A Glycosylation Site, $_{60}$SGTS$_{63}$, of p67 Is Required for Its Ability To Regulate the Phosphorylation and Activity of Eukaryotic Initiation Factor 2α†

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ABSTRACT: Eukaryotic initiation factor 2- (eIF2-) associated glycoprotein p67 blocks eIF2α phosphorylation by kinases, and its N-terminal 1−97 amino acid segment can induce efficient translation. To investigate whether glycosylation at the serine/threonine clusters at this region is important in protein synthesis, we selected $_{27}$TSST$_{30}$ and $_{60}$SGTS$_{63}$ clusters for further analysis. By site-directed mutagenesis, $_{27}$TSST$_{30}$ and $_{60}$SGTS$_{63}$ clusters were substituted with $_{27}$AAGA$_{30}$ and $_{60}$AGAA$_{63}$ amino acid residues in full-length p67, and their EGFP fusions were constitutively expressed in rat tumor hepatoma cells (KRC-7). The $_{60}$AGAA$_{63}$ mutant blocked eIF2α phosphorylation less than either wild-type p67 or the $_{27}$AAGA$_{30}$ mutant. The $_{60}$AGAA$_{63}$ mutant also showed a low level of protein synthesis rate, a lower level of glycosylation, increased turnover rate, and weaker binding to eIF2α. These results suggest that glycosylation within the $_{60}$SGTS$_{63}$ sequence of p67 plays an important role in its stability and thus its regulation of protein synthesis by modulating the phosphorylation of the α-subunit of eIF2.

The initiation of protein synthesis is the key step for the regulation of translation and thus plays an important role in the regulation of gene expression (*1*). Several protein factors are involved in this process (*1*). The activities of these protein factors are modulated by their posttranslational modifications such as phosphorylation and glycosylation (*2*, *3*). Phosphorylation of the proteins is the hallmark modification that is involved in various cellular events and that regulates cell growth and proliferation. Several kinases are involved in the phosphorylation of proteins, and the activities of kinases are negatively regulated by phosphatases (*4*−*7*). O-Linked glycosylation, namely, the attachment of the simple monosaccharide O-GlcNAc,[1] is found on many proteins involved in DNA replication, transcription, cell signaling, and protein translation (*8*−*10*). Although the consensus motif for the O-GlcNAc site has not been identified, it is clear that the hydroxyl group of the serine/threonine amino acid residues is the site for this modification (*11*, *12*). Several classes of proteins including hormone receptors, kinases and signaling molecules, cytoskeletal components, nuclear pore proteins, oncogenes, transcription factors, tumor suppressors, viral proteins, and protein synthesis initiation factors are modified by O-GlcNAc (*13*). O-GlcNAc-modified proteins participate in multiprotein complex formation as well as in protein stability (*13*−*16*).

Protein synthesis initiation in mammals is regulated by the phosphorylation of the smallest α-subunit of eukaryotic initiation factor 2 (eIF2) (*2*). The phosphorylation of eIF2α is controlled by the cellular glycoprotein p67, which is associated with eIF2 (*17*−*24*). Purified p67 is modified by O-linked glycosylation, and this glycosylation is required for its ability to block eIF2α phosphorylation (*17*, *19*, *20*). On the basis of our biochemical data, earlier we reported that p67 has multiple O-glycosylation sites (*17*). Recently, we reported that the N-terminal 1−97 amino acid segment of p67 showed higher activity in blocking eIF2α phosphorylation when this segment was constitutively expressed in mammalian cells. However, the glutathione *S*-transferase fusion of this segment of p67 did not show this activity in in vitro phosphorylation assays (*23*). These results indicated that posttranslational modification such as O-glycosylation at this segment is possibly needed for its function. Three serine/threonine clusters with sequences $_{8}$SS$_{9}$, $_{27}$TSST$_{30}$, and $_{60}$SGTS$_{63}$ are present at the N-terminal 1−97 amino acid segment of p67 (*23*, *25*). Among these clusters, $_{29}$ST$_{30}$ and $_{60}$S are conserved in mammals (*3*, *23*). In this study, we addressed the question of whether $_{27}$TSST$_{30}$ and $_{60}$SGTS$_{63}$ sites are involved in O-glycosylation, and if glycosylated, what the roles of the O-glycosylation in protein synthesis are. We find that the $_{60}$SGTS$_{63}$ amino acid sequence is involved in in vivo glycosylation and that this glycosylation is required for the stability of p67.

MATERIALS AND METHODS

All chemicals used in this study were obtained from Sigma Chemicals (St. Louis, MO), Merck (Darmstadt, Germany), ICN Biomedicals, Inc. (Aurora, OH), Fisher Chemicals (Fair Lawn, NJ), or GIBCO-BRL (Rockville, MD). All enzymes used in this study were purchased from New England Biolabs

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[1] Abbreviations: p67, eukaryotic initiation factor 2-associated 67 kDa glycoprotein; eIF2α, α-subunit of eukaryotic initiation factor 2 (eIF2); p67-DG, p67 deglycosylase; GlcNAc, *N*-acetyl-*β*-D-glucosamine; EGFP, enhanced green fluorescent protein; GST, glutathione *S*-transferase.

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(Beverly, MA). Different molecular mass markers were purchased from Bio-Rad. [^{35}S]Methionine, [^{3}H]UDP-galactose, and [^{3}H]UDP-glucosamine were purchased from Amersham Corp. Galactosyltransferase was purchased from Sigma Chemicals.

Site-Directed Mutagenesis and Subcloning into Mammalian Expression Vector. An ~1.4 kb cDNA insert containing the entire p67 coding region was obtained by PCR using appropriate forward and reverse primers (5′ TCCCCCGGGTGATGGCGGGCGTGGAAGAG 3′ and 5′ TCCCCCGGGAAGTTTTAATAGTCATCTCCTC 3′, respectively) and the pGEM-p67 as a template (*25*). The resulting DNA fragment was gel purified, digested with *Sma*I, and ligated in M13mp18 (Stratagene) at the *Sma*I site. A single-stranded uracil template was made, and annealing reactions were performed with mutant oligonucleotides, for example, PGTSST-AAGA (5′ CTTTTCTTCTTGGCGGCTTCCTCGGCCGCGCCGGCGGCTCCCTCTTCCCTGTCGTCTGGATCCAG 3′) and PSGTS-AGAA (5′ CTTGAACTATTTCTTCGCCCTCGGCGTCACCTGCTTCATCGTTTTG 3′) for substituting $_{27}$TSST$_{30}$ and $_{60}$SGTS$_{63}$ with $_{27}$AAGA$_{30}$ and $_{60}$AGAA$_{63}$ sequences, respectively. Positions for targeted mutations at the mutant oligonucleotides are underlined. To detect mutations at $_{27}$TSST$_{30}$ and $_{60}$SGTS$_{63}$ sites, sequence primers PGTSSTSP (5′ CTTTATCAAGTTCTTGTTGCC 3′) and K2SP (5′ CCAGTGCTTGTCTCTCC 3′), respectively, were used in DNA sequencing reactions. The oligonucleotides used in this study were purchased from either GIBCO/BRL or Sigma-Genesis. DNA sequencing was performed by sequenase kit version 2.0 (US Biochemicals). The cDNA inserts containing mutations at p67 were isolated from the RF form of M13mp18 after digestion with *Xma*I and ligated at the *Xma*I site of pEGFP-C3 vector (Clontech, Palo Alto, CA). Plasmids with sense orientation were selected for further analysis. The specific fusion junction between mutant cDNA and enhanced green fluorescent protein (EGFP) was confirmed by DNA sequencing. All procedures for manipulation of recombinant DNA were either published earlier (*23*) or followed from *Molecular Cloning, a Laboratory Manual* (*26*), and *Current Protocols in Molecular Biology* (*27*).

Cell Culture, Generation of Stable Cell Lines, Cell Lysate Preparation, Western Blot Analysis, Immunoprecipitations, Antibody Stripping, and Metabolically Labeling Cells with [^{35}S]Methionine and [^{3}H]UDP-glucosamine. Several experimental procedures used in this study were previously described (*23*). In brief, KRC-7 cells were grown, transfected with pEGFP-C3 vector for either the expression of EGFP alone or its in-frame fusion proteins with either wild-type p67 or its mutant forms $_{27}$AAGA$_{30}$ and $_{60}$AGAA$_{63}$, and stable cell lines were generated following the procedures as described (*23*, *26*−*28*). After selection with G418, more than 200 colonies constitutively expressing fusion proteins were pooled, and stable cell lines were maintained in G418. Cell lysates were prepared, and Western blotting experiments were carried out following the procedures as described (*23*). The stripping of the antibody from the nitrocellulose filter was performed following the procedures as described (*23*). For labeling metabolically with [^{35}S]methionine, cells from different lines were seeded at a low density (50−60%), and growth medium was replaced with methionine-deficient medium containing dialyzed serum and grown for 30 min. [^{35}S]Methionine (300 μCi/mL) was added to these cells and allowed to grow at 37 °C in a CO_2 incubator for an additional 2.5 h. For labeling with [^{3}H]UDP-glucosamine, similar procedures were followed except 100 μCi/mL [^{3}H]UDP-glucosamine was added to the medium and incubated at 37 °C in a CO_2 incubator for 16 h. After labeling, cells were harvested, and lysates were prepared. For immunoprecipitation experiments, 5 μg of polyclonal eIF2α antibody was bound to protein A−agarose, free antibody was removed, and radiolabeled cell lysates containing 500 μg of proteins were added to antibody-bound protein A−agarose beads and mixed by gently shaking at 4 °C for 12−16 h. The unbound proteins were removed by brief centrifugation and extensively washed with washing buffer, and beads were resuspended with 5× SDS−PAGE loading buffer. The mixture was then boiled for 10 min and microcentrifuged briefly, and the clear supernatant was loaded on 15% SDS−PAGE followed by either fluorography or immunoblot analysis. The procedures for fluorography were as previously described (*24*). The immunoblotting experiment with monoclonal antibody against O-linked GlcNAc was performed following the procedures as described (*29*).

WGA Binding Assay. Similar immunoprecipitation experiments with monoclonal EGFP antibody were performed as described above, and WGA binding assays with or without GlcNAc were carried out following the procedures as described (*17*, *22*).

Pulse−Chase Experiment. Cells from different lines were labeled metabolically with [^{35}S]methionine for 2.5 h following the procedures as described above. The radiolabeled methionine was removed from the cells by repeated washing with growth medium, and then the cell was allowed to grow with normal growth medium. Cells were harvested during different times of chase, and lysates were prepared. For immunoprecipitation experiments with either polyclonal antibodies against p67 or eIF2α, 500 μg of total protein samples was taken, and similar procedures were followed as described above.

Antibodies. Preparation and characterization of polyclonal antibodies against p67, p67 deglycosylase, and eIF2α have been described (*18*, *21*). Goat polyclonal antibody against eIF2α(C-20) and monoclonal antibody against EGFP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and monospecific polyclonal anti-eIF2α(P) antibody was purchased from Research Genetics (Huntsville, AL). Anti-mouse, -rabbit, or -goat secondary antibodies against whole immunoglobulins conjugated with horseradish peroxidase were purchased from Amersham Corp. or Sigma Chemicals. For immunoprecipitation experiments, monoclonal antibody against EGFP was purchased from Clontech (Palo Alto, CA). The O-linked GlcNAc-specific monoclonal antibody (CTD110.6) was a gift of Dr. Gerald W. Hart (Johns Hopkins University School of Medicine, Baltimore, MD) (*29*).

RESULTS

To test whether $_{27}$TSST$_{30}$ and $_{60}$SGTS$_{63}$ serine/threonine clusters are involved in O-GlcNAc modification, we substituted these with $_{27}$AAGA$_{30}$ and $_{60}$AGAA$_{63}$ residues, respectively, in full-length rat recombinant p67 (Figure 1A) by site-directed mutagenesis. The mutants were then subcloned into pEGFP-C3 vector to obtain in-frame fusion with enhanced green flouroscence protein (EGFP). These EGFP fusion

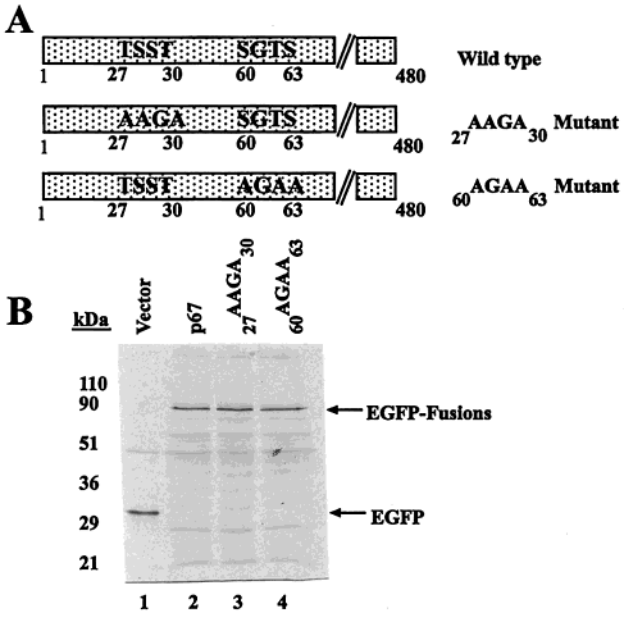

FIGURE 1: Expressions of wild-type rat recombinant p67 and its mutants $_{27}$AAGA$_{30}$ and $_{60}$AGAA$_{63}$ in KRC-7 cells. (A) Schematic diagram of the wild-type sequence with AAGA substitution at $_{27}$TSST$_{30}$ and AGAA substitution at $_{60}$SGTS$_{63}$ of rat recombinant p67. (B) Immunoblotting analyses for the levels of EGFP and its fusion proteins wild-type p67 and its mutants $_{27}$AAGA$_{30}$ and $_{60}$AGAA$_{63}$ in KRC-7 cells. KRC-7 cells constitutively expressing EGFP vector alone (vector, lane 1), EGFP fusions of rat recombinant p67 (p67, lane 2), or its mutant forms $_{27}$AAGA$_{30}$ (lane 3) and $_{60}$AGAA$_{63}$ (lane 4) were grown in normal growth conditions. Cell lysates were prepared, and 100 μg of total protein samples were analyzed with 15% SDS−PAGE followed by immunoblotting with monoclonal antibody against EGFP. The positions corresponding to the EGFP and EGFP fusions of p67 and its mutant forms are indicated. Molecular mass markers are shown at the left. Some of the faster migrating bands that were detected by this antibody might represent the degradation products of the EGFP fusion proteins or nonspecific interactions of EGFP antibody with cellular proteins. This experiment was performed three times with similar results.

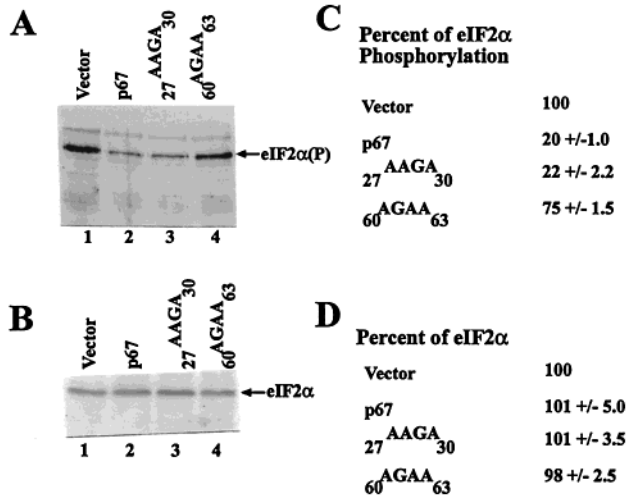

FIGURE 2: Phosphorylation of eIF2α in KRC-7 cells constitutively expressing $_{27}$AAGA$_{30}$ and $_{60}$AGAA$_{63}$ mutants. KRC-7 cells constitutively expressing EGFP (vector), EGFP fusions of rat recombinant p67 (p67), and its mutants $_{27}$AAGA$_{30}$ and $_{60}$AGAA$_{63}$ were grown. Cell lysates were prepared; 100 μg of total protein samples was analyzed with 15% SDS−PAGE and immunoblotted with polyclonal antibodies against the phosphorylated form of eIF2α (A) and with polyclonal antibodies against the unphosphorylated form of eIF2α (B). Positions corresponding to phosphorylated eIF2α and total eIF2α protein are indicated as eIF2α(P) and eIF2α, respectively. The intensities of protein bands corresponding to the phosphorylated and total eIF2α protein were measured and expressed in terms of percent, taking the vector as 100% (panels C and D, respectively). These experiments were repeated in three different preparations of cell lysates, and one representative experiment is displayed. The standard errors are indicated next to the percent of eIF2α phosphorylation or total eIF2α.

proteins along with controls, such as EGFP alone or its in-frame fusion of wild-type rat recombinant p67, were constitutively expressed in KRC-7 cells, and their levels of expression were examined on a Western blot (Figure 1B). Both mutants and the wild-type p67 express equally well.

Next, we determined the levels of the phosphorylated form of eIF2α in extracts from these cells (Figure 2). We found that constitutive expression of the $_{60}$AGAA$_{63}$ mutant in KRC-7 cells resulted in a loss of p67's ability to block eIF2α phosphorylation as compared to its wild-type protein, and under similar conditions the $_{27}$AAGA$_{30}$ mutant did not show any significant effect (Figure 2A, compare lanes 2−4 with lane 1). Consistent with our previous findings, the wild-type p67 showed ∼5-fold increased levels of phosphorylation inhibitory activity as compared to the vector, and this activity was lost by almost 75% when the $_{60}$SGTS$_{63}$ sequence was substituted with $_{60}$AGAA$_{63}$ in full-length p67 (Figure 2A,C). The levels of eIF2α in these cell lines remained unchanged (Figure 2B,D). These results indicate that AGAA substitution at the $_{60}$SGTS$_{63}$ sequence of p67 results in a significant loss of p67's ability to prevent eIF2α phosphorylation.

Since the rate of protein synthesis in mammalian cells depends on the status of eIF2α phosphorylation, and that in turn depends on the activity of p67 (*2*, *20*−*24*), we then examined the protein synthesis rate in cells expressing p67 mutants and compared that with the rate of controls (Figure 3). We found that the rate of protein synthesis in cells constitutively expressing the $_{60}$AGAA$_{63}$ mutant was >40% lower than that of the basal level (Figure 3, compare panel vector with that in $_{60}$AGAA$_{63}$) and >6-fold lower than that of wild-type p67 (compare panel $_{60}$AGAA$_{63}$ with that in p67). A similar mutation at the $_{27}$TSST$_{30}$ site did not show any change in overall rate of protein synthesis compared to wild-type protein (compare panel $_{27}$AAGA$_{30}$ with p67). The fluorographic results as shown in Figure 3B were consistent with the overall rate of protein synthesis in these cell lines (Figure 3A).

Previously, we reported that the ability of p67 to block eIF2α phosphorylation depended upon the level of its glycosylation (*17*, *19*, *20*). We therefore examined the level of glycosylation of the $_{27}$AAGA$_{30}$ and $_{60}$AGAA$_{63}$ mutants in KRC-7 cells by metabolically labeling with [^{3}H]glucosamine and compared it with controls such as EGFP alone or EGFP fusion of wild-type p67 (Figure 4). Our data revealed that the level of glycosylation of the $_{60}$AGAA$_{63}$ mutant was at least 3.6-fold lower than that of wild-type p67 (compare panels $_{60}$AGAA$_{63}$ and p67 in Figure 4 A,C) and slightly higher than the background level (compare lane 4 with lane 1 in Figure 4A and panel $_{60}$AGAA$_{63}$ with vector in Figure 4C). The level of glycosylation of the $_{27}$AAGA$_{30}$ mutant, however, remained unaffected as compared to the wild-type protein (compare lane 2 with lane 3 in Figure 4A and panel $_{27}$AAGA$_{30}$ with p67 in Figure 4C). These results suggest that

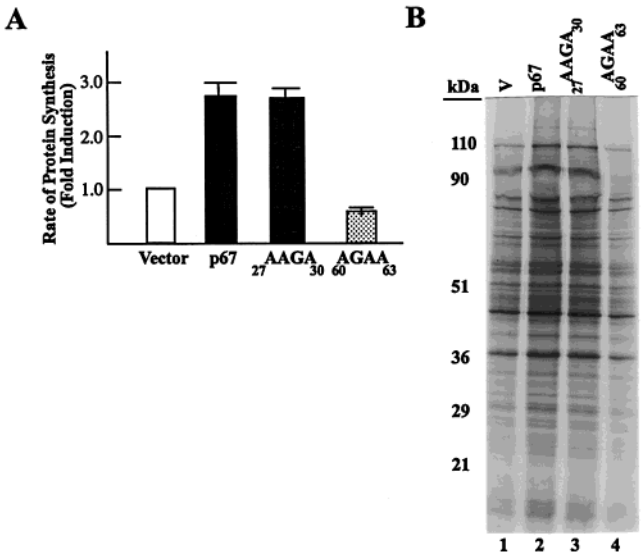

FIGURE 3: Rate of protein synthesis in KRC-7 cells constitutively expressing EGFP alone and EGFP fusion proteins of wild-type p67 and its mutants $_{27}AAGA_{30}$ and $_{60}AGAA_{63}$. (A) KRC-7 cells constitutively expressing EGFP and EGFP fusions of p67 and mutants $_{27}AAGA_{30}$ and $_{60}AGAA_{63}$ were grown for 72 h after culturing. Cells were metabolically labeled with [^{35}S]methionine, cell lysates were made, and protein concentrations were measured by the Bio-Rad assay kit with bovine serum albumin as a standard. Total labeled protein samples (10 μg) were spotted on a Whatmann filter, dried, and counted in a scintillation counter. The rate of protein synthesis was expressed by the incorporation of the [^{35}S]methionine as compared to cells expressing vector alone (taken as 1). The open bar represents the rate of protein synthesis in cells expressing EGFP (vector). The black-striped bar, gray bar, and black-dotted bar represent that of cells expressing EGFP fusions of wild-type p67 and $_{27}AAGA_{30}$ and $_{60}AGAA_{63}$ mutants, respectively. The results are from three different experiments measured in triplicate, with error bars representing standard deviations from the means. (B) While cells from the above lines were growing (for 72 h after culturing), they were metabolically labeled with [^{35}S]methionine, and 25 μg of radiolabeled protein samples was analyzed with 15% SDS−PAGE followed by fluorography. Lanes: 1, vector; 2, p67; 3, $_{27}AAGA_{30}$; and 4, $_{60}AGAA_{63}$. Molecular mass markers are shown at the left.

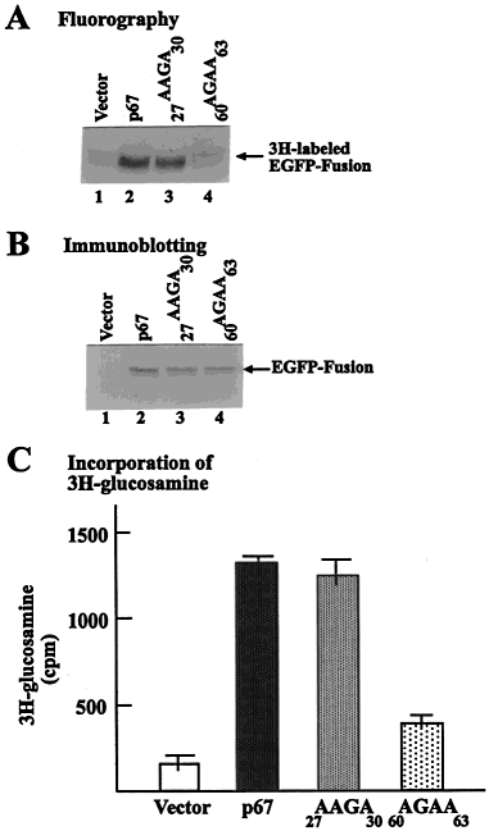

FIGURE 4: Determination of the glycosylation of EGFP fusion proteins of p67 and its mutants $_{27}AAGA_{30}$ and $_{60}AGAA_{63}$. KRC-7 cell lines constitutively expressing EGFP alone (vector), EGFP fusion proteins of p67 (p67), and its mutant derivatives $_{27}AAGA_{30}$ and $_{60}AGAA_{63}$ were metabolically labeled with [^{3}H]UDP-glucosamine. EGFP or EGFP fusion proteins from 500 μg of total proteins were immunoprecipitated with monoclonal antibody against EGFP and subjected to fluorography (A). Panel B shows the corresponding Western blot with EGFP monoclonal antibody from the similar experiment shown in panel A. The ^{3}H-labeled EGFP fusion protein bands shown in panel A were cut out and counted by a scintillation counter. The counts were expressed in cpm and plotted on the graph (C). The results are from three different experiments measured in triplicate, with error bars representing standard deviations from the means. As a control, EGFP protein bound in protein A−agarose beads (vector) was used to detect nonspecific binding of radiolabeled proteins.

the $_{60}AGAA_{63}$ mutant is efficiently O-GlcNAc-modified in mammalian cells.

To further verify that the $_{60}AGAA_{63}$ mutant is not efficiently glycosylated by O-GlcNAc moieties, we immunoprecipitated its EGFP fusion and that of wild-type p67 with EGFP monoclonal antibody and allowed them to bind to wheat germ agglutinin (WGA). The WGA binding was competed with excess GlcNAc. WGA binds to O-GlcNAc moieties, which are present in proteins (*17*, *19*, *22*), and free GlcNAc is a competitive inhibitor of this lectin. In these experiments, we find that wild-type p67 binds to WGA, and this binding is significantly inhibited in the $_{60}AGAA_{63}$ mutant. In addition, the binding of WGA to EGFP fusions of p67 or the $_{60}AGAA_{63}$ mutant is fully competed with the competitive inhibitor GlcNAc, indicating the specific binding of WGA (data not shown). A similar immunoprecipitation experiment was performed with EGFP monoclonal antibody, and the EGFP fusion proteins of wild-type p67 and its mutant $_{60}AGAA_{63}$ were identified with the O-linked GlcNAc-specific antibody (Figure 5). Our results suggest that the wild-type p67 is glycosylated by O-linked GlcNAc moieties, and the detection of this glycosylation is significantly decreased in its $_{60}AGAA_{63}$ mutant (compare lane 2 with lane 3). To verify further whether the $_{60}AGAA_{63}$ mutant had reduced a level of O-GlcNAc modification, the immunoprecipitates of p67 and this mutant were subjected to galactosylation with galactosyltransferase and [^{3}H]UDP-galactose as a substrate, following the procedures as described previously (*11*). The radiolabeled proteins were analyzed with SDS−PAGE and fluorographed. After exposing the film for 7 days, we detected very faint signals. Nonetheless, we found that the wild-type p67 was labeled with [^{3}H]galactose whereas its $_{60}AGAA_{63}$ mutant had significantly less labeling with the substrate (data not shown). These results further support our findings, as mentioned earlier, that indeed the $_{60}SGTS_{63}$ is a major site for attachment of O-GlcNAc modification in p67. In summary, the data presented in Figures 2−5 indicate that substitution of the $_{60}SGTS_{63}$ sequence with $_{60}AGAA_{63}$ residues in p67 led to a lower level of glycosylation and possibly cause higher levels of eIF2α phosphorylation and thus a lower rate of overall protein synthesis.

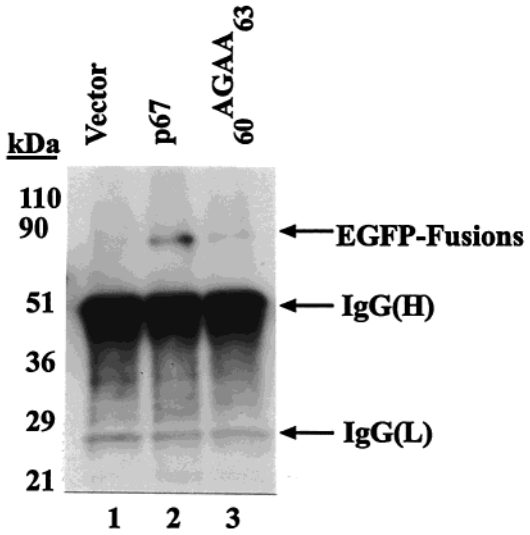

FIGURE 5: Western blot analysis with O-linked GlcNAc-specific antibody. Similar immunoprecipitation reactions to those described in the legend to Figure 5 were performed with EGFP monoclonal antibody and analyzed with 15% SDS−PAGE, followed by binding with O-linked GlcNAc-specific monoclonal antibody, and color was developed with ECL. Lanes: 1, vector; 2, EGFP fusion of wild-type p67; 3, EGFP fusion of $_{60}$AGAA$_{63}$ mutant. Molecular mass markers are shown at the left. The experiment was repeated two times with similar results. Immunoglobulin heavy chain and light chain are indicated as IgG(H) and IgG(L), respectively, and shown by arrows.

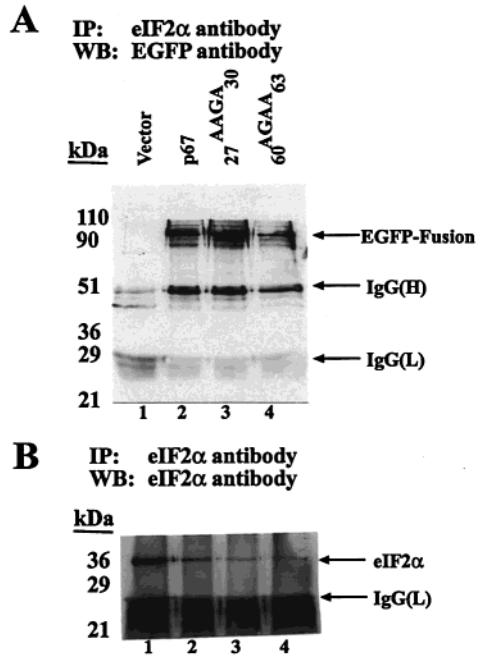

FIGURE 6: Analysis of the protein−protein interactions with p67 and its mutant forms $_{27}$AAGA$_{30}$ and $_{60}$AGAA$_{63}$ with eIF2α. (A) KRC-7 cells constitutively expressing EGFP alone (vector, lane 1), EGFP fusion proteins of wild-type p67 (p67, lane 2), and its mutant forms $_{27}$AAGA$_{30}$ (lane 3) and $_{60}$AGAA$_{63}$ (lane 4) were grown, and cell lysates were prepared. Total protein samples (500 μg) were used for immunoprecipitations with anti-eIF2α (5 μg) followed by immunoblotting with monoclonal antibody against EGFP (1:1000 dilution). As a control, protein A−agarose beads bound to EGFP were used to detect any nonspecific binding of cellular proteins. The antibodies from the blot were stripped out and immunoblotted with polyclonal antibody against eIF2α (B). Arrows indicate EGFP fusion proteins and eIF2α. Molecular mass markers are shown at the left. Immunoglobulin heavy chain and light chain are indicated as IgG(H) and IgG(L), respectively, and shown by arrows.

p67 copurifies with eIF2 and regulates its phosphorylation (*3*, *18*). Substitution of the amino acid sequence at the lysine-rich domain I of p67 with specific amino acid residues inhibits its activity (*23*). We therefore wanted to test the hypothesis that p67 might bind to eIF2α and the O-GlcNAc moieties of p67 are required for p67−eIF2α interaction. To test this hypothesis, eIF2α was immunoprecipitated from cell extracts. The immunoprecipitates were then analyzed with SDS−PAGE followed by Western blot with monoclonal antibody against EGFP (Figure 6A). The EGFP fusions were detected in cell extracts from KRC-7 cells constitutively expressing its fusion with wild-type p67 (lane 2) or its mutants $_{60}$AGAA$_{63}$ (lane 3) and $_{27}$AAGA$_{30}$ (lane 4). The EGFP fusion of the $_{60}$AGAA$_{63}$ mutant coprecipitated less efficiently than that of wild-type p67 and the $_{27}$AAGA$_{30}$ mutant (compare lanes 2 and 3 with lane 4). This is not due to the lower amount of antibody used for the experiments because the levels of heavy chain and light chain of IgG remained unchanged in these experiments (Figure 6A). To check whether these immunoprecipitation assays pulled down equal amounts of eIF2α, the EGFP antibody from the blot was stripped out and reprobed with polyclonal eIF2α antibody (Figure 6B). Although a >3-fold excess of eIF2α was precipitated by its antibody from cell extract obtained from cells constitutively expressing vector alone (lane 1) as compared to that of EGFP fusions with wild-type p67 (lane 2), the $_{27}$AAGA$_{30}$ mutant (lane 3), and the $_{60}$AGAA$_{63}$ mutant (lane 4), its level remained basically unchanged in the later three cell lines (compare lane 1 with lanes 2−4 in Figure 6B). These results suggest that the lesser amount of EGFP fusion of the $_{60}$AGAA$_{63}$ mutant coprecipitated with eIF2α was not due to the lesser amount of eIF2α. This, however, could be either because of weaker interaction of this p67 mutant with eIF2α or because this mutant has a higher turnover rate as compared to wild-type p67.

To test the turnover rate, KRC-7 cells constitutively expressing either wild-type p67 or the $_{60}$AGAA$_{63}$ mutant were labeled metabolically with [^{35}S]methionine and chased with cold methionine for several hours. pP67 was immunoprecipitated from the respective cell lysates, and both endogenous p67 and its EGFP fusion were detected by fluorography (Figure 7A). Although, the levels of endogenous p67, which is present in >20-fold excess as compared to its EGFP fusions, and EGFP fusion of wild-type p67 remained unchanged, the EGFP fusion of the $_{60}$AGAA$_{63}$ mutant had a higher rate of turnover than its wild-type protein (compare lanes 1−3 with lanes 4−6). The wild-type p67 was detected even after 16 h of chase (data not shown), whereas that of the $_{60}$AGAA$_{63}$ mutant could not be detected even after 2 h of chase (Figure 7A). In addition, the level of this mutant at 0 h of chase was slightly low compared to that of wild-type p67 [compare lanes 1 in both panels p67 and p67 (mut), Figure 7A]. This lower level of the $_{60}$AGAA$_{63}$ mutant was not due to the total protein immunoprecipitated, because the EGFP fusions of wild-type p67 and its $_{60}$AGAA$_{63}$ mutant remained basically unchanged (Figure 7B, compare lanes 1−3 with lanes 4−6). These results therefore suggest that the wild-type p67 is very stable compared to its $_{60}$AGAA$_{63}$ mutant, which has a higher turnover rate. We have

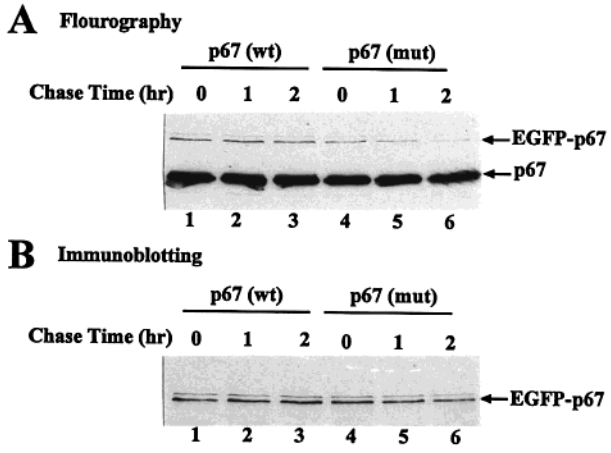

FIGURE 7: The $_{60}$AGAA$_{63}$ mutant has a higher turnover rate than wild-type p67. Cells from wild-type p67 and $_{60}$AGAA$_{63}$ mutant lines were metabolically labeled with [^{35}S]methionine for 2.5 h and chased for 0 (lanes 1 and 4, panel A), 1 (lanes 2 and 5), and 2 h (lanes 3 and 6) with unlabeled methionine. Cells were lysed, and 500 μg of total protein samples was immunoprecipitated with p67 polyclonal antibody and subjected to fluorography (A). Panel B shows the corresponding Western blot from the experiment similar to that shown in (A), probed with monoclonal EGFP antibody. The positions of endogenous p67 and EGFP fusion of wild-type p67 or the $_{60}$AGAA$_{63}$ mutant are indicated by arrows. A slower migrating protein appearing as a doublet with EGFP fusions was also detected by both p67 and EGFP antibodies from the protein samples analyzed, and it is present in minor quantity.

also performed similar experiments with the $_{27}$AAGA$_{30}$ mutant and found no difference in stability between wild-type p67 and this mutant (data not shown).

DISCUSSION

Many nuclear and cytoplasmic proteins are modified by O-GlcNAc moieties at the serine/threonine residues (*8*, *13*, *30*). These or adjacent sites are often modified with phosphate group by kinases (*13*, *15*, *30*−*32*). Mapping the sites of O-GlcNAc modification is a difficult task, and proteins, which show reciprocity between O-GlcNAc and O-phosphate, make it even harder to pinpoint the sites of modification. This is because a preparation of such a protein contains a mixed population of both modifications along with its unmodified form. Recently, Wells et al. used a mass spectrometry-based method to identify sites modified by O-GlcNAc that relies on mild β-elimination followed by Michael addition with dithiothreitol. This method successfully identified the O-GlcNAc sites in synapsin I, lamin B receptor, and nucleoporin Nup155 (*33*). Nonetheless, site mapping allows a test for the function of such modification by site-directed mutagenesis. We wanted to identify the potential glycosylation site(s) on p67 that could be involved in its ability to block eIF2α phophorylation. Since there is no consensus sequence for such modification, we have taken a molecular biology approach and combined with it the in vivo functional assay for p67 activity. Using these methods, we identified an N-terminal 1−97 amino acid segment that increases p67's activity to block eIF2α phosphorylation only when it is constitutively expressed in mammalian cells, where it is glycosylated (*23*). This then prompted us to test for potential glycosylation at the serine/threonine clusters present within this amino acid segment. We have changed the amino acid residues at the conserved sequences $_{27}$TSST$_{30}$ and $_{60}$SGTS$_{63}$ to $_{27}$AAGA$_{30}$ and $_{60}$AGAA$_{63}$, respectively, in full-length p67 (Figure 1). These mutants were constitutively expressed in rat tumor hepatoma cells. The p67's activity to block eIF2α phosphorylation, rate of protein synthesis, glycosylation, binding with eIF2α, and the turnover rates of wild-type p67 and its mutants were measured in these cell lines. Our data showed that the $_{60}$SGTS$_{63}$ site is modified with an O-GlcNAc moiety, and this glycosylation plays an important role in its stability and, thus, in regulation of eIF2α phsophorylation.

Several studies indicate that the O-GlcNAc modification on proteins plays an important role in the regulation of various cellular events such as DNA replication, transcription, cell signaling, and translation (*8*−*10*). For example, RNA polymerase II has a CTD domain at its C-terminus modified with an O-GlcNAc moiety. This modification possibly leads to the assembly of several transcription factors into the TATA box. Once the preinitiation complex is formed, these glycosyl residues are removed by deglycosylase activity, and the CTD kinases possibly phosphorylate the same sites or adjacent sites. At this stage, the transcription machinery changes to elongation mode and continues RNA synthesis (*31*). The CTD domain of RNA pol II has clusters of serine/threonine residues. The N-terminal 1−97 amino acid segment of p67 has three serine/threonine clusters (*3*, *23*), and this study showed that one such cluster with sequence $_{60}$SGTS$_{63}$ is one of the glycosylation sites. To test for the requirement of glycosylation at this site, we examined whether p67 binds to eIF2α (*18*) to explain whether this binding is essential for protection of eIF2α phosphorylation activity. Our yeast two-hybrid assays for identifying the interaction between these two proteins revealed that this interaction is weak whereas that with eIF2γ was strong (manuscript in preparation), indicating that the interaction between p67 and eIF2α may be through posttranslation modification such as O-GlcNAcation, which might be absent in yeast. In addition, substitution of the lysine-rich domain I with the NMKSGNKTQ sequence in p67 abolished its activity significantly in KRC-7 cells (*23*), indicating the possibility of binding the lysine-rich domain I of p67 and eIF2α. However, we could not detect such interaction in our yeast two-hybrid assay (data not shown). We therefore performed co-immunoprecipitation assays and detected the efficient binding between eIF2α and p67 in KRC-7 cells. This binding was reduced in cells constitutively expressing the $_{60}$AGAA$_{63}$ mutant (Figure 6). Although the reduced level of O-GlcNAc modification at the $_{60}$SGTS$_{63}$ site correlated with the reduced binding with eIF2α, the endogenous p67 level in this mutant cell line did not change (Figure 7). This is indeed due to the higher turnover rate of the $_{60}$AGAA$_{63}$ mutant as compared to wild-type p67 (Figure 7). These results suggest that O-GlcNAc modification at the $_{60}$SGTS$_{63}$ site is involved in the turnover of p67. Although the levels of expression of EGFP fusions of wild-type p67 and its $_{27}$AAGA$_{30}$ and $_{60}$AGAA$_{63}$ mutants were <20-fold as compared to endogenous p67 (Figure 7A), the $_{60}$AGAA$_{63}$ mutant had a lower level of O-GlcNAc modification (Figure 4), and subsequently KRC-7 cells constitutively expressing this mutant had higher levels of eIF2α phosphorylation (Figure 2). In contrast, a similar mutation at $_{27}$TSST$_{30}$ did not show any effect on eIF2α phosphorylation, and it was efficiently glycosylated (Figures 2 and 4). These results point out the importance of

the O-GlcNAc modification at the $_{60}$SGTS$_{63}$ site. In addition, the $_{60}$AGAA$_{63}$ mutant had a higher rate of turnover as compared to wild-type p67 (Figure 7). This may explain its inefficient binding with eIF2α (Figure 6). It is intriguing, however, that a small population of the $_{60}$AGAA$_{63}$ mutant can compete with the large excess of wild-type p67 and possesses a dominating effect on eIF2α phosphorylation in mammalian cells. This could be achieved possibly by targeting a key cellular factor that is involved in eIF2α phosphorylation or by inducing the dissociation of eIF2-bound p67 more efficiently and interfering with binding to eIF2γ. Another possibility could be that the $_{60}$AGAA$_{63}$ mutant converted the wild-type p67 into an inactive form via an unknown mechanism. Our present study on protein−protein interaction will possibly provide clues to these important unanswered questions. Among several functions of O-GlcNAc modification in proteins, one is to modulate the turnover and protein−protein interactions (*13*−*16*). For example, estrogen receptors have O-GlcNAc modification sites close to the PEST sequence. The latter sequence motif has been proposed as the signal responsible for rapid degradation (*34*), and O-GlcNAc modification at these sites might increase the turnover rate of the molecule (*15*, *35*). Although we do not find any PEST sequence nearer to the $_{60}$SGTS$_{63}$ site of p67, the lysine-rich domain I ($_{36}$KKKRRKKKK$_{44}$) is very close to this site and is required for its activity (*23*). The two arginine residues within this domain could be the sites for ubiquitin-mediated degradation in the proteosome.

Posttranslation modifications such as phosphorylation and glycosylation require the same or adjacent serine/threonine sites (*13*). The activities of the kinases and phosphatases regulate the status of the phosphorylation at these sites in proteins (*8*, *13*, *30*). In contrast, the activities of *O*-glycosyltransferases (OGTs) and deglycosylases (O-GlcNAcase) regulate the level of O-GlcNAc modification in proteins (*8*, *36*−*39*). We have detected higher levels of the p67-specifc OGT activity in mouse brain as compared to liver and higher levels of deglycosylase activity in liver as compared to mouse brain (data not shown). Although we have not purified such OGT, we have succeeded in purifying p67−DG from rabbit reticulocytes to homogeneity (*22*). Since there are reciprocal relationships between glycosylation by OGT and phosphorylation by kinases, it is not clear from our present study whether this relationship exists in p67. The $_{60}$SGTS$_{63}$ site of p67 is modified by O-GlcNAc, and the same amino acid residues might be involved in phosphorylation by kinase(s). If it does, the specific kinase that phosphorylates p67 will reveal the detailed mechanism of the regulation of eIF2α phosphorylation in protein synthesis initiation.

Our earlier study indicated that the glycosylation of the N-terminal 1−97 amino acid segment of p67 was necessary for its ability to block eIF2α phosphorylation (*23*). This study identified such a site for glycosylation and determined its importance in its activity. On the basis of our biochemical studies, we reported that p67 might have at least 10 putative O-GlcNAc modification sites (*17*). Recently, we also found that constitutive expression of the N-terminal 1−35 amino acid segment in KRC-7 cells did not show any effect on eIF2α phosphorylation (data not shown), indicating that O-GlcNAcation, if present at the $_{8}$SS$_{9}$ cluster, did not have any role in p67's activity. Taken together, these findings indicate that the rest of the serine/threonine sites are possibly present at the 98−480 amino acid segment of p67, and O-GlcNAc modifications at these sites might also be involved in the regulation of the eIF2α phosphorylation as well.

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REFERENCES

1. Merrick, W. C., and Hershey, J. W. B. (2000) in *Translational control of gene expression* (Hershey, J. W. B., Mathews, M. B., and Sonenberg, N., Eds.) pp 33−88, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. Hershey, J. W. B. (1991) *Annu. Rev. Biochem. 60*, 717−755.
3. Datta, B. (2000) *Biochimie 82*, 95−107.
4. Brown, E. J., and Schreiber, S. L. (1996) *Cell 86*, 517−520.
5. Hunter, T., and Pines, J. (1994) *Cell 79*, 573−582.
6. Mendez, R., Myers, M. G., Jr., White, M. F., and Rhoads, R. E. (1996) *Mol. Cell. Biol. 16*, 2857−2864.
7. Hengst, L., and Reed, S. I. (1996) *Science 271*, 1861−1864.
8. Wells, L., Vosseller, K., and Hart, G. W. (2001) *Science 291*, 2376−2378.
9. Comer, F. I., and Hart, G. W. (2000) *J. Biol. Chem. 275*, 29179−29182.
10. Cheng, X., and Hart, G. W. (2001) *J. Biol. Chem. 276*, 10570−10575.
11. Torres, C.-R., and Hart, G. W. (1984) *J. Biol. Chem. 259*, 3308−3317.
12. Holt, G. D., and Hart, G. W. (1986) *J. Biol. Chem. 261*, 8049−8055.
13. Hart, G. W. (1997) *Annu. Rev. Biochem. 66*, 315−335.
14. Han, I., Roos, M. D., and Kudlow, J. E. (1996) *J. Cell. Biochem. 68*, 50−61.
15. Cheng, X., Cole, R. N., Zaia, J., and Hart, G. W. (2000) *Biochemistry 39*, 11609−11620.
16. Roos, M. D., Xie, W., Su, K., Clark, J. A., Yang, X., Chin, E., Paterson, A. J., and Kudlow, J. E. (1998) *Proc. Assoc. Am. Phys. 110*, 422−432.
17. Datta, B., Ray, M. K., Chakrabarti, D., Wylie, D. E., and Gupta, N. K. (1989) *J. Biol. Chem. 264*, 20620−20624.
18. Datta, B., Chakrabarti, D., Roy, A. L., and Gupta, N. K. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3324−3328.
19. Chakraborty, A., Saha, D., Bose, A., Chatterjee, M., and Gupta, N. K. (1994) *Biochemistry 33*, 6700−6706.
20. Ray, M. K., Chakraborty, A., Datta, B., Chattopadyay, A., Saha, D., Bose, A., Kinzy, T. G., Hileman, R. E., Merrick, W. C., and Gupta, N. K. (1993) *Biochemistry 32*, 5151−5159.
21. Datta, B., and Datta, R. (1999) *Exp. Cell Res. 246*, 376−383.
22. Datta, B., Datta, R., Mukherjee, S., and Zhang, Z. (1999) *Exp. Cell Res. 250*, 223−230.
23. Datta, R., Choudhury, P., Bhattacharya, M., Leon, F. S., Zhou, Y., and Datta, B. (2001) *Biochimie 83*, 919−931.
24. Datta, R., Tammali, R., and Datta, B. (2002) *Exp. Cell Res*. (in press).
25. Wu, S., Gupta, S., Chatterjee, N., Hileman, R. E., Kinzy, T. G., Denslow, N. D., Merrick, W. C., Chakrabarti, D., Osterman, J. C., and Gupta, N. K. (1993) *J. Biol. Chem. 268*, 10796−10801.
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
27. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1992) *Current Protocols in Molecular Biology*, Vol. 1, Section 8.1, John Wiley & Sons, New York.
28. Datta, B., Li, B., Choubey, D., Nallur, G., and Lengyel, P. (1996) *J. Biol. Chem. 271*, 27544−27555.

29. Comer, F. I., Vosseller, K., Wells, L., Accavitti, M. A., and Hart, G. W. (2001) *Anal. Biochem. 293*, 169−177.
30. Hanover, J. A. (2001) *FASEB J. 15*, 1865−1876.
31. Comer, F. I., and Hart, G. W. (2001) *Biochemistry 40*, 7845−7852.
32. Griffith, L. S., and Schmitz, B. (1999) *Eur. J. Biochem. 262*, 824−831.
33. Wells, L., Vosseller, K., Cole, R. N., Cronshaw, J. M., Matunis, M. J., and Hart, G. W. (2002) *Mol. Cell. Proteomics 1*, 791−804.
34. Rechsteiner, M., and Rogers, S. W. (1996) *Trends Biochem. Sci. 21*, 267−271.
35. Jiang, M.-S., and Hart, G. W. (1997) *J. Biol. Chem. 272*, 2421−2428.
36. Vosseller, K., Wells, L., and Hart, G. W. (2001) *Biochimie 83*, 575−581.
37. Wells, L., Gao, Y., Mahoney, J. A., Vosseller, K., Chen, C., Rosen, A., and Hart, G. W. (2002) *J. Biol. Chem. 277*, 1755−1761.
38. Gao, Y., Wells, L., Comer, F. I., Parker, G. J., and Hart, G. W. (2001) *J. Biol. Chem. 276*, 9838−9845.
39. Yang, X., Zhang, F., and Kudlow, J. E. (2002) *Cell 110*, 69−80.

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