell lysate proteins from cells infected with VTF7-3 virus only. *B*, the same cell lysates as in *A* were analyzed by Western blot with anti-GST tag antibody. *C*, GST-mGFAT2 was purified on glutathione beads, and purified protein was eluted with 20 mM reduced glutathione. *Lane 1* shows the starting material was cell lysate from cells infected by both GST-mGFAT and VTF7-3 vaccinia viruses. The GST-mGFAT fusion protein was purified on glutathione beads followed by separation of the protein by 10% SDS-PAGE and staining with Coomassie Blue. *Lane 2* shows the starting material was cell lysate from cells infected with only VTF7-3 vaccinia virus. Purification and analysis was the same as *lane 1*. *D*, to express His-mGFAT2, BSC40 cells were transfected with plasmid pTM3His-mGFAT2 (*D, lane 1*) or vector control pTM3 (*D, lane 2*) following infection by VTF7-3 virus. The cell lysates were analyzed by Western blot with anti-His tag antibody.



purified protein at the same molecular weight was barely visible on a Coomassie Blue-stained gel (data not shown).

Kinetics of mGFAT2 Enzymatic Activity

A spectrophotometric assay was used to determine whether the purified GST-mGFAT2 fusion protein showed GFAT activity. The concentration of both substrates, fructose 6-phosphate (Fig. 2*A*) and glutamine (Fig. 2*B*), was varied while the other substrate was kept at 10 mM (saturation concentration) and the initial rates of the reactions were determined. When the reciprocal of the rate of the reaction was plotted against the reciprocal of the substrate concentration (Fig. 2, *lower panels*), a linear plot was obtained (Lineweaver-Burke plot). From these linearized results, the K_m values were obtained for both substrates. The result showed that the purified recombinant GST-mGFAT2 did have GFAT activity like GFAT1. The K_m of GST-mGFAT2 for fructose 6-phosphate was 0.8 mM, and K_m for glutamine was 1.2 mM (Fig. 2). Previous studies have measured the kinetic characteristics of GFAT1 by different assay methods and showed the K_m values range between 0.2 and 1.56 mM for fructose 6-phosphate and 0.4 and 3.8 mM for glutamine (25, 26). The K_m values of GST-mGFAT2 for both of its two substrates measured in this study fall within these ranges.

PKA Treatment Induces an Increase of mGFAT2 Activity In Vitro

From the previous studies on hGFAT1 (human GFAT1), we found that phosphorylation of recombinant protein GST-hGFAT1 by PKA at its serine 205 blocks the enzyme activity, and serine 235, the other potential phosphorylation site of hGFAT1, is neither necessary nor sufficient for the inhibition (22). Alignment of different GFATs

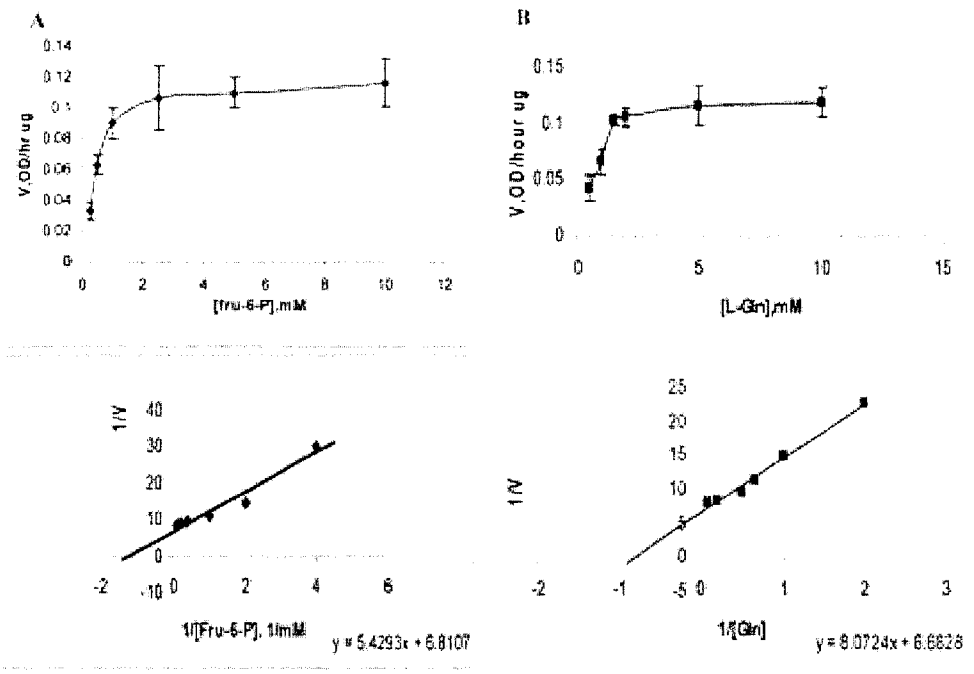


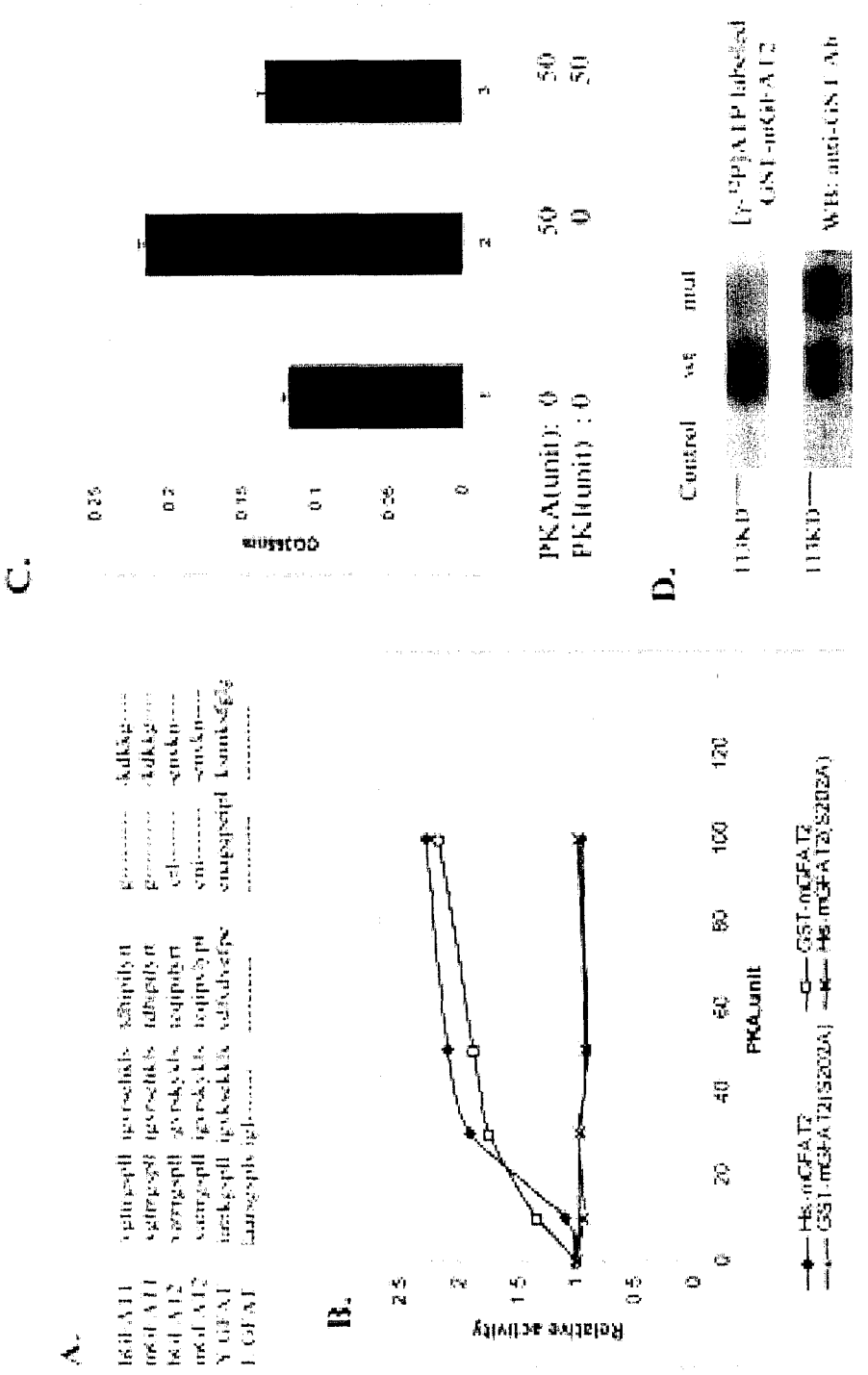
Figure 2. K_m determinations of recombinant purified GST-mGFAT2 for fructose 6-phosphate (*A*) and glutamine (*B*). The K_m values were determined by measuring the enzyme activity (initial rate of enzyme reaction) at different concentrations of one substrate while holding the other substrate at a constant and saturating concentration (10 mM) (*upper panels*). The K_m values were derived from linearized double reciprocal plots (*lower panels*). Each point represents the mean of three determinations ± S.D. The measured K_m values of purified GST-mGFAT2 for fructose 6-phosphate (*Fru-6-P*) and glutamine (*L-Gln*) were 0.8 and 1.2 mM, respectively.

shows this serine is a very conserved from *E. coli* to human (Fig. 3*A*) and that the RRGS is a potential recognition site of PKA. The counterpart of serine 235 of GFAT1 is not present in GFAT2 (Fig. 3*A*). No other potential PKA phosphorylation sites were present in either protein. The presence of only a single site in GFAT2 made analysis simpler than for GFAT1. As for GFAT1, recombinant purified mGFAT2 fusion protein was tested to determine whether it can be phosphorylated and its activity regulated by PKA. Indeed, our results showed that GST-GFAT2 can be phosphorylated by the catalytic subunit of PKA (Fig. 3*D*), but unlike recombinant GST-GFAT1 that is inhibited by this phosphorylation, both purified fusion proteins GST-mGFAT2 and His-mGFAT2 were stimulated approximately 2.2-fold by this modification (Fig. 3, *B* and *C*). When a PKA inhibitor was added into the reaction with the catalytic subunit of PKA, the activation of mGFAT2 by PKA was substantially reduced (Fig. 3*C*). According to the homology around the single PKA phosphorylation site of mGFAT2, its serine 202 was replaced by an alanine. Not only did this mutation abrogate the ability of mGFAT2 to be phosphorylated (Fig. 3*D*), it also prevented the change in activity that was observed for the wild type enzyme (Fig. 3*B*), regardless of whether the N-terminal tag was GST or His. Thus, the two isoforms of GFAT can be phosphorylated by PKA at a comparable site; yet the enzymatic activity of one is inhibited, whereas the other is simulated.

UDP-GlcNAc Partially Inhibits the Activity of Recombinant mGFAT2

It has been reported that GFAT1 can be feedback-inhibited by UDP-GlcNAc, the final product of the hexosamine biosynthetic pathway (26, 27). GFAT2 was also found to be inhibited in a dose-dependent manner by UDP-GlcNAc, but the maximal inhibition of its activity was only 15% (Fig. 4). The inhibition appears specific to the sugar because

Figure 3. Phosphorylation of recombinant mGFAT2 fusion proteins by PKA increases enzyme activity. *A*, conserved putative PKA phosphorylation site in the alignment of amino acids sequences of hGFAT1 and hGFAT2, and mGFAT1 and mGFAT2, yeast (*Saccharomyces cerevisiae*) GFAT (*Y.GFAT*) and *E. coli* GFAT (*E.GFAT*). The alignment was performed using the GCG software package licensed to the University of Alabama at Birmingham. Gaps are introduced where necessary and presented by *dashes* (—). *B*, the effect of mutations from serine 202 to alanine 202 on the relative activity of recombinant mGFAT2 purified using either the GST tag or His tag to which it was fused. For all forms of mGFAT, the dependence of the GFAT activity on the amount of PKA added was determined. Each point represents the mean of three determinations. *C*, activity of GST-mGFAT2 treated with 50 units of PKA or PKA and PKA inhibitor. The enzyme activity was measured as before after the addition of the indicated reagents. Each value represents the mean of three determinations ± S.D. *D*, PKA was able to modify GST-mGFAT2 but not its mutant. Cells were infected with either GST-mGFAT2 and VTF7-3 viruses or VTF7-3 virus alone (control). The GST-tagged protein was then purified and subjected to phosphorylation (*top panel*) by incubation with PKA and [-^{32}P]ATP. No label was incorporated into the control, but wild type (*wt*) GST-mGFAT was labeled. The mutation (*mut*) at the 202 position abrogated the labeling. The wild type and mutant samples contained the same amount of GST fusion protein, although the control sample contained no fusion protein when analyzed by Western blot (*WB*) using anti-GST antibody (*Ab*) (*lower panel*).



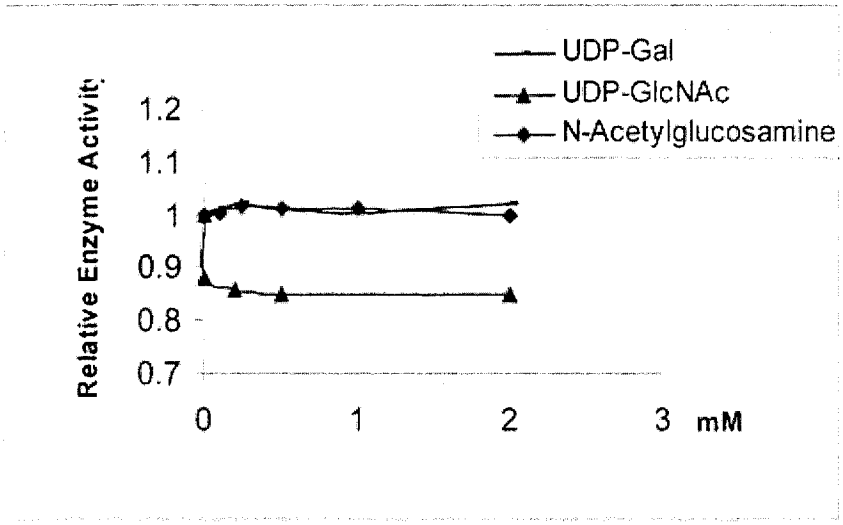


Figure 4. UDP-GlcNAc modestly inhibited recombinant GST-mGFAT2 activity. The enzyme activity was measured at different concentrations of UDP-galactose (UDP-Gal, *square*), UDP-GlcNAc (*triangle*), and *N*-acetylglucosamine (*diamond*). Each data point represents the mean value of three independent experiments.

neither UDP-galactose nor GlcNAc without a UDP moiety was able to inhibit the enzyme activity. GFAT1 appears to be more influenced by UDP-GlcNAc because its inhibition was observed to be greater at 51% (27) and 80% (26), respectively.

mGFAT2 is Probably Not O-GlcNAc-modified

One mechanism by which GFAT activity might be feedback-inhibited through increasing quantities of UDP-GlcNAc is by modification with *O*-GlcNAc. This post-translational modification is known to alter the activity of other proteins, such as Sp1, and it has been shown that OGT activity is substrate concentration-dependent. To test this idea, we attempted to modify GST-mGFAT2 with recombinant GST-OGT. Before treating GST-mGFAT2 with GST-OGT, the fusion protein, although bound to glutathione beads, was pretreated with a general phosphatase to preclude possible blockage of *O*-GlcNAc modification by a potential phosphorylation at the same site; however, no modification of GST-mGFAT2 could be detected with RL2 antibody either before or after exposure to OGT (Fig. 5). Furthermore, OGT exposure did not change GST-mGFAT2 activity (data not shown). Although it remains possible that mGFAT2 is modified by *O*-GlcNAc and that this modification is hidden from detection by the RL2 antibody, it is more likely that it is not modified by OGT. Thus, product inhibition by UDP-GlcNAc of mGFAT2 may be allosteric.

Discussion

The GFAT2 cDNA was cloned and designated as GFAT solely on the basis of sequence homology to GFAT1 (18). Here, we demonstrate for the first time that GFAT2 has GFAT activity. Thus, there are now two enzymes that can sample some of the glucose

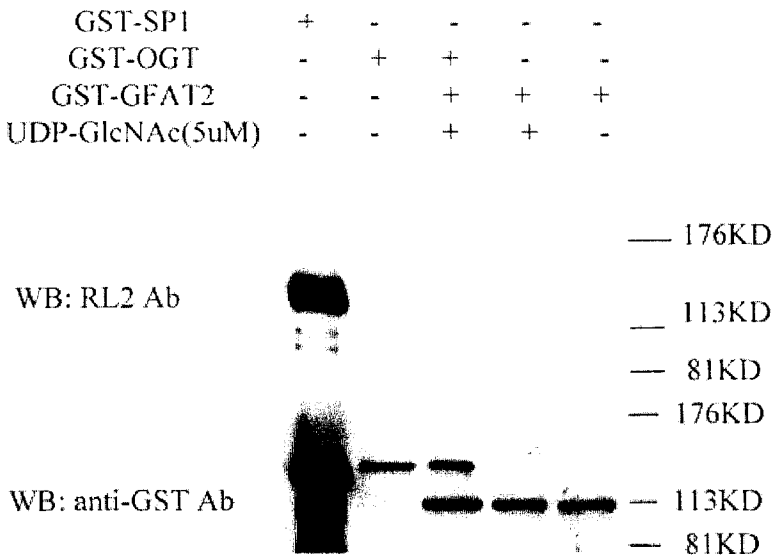


Figure 5. mGFAT2 may not be modified by *O*-GlcNAc. The RL-2 monoclonal antibody recognizes Sp1 (*lane 1*), a protein modified by *O*-GlcNAc (30). GST-mGFAT2, after treatment with phosphatase, was either untreated or treated with GST-OGT. The fusion protein was then analyzed by Western blots. Although loading was assured when the fusion proteins were analyzed with a GST antibody (*lower panel*), GST-mGFAT was not detected with the RL-2 antibody (*upper panel*) either before or after treatment with GST-OGT. The + or – indicates the reagents in the reaction.

presented into the cell and divert it through the hexosamine pathway (27). Because the tissue distribution of the two GFAT proteins differs, the enzymes may provide for the specific needs of the tissue. For example, we showed previously that GFAT1 is inhibited by phosphorylation by PKA. Antisense reduction of GFAT1 in the βcells of transgenic mice makes these cells resistant to streptozotocin apoptosis (5) and so does the incretin Glp-1 (28, 29), of which the signal to the βcell is transduced by cAMP. The isolated islets from these transgenic mice also secrete more insulin under glucose stimulation than do the islets of their littermates (7), again mimicking the effect of the incretins on insulin secretion. Conversely, an increase in the *O*-GlcNAc content of βcells inhibits insulin secretion (7). Thus, in βcells, the natural down-regulation of GFAT1 activity by cAMP or the unnatural down-regulation of the enzyme by an antisense strategy both provide less substrate for *O*-GlcNAc transferase via the hexosamine pathway and may play some role in insulin secretion.

The GFAT2 homology to GFAT1 extends to a homologous PKA phosphorylation site in GFAT2. The putative serine 202 site in GFAT2 is near the serine 205 site observed in GFAT1. Because of our elucidation of the role of cAMP in GFAT1 activity, we investigated this potential phosphorylation site and found that GFAT2 is also modified by PKA, but instead of inhibiting enzymatic activity of GFAT2, phosphorylation by this kinase stimulated its activity. Mutagenesis of the putative phosphorylation site not only abrogated the modification but also blocked the ability of PKA to stimulate the enzymatic activity of GFAT2. This ~2-fold stimulation effect of PKA was independent of the epitope tag on the N terminus because both GST-GFAT2 and His-GFAT2 behaved identically. Therefore, the potential dimerization that might occur with the GST tag and does not occur with the His tag rules out tag-induced dimerization as a cause of this effect on

GFAT2. Interestingly, when GFAT1 was His-tagged, PKA still inhibited the enzymatic activity (data not shown) (22), also ruling out the potential role of the N-terminal tag in this isozyme. Because the tissue distribution of GFAT2 differs from that of GFAT1 (18), we believe this stimulation of activity by PKA could subserve a different function in these tissues of the hexosamine pathway under cAMP stimulation.

Although the GFAT enzymes used in these experiments were recombinant and tagged, studies of the enzyme purified from rat liver (17), *Candida albicans* (30), and *Drosophila* (31) all show stimulation of GFAT activity by PKA. Because the two mammalian GFATs are homologous and similar in size, it remains possible that GFAT2 was purified, thereby accounting for the stimulation by PKA that was identical to that which we observed for GFAT2. Interestingly, the same interchange of GFATs may have occurred in *Drosophila*, which also appears to have two isozymes. The only recombinant GFAT tested was highly expressed in the chitin-synthesizing organs, and it is stimulated by PKA. A careful examination shows that the tested GFAT is more similar to GFAT2 than GFAT1, despite the title of the report (31). Studies on the other *Drosophila* GFAT would be helpful. The GFAT in fungus, which synthesizes chitin, unlike that in mammals, is also stimulated by PKA. The evolution of an isozyme that is inhibited by PKA must have come approximately in multicellular organisms in which the diversification of the tissues requires differential regulation of the hexosamine pathway by cAMP. Studies on how homologous enzymes containing homologous phosphorylation sites can have opposite regulation could provide insight into the regulatory mechanisms of GFAT.

Although the GFAT proteins differed with respect to the effects of PKA phosphorylation, they were much more similar with respect to product inhibition. Both enzymes were inhibited by UDP-GlcNAc, although the inhibition was partial and appeared

greater for GFAT1 (27) than for GFAT2. Modification of GFAT2 by *O*-GlcNAc was not detected with the RL-2 antibody, suggesting that the inhibition by UDP-GlcNAc resulted from an allosteric change in GFAT2. This product inhibition and the inhibition of GFAT1 but not GFAT2 by cAMP provide strong caveats for the use of GFAT1 in transgenic animals. For example, overexpression of GFAT1 in the βcells of transgenic mice may result in increased flux through the hexosamine pathway of isolated βcells. When these cells are *in vivo*, however, where modulation of cAMP by incretins like Glp-1 might occur, GFAT1 might be inhibited during meals at a time when both incretins and product inhibition turn off the presumptive conversion of glucose to glucosamine. The finding of a GFAT isoform that is oppositely regulated by cAMP and more modestly regulated by its product, UDP-GlcNAc, will provide the opportunity to create transgenes that much more effectively alter the hexosamine flux in these and other cells.

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OVEREXPRESSION OF THE DOMINANT NEGATIVE SPLICE VARIANT OF NUCLEAR AND CYTOPLASMIC *O*-GlcNAcase AND ACETYLTRANSFERASE (NCOAT): GK-NCOAT RESULTS IN CELL APOPTOSIS

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Format adapted for dissertation

Abstract

The addition of *O*-linked ß-*N*-acetylglucosamine (*O*-GlcNAc) to serine and threonine residues is a post-translational modification of nucleocytoplasmic proteins. This modification plays important roles in regulating gene transcription as well as proteasome activity. OGT and NCOAT are responsible for the addition and removal of this modification from proteins. We cloned a splice variant of NCOAT from the GK (Goto-Kakizaki) rat, a model animal for diabetes. This variant deletes amino acids 250 to 345, resulting in a 90 kDaprotein with a removal of exon 8 from the wild type form. *In vitro* experiments show that this splice variant (GK-NCOAT) loses the *O*-GlcNAcase activity but retain the histone acetyltransferase (HAT) activity. In this study, we analyzed the role of GK-NCOAT *in vivo* by transfecting it into mammalian cells. We found that overexpression of GK-NCOAT results in an increase in the cellular *O*-GlcNAc modification level, decreased proteasome activity, and cell apoptosis. Our results suggest that GK-NCOAT may act as a dominant negative to the wild type NCOAT O-GlcNAcase, blocking its function. Since the *O*-GlcNAcase is required to offset the action of OGT, which also mediates the inhibition of the proteasome, cell death may result from the blockage of *O*-GlcNAc metabolism and proteasome inactivation. These results are consistent with our previous observations that the blockage of O-GlcNAc removal with the chemical streptozotocin (STZ) results in pancreatic ß cell and neuronal cell apoptosis.

Introduction

O-linked ß-*N*-acetylglucosamine (*O*-GlcNAc) is an abundant and dynamic post-translational modification that occurs on serine or threonine residues of cytosolic and nuclear proteins and is analogous to phosphorylation. It has been showed that *O*-GlcNAc

modification plays an important role on gene transcription (1), translation (2), protein transportation (3), and cell signaling (4, 5). Since *O*-GlcNAc was described twenty years ago, the proteins identified as having this modification have reached over 100, including transcription factors, cytoskeletal components, metabolic enzymes, and signaling components (6, 7).

OGT and NCOAT/*O*-GlcNAcase are the enzymes responsible for the dynamic glycosylation and deglycosylation of proteins (8-11). OGT and NCOAT are necessary for survival at the single-cell level. The OGT gene maps to Xq13, a locus commonly associated with neurodegenerative diseases (12). The NCOAT gene is localized to 10q24, which maps to a locus for late onset of Alzheimer's disease (13, 14). Like OGT, NCOAT is found in all tissues examined, with the highest expression in brain, placenta, and pancreas (11). It has been shown that NCOAT processes both an *O*-GlcNAcase activity, which is responsible for the removal of *O*-GlcNAc modification on nuclear and cytosolic proteins, and an intrinsic histone acetyltransferase (HAT) activity. The domains for the O-GlcNAcase activity and HAT activity reside in the N terminus and C terminus of NCOAT, respectively (10). NCOAT can be cleaved by caspase-3, which generates a 65-kDa C-terminal fragment, but this has no effect on O-GlcNAcase activity *in vitro*. This finding may suggest a role for NCOAT in apoptosis (15). The O-GlcNAcase activity of NCOAT can be blocked by the chemical inhibitors *O*- (2-acetamido-2-deoxy-D-glucopyranosylidene) amino-*N*-phenylcarbamate (PUGNAc) and streptozotocin (STZ) (16-18).

The full-length NCOAT protein is about 130 kDa and is primarily found in the cytosolic fraction. A 75-kDa splice variant of NCOAT lacking a portion of the C terminus resides mainly in the nucleus (15,19,20). Recently in our laboratory, two new splice vari-

ants, named GK-NCOAT and SD-NCOAT, were cloned from the Goto Kakazaki (GK) rat and the Sprague-Dawley (SD) rat, respectively. These variants were expressed as proteins of 90 kDa for the GK variant due to the deletion of amino acids 250-345; and 84 kDa for the SD variant due to the deletion of amino acids 250-398. Both of these variants were found to lack hexosaminidase (*O*-GlcNAcase) activity but still have the complete HAT activity as the full-length enzyme (10).

In this study, in order to investigate the *in vivo* role of the splice variant GK-NCOAT, we transiently transfected mammalian cells with plasmids that can overexpress the GK-NCOAT protein and found that the GK-NCOAT can act as a dominant negative form of wild type NCOAT *in vivo* by blocking its *O*-GlcNAcase activity, thereby elevating cellular *O*-GlcNAc levels. We also found that proteasome activity in those cells with increased cellular *O*-GlcNAc modification, and these cells eventually underwent apoptotic cell death. This is the first time genetic methods have been used to prove that blockage of *O*-GlcNAc metabolism in vivo leads to cell apoptosis; previously, this had been shown in pancreas and brain by using STZ, a chemical inhibitor of NCOAT (17,21). These results indicate that this GK-NCOAT may be a genetic tool for further *O*-GlcNAc modification study in transgenic animals.

Experimental Procedures

Cell Culture

NIH 3T3 cells and CHO cells were grown in Dulbecco's modified Eagle's medium with 10% newborn calf serum (Invitrogen); 293 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Invitrogen). These media included 100

μg/ml penicillin and 50 μg/ml gentamicin. All cells grew at 37 °C in a humidified incubator with 7.5% CO_2.

Cloning

The vector plasmid pEGFP-C1 (Clontech) was used to construct plasmids that express green fluorescence protein (GFP) tagged fusion proteins. The pEGFP-WT-NCOAT and pEGFP-GK-NCOAT were made as described (unpublished data).

The vector plasmid pRFP was a kind gift from Dr. Chow. This vector plasmid originated from the plasmid pEGFP-C1 with a replacement of the GFP tag coding sequence in pEFGP-C1 with coding sequence of the red fluorescent tag RFP (accession number AF506027). The vector pRFP has same enzyme restriction sites and reading frame as pEGFP-C1 and can express RFP-tagged fusion proteins. Full-length NCOAT was cloned from F344 rat brain cDNA by PCR using oligo "5'-CATGGATCCTCTAGAATGGTGCAGAAGGAGAGTCAAGCG-3'"and oligo "5'-CATGGTACCTCTAGAGTCGACTCACAGGCTTCGACCAAG-3'", and the total cDNA sequence (2750 base pairs) was cloned into Bluescript KS between the BamHI and KpnI site. Full- length wild type NCOAT was cut out with XbaI and inserted into the pRFP vector plasmid at its XbaI site, and a clone with the correct direction insert was selected to express RFP-WT-NCOAT fusion proteins in mammalian cells. The plasmid pRFP-WT-NCOAT was cut with BamHI and BglII and religated to delete the restriction enzyme sites between the BamHI and BglII in the original construct. To make the plasmid pRFP-GK-NCOAT that expresses RFP-GK-NCOAT fusion proteins, the small piece of DNA sequence between the XmaI and EcoRI of wild type NCOAT in the modified

pRFP-WT-NCOAT plasmid was replaced with the sequence between these two sites of GK-NCOAT.

The plasmid pCMV-flag (Sigma) was used as a parent plasmid to make the construct that expresses flag-tagged GK-NCOAT using the CMV promoter. GK-NCOAT cDNA was amplified by PCR with the primer: "5'-CATGGATCCTCTAGAATGGTGCAGAAGGAGAGTCAAGCG-3'" and "5'-CGGGATCCTCACAGGCTTCGACCAAG-3'". The PCR product was digested with BamHI and XbaI and inserted into the vector pCMV-flag between these two sites. This plasmid was used to express flag-tagged GK-NCOAT protein in mammalian cells, and we named it pCMV-flag-GK-NCOAT. The plasmid pCMV-flag-GK-NCOAT and WT-NCOAT DNA were cut with restriction enzymes ApaI and BamHI; the resulting short fragment of GK-NCOAT was replaced by the fragment of WT-NCOAT, which made a new plasmid, named pCMV-flag-WT-NCOAT, to express WT-NCOAT.

For plasmid pcDNA-GST-P62, the first step was to insert the GST tag sequence into the parent plasmid pcDNA3.1HisC (Invitrogen) between Hind III and EcoRI sites to make a new vector plasmid, pcDNA-GST; then the P62 coding sequence was inserted into pcDNA-GST between the EcoRI and XhoI sites. All plasmids were confirmed by sequencing.

Immunostaining

Lipofectamine2000 (Invitrogen) were used to transfect NIH3T3 cells with plasmid pEGFP-C1/pRFP, pEGFP-WT-NCOAT/pRFP-WT-NCOAT, or pEGFP-GK-NCOAT/pRFP-GK-NCOAT according to the manufacturer's directions. After incubation for 40 or 64 hours after the transfection, the cells were fixed in 4% paraformaldehyde,

washed with PBS, and stained with RL2 mouse monoclonal antibody (1:100). The secondary antibody used in this experiment was Alexa Fluor 594 (red) or 488 (green) chicken anti-mouse IgG (H+L) (Molecular Probes, Eugene, Oregon, USA). The CHO cells were transfected with plasmids pRFP or pRFP-GK-NCOAT using Targefect F1 reagent (Targeting System, Santee, CA, USA) according to the manufacturer's directions. After incubation for 64 hours, the cells was fixed in 4% paraformaldehyde (pH 7.4) and stained with activated caspase-3 mouse monoclonal antibody (1:250) (Cell Signaling). The secondary antibody used in this experiment was Alexa Fluor 488 goat anti-mouse IgG (H+L) (Molecular Probe, Eugene, Oregon, USA). All cells were stained with DAPI (Sigma, St. Louis, MO, USA) before the slides was mounted.

Western Blot

293 cells were cotransfected by pEGFP-C1 or pEGFP-WT-NCOAT or pEGFP-GK-NCOAT with plasmid pcDNA-GST-P62. Forty hours later, the cells were harvested and lysed in 293 cell extraction buffer: 1% (v/v) Triton X-100, 150 mM NaCl, 10% (v/v) glycerol, 50 mM Tris-HCl (pH 8.0), 100 mM NaF, 2 mM EDTA, and protease inhibitor cocktail (Roche). After centrifugation at 14,000 rpm at 4 ^{o}C for 10 minutes, the upper layer of the cell extract was incubated with Glutathione-Sepharose beads at 4^{o}C for 60 minutes to purify the GST-P62 fusion protein. The beads were collected and washed three times with 293 cell extraction buffer. After the washing, 2×sample buffer was added to the beads, and the samples were boiled for 5 minutes. An equal volume of each sample was loaded into 8% SDS-PAGE for analysis. The mouse monoclonal antibody CTD110.6 used in this experiment was a kind gift of Dr. Marchase. Like RL2, CTD110.6 is another antibody that can recognize the O-GlcNAc modification on protein (22). The blocking

buffer used in CTD110.6 blots was 1% casein/PBS plus 0.01% Tween-20, and CTD110.6 was diluted 1:3000 in the blocking buffer for the working solution. The membrane was first blotted with CTD110.6 and then stripped and reblotted with anti-GST mouse monoclonal antibody (1:2000 diluted) (Sigma).

Terminal Deoxynucleotide Transferase-mediated dUTP Nicked-end Labeling (TUNEL) Assay

CHO cells were transfected with plasmid pRFP or pRFP-GK-NCOAT using Targefect F1 reagent. After incubation for 64 hours, the cells were collected for TUNEL assay. The TUNEL assay was performed using an *in situ* cell death detection kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's protocol, and was analyzed by fluorescence microscope.

In Vivo Proteasome Activity Assay

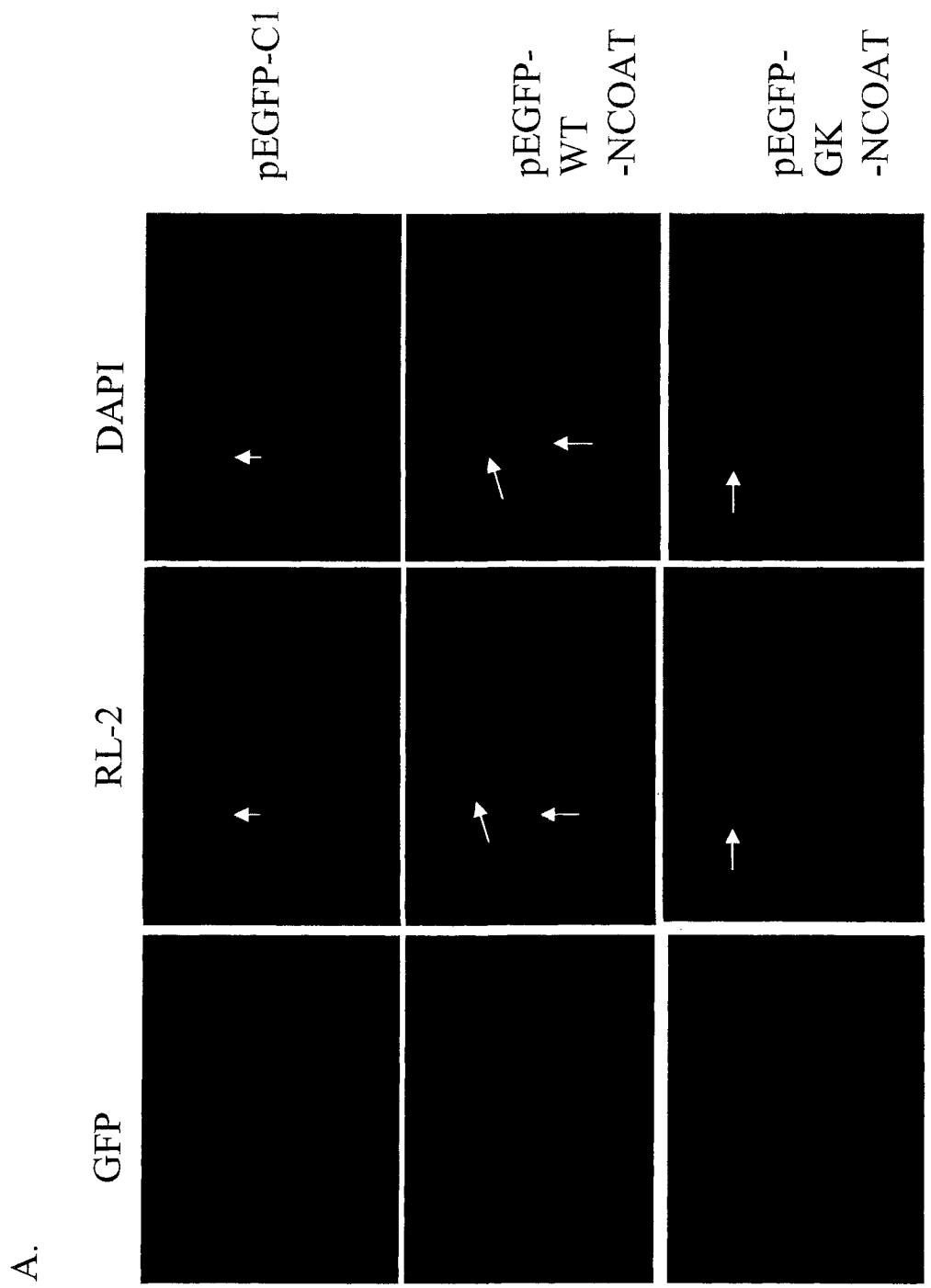
Targefect F1 reagent was used to cotransfect *in vivo* proteasome activity reporter plasmid pCMV-GFP-CL1 with the plasmids pCMV-flag, pCMV-flag-WT-NCOAT, or pCMV-flag-GK-NCOAT into 293 cells along with the plasmid pCMV-β-gal to check the transfection efficiency using β-gal assay. Forty hours later, the cells were collected and lysed in 293 cell extraction buffer. After centrifugation at 14,000 rpm at 4°C for 10 minutes, Fluorescence (excitation 470 nm, emission 535 nm) of the upper layer of the cell extract (900 μl) was measured using a Turner Qantech Digital Fluorometer (Barnstead International).

Results

GK-NCOAT is a Dominant Negative Form of Wild Type NCOAT in Vivo

GK-NCOAT is an alternative splice form of the wild type NCOAT. It was cloned from the brain of Goto-Kakazaki rat, so we named the protein as GK-NCOAT. It is an approximately 90 kDa protein that lacks the amino acids 250-345 of the wild type NCOAT that correspond to exon 8. *In vitro* experiments showed that NCOAT processes both *O*-GlcNAcase activity and histone acetyltransferase (HAT) activity. GK-NCOAT still has complete HAT activity, but loses *O*-GlcNAcase activity (10). In order to investigate the in vivo role of this RNA splice variant, we made plasmids that can express, under the CMV promoter, wild type and GK-NCOAT protein fused with a GFP tag on their N terminus. We transiently transfected these plasmids into NIH3T3 cells to overexpress these fusion proteins and performed the immunofluorescence staining with RL2 antibody, which can specifically detect *O*-GlcNAc modified proteins (23,24). We found that the cells transfected with vector control plasmid pEGFP-C1 had no difference in RL2 staining compared to the nontransfected cells in the same plates (Fig. 1*A, top panels*); the cells transfected to express GFP-WT-NCOAT fusion protein showed much weaker RL2 staining signal than the surrounding nontransfected cells (Fig.1*A, middle line panels*). However, the cells transfected with the plasmid pEGFP-GK-NCOAT had elevated RL2 staining signal, particularly in the nucleus, compared with the nontransfected cells on the same plate (Fig. 1*A*, *bottom panels*). There was an obvious elevation of the *O*-GlcNAc modification level, especially in the nucleus, which might be because the nuclear pore complex proteins like NUP62, POM121, and NUP180 are usually heavily *O*-GlcNAc modified (3) and because many transcriptional factors like Sp1, P53, and NF-κb are *O*-GlcNAcylated.

Figure 1. Overexpressing GK-NCOAT results in elevated cellular O-GlcNAc modification level. (A) Immunostaining of NIH3T3 cells with RL2 antibody. NIH3T3 Cells were transfected with lipofectmine2000 (Invitrogen), incubated for 40 hours in DMEM plus 10% newborn bovine calf serum after transfection. Then cells were fixed in 4% paraformaldehyde, washed with PBS and immunostained with RL-2 antibody. Cells with green Fluorescence are the transfected cells. RL2 staining shows the cellular O-GlcNAc modification. DAPI staining shows nucleus of the cells. Top panels are the cells transfected with pEGFP-C1, middle panels are cells transfected with pEGFP-WT-NCOAT and bottom panels are cells transfected with pEGFP-GK-NCOAT. (B) Same experiments except that cells were transfected with pRFP, pRFP-WT-NCOAT and pRFP-GK-NCOAT respectively. Arrows point to the transfected cells.



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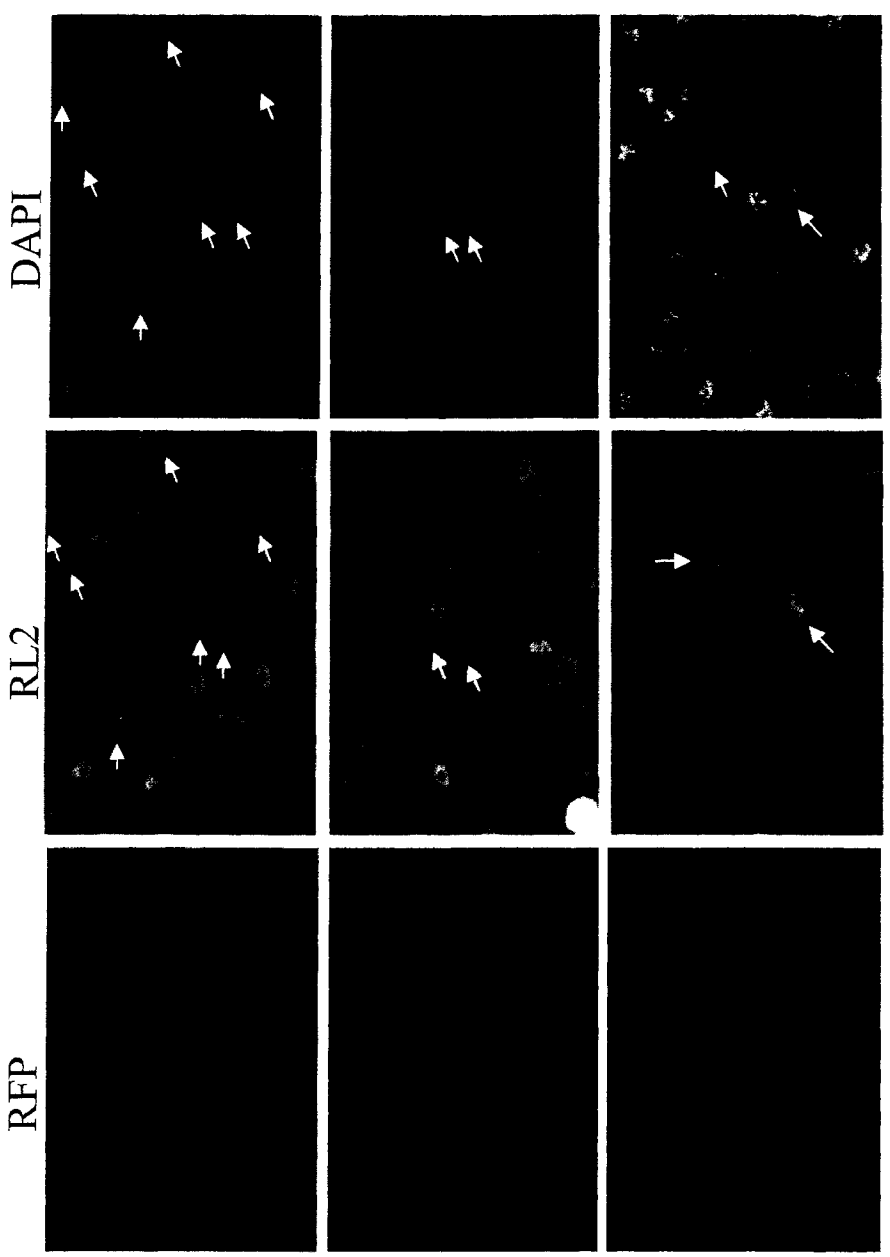

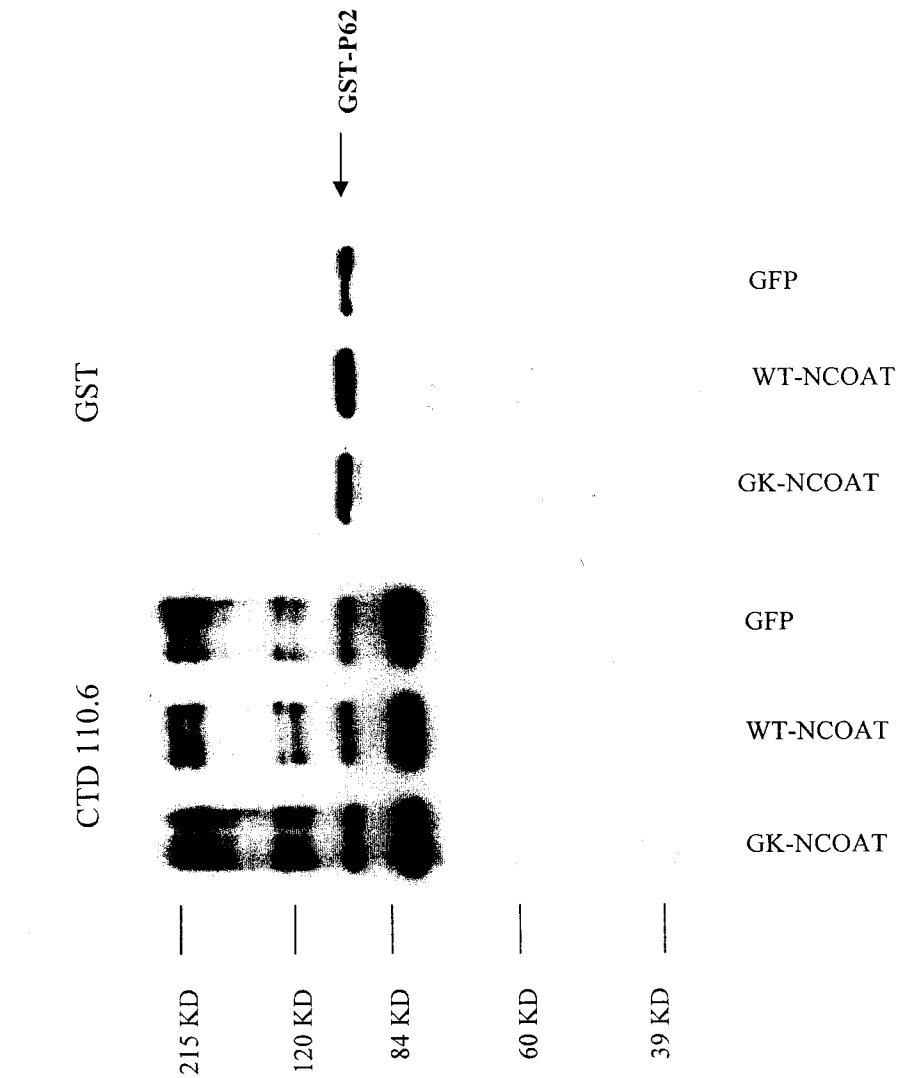

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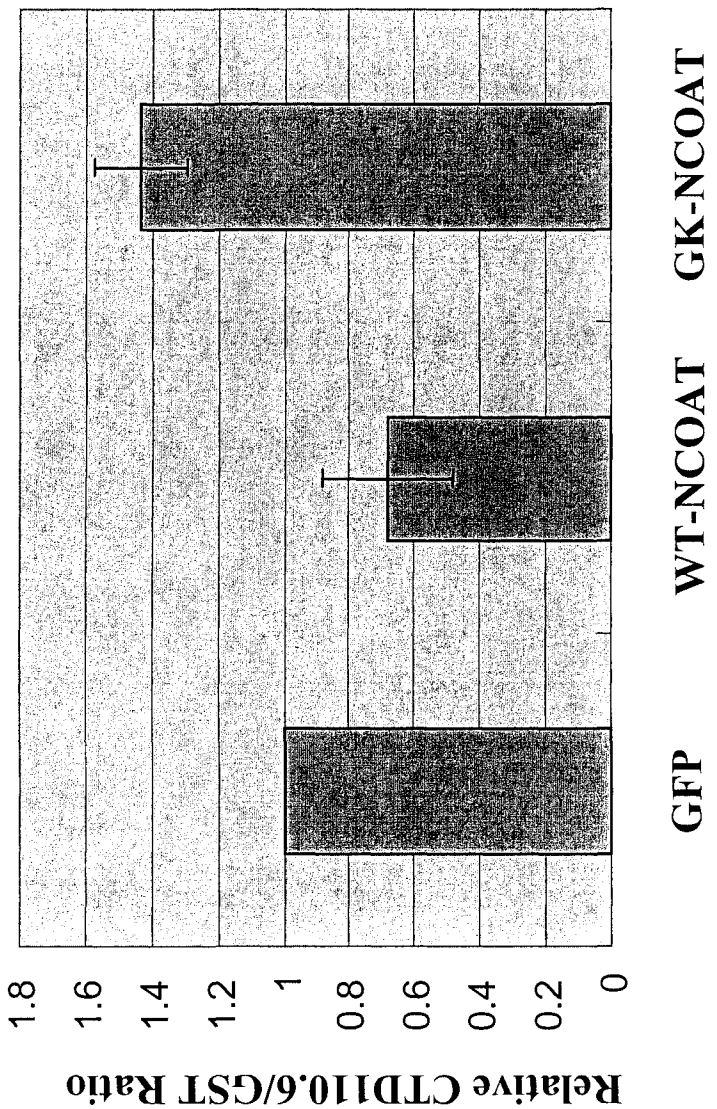


Using RFP tagged-proteins, we repeated the same experiments with similar results as showed in Fig. 1*B*.

In addition to fluorescence immunostaining, we also used western blotting to quantitate the *O*-GlcNAc levels under those conditions. Since the *O*-GlcNAc modification is an abundant post-translational modification in the cell and since transfection efficiency could not be 100% using plasmid transient transfection, we co-expressed a GST-P62 fusion protein as a reporter with GFP or GFP-NCOATs (WT or GK) in 293 cells to check these cells' O-GlcNAc level. The results are shown in Fig. 1*C*: The right panel is the CTD 110.6 blot and shows O-GlcNAc level of purified GST-P62 and the other co-purified background proteins. (The antibody CTD110.6 is more sensitive than RL2 at detecting the p62 protein's O-GlcNAc modification level.). The right panel is the anti-GST blot of the same membrane. The arrow points to the purified GST-P62 bands. The intensity of those bands was quantitated using the software ImageJ 1.33. (Download from http://rsb.info.nih.gov/ij/download.html) The CTD110.6: GST ratio of every GST-P62 band was calculated by dividing the intensity value of the band on the CTD110.6 blot by the intensity value of the corresponding band on the GST blot. The relative CTD110.6: GST ratio of every GST-P62 band on those two blots can be used to compare the O-GlcNAc modification level of GST-P62 under those three conditions. Fig. 1*D* shows the average relative CTD110.6: GST ratio from three experiments under these conditions. The results of these experiments showed that, compared with GFP control, overexpressing WT-NCOAT reduced the cellular O-GlcNAc level to about 30%, but overexpressing GK-NCOAT caused around a 40% increase of cellular O-GlcNAc levels; and by extending the incubation, these values would likely increase with the O-GlcNAc modification's accumulation.

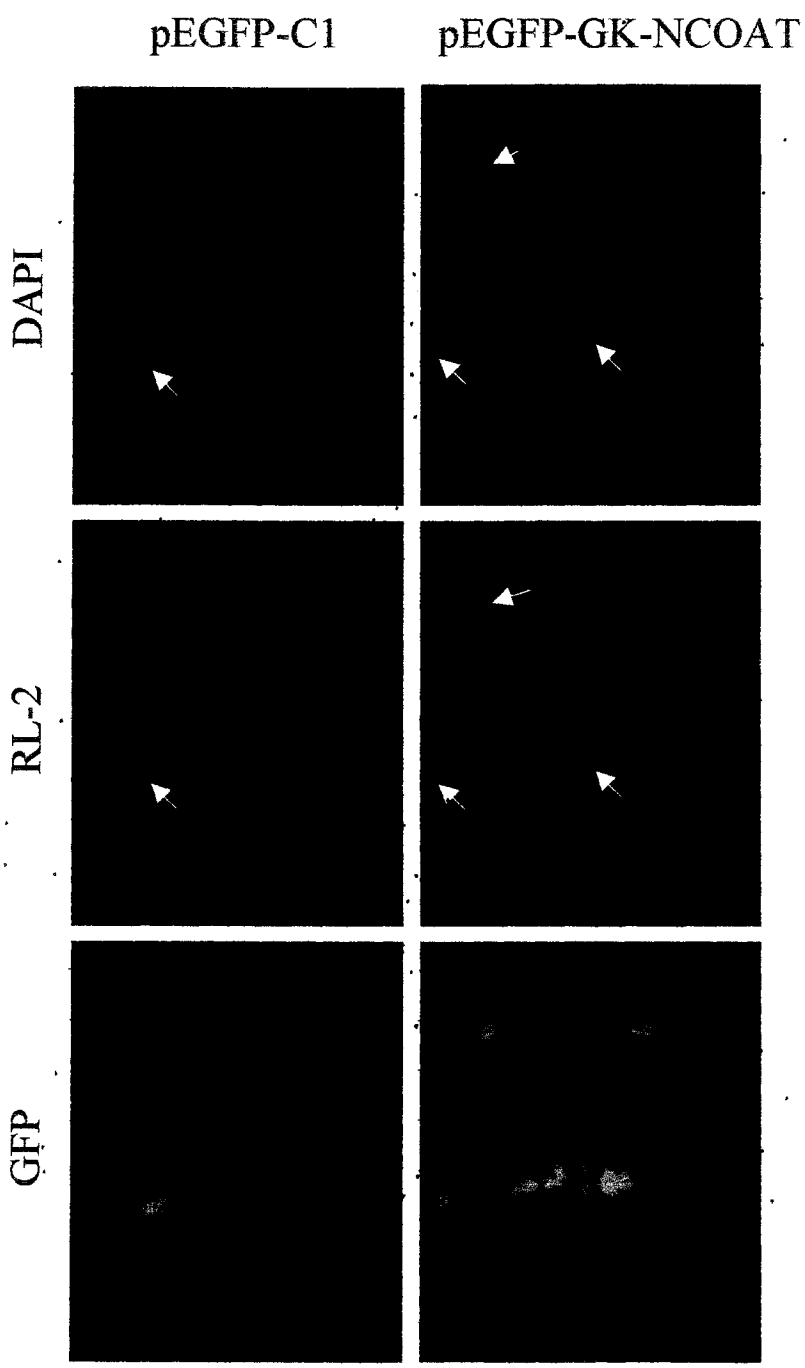
These results suggest that overexpressed GK-NCOAT, which lacks exon 8 and has no O-GlcNAcase activity but retains HAT activity, might be a dominant negative form of wild type NCOAT, blocking its *O*-GlcNAcase enzymatic activity and causing the cellular *O*-GlcNAc modification accumulation.

In addition, our laboratory recently found that NCOAT forms a stable complex with OGT in the nucleus and that this OGT/NCOAT complex sits on gene promoters to regulate gene transcription. The GK-NCOAT, although missing the exon 8, can also form this complex with OGT, but this complex is dysfunctional since the GK-NCOAT lacks the O-GlcNAcase activity (unpublished data). In addition, it has also been reported that, when native NCOAT was purified from cow brain, it was also found in a complex with about 10 other proteins, including HSP100, HSC70, and dihydropyriminidase-related protein-2 (DRP-2) (15). Therefore, *in vivo* the splice variant GK-NCOAT may also play a dominant negative role in interrupting the normal functions of these complexes.

Overexpression of GK-NCOAT Results in Cell Death

Forty hours after transfecting the plasmid-GK-NCOAT into NIH3T3 cells, we found the elevated cellular O-GlcNAc modification level as described above. When the incubation time was extended to 64 hours (one more day) after the transfection, the cells were fixed, and similar RL2 staining was performed; the results are shown in Fig. 2. The cells transfected with pEGFP-C1 (vector plasmid) had the same RL2 staining signal, morphology, and intact nuclei as the nontransfected cells in the same plate (*top panels*). However, the cells overexpressing GK-NCOAT had small and bright RL2 staining in the nuclei, and there were no clear DAPI stained nuclei, which means the nuclei of those transfected cells were condensed, shrunken and broken. These cells on the whole, were

Figure 2. Overexpressing GK-NCOAT causes cell death. NIH3T3 cells were treated and stained similarly as cells showed in Figure 1A except that extended the incubation time to 64 hours (1 more day) after the transfection. Arrows point to the transfected cells.



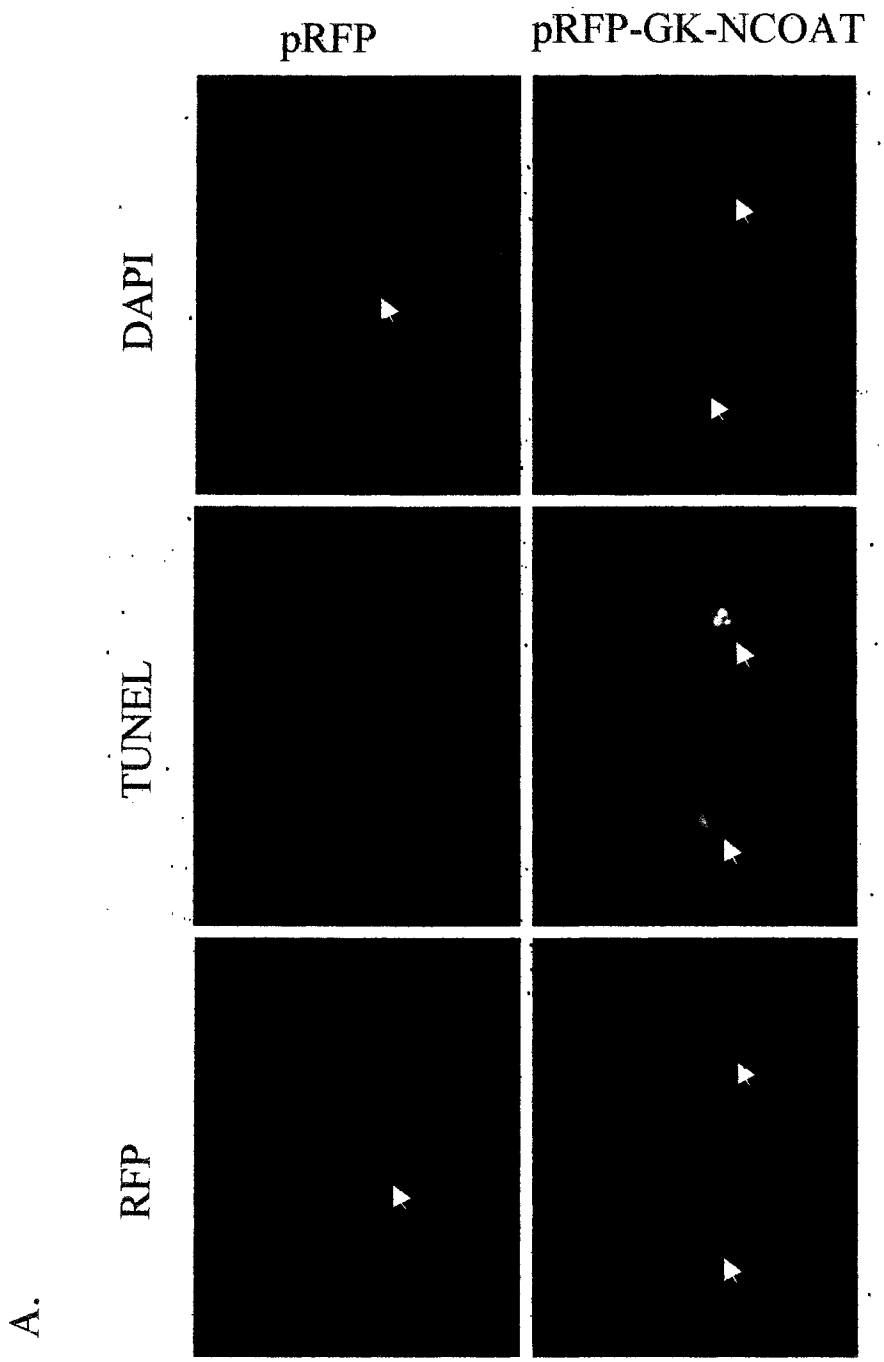
also much smaller than the surrounding nontransfected cells. Some of these cells also seemed to have broken cell membranes (Fig. 2, *bottom panels*). This staining pattern suggests that the cells overexpressing GK-NCOAT died. Those cells overexpressing WT-NCOAT had the same cell shape and intact nuclei as the nontransfected cells; however, that their RL2 staining signals were so weak that they could not be seen under the microscope (data not shown).

Under the same conditions, however, with cell serum starvation overnight after the transfection, we also observed the similar cell death of the cells transfected by pEGFP-GK-NCOAT, even at 40 hours after the transfection. The cells transfected by pEGFP-C1 were as viable as nontransfected cells (data not shown). This suggests that the absence of some nutritional factors or stress in GK-NCOAT overexpressing cells accelerated the cell death.

The Death of Cells Overexpressing GK-NCOAT is due to Apoptosis

From the observation described above, we know that overexpressing GK-NCOAT can result in cell death. In order to address if this death occurred by apoptosis, we did the TUNEL assay. Since the TUNEL assay kit (Roche) uses green fluorescence reagent, for this experiment, we made the plasmid that expresses the RFP tagged GK-NCOAT fusion protein under the CMV promoter. We transfected vector control plasmid pRFP and pRFP-GK-NCOAT into CHO cells, 64 hour later, the TUNEL assay was performed according to the manuscript. As showed in Fig. 3*A*, the cells transfected with pRFP showed no TUNEL staining and look normal in cell shape and nucleus (*top panel*). However, the cells transfected with pRFP-GK-NCOAT shows positive TUNEL staining, and it is clear that the nuclei of the cells were broken into several parts after TUNEL and DAPI staining.

Figure 3. Overexpressing GK-NCOAT results in cell apoptotic death. CHO cells were transfected with pRFP and pRFP-GK-NCOAT with Targefect F1 reagent. 64 hours later after transfection, cells were fixed for TUNEL assay analysis (3A) and activated caspase-3 immunostaining (3B). Arrows point to the transfected cells.



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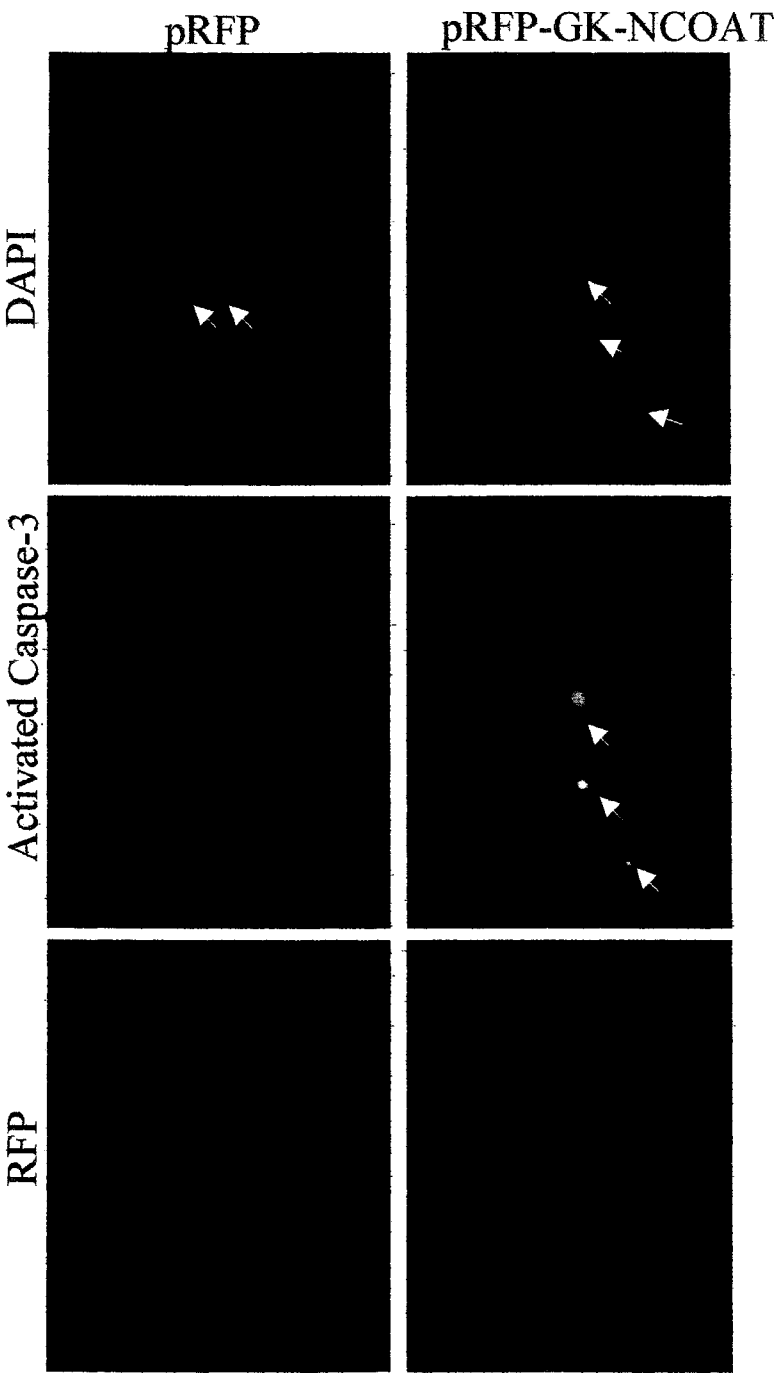

When we counted the TUNEL positive cells, we found that 2 of the 61 red cells tranfected with pRFP were TUNEL positive, and that 39 of 47 cells transfected with pRFP-GK-NCOAT were TUNEL positive, the two groups had a significant difference and $p < 0.0001$. From these results, we can conclude that overexpressing GK-NCOAT can cause cell apoptosis.

Besides the TUNEL assay, we also performed the immunostaining with activated caspase-3 antibody (Cell Signaling) on similarly treated CHO cells. This antibody only recognizes the cleaved and activated caspase-3 (17/19 kDa). The results showed that, in cells overexpressing GK-NCOAT, caspase-3 could be activated (green fluorescence in Fig. 3*B*, *the bottom panel*), and we can clearly see apoptosomes from the activated caspase-3 and DAPI staining.

Proteasome Activity is Impaired in Cells Overexpressing GK-NCOAT

It was been shown that proteasome activity can be regulated by O-GlcNAc modification on its Rpt2/S4 subunit of the 19S cap (25). Blocking the O-GlcNAc modification removal by STZ in the cerebrum causes cerebral proteasome function to decrease and ubiquitin, and p53 to accumulate, and some hippocampal cells to undergo apoptosis (21). Since overexpressed GK-NCOAT can play a dominant negative role and block O-GlcNAc removal, we wanted to know if the proteasome activity was impaired in those GK-NCOAT overexpressed cells. GFP-Degron is a fusion protein consisting of a short protein sequence named the CL1 degron fused to the C terminus of GFP. The CL1 degron specifically targets the normally stable GFP for efficient clearance by the ubiquitin proteasome system (UPS), and can be used as a reporter protein of proteasome activity in vivo (25,26). We co-transfected the plasmid pCMV-GFP-CL1 with pCMV-flag, pCMV-

flag-WT-NCOAT, or pCMV-flag-GK-NCOAT into 293 cells. Forty hours after transfection, a cell lysate was prepared to measure the green fluorescence as described in the manuscript. Compared with the relative GFP value of samples that were co-transfected with the vector control, pCMV-flag, the sample co-transfected with pCMV-flag-GK-NCOAT shows a 25% increase, and the sample co-transfected with pCMV-flag-WT-NCOAT shows a 27% decrease (Fig. 4.). Data were analyzed by ANOVA, followed by multiple comparisons between the means using the least-significant-difference test, and a probability if $p < 0.05$ was considered to indicate statistical significance. These data suggest that the activity of the proteasome in cells overexpressing GK-NCOAT decreases.

Discussion

NCOAT is the only nuclear and cytosolic enzyme encoded in the genome that processes the O-GlcNAcase activity to remove this sugar from protein, and it recently has been shown to be a bifunctional enzyme that contains a domain with intrinsic HAT activity. In the full-length NCOAT, the O-GlcNAcase domain and histone acetyltransferase domain reside in the N terminus and the C terminus of the protein, respectively.

GK-NCOAT is a splice variant of NCOAT cloned from the Goto-Kakazaki rat, which is a diabetic animal model. GK-NCOAT lacks the exon 8 of the wild type NCOAT and results in the loss of O-GlcNAcase activity but it retains complete HAT activity. To study the physical role of this splice variant GK-NCOAT, we transiently transfected a plasmid into mammalian cells to overexpress GK-NCOAT. The results showed that in cells overexpressing GK-NCOAT, there was an increase in the cellular O-GlcNAc modification level, a decrease in proteasome activity, and these were followed by apoptotic cell death. We observed the apoptotic death in both NIH 3T3 cells and CHO cells when

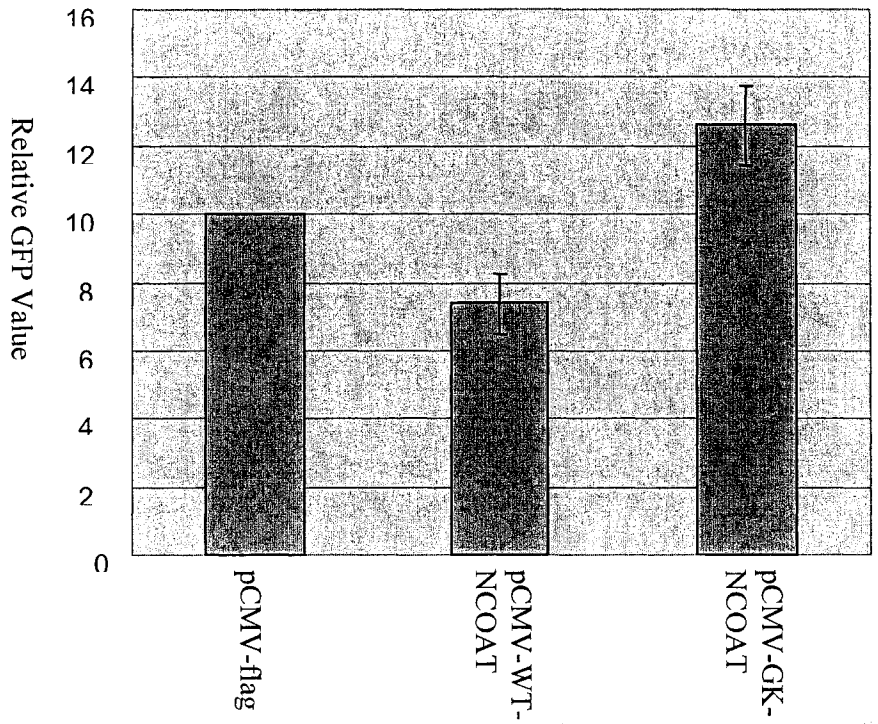

Figure 4. Overexpressing GK-NCOAT decreased the proteasome activity. 293 cells were cotransfected pCMV-GFP-CL1 and pCMV-β-Gal with pCMV-flag, pCMV-flag-WT-NCOAT and pCMV-flag-GK-NCOAT. 40 hours later, cells were lysed and cell extract were made for green fluorescence measurement. The reading values of GFP activity were balanced by transfection efficiency and total protein level.

GK-NCOAT was overexpressed in them. This suggests that the apoptosis caused by overexpressing GK-NCOAT may not be a cell line-specific phenomena.

Since *in vitro* experiments have shown that GK-NCOAT lacks the *O*-GlcNAcase activity, the overexpressed GK-NCOAT may play a dominant negative role to block wild type NCOAT normal function in vivo, leading to a decrease in *O*-GlcNAc removal and the accumulation of protein *O*-GlcNAc modification in a cell. To test this idea, we did immunofluorescence staining on the cells to check the cellular *O*-GlcNAc modification level with RL2 antibody. As showed in Fig. 1*A-D*, the O-GlcNAc modification level of cells transfected with the plasmid GK-NCOAT is higher than the level in nontransfected cells on the same plate. These results are consistent with the idea that the GK-NCOAT can act as a dominant negative. The cells overexpressing wild type NCOAT protein show a dramatic decrease in *O*-GlcNAc modification.

In addition to observing the elevated *O*-GlcNAc modification level, we also observed a decrease in proteasome activity in those cells that overexpress GK-NCOAT. It has been shown that *O*-GlcNAc modification can regulate the proteasome activity: The Rpt2 ATPase in the mammalian proteasome 19S cap is modified by *O*-GlcNAc; and as it increases in *O*-GlcNAc modification, proteasome activity decreases. This mechanism may couple the proteasome to the general metabolic state of the cell. The *O*-GlcNAc modification of the proteasome may allow the organism to respond to its metabolic needs by controlling the availability of amino acids and regulatory proteins (25). Overexpressed GK-NCOAT blocked the removal of the *O*-GlcNAc modification and resulted in cellular protein *O*-GlcNAc accumulation. As one of the *O*-GlcNAc modified substrates, the proteasome would have the increase in *O*-GlcNAc modification level, which would cause the proteasome activity to decrease. The proteasome is the primary place for protein degrega-

tion, and its activity down-regulation may lead to accumulation of some proteins that have synthesis levels that are relatively constant and whose cellular protein levels are mainly controlled by their degradation, like P53 (27,28), cyclins (29), ß-catenin (30). These proteins play important roles in cell cycle progression, apoptosis and developmental gene expression. Failure to degrade these proteins would lead to their accumulation, producing profound effects on cellular functions. These factors may count for our observation of the apoptotic death in cells that had impaired *O*-GlcNAc metabolism via the overexpression of the splice variant GK-NCOAT.

Besides the proteasome, a large number of *O*-GlcNAc modified proteins are transcription factors, including CREB, Sp1, and so on. *O*-GlcNAc modification on CREB interrupts its interaction with TAFII 130 and reduces CREB's transcriptional capability (5). *O*-GlcNAc modification on Sp1 also decreases its transcriptional activity (24). The *O*-GlcNAc modification on these transcription factors will lead to downstream gene expression change; for example, high glucose or the overexpression of OGT will result in a cellular O-GlcNAc modification level increase and a Sp1 transcriptional activity decrease. The sarcoendoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) gene encodes a protein that has a critical role in myocardial Ca^{2+} cycling. There are several Sp1 recognition sites and other *O*-GlcNAcylated transcription factor binding sites on the promoter of SERCA2a gene. High glucose or overexpression of OGT causes SERCA2a mRNA and protein levels to decrease, which would contribute to the impaired cardiac myocyte function and the development of diabetic cardiomyopathy (31).

OGT, the enzyme that adds *O*-GlcNAc modification to proteins, interacts with a histone deacetylase complex by binding to the corepressor mSin3A, to repress transcription in parallel with histone deacetylation (1). NCOAT, the enzyme responsible for re-

moval *O*-GlcNAc from proteins, possesses a histone acetyltransferase activity (10). Recent research in our laboratory showed that the OGT and NCOAT form a complex in the nucleus and that this complex sits in gene promoters to regulate transcription (unpublished data). GK-NCOAT is a naturally occuring splice variant found in GK rats, and it lacks the exon 8 of the full length NCOAT and has no *O*-GlcNAcase activity but retains HAT activity. It has been shown that GK-NCOAT also can form a complex with OGT, and that as a result, GK-NCOAT also may perform a dominant negative role to replace wild type NCOAT in this OGT-NCOAT transcriptional complex and impair the complex's normal function in vivo. This would cause multigene transcription mis-regulation and eventually lead to cell apoptosis.

In summary, NCOAT has important and multiple roles *in vivo*, and overexpressing GK-NCOAT results in elevated cellular *O*-GlcNAc modification, a decrease in proteasome activity, and cell apoptotic death. The cell apoptotic death is caspase-dependent apoptosis since we can observe cleaved caspase-3 in these apoptotic cells. A detailed mechanism underlying the cell apoptosis is not clear. From our observation of elevated cellular *O*-GlcNAc levels and impaired proteasome activity, we believe the cell apoptosis process is as follows: Overexpressing GK-NCOAT causes a disruption in *O*-GlcNAc metabolism and an accumulation of cellular *O*-GlcNAc modification. These would necessarily impair many protein functions like transcription factors and would disrupt gene transcriptional regulation, which would initiate caspase signal and apoptotic death.

GK-NCOAT is a splice variant of NCOAT cloned from brain tissue of the Goto-Kakizaki rat. The tissue distribution of this splice variant thoughout the body has not yet been investigated. Whether these phenomena also happen in vivo requires further study. However, from the results in this study, we know that GK-NCOAT may act as a domi-

nant negative of the wild type NCOAT function and result in an imbalanced protein O-GlcNAc modification and cell apoptosis. The administration of STZ, an inhibitor of NCOAT, in mice pancreas and brain also causes ß cell and neuronal apoptosis (17,21). Now with the use of a genetic method to block NCOAT, we also found that disrupting O-GlcNAc metabolism leads cell to apoptosis. This splice variant, GK-NCOAT, may also be used as a transgene in animal models for further studies.

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SUMMARY

Characterization of Mouse GFAT2

When the cDNA of GFAT2 was cloned, the protein was named GFAT2 only because of its sequence homology to GFAT1 (19). Our study demonstrated for the first time that GFAT2 has GFAT activity. The *Km* values of mouse GFAT2 have also been checked here: The *Km* values for fructose-6-phosphate and glutamine are 0.8 mM and 1.2 mM, respectively. It suggests that GFAT2 has higher affinity to fructose-6-phosphate than to glutamine. Several groups have investigated *Km* values for GFAT1 with different assays, and the ranges of *Km* values for fructose-6-phosphate and glutamine are 0.2-1.56 mM and 0.4-3.8 mM (14,18). The *Km* values for both of the GFAT2 substrates measured in this study fall within these ranges. Although every *Km* value is checked under a certain condition (change of any reaction conditions like temperature, pH value, or salt concentrations of the buffer may result in another *Km* value), these results suggest that GFAT1 and GFAT2 have similar enzyme activity. Now there are two known enzymes that can be rate-limiting in the hexosamine pathway that was proposed as a nutrient sensor and has been implicated in insulin resistance (1,2). Under hyperglycemic conditions, muscle cells develop insulin resistance, which can be prevented by inhibiting the activity of GFAT (64). Overexpressing GFAT in transgenic animals increases the hexosamine flux and results in many effects that mimic type II diabetes: Muscles become insulin resistant; the liver synthesizes excess fatty acid; and pancreas ß cells secrete excess insulin, leading to

hyperinsulinemia (9). On the base of these observations, GFAT is studied by many investigators as a molecular target for diabetes treatment.

The two GFAT proteins have different tissue distributions, which may provide for the specific needs of the tissue. GFAT2 conserves the homologous PKA phosphorylation site seen in GFAT1, which is serine 202 in GFAT2 and serine 205 in GFAT1. Because cAMP has a role in GFAT1 activity, we treated purified GST-GFAT2 fusion protein with protein kinase A and found that GFAT2 is also modified by PKA. However, instead of inhibiting the enzymatic activity of GFAT2, phosphorylation by this kinase stimulated GFAT2's activity about 2 folds. Mutagenesis of the putative phosphorylation site not only abrogated the modification but also blocked the ability of PKA to stimulate the enzymatic activity of GFAT2. Because the tissue distribution of GFAT2 differs from that of GFAT1 (19), this stimulation of activity by PKA could serve a different function of the hexosamine pathway under the cAMP signal in these tissues. Thus, *in vivo*, the same cAMP signal may cause a different response of GFAT isozymes in different tissues. This is not a singular case in glucose metabolism. Another similar example is PFK-2/FBPase-2, the bifunctional enzyme that synthesizes and degrades fructose-2, 6-biphosphate. The cAMP signal controls the PFK-2/FBPase-2 enzyme system in heart in the opposite way to that in liver. Thus, increased cAMP stimulates glycogen breakdown and gluconeogenesis, resulting in glucose export in liver; however, in heart, increased cAMP stimulates glycogen breakdown and glycolysis, resulting in glucose consumption. Consequently, cAMP signal initiated by hormone as well as insulin control the general glucose level. Here, we found the different regulation on GFAT2 by PKA phosphorylation compared with GFAT1, but the detailed physiological significance of this phenomenon is still unclear. To find the an-

swer, the function of the hexosamine pathway in different tissues needs be studied further.

Although the GFAT proteins differed with respect to PKA phosphorylation, they were quite similar with respect to product inhibition. Both enzymes were inhibited by UDP-GlcNAc, which is the end product of the hexosamine biosynthesis pathway, although the inhibition was partial and appeared greater for GFAT1 (17) than for GFAT2. *O*-GlcNAc modification on GFAT2 was not detected with the RL-2 antibody, suggesting that the inhibition by UDP-GlcNAc resulted from an allosteric change in GFAT2. The finding of a GFAT isoform that is oppositely regulated by cAMP and more modestly regulated by its product, UDP-GlcNAc, will provide the opportunity to create a transgene that much more effectively alters the hexosamine flux in these and other cells.

Splice Variant of NCOAT: GK-NCOAT

OGT and NCOAT are the enzymes responsible for adding and cleaving *O*-GlcNAc modification to or from protein. At first, NCOAT was named *O*-GlcNAcase because it was capable of removing *O*-GlcNAc modification. It is the only enzyme encoded in the genome that processes *O*-GlcNAcase activity. Recently, an intrinsic histone acetyltransferase (HAT) activity has been discovered in this protein. Thus, it was renamed as Nuclear and Cytoplasmic *O*-GlcNAcase and Histone acetylTransferase (NCOAT) because it is a bifunctional enzyme having two different enzyme activities. In full length NCOAT, the *O*-GlcNAcase domain and histone acetyltransferase domain reside in N terminus and C terminus of the protein, respectively.

GK-NCOAT is a splice variant of NCOAT cloned from the Goto-Kakazaki rat, which is a diabetic animal model. GK-NCOAT lacks the exon 8 of wild type NCOAT, which results in a lack of *O*-GlcNAcase activity, while it keeps complete HAT activity. At the time the GK-NCOAT was found and cloned, we were developing a transgenic mouse model proposed in the Introduction of this dissertation that had its *O*-GlcNAc metabolism and proteasome function impaired specifically in the brain. Then we wished to address if GK-NCOAT plays a dominant negative role in vivo to block endogenous *O*-GlcNAcase. If it is able to do so, then GK-NCOAT can be used as a genetic tool in transgenic mouse to study of *O*-GlcNAc metabolism in brain and to address other relative questions, including proteasome function, protein aggregate accumulation, synaptosomal transport, and so on. To accomplish this aim, we needed to test this dominant negative idea on GK-NCOAT in cultured cells before making GK-NCOAT as a transgene.

To study the physical role of this splice variant, we transiently transfected the plasmid into mammalian cells to overexpress GK-NCOAT. The results showed that in cells, overexpressing GK-NCOAT, there was an increase in the cellular *O*-GlcNAc modification level, and a decrease in proteasome activity, and which were followed by cell apoptotic death. Since the apoptotic death caused by GK-NCOAT was observed in both NIH 3T3 cells and CHO cells, it may not be a cell line specific phenomenon.

Immunofluorescence staining with the RL2 antibody was done to check the general cellular *O*-GlcNAc modification level in cells. In order to confirm the observations from immunofluorescence staining experiments and to quantitate the difference of cellular *O*-GlcNAc levels caused by the transfected gene (NCOAT or GK-NCOAT), western immunoblotting were also done. In these experiments, the reporter molecule P62, which

is a nuclear pore complex protein heavily modified with *O*-GlcNAc, was co-expressed in cells containing those transfected genes. After the cells were incubated, P62 was purified from whole cell lysates, following by western blot analysis of *O*-GlcNAc modification level of P62 in each condition. Both experiments showed that overexpressing GK-NCOAT increases the cellular *O*-GlcNAc modification level. In contrast, the cells over-expressing wild type NCOAT protein showed a dramatic decrease in cellular *O*-GlcNAc modification. This result is consistent with the idea that GK-NCOAT may be a dominant negative to block endogenous *O*-GlcNAcase and causes cellular *O*-GlcNAc accumulation.

O-GlcNAc transferase (OGT), the enzyme responsible for the addition of *O*-GlcNAc modification to proteins, interacts with a histone deacetylase complex by binding to the co-repressor mSin3A to repress transcription in parallel with histone deacetylation (42). NCOAT, the enzyme responsible for removal of *O*-GlcNAc from proteins, processes a histone acetyltransferase activity (27). Recently, unpublished research in our laboratory showed that the OGT and NCOAT form a complex in the nucleus, which sits on a gene's promoter to regulate gene transcription together. GK-NCOAT also can form a similar complex with OGT, but this complex is dysfunctional because it lacks *O*-GlcNAcase activity. This finding indicates that GK-NCOAT is also a dominant negative that replaces wild type NCOAT in this OGT-NCOAT transcriptional complex and causes impairment of impairs the normal complex's function *in vivo* and mis-regulation of multigene transcription.

When we extended the incubation of cells overexpressing GK-NCOAT, caspase-dependent cell apoptosis was observed; however, the same treatment of the cells that overexpress vector control or wild type NCOAT did not yield this result. The details of

the mechanism underlying the cell apoptosis are not clear. The elevated cellular O-GlcNAc level and the misfunctional GK-NCOAT: OGT complexes in these cells may count for the cell death.

Excess *O*-GlcNAc modification on many proteins has been showed to impair their normal functions, including that of the proteasome, the organelle responsible for degrading denatured, misfolded, or excess proteins (24,45). *O*-GlcNAc modification regulates proteasome activity in a reversible way: OGT adds *O*-GlcNAc modification on Rpt2, one of the ATPases in the mammalian proteasome 19S cap, which decreases proteasome activity; however, removal of the modification from Rpt2 by NCOAT recovers the proteasome activity. This mechanism may couple the proteasome to the general metabolic state of the cell. The *O*-GlcNAc modification of the proteasome may allow the organism to respond to its metabolic needs by controlling the availability of amino acids and regulatory proteins (45). In cells that overexpress GK-NCOAT, we also observed decreased proteasome activity. Overexpressed GK-NCOAT blocked the removal of *O*-GlcNAc modification and resulted in cellular O-GlcNAc accumulation. One of the *O*-GlcNAc modified substrates, the proteasome, may have an increase in *O*-GlcNAc modification level, which would cause the proteasome activity to decrease. The proteasome is the premier place for protein degradation. The down-regulation of its activity may lead to accumulation of some proteins with synthesis levels that are relative constant and with cellular levels that are mainly controlled by its degradation, like P53 (101), cyclins (102), and ß-catenin (103). These proteins play important roles in cell cycle progression, apoptosis, and developmental gene expression, and failure to degrade these proteins would lead to their accumulation and profound effect on cellular functions. These factors may account for our

observation of the apoptotic death in cells that follows impaired *O*-GlcNAc metabolism and proteasome function caused by overexpressing the splice variant GK-NCOAT.

Like the proteasome, many transcription factors can also be *O*-GlcNAcylated (104). For example, *O*-GlcNAc modification on CREB interrupts its interaction with TAFII 130 and reduces the CREB transcriptional capability (41). *O*-GlcNAc modification on Sp1 decreases its transcriptional activity (24). The downregulation of the transcriptional activation capability of these factors by *O*-GlcNAc modification may lead to a change of downstream gene expression. It has been showed that sarcoendoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) gene expression is influenced by *O*-GlcNAc modification: On the promoter of the SERCA2a gene, which encodes a protein that has a critical role in myocardial Ca^{2+} cycling, there are several Sp1 and other transcription factors recognition sites. High glucose or overexpression of OGT causes the accumulation of *O*-GlcNAc modification on cellular proteins, including Sp1, which inhibits its transcription activation activity. As a result, SERCA2a mRNA and protein levels decrease, and these decreases contribute to impaired cardiac myocyte function and the development of diabetic cardiomyopathy (105).

In summary, NCOAT has such important and multiple roles *in vivo* and overexpressing GK-NCOAT results in elevated cellular *O*-GlcNAc modification, decreased proteasome activity, and apoptotic cell death. From these results, we know that GK-NCOAT may be a dominant negative form blocking wild type NCOAT function and resulting in an imbalanced *O*-GlcNAc modification level and cell apoptosis. The administration of STZ, an inhibitor of NCOAT on mice pancreas and brain, also causes ß cell and neuronal apoptosis (62,63). With the use of a genetic method to block NCOAT in cultured cells,

we also found that disrupting *O*-GlcNAc metabolism leads to cell apoptosis. This splice variant, GK-NCOAT, may also be used in transgenic animal models for further studies.

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Title of Dissertation Blockage of O-GlcNAc Metabolism by Overexpressing a Dominant-Negative Splice Variant of NCOAT Results in Cell Apoptosis

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 student are such that she may be recommended for the degree of Doctor of Philosophy.

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