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N-Glycosylation of ATF6β Is Essential for Its Proteolytic Cleavage and Transcriptional Repressor Function to ATF6α

Dongyin Guan,[1] Hao Wang,[2] Veronica E. Li,[3] Yingying Xu,[1] Min Yang,[1] and Zonghou Shen[1*]

[1] *Department of Biochemistry and Molecular Biology, Shanghai Medical College, Fudan University, 130 Dong-An Road, Shanghai 200032, People's Republic of China*

[2] *Department of Anesthesiology, Zhongshan Hospital, Fudan University, 180 Fenglin Road, Shanghai 200032, People's Republic of China*

[3] *SUNY Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, New York 11203*

ABSTRACT

Activating transcription factor 6 (ATF6), a member of the ATF/CREB family of transcription factors, has two isoforms of 90-kDa (p90ATF6α) and 110-kDa (p110 ATF6β) as endoplasmic reticulum (ER) transmembrane glycoprotein. ATF6β contains five evolutionarily conserved N-linked glycosylation sites and is a key transcriptional repressor of ATF6α, which contribute to regulating the strength and duration of ATF6-dependent ER stress response (ERSR) gene induction. Although it is well established that p110ATF6β can be cleaved and generate a nuclear form of 60-kDa (p60ATF6β) that inhibits ATF6α-mediated ERSR genes activation, the functional significance of p110 ATF6β N-linked glycosylation is unknown. Herein, we found that the fully unglycosylated ATF6β cannot be proteolytic cleaved, be detectable in nucleus after dithiothreitol treatment, and repress the transcriptional activity of ATF6α. These results provide the first evidence that unglycosylated ATF6β may directly facilitate the expression of ERSR genes by losing its repressor function to ATF6α. J. Cell. Biochem. 108: 825–831, 2009.

KEY WORDS: ATF6β; N-GLYCOSYLATION; ER STRESS RESPONSE

Disturbances in the normal functions of the endoplasmic reticulum (ER) lead to an evolutionarily conserved cell stress response, termed the unfolded protein response (UPR), which is an intracellular signaling pathway from ER to the nucleus. This signaling pathway activates the transcription of appropriate genes whose products are required to cope with the disturbance, leading to the maintenance of homeostasis [Schroder and Kaufman, 2005; Xu et al., 2005].

The product of the G13/CREB-RP gene (G13) is a type II transmembrane glycoprotein in the ER. Because of its high degree of homology to ATF6α, G13 has been named ATF6β [Haze et al., 2001]. Either as a homodimer or as a heterodimer with ATF6α, ATF6β binds to the ER stress response (ERSR) element to active UPR target genes [Yoshida et al., 2001]. Compared with ATF6α, ATF6β possesses very low specific transcriptional activity and, most likely by virtue of this characteristic, ATF6β can serve as a repressor of ATF6α-mediated ERSR genes induction and regulate the strength and duration of ATF6α-mediated ERSR genes activation during the ERSR [Thuerauf et al., 2004, 2007]. ATF6β is a 703-amino acid glycoprotein with the electrophoretic mobility of a 110-kDa protein (p110ATF6β). ER stress induces proteolysis of the membrane-bound p110ATF6β, releasing the soluble amino portion of p60ATF6β. The p60ATF6β relocates to the nucleus and binds to a wide variety of ER stress-inducible promoters, of which glucose regulated protein 78 (GRP78)/Bip is the most well characterized [Haze et al., 2001; Thuerauf et al., 2002; Okada et al., 2003].

N-glycosylation is known to play a pivotal role in protein folding, protein stabilization, quality control, and degradation [Rudd and Dwek, 1997; Helenius and Aebi, 2004]. In our previous work, blocking of N-acetylglucosaminyltransferase V (GnT-V), which

Abbreviations used: ATF6, activating transcription factor 6; GRP78, glucose regulated protein 78; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ERSR, ER stress response; Tg, thapsigargin.

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*Correspondence to: Zonghou Shen, Department of Biochemistry and Molecular Biology, Shanghai Medical College, Fudan University, 130 Dong-An Road, Shanghai 200032, People's Republic of China. E-mail: zhshen@shmu.edu.cn

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catalyzes the formation of β-1,6-GlcNAc linkage in the complex-type N-glycans of C2 C2, 6 tri-antenna or C2, 4 C2, 6 tetra-antenna, induces ER stress in human hepatocarcinoma 7,721 cells [Fang et al., 2006]. Blocking of α1,2-mannosidase, which catalyzes the trimming of the "high mannose" chains involving four α1,2-linked mannose residues and generates Man5GlcNAc2, also induces ER stress in human hepatocarcinoma 7,721 cells [Lu et al., 2006]. It is reported that p90ATF6α mutants targeting single or multiple N-glycosylation sites show higher constitutive transcriptional activity than wild-type [Hong et al., 2004]. So, N-glycosylation may play an important role in ERSR.

In the ER lumen, human p110ATF6β exists constitutively as a glycosylated protein. It contains five potential N-glycosylation sites at Asn residues 476, 505, 610, 627, and 676 on the basis of amino acid sequence analysis (GenBank Accession Q99941). The five potential N-glycosylation sites also lie at Asn residues 473, 502, 607, 624, and 673 in mouse p110ATF6β (GenBank Accession O35451). To the best of our knowledge, the functional significance of p110ATF6β N-linked glycosylation is unknown. So we obliterated each potential N-glycosylation sites of human p110ATFβ by substituting Gln for Asn. This method is generally considered to lead to the least perturbation of protein function. The mutated cDNAs with FLAG tag were expressed in HeLa cells. In present study, we found that unglycosylated all potential N-glycosylation sites, but not single sites, mutant can block the cleavage of p110ATF6β. Further studies revealed that N-glycosylation of p110ATF6β may affect the repressor function to ATF6α.

MATERIALS AND METHODS

PLASMIDS CONSTRUCTION AND SITE-DIRECTED MUTAGENESIS

The plasmid pcDNA3.1-ATF6α and pcDNA3.1-ATF6β containing human full-length ATF6α and ATF6β cDNA were kindly supplied by Kazutoshi Mori (Kyoto University)[Haze et al., 2001]. To distinguish from endogenetic ATF6β, we added FLAG tag at the N terminus as described in Figure 1A. Then we used pcDNA3.1-FLAG-ATF6β as template to construct various mutants, which targeted to destroy the N-glycosylation sites, using a QuikChange site-directed mutagenesis kit (Stratagene). The mutated base in the construct was confirmed by DNA sequencing.

pGL3-GRP78promoter-Luc encodes the GRP78 promoter from −275 to +7 driving luciferase, and its construction has been described previously [Thuerauf et al., 2004]. The GRP78 promoter was created by PCR using HeLa genomic DNA as a template and cloned into pBluescript II KS plasmid using *Eco*RI and *Sal*I restriction sites. Then pKS-GRP78promoter-Luc was digested with *Sac*I and *Xho*I and the digested fragment was ligated into the vector pGL3 (Promega).

CELL CULTURE AND TRANSFECTION

HeLa cells and HEK-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10%(v/v) fetal calf serum. Cells were maintained in an incubator under air/CO_2 (19:1) at 37°C. HeLa and HEK-293 cells were transfected with the plasmid by Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacture's instructions.

RT-PCR

Following transfection with wild-type ATF6β and various mutant plasmids, HeLa cells were incubated in 10% fetal calf serum for 48 h. Total RNA (5 μg) extracted from HeLa cells with Trizol reagent (Invitrogen) according to the manufacture's instructions were used as templates for cDNA synthesis. Reverse transcription was carried out by ReverTra Ace (Toyobo) and then cDNAs were subjected to PCR. Primers used for PCR were as follows: ATF6β forward primer 5′-CCGATCTTCCCAGATCTTCAG-3′ reverse primer 5′-CACCCTG-GATGAGGACAACTG-3′ β-actin forward primer 5′-TGGGCATG-GGTCAGAAGGAT-3′ and reverse primer 5′-AAGCATTTGCGGTG-GACGAT-3′. The PCR products were electrophoresed on agarose gel, visualized by ethidium bromide (EB) staining on an image system.

WESTERN BLOTTING

Whole cell extracts were prepared by lysing cells with 1× SDS lysis buffer containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, 10 mM NaF, 2 mM Na_3VO_4, and 5 μg/ml leupeptin. Lysates were then collected by centrifugation at 4°C. Then 50 μg of proteins were loaded into 12% SDS–polyacrylamide gels for electrophoresis and they were transferred to polyvinylidene difluride (PVDF) membranes. The membranes were blocked with 5% nonfat milk power for 1 h at room temperature and then were incubated overnight with primary antibody (anti-FLAG monoclonal antibody (Rockland) or anti-GRP78 monoclonal antibody (Cell Signaling Technology)) at 4°C. Following washing and incubation with horseradish peroxidase-conjugated secondary antibody (Kang-Chen Biotech) for 2 h at room temperature, the membranes were washed, detected by a Pierce chemiluminescent substrate (Rockford), and exposed to X-ray film.

INDIRECT IMMUNOFLUORESCENCE ANALYSIS

Following transfection with wild-type ATF6β or fully unglycosylated ATF6β (Quintuple M) plasmids, HeLa cells were incubated in 10% fetal calf serum for 42 h. Then the transfected cells treated with dithiothreitol (10 mM) for 0, or 0.5, and 1.5 h. After washing twice with PBS, the cells were fixed with 0.4% paraformaldehyde, washed with PBS, permeabilized with 0.1% bovine serum albumin and 0.2% Triton X-100 in 1× TBST, and blocked with 5% bovine serum albumin/TBST for 1 h. For detection of wild-type or mutated ATF6β, cells were incubated with anti-FLAG monoclonal antibody (dilution 1:200) for 1 h at 37°C. Then after washing twice with TBST, cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody (dilution 1:100, KPL). To detect nucleus, HeLa cells were incubated with Hoechst 33258 (5 μg/ml) at room temperature for 10 min. Images were captured and analyzed with a fluorescent microscope (OLYMPUSB, IX51).

LUCIFERASE ASSAYS

HeLa cells cultured in 24-well plates were transfected with 0.4 μg reporter plasmid carrying the firefly luciferase gene, driven by the GRP78 promoter, and 0.04 μg pRL-SV40, as an internal control, in the presence of 0.4 μg wild-type ATF6β or various mutant plasmids, indicated in Figure 4. After transfected for 24 h, HeLa cells induced with or without 300 nM thapsigargin (Tg) for 16 h. The transfected cells were lysed and assayed for firefly and Renilla luciferase activity

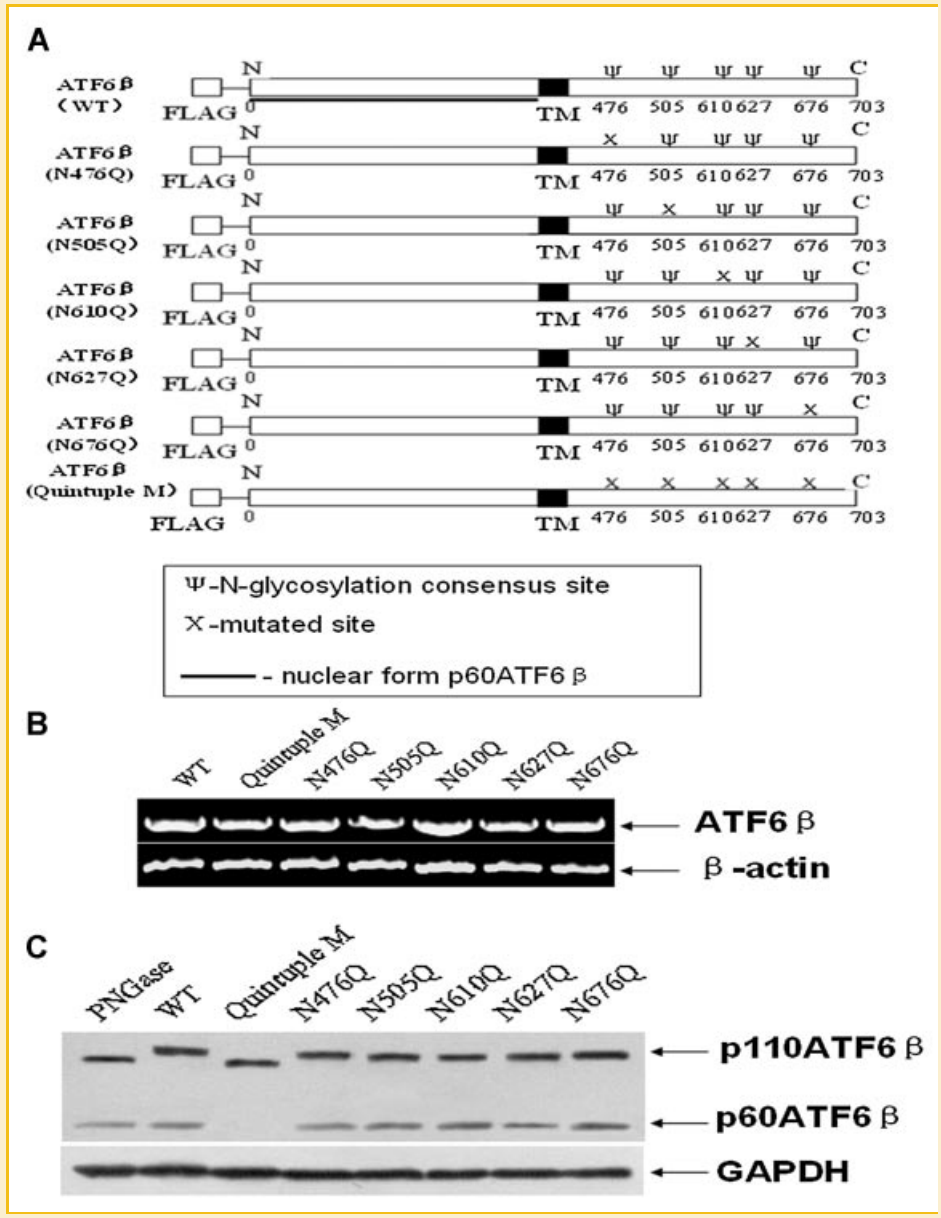

Fig. 1. Express ATF6β wt and mutant molecules in HeLa cells. A: Schematic drawing of wild-type p110ATF6β, and glycosylation-deficient mutants targeting to single or all five N-glycosylation sites. TM, transmembrane domain. The pcDNA3.1 vectors used for the transfections contained the FLAG tagged full-length ATF6β with various N-glycosylation sites. B: The mRNA levels of p110ATF6β in wt and mutants were detected by RT-PCR. C: The protein levels of p110ATF6β in wt and mutants were detected by Western blotting. Took PNGase F treated p110ATF6β wt as the fully nonglycosylated form control.

using the dual luciferase kit (Promega). The results were normalized to the Renilla luciferase activity of the internal control. Each experiment was repeated four times. The average and the SE are shown.

RESULTS

EXPRESSION OF WILD-TYPE ATF6β AND MUTANT MOLECULES IN HELA CELLS

To examine the functional importance of the individual glycosylation sites of the ATF6β, five N-glycosylation-deficient mutants were generated (Fig. 1A) and were transiently expressed in HeLa cells. PNGase F is an amidase that cleaves most N-glycans, including high mannose, hybrid, and complex structures, at the asparagine residues [Maley et al., 1989]. So, we take PNGase F treated p110ATF6β wt as the fully nonglycosylated form control. If Asn476, Asn505, Asn610, Asn627, and Asn676 is glycosylated, each of the N476Q, N505Q, N610Q, N627Q, and N676Q mutants should exhibit an electrophoretic mobility faster than the fully glycosylation form (p110ATF6β wt) but slower than the fully nonglycosylated form. As shown in Figure 1C, each of the five mutants, targeting to single site, exhibited an electrophoretic mobility faster than the fully glycosylation form (lane 2) and slower than the fully nonglycosylated form (lane 1). But the bands of these five mutants are more

close to the band of fully glycosylation than to the band of fully nonglycosylated form, which indicated that these five mutants are slightly glycosylated. To better investigate the role of N-glycosylation to p110ATF6β, Quintuple M was created, which contains all five mutations (N476Q, N505Q, N610Q, N627Q, and N676Q). As shown in Figure 1C, Quintuple M exhibited same electrophoretic mobility to the fully nonglycosylated form. Interesting, the protein of p60ATF6β in Quintuple M was undetectable. But the mRNA levels of ATF6β wt and all ATF6β mutants were similar (Fig. 1B). Similar results were observed in HEK-293 cells (data not shown).

N-GLYCOSYLATION IS ESSENTIAL FOR THE PROTEOLYTIC CLEAVAGE OF ATF6β

ATF6β can be further activated in response to ER stress. To investigate that whether p60ATF6β of Quintuple M can be detectable in ER stress condition, after ATF6β wt and Quintuple M were transiently transfected in HeLe cells for 12 h, 300 nM Tg, a typical inducer of ERSR, was added for 36 h. Then the total protein lysates were collected and subjected to Western blotting analysis. As show in Figure 2A, p60ATF6β was also undetectable in both Quintuple M with or without Tg treatment.

There are two possibilities for the absence of p60ATF6β in Quintuple M. One possibility is that most of p60ATF6β in Quintuple M degraded. Or unglycosylated p110ATF6β blocked its proteolytic cleavage. In mammalian cells, proteasome and lysosome provide the important pathways for most protein degradation [Lee and Goldberg, 1998]. To determine the first possibility, 50 μM MG132, inhibitor of proteasomes, and 40 μM chloroquine, inhibitor of lysosome, were added for 4 h, after ATF6β wt and Quintuple M were transiently transfected in HeLa cells for 36 h. As shown in Figure 2C,D, p60ATF6β was undetectable in both Quintuple M with or without Tg treatment. This evidence indicates that N-glycosylation of ATF6β is essential for its proteolytic cleavage.

FULLY UNGLYCOSYLATION AFFECTS THE TRANSCRIPTIONAL REPRESSOR FUNCTION OF p110ATF6β

To examine the functional consequence of the fully unglycosylated p110ATF6β, we first compared the cellular distribution of ATF6β of Quintuple M and wild-type through indirect immunofluorescence analysis. As shown in Figure 3A, at time 0 in control cell, most of ATF6β was present in perinuclear structures that were distinct from the nucleus stained with hoechst 33258, (hoechst 33258 is a specific marker for nucleus) as expected. After 0.5 h of treatment with dithiothreitol (5 mM), ATF6β had become concentrated in nucleus. After 1.5 h of treatment with dithiothreitol, there was higher concentration of ATF6β in the nucleus. In cells transfected with Quintuple M, there were very faint or even undetectable ATF6β in nucleus (Fig. 3B). We also analyzed the HeLa cells extracts by Western blotting. As show in the Figure 3C, p60ATF6β of Quintuple M cannot be detectable with or without dithiothreitol treatment. These results indicate that the fully unglycosylated p110ATF6β cannot relocate in nucleus.

It is reported that both ATF6α and ATF6β can bind to a wide variety of ER stress-inducible promoters, of which GRP78/Bip is the most characterized. To further investigate the functional consequence of the fully unglycosylated p110ATF6β, HeLa cells were cotransfected with a luciferase reporter gene driven by the GRP78 promoter and equal amounts of the expression vector for pcDNA3.1, ATF6α, ATF6β, or Quintuple M, and took Renilla luciferase gene as the internal control. As shown in Figure 4, as expected, compared with vector, ATF6β exhibited a similar

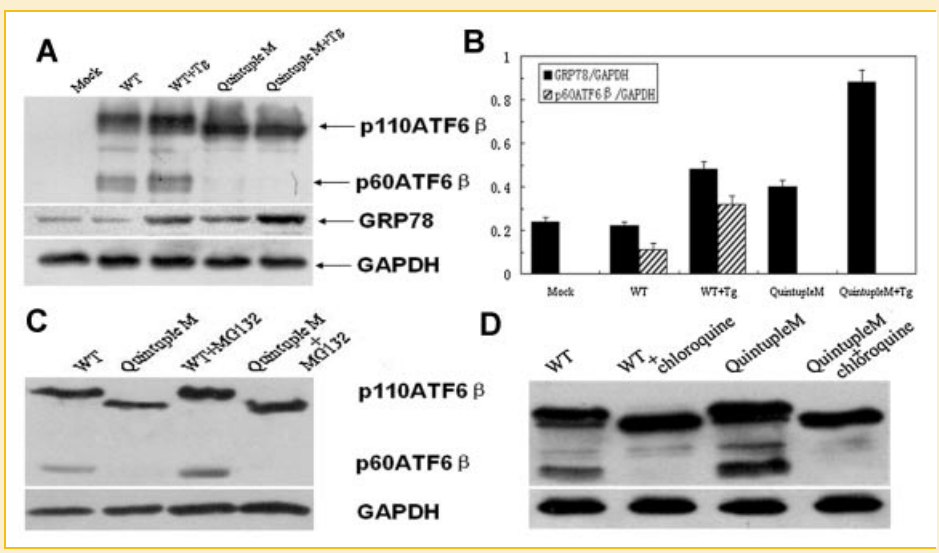

Fig. 2. N-Glycosylation is essential for the proteolytic cleavage of ATF6β (A,B). HeLa cells were transfected with p110ATF6β wt or Quintuple M or an empty vector. After 12 h, the cells were treated with 300 nM thapsigargin (Tg) for 36 h. The cell lysates were then prepared for Western blotting analysis. A typical blot (A) and densitometric scans of triplicate blots (B) are shown. Values are presented as mean ± SD. C,D: HeLa cells were transfected with fully glycosylation form (p110ATF6β wt) or unglycosylated form (Quintuple M). After 36 h, the cells were treated with 50 μM MG132 (C) or 40 μM chloroquine (D) for 36 h. The cell lysates were then prepared for Western blotting analysis.

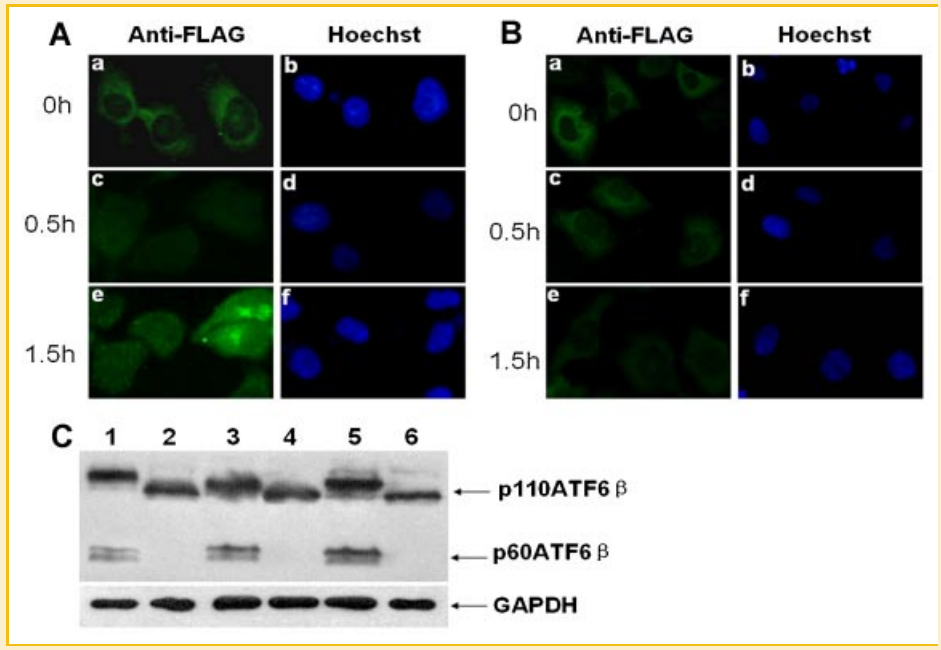

Fig. 3. Location of p110ATF6β wt and Quintuple M (A,B). Indirect Immunofluorescent analysis of HeLa cells treated with dithiothreitol. A: Location of p110ATF6β wt. B: Location of Quintuple M. HeLa cells untreated (a,b) or treated with 5 mM dithiothreitol for 0.5 h (c,d) or 1 h (e,f) were stained with anti-FLAG antibody (a,c,e) or hoechst 33258 (b,d,f) (original magnification 200×). C: Western blotting analysis of HeLa cells treated with dithiothreitol. HeLa cells transfected with p110ATF6β wt (1,3,5) and Quintuple M (2,4,6) untreated (1,2) or treated with 5 mM dithiothreitol for 0.5 h (3,4) or 1 h (5,6) were prepared for Western blotting. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

transcriptional activity in unstressed cells, whereas ATF6β exhibited a low transcriptional activity in stressed cells. ATF6β inhibited ATF6α-mediated GRP78 promoter activation. However, Quintuple M exhibited a similar transcriptional activity as vector and did not block ATF6α-mediated GRP78 promoter activation, which is consistent with the Western blotting result shown in Figure 2A,B.

DISCUSSION

ERSR, which is a common cellular response triggered by dysfunction of ER, has been implicated in health and diseases including diabetes, inflammation, neurodegenerative disorders, and cancer [Schroder and Kaufman, 2005; Xu et al., 2005; Yoshida, 2007]. A central transcriptional factor that regulates ERSR is ATF6α. ATF6β serves as

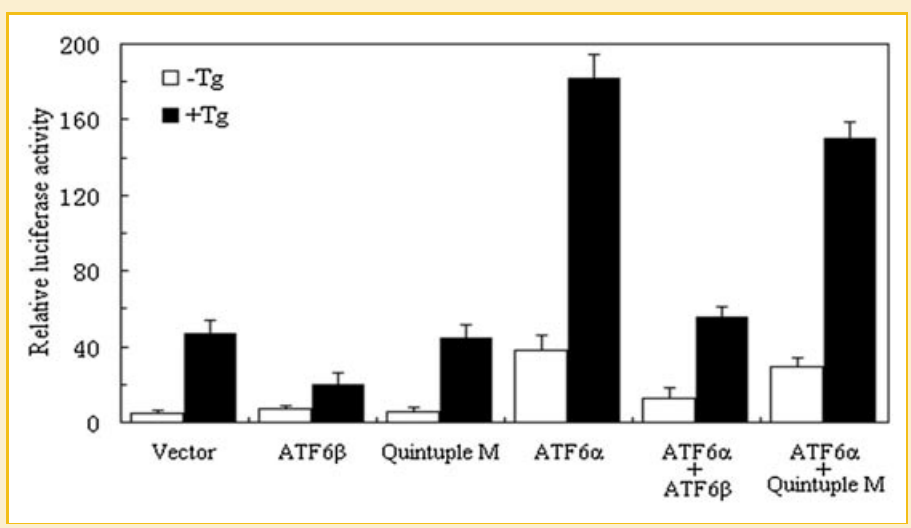

Fig. 4. Transcriptional repress effect of ATF6β or Quintuple M to ATF6α on Tg-activated GRP78 Promoter-Luc. HeLa cells were cotransfected with 0.4 μg GRP78 Promoter-Luc and 0.04 μg pRL-SV40, in the presence of 0.4 μg wild-type ATF6β or various mutant plasmids as indicated. After 24 h, HeLa cells induced with or without 300 nM Tg for 16 h, and then were lysed and assayed for firefly and Renilla luciferase activity. Values from quadruple experiments are presented as mean ± SD.

transcriptional repressor functioning in part to regulate the strength and duration of ATF6α-mediated ERSR genes induction during the ERSR. It is reported that underglycosylation of ATF6α shows higher constitutive transcriptional activity than the fully glycosylation ATF6α [Shen et al., 2002; Thuerauf et al., 2004, 2007]. The full-length form of ATF6β also exists constitutively as a glycosylated protein, and the function of this modification is not known.

To investigate the role of glycosylation of ATF6β, we constructed several mutants, in which single or multiple N-glycosylation sites are destroyed. We found that the fully unglycosylated ATF6β cannot be proteolytic cleaved. This may be can explain that why p110 ATF6β contains N-linked glycosylation sites within its ER luminal domain and why these N-glycosylation sites are evolutionarily conserved between human and mouse p110 ATF6β. But the question that why the fully unglycosylated ATF6β cannot be proteolytic cleaved needs more investigation. One straightforward explanation is that the states of N-glycosylation affect the protein topology or protein–protein interaction, which required for its processing. Another nonmutually exclusive possibility is that Quintuple M prevents normal localization of ATF6β and as such mutant cannot be available for processing. The colocalization of ATF6β and 58K-like protein/formiminotransferase cyclodeaminase (FTCD), a marker for the Golgi complex, was investigated. Because ATF6β must relocate to the Golgi apparatus to be proteolytic cleaved. But, there was no significant difference in distribution of ATF6β between wild-type and Quintuple M plasmids transfected HeLa cells (data not shown). Other mechanisms could be more complex and involve novel pathways, because the mechanism of the processing of ATF6β is not fully understood. It is reported that, in response to tunicamycin, the cleavage of ATF6α was shown to be maximal after approximately 3–4 h, whereas the cleavage of ATF6β did not reach a maximum until 8 h after tunicamycin treatment [Haze et al., 2001; Thuerauf et al., 2004, 2007]. The different induction time of processing indicates that there may be a mechanism to control the strength and duration of the processing of ATF6α and ATF6β.

Next, we found that the unglycosylated ATF6β cannot be relocated in nucleus and losts the repressor function to ATF6α. It is of interest to consider the consequences of blocking the transcriptional repressor function of the unglycosylated ATF6β. In normal condition, ATF6β act as the repressor of ATF6α, which inhibits the expression of ERSR genes. In unglycosylated states, ATF6α enhances its transcriptional activity, whereas ATF6β lost its repressor function, which facilitates the expression of ERSR genes. So, the N-glycosylation of ATF6α and ATF6β are correlative to the expression of ERSR genes.

Actually, there are other examples of unglycosylated proteins losing function. E-cadherin mediates calcium-dependent cell–cell adhesion between epithelial cells. It is reported that removal of the N-glycans of E-cadherin caused a significant decrease in calcium-dependent cell–cell adhesion accompanied with elevated cell migration [Zhao et al., 2008]. Fully unglycosylated of human gastric lipase reduced the specific enzymatic activity of recombinant HGL, measured on short- and long-chain triglycerides, to about 50% of normal values [Wicker-Planquart et al., 1999]. Removal of N-linked glycans from secreted cryptococcal PLB1 leads to loss of enzyme activity [Turner et al., 2006]. Disturbances of protein glycosylation also affect the protein function. The overexpression of GnT-V in gastric cancer cells leads to severe peritoneal dissemination in athymic mice, which can be attributed to the increased expression of matriptase. This increase was due to the acquired resistance of matriptase to degradation, since it is glycosylated by GnT-V and a corresponding increase in the active form [Ihara et al., 2002].

In summary, our study uncovered that the unglycosylated ATF6β cannot be proteolytic cleaved and losts its repressor function to ATF6α. Most studies reported that URP triggered by unglycosylated protein is because of the unfold protein accumulation in ER lumen [Lecca et al., 2005; Lehrman, 2006; Zhang and Kaufman, 2006]. Our study provides a novel mechanism that unglycosylated ATF6β may directly facilitate the expression of ERSR genes. Further studies examining the unglycosylated forms of other glycoprotein, which involved in ERSR, will be required to fully appreciate the role of N-glycosylation in ER stress.

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