Underglycosylation of ATF6 as a Novel Sensing Mechanism for Activation of the Unfolded Protein Response

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ATF6 is a key transcriptional activator of the unfolded protein response (UPR), which allows mammalian cells to maintain cellular homeostasis when they are subjected to a variety of environmental and physiological stresses that target the endoplasmic reticulum (ER). ATF6, a 90-kDa ER transmembrane protein, contains three evolutionarily conserved N-linked glycosylation sites within its carboxyl luminal domain. Although it is well established that p90ATF6 activation requires transient transport from the ER to the Golgi, where it is cleaved by the S1P/S2P protease system to generate a nuclear form p60ATF6 that acts as a transcriptional activator, the functional significance of p90ATF6 N-linked glycosylation is unknown. Here we show that ER Ca\(^{2+}\) depletion stress, a triggering mechanism for the UPR, induces the formation of ATF6(f), which represents de novo partial glycosylation of newly synthesized p90ATF6. By mutating a single amino acid within the N-linked glycosylation site closest to the carboxyl terminus of p90ATF6, we recreated ATF6(f). This mutation sharply reduces p90ATF6 association with calreticulin, a major Ca\(^{2+}\)-binding chaperone for N-glycoprotein. We further determined that ATF6(f) exhibits a faster rate of constitutive transport to the Golgi, resulting in a higher level of p60ATF6 in the nucleus and stronger transactivating activity in the absence of ER stress. Additional analysis of p90ATF6 mutants targeting single or multiple N-linked glycosylation sites also showed higher constitutive trans-activating activity than wild type ATF6. Because accumulation of underglycosylated proteins in the ER is a potent inducer for the UPR, these studies uncover a novel mechanism whereby the glycosylation status of p90ATF6 can serve as a sensor for ER homeostasis, resulting in ATF6 activation to trigger the UPR.

The discovery that ATF6 is a key transcriptional activator of endoplasmic reticulum (ER\(^{1}\))-resident molecular chaperones and folding enzymes in the unfolded protein response (UPR) has revealed novel molecular mechanisms employed by mammalian cells to respond to stresses that perturb ER homeostasis. ATF6, a 670-amino acid glycoprotein with the electrophoretic mobility of a 90-kDa protein (p90ATF6), is constitutively expressed in a variety of mammalian cells (1). It contains a single transmembrane domain with 272 amino acids oriented in the ER lumen. ER stress induces proteolysis of the membrane-bound p90ATF6, releasing the soluble amino portion of p60ATF6, which relocates to the nucleus and activates the transcription of a wide variety of ER stress-inducible promoters, of which Grp78/BiP is the most well characterized (2–4). Conservation of protease cleavage sites led to the discovery that Golgi-localized S1P/S2P proteases that process sterol response element binding proteins in response to cholesterol deprivation also process ATF6 in response to ER stress (4). Specific luminal domains of p90ATF6 have been mapped which are required for translocation to the Golgi (5). It was further determined that GRP78 retains p90ATF6 in the ER by inhibiting its Golgi localization signals (6). However, whether other proteins also contribute to the ER retention of p90ATF6 and how ER stress causes ATF6 transit to the Golgi remain to be determined.

p90ATF6 exists constitutively as a glycosylated protein. The consensus sequence for N-linked glycosylation sites consists of the Asn-X-Ser/Thr, where X denotes any amino acid except proline (7). The ER luminal domain of human p90ATF6 contains three such sites at 472, 584, and 643 amino acids, and the three sites are also conserved in mouse p90ATF6. The fully glycosylated form of ATF6, a 670-amino acid protein, exhibits an electrophoretic mobility of 90 kDa in denaturing SDS-gels in part because of the glycosylation modifications. N-Linked glycosylation has been shown to play a pivotal role in protein folding, sorting, and transport (8). Thus far, the functional significance of N-linked glycosylation of p90ATF6 is not understood. Interestingly, blockage of N-linked protein glycosylation by treating cells with reagents such as tunicamycin (Tu) results in malfolded protein formation in the ER, ATF6 activation, and the onset of UPR. In Tu-treated cells, p90ATF6 is completely non-glycosylated (2). In cells treated with DTT, a small fraction of p90ATF6 becomes O-linked glycosylated in the Golgi (6). In cells treated with thapsigargin (Tg), which depletes the ER Ca\(^{2+}\) store by specific inhibition of the ER Ca\(^{2+}\)-ATPase (9), we reported earlier that in kinetics in parallel with activation of the UPR, p90ATF6 showed an additional appearance of a faster migrating form distinct from the completely nonglycosylated form ATF6 (3). While searching for the nature and functional significance of this new form of p90ATF6, we discovered that ER Ca\(^{2+}\) depletion stress induces the formation of a newly synthesized, par-
secretion of ATF6 is required for the activation of the UPR. This process is mediated by the translocation of the ATF6 precursor (p90ATF6) from the ER to the Golgi apparatus, where it is glycosylated and proteolytically cleaved into its active form (p50ATF6). The resulting polypeptide contains an N-terminal transactivation domain that enables it to translocate into the nucleus and activate transcription of UPR target genes.

The expression of p90ATF6 was assessed using Western blot analysis. The transfected cells were grown in normal culture conditions or in the presence of tunicamycin, a drug that inhibits N-linked glycosylation. The efficiency of transfection was monitored using a reporter gene construct that encodes firefly luciferase under the control of a minimal promoter. The cell extracts were assayed for luciferase activity, and the results were normalized to the internal control (β-galactosidase activity) to correct for transfection efficiency.

TRAFC6 activation to trigger the UPR.

p90ATF6 can serve as a sensor for ER homeostasis, resulting in a higher constitutive transactivating activity than wild-type ATF6. Because accumulation of underglycosylated proteins in the ER is a potent inducer for the UPR, these studies uncover a novel mechanism whereby the glycosylation status of p90ATF6 can serve as a sensor for ER homeostasis, resulting in ATF6 activation to trigger the UPR.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions—CHO-7, NIH3T3, and 293T cells were maintained in high glucose Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin-neomycin antibiotics at 37 °C. The CHO-7 and 293T-defective M19 cells were generously provided by Dr. Joseph L. Goldstein (University of Texas, Southwest Center for Medical Education, Dallas) and have been described previously (4). For stress induction, cells were grown to 80% confluence and treated with 300 μM Tg or 1.5 μg/mL Tu for various time intervals as indicated. Tg, Tu, and cycloheximide (CHX) were purchased from Sigma. Endoglycosidase H was purchased from Roche Applied Science.

Plasmids—The plasmid pCGN-ATF6 containing hemagglutinin epitope (HA)-tagged full-length human ATF6 driven by the cytomegalovirus promoter was provided by Dr. Ron Prywes (Department of Biological Science, Columbia University) and its construction has been described previously (1). pTK-HSV-ATF6 was provided by Dr. Joseph L. Goldstein, and its construction has been described previously (4). The site-specific mutants pCGN-ATF6(T645I), pCGN-ATF6(T586I), pCGN-ATF6(T474I), pCGN-ATF6(DoubleM), pCGN-ATF6(TripleM), and pTK-HSV-ATF6(T645I) were constructed using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutated base in the construct was confirmed by DNA sequencing. The construction of the −169/Luc reporter gene, which spans from −169 to −29 of the rat Grp78 promoter with all three endoplasmic reticulum stress elements, has been described previously (11, 12).

Transection and Assay of Reporter Gene Activity—CHO-7, 293T, CHO-7, or M19 cells were grown to 60–80% confluence. One μg of the −169/Luc reporter gene was cotransfected with various amounts of pCGN-ATF6 or pTK-HSV-ATF6 plasmids using PolyFect or SuperFect reagents (Qiagen, Valencia, CA). Empty vector was added to adjust the total amount of plasmids to be the same, and the β-galactosidase reporter gene driven by the cytomegalovirus promoter was used as an internal control for transfection efficiency as described previously (3). The transfected cells were either grown in normal culture conditions or subjected to Tg and Tu treatment. After drug treatment, cell lysates were prepared for Western blots or luciferase assays, which were performed using the Luciferase Assay System (Promega, Madison, WI). Each transfection was performed in duplicate and repeated three or four times.

Western Blotting—Conditions for Western blot analysis of endoglycosylated ATF6 using a rabbit polyclonal ATF6 antibody, HA-ATF6, and β-actin have been described previously (3). For the detection of endoglycosylated ATF6 using a mouse monoclonal antibody against the amino-terminal 273 amino acids of ATF6 (Imgenex, San Diego), the primary antibody was diluted at 1:500 in 5% milk and incubated with the transferred membranes at 4 °C overnight. The secondary antibody used was horseradish peroxidase-conjugated goat-anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:3,000. For the detection of H9262 HVATF6, the mouse monoclonal anti-HSV tag (Novagen, Madison, WI) antibody was used as the primary antibody at a dilution of 1:1,000. Western blot analysis of Grp78 was performed using an anti-Grp78 mouse monoclonal antibody (StressGen, Victoria, BC, Canada) used as the primary antibody at a dilution of 1:1,500. For detection of CTR, the anti-CTR mouse monoclonal antibody (Calbiochem) was used at a dilution of 1:1,000. For detection of calnexin (CNX), the anti-CNX rabbit polyclonal antibody (StressGen) was used at a dilution of 1:2,000.

Biochemical Fractionation—293T cells grown in 150-mm-diameter dishes to 70% confluence were transfected with various pTK-HSV-ATF6 expression plasmids. The conditions for the preparation of soluble nuclear protein have been described previously (3). For the isolation of microsomes, 293T cells transfected with pCGN-ATF6 or pCGN-ATF6(T645I) expression plasmids were processed for preparation of microsomes as described (13).

Coimmunoprecipitation Assays—The conditions for coimmunoprecipitation have been described previously (3). 200 μg of microsome extract from each sample were incubated with protein A-Sepharose beads (Sigma) and 10 μl of mouse anti-HA monoclonal antibody (Santa Cruz Biotechnology) or 10 μl of rabbit anti-CRT polyclonal antibody (Biomedical Technologies). After the incubation, the beads were washed four times. The immunoprecipitates were resolved by SDS-PAGE and then subjected to silver staining with a SilverQuest silver staining kit (Invitrogen) or Western blotting.

Northern Blotting—The methods for total cellular RNA extraction and Northern blot hybridizations have been described (14). The cDNA probes used for the detection of Grp78 and glyceraldehyde-3-phosphate dehydrogenase have been described previously (15). RT-PCR Conditions—RT-PCR was performed using the SuperScript™ Preamplification System (Invitrogen). For each reaction, 5 μg of total RNA and 1 μl of 0.5 μg/μl oligo(dT) were incubated in sterilized diH2O for total volume of 12 μl at 70 °C for 10 min, then on ice for 5 min, and spun down. Four μl of 5× first strand buffer, 2 μl of 0.1 μM DTT, 1 μl of 10 mM dNTP were added subsequently and incubated in a 42 °C water bath for 2 min. 200 units of SuperScript reverse transcriptase were added. The reactants were incubated at 42 °C for 1 h and then inactivated at 70 °C for 5 min. Two μl of RT product was subjected to PCR using AmpliTaq Gold™ (PerkinElmer Life Sciences) at a final magnesium concentration of 1.5 mM. The PCRs were first heated for 3 min at 94 °C. The PCR cycle was 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. After the completion of 45 PCR cycles, the mixture was incubated for 7 min at 72 °C. The PCRs were performed using GeneAmp PCR system 2400 (PerkinElmer Life Sciences). The primers used were: 5′-ATF-5′, 5′-GGGATCCAGCTGAAGACA-3′ (base 1121–1141); 5′-ATF-TM, 5′-CTCTTTAGCAGCACAGAAATCCTAG-3′ (base 1297–1317 of the ATF6 coding sequence); 5′-HindIII-hATF, 5′-AACCACAACTGAGCCTGGTGGT-3′ (base 4–21 of the ATF6 coding sequence with the addition of the HindIII site at the 5′ end); 5′-BamHI-hATF, 5′-CTTGAGCACGTGGC CCTGTTAGTACGAC-3′ (base 996–1010 of the ATF6 coding sequence with the addition of the BamHI site at the 5′ end).

Immunoassay—The CHO-7 cells were plated in 8-well Tek-II slides (Naige-Nuc, Naperfield, IL) at 40% confluence and transfected with either HA-ATF6 or HA-ATF6(T645I) using Polyfect reagent. 36 h after transfection, the cells were treated with either 10 μM DTT (tunicamycin, the Applied Science) or 2.5 μg/mL brefeldin A (Sigma) in 4% formaldehyde. The slides were incubated with anti-HA rabbit polyclonal antibody (Santa Cruz Biotechnology) at a 1:500 dilution in TBS-Tween buffer with 1% bovine serum albumin, followed by incubation in 4 μg/mL of PBS of anti-rabbit polyclonal Texas Red-conjugated secondary antibody (Vector Laboratories, Burlingame, CA). Golgi staining was accomplished by incubating the fixed cells with a 1:500 dilution of fluorescence-conjugated anti-Golgi antibody (BD Biosciences). The cells were visualized on a Zeiss confocal microscope at 400× magnification. Five images of each time point containing at least 250 cells were taken and numbered to facilitate a blind controlled observation. Cells containing Golgi-localized HA-ATF6 were counted and confirmed by two separate investigators. The experiment was repeated twice.

RESULTS

ER Ca2+ Depletion Induces a New Form of Full-length ATF6—Upon treatment of NIH3T3 cells with Tg, the level of Grp78 mRNA remained low during the first 2 h and gradually increased to a high level that was sustained up to 16 h (Fig. 1A). The steady-state level of endogenous p90ATF6 as detected by a polyclonal antibody showed a transient reduction during the first 2 h of Tg treatment; however, the level of p90ATF6 was replenished by 4 h and by 8 h, the amount of ATF6 was elevated instead, correlating with sustained high level induction of Grp78 mRNA (Fig. 1B). In addition, the replenished form of p90ATF6 appeared to consist of a doublet band, referred to below as ATF6 and ATF6(f). Longer electrophoresis time further resolved the p90ATF6 doublet into two distinct conditions.
conserved N-linked glycosylation sites within its ER luminal domain, and in nonstressed cells p90ATF6 is a glycosylated protein (Fig. 3A). One mechanism for ATF6(f) formation is that Tg treatment results in partial glycosylation of a fraction of p90ATF6. In this scenario, ATF6(f) should exhibit an electrophoretic mobility faster than the fully glycosylated form but slower than the fully nonglycosylated form. If the new form of p90ATF6 is localized in the ER or cis-Golgi, it should be sensitive to endoglycosidase H, which removes simple, high mannos N-linked oligosaccharide characteristic of ER localization but cannot remove N-linked glycans that have been processed in the trans-Golgi (16). Alternatively, if Tg results in a subpopulation of p90ATF6 relocated to the trans-Golgi where it undergoes further glycosylation modification, ATF6(f) should be endoglycosidase H-resistant. In both cases, blockage of p90ATF6 N-glycosylation by treatment of cells with Tu should eliminate ATF6(f). If the new form of p90ATF6 is caused by other mechanisms unrelated to N-glycosylation, it should persist upon simultaneous treatment with Tg and Tu. To test these different mechanisms, HA-tagged ATF6 was transfected into COS-7 cells followed by treatment of Tg or Tu alone or a combination of both Tg and Tu.

Four lines of evidence support the partial N-linked glycosylation mechanism in the ER. First, ATF6(f) exhibited an electrophoretic mobility faster than the fully glycosylated form in nonstressed cells but slower than the nonglycosylated form of p90ATF6 generated by Tu, referred to as ATF6(ψ) (Fig. 3B). Second, simultaneous treatment of Tg and Tu reduced the p90ATF6 doublet to a single band with electrophoretic mobility identical to ATF6(ψ), which indicates that the critical mechanism for ATF6(f) formation is N-linked glycosylation inhibition. Third, both the fully glycosylated form and ATF6(f) were reduced to the nonglycosylated form upon endoglycosidase H treatment (Fig. 3B), thus ATF6(f) is not caused by altered glycosylation in the trans-Golgi.

Lastly, we generated ATF6(f) by site-specific mutation of the glycosylation site closest to the carboxyl end of this site was chosen because progressive deletion of the carboxyl end of p90ATF6 showed that the new form of p90ATF6 was largely eliminated when this site was trimmed, whereas the sites at amino acids 472 and 584 remained intact (data not shown). Mutant T645I was created where the threonine residue at 645 was changed to isoleucine (Fig. 3A). N-Glycosylation occurs on the ER luminal side of the membrane and requires the protein recognition sequence Asn-X-Ser/Thr. An asparagine residue not followed by a serine or threonine as shown will not be recognized by the oligosaccharyl transferase, a key enzyme in N-linked glycosylation mechanism, and therefore will not be glycosylated (17). The wild type or the T645I mutant was transfected into COS-7 cells, and the cell lysates were prepared after Tg treatment. As predicted, in nonstressed cells, the electrophoretic mobility of T645I was faster than the fully glycosylated HA-ATF6 (Fig. 3C, compare lanes 1 and 3) but identical to HA-ATF6(f) generated by Tg treatment (compare lanes 2 and 3). Upon Tg treatment, T645I remained largely as one band (Fig. 3C, lane 4). As in the case for wild type p90ATF6, treatment of T645I with Tu alone or Tu in combination with Tg resulted in the fully nonglycosylated form (Fig. 3D).

To address further whether ATF6(f) is formed by partial de-glycosylation of full glycosylated p90ATF6 or de novo partial glycosylation of newly synthesized p90ATF6, CHX was used to inhibit protein synthesis in control and Tg-treated cells. As shown in Fig. 3E, Tg-induced formation of ATF6(f) was blocked by CHX pretreatment, suggesting that ATF6(f) represents de novo partial glycosylation of newly synthesized
p90ATF6 rather than partial deglycosylation of fully glycosylated p90ATF6, which should not be affected by CHX treatment.

**T645I Mutation Results in a Higher Constitutive Rate of Transport to the Golgi**—To examine the functional consequence of partial glycosylation of p90ATF6, we first compared the cellular distribution of the mutant and wild type protein through confocal microscopy. COS-7 cells were transfected with either HA-ATF6 (wild type) or T645I. In both cases, immunofluorescence of the HA tag showed that although the majority of both proteins were localized in the perinuclear region consistent of the ER, constitutive relocalization to the Golgi, as revealed by colocalization with a specific Golgi marker, could also be detected. Examples of HA-ATF6 scored as ER and Golgi staining in the transfected cells are shown in Fig. 4, A and B. In nonstressed cells, Golgi location was detected in about 2% of the cells transfected with wild type ATF6. Cells transfected with the T645I mutant showed about 5.5% of ATF6 localized in the Golgi, representing about a 2.5-fold increase compared with wild type ATF6 (Fig. 4C). Upon treatment with DTT, a potent UPR inducer used previously to demonstrate ATF6 relocalization to the Golgi (6), both wild type and the T645I mutant showed increased transit to the Golgi; by 30 min of DTT treatment, the difference between the mutant and wild type protein was marginal (Fig. 4C). Upon treatment with DTT, a potent UPR inducer used previously to demonstrate ATF6 relocalization to the Golgi (6), both wild type and the T645I mutant showed increased transit to the Golgi; by 30 min of DTT treatment, the difference between the mutant and wild type protein was marginal (Fig. 4C). As expected, the relocalization of p90ATF6 from the ER to Golgi was suppressed substantially in cells treated with brefeldin A, which causes redistribution of the Golgi to the ER (Fig. 4D).

**CRT Association with ATF6 Is Sharply Reduced in the T645I Mutant**—To examine possible contributing factors for the higher constitutive translocation to the Golgi for the T645I mutant, wild type HA-ATF6 or the T645I mutant was transfected into 293T cells, and microsome fractions were prepared. Coimmunoprecipitation followed by silver staining was used to identify protein partners that showed differential interaction between the wild type and mutant HA-ATF6 (Fig. 5A). Although the signal was weak, this approach yielded a candidate protein around 65 kDa that associated with the wild type ATF6 but was hardly detectable with the T645I mutant. The common band at 90 kDa was confirmed to be HA-ATF6 by Western blot, and the common band at 78 kDa showed electrophoretic mobility identical to that of GRP78 (Fig. 5A and data not shown). Although the amount of the p65 band was insufficient for direct sequencing, its electrophoretic mobility is similar to CRT, a 63-kDa Ca\(^{2+}\)-binding protein that also serves as chaperone for glycoproteins in the ER lumen.

To test directly the interaction between CRT and the two forms of p90ATF6, microsomal fractions were prepared from 293T cells that were transfected with either HA-ATF6 or T645I. Using anti-HA antibody as the immunoprecipitating antibody followed by Western blot with anti-CRT antibody, we observed a substantial decrease in the amount of CRT interacting with T645I compared with wild type ATF6 (Fig. 5B). Thus, CRT exhibits the same binding property as the 65 kDa band identified by silver staining. Reprobing the same filter with anti-HA antibody showed that the amount of HA-ATF6 immunoprecipitated with anti-CRT antibody is similar to that precipitated with anti-HA antibody, indicating that the decrease in CRT associated with T645I is specific and not due to nonspecific binding.
FIG. 3. Blockage of N-linked glycosylation eliminates Tg-induced ATF6 doublet formation. A, schematic drawing of wild type (wt) p90ATF6 and the T645I mutant. TM, transmembrane domain. The pCGN vectors used for the transfections contained the cytomegalovirus promoter driving the expression of HA-tagged full-length ATF6 (670 amino acids) with three N-linked glycosylation sites or HA-tagged ATF6(T645I) with only two N-linked glycosylation sites. B, COS-7 cells transfected with pCGN-ATF6 were either not treated (lanes 1 and 5) or treated with Tg (lanes 2 and 6) or Tu (lanes 3 and 7) or a combination of Tg and Tu (lanes 4 and 8) for 16 h, as indicated. Half of the whole cell lysates were subjected to endoglycosidase H digestion, and then all of the samples were analyzed by immunoblotting using anti-HA-Tag antibody. The positions of HA-ATF6, HA-ATF6(T645I), and HA-ATF6(Δ2) are indicated. C, COS-7 cells transfected with either pCGN-ATF6 or pCGN-ATF6(T645I) were not treated (lanes 1 and 3) or treated with Tg for 16 h (lanes 2 and 4). Whole cell lysates were subjected to immunoblotting using anti-HA-Tag antibody. D, COS-7 cells transfected with pCGN-ATF6 or pCGN-ATF6(T645I) were either not treated (lanes 1 and 5) or treated with Tg (lanes 2 and 6) or Tu (lanes 3 and 7) or with a combination of Tg and Tu (lanes 4 and 8) for 16 h, as indicated. Whole cell lysates were subjected to immunoblotting using anti-HA antibody. The positions of HA-ATF6, HA-ATF6(T645I), and HA-ATF6(Δ2) generated from the wild type (WT) p90ATF6 are indicated on the left, and the positions of HA-ATF6(Δ2) and HA-ATF6(Δ2) generated from the T645I mutant are indicated on the right. E, COS-7 cells transfected with pCGN-ATF6 were either not treated (lanes 1 and 3) or treated with Tg for 4 h (lanes 2 and 4), in the absence (lanes 1 and 2) or presence of 10 μg/ml CHX (lanes 3 and 4) for 4.5 h. Whole cell lysates were subjected to immunoblotting using anti-HA antibody.

FIG. 4. T645I mutation results in higher rate of Golgi translocation. A, COS-7 cells transfected with HA-ATF6 (wild type) and cultured under normal conditions. Cells were fixed and incubated with anti-HA and anti-Golgi primary antibodies and then stained with rhodamine-conjugated and fluorescein isothiocyanate secondary antibodies. HA-ATF6 is shown as red fluorescence with normal perinuclear/ER localization, and Golgi staining appears green. B, an example of Golgi-localized HA-ATF6. Cells were prepared exactly as in A but were treated with 10 mM DTT for 20 min. The presence of the color yellow in the merged images occurs where there are proteins staining in the same area of the cell. Cells transfected with the T645I mutant showed the same staining patterns for ER and Golgi localization. C, cells transfected with either HA-ATF6 (WT) or the T645I mutant were treated with 10 mM DTT for the times indicated. Cell images were captured using a Zeiss confocal microscope and randomized, and the cells containing HA staining in the Golgi were counted and expressed as a percentage of total transfected cells. D, same as C except transfected cells were either not treated (Ctrl) or treated with 2.5 mg/ml brefeldin A (BFA) for 6 h.
with anti-HA antibody confirmed that equivalent amounts of wild type and mutant HA-ATF6 were immunoprecipitated. Further, Western blot analysis with microsome extracts confirmed that the endogenous level of CRT was also equivalent in cells expressing the wild type and the mutant ATF6 (Fig. 5B). The reduction in binding to CRT is specific because ATF6 binding to CNX, another ER chaperone with affinity for newly synthesized monomeric glycoproteins (18), was not affected by the T645I mutation (Fig. 5C). Further, in agreement with the silver stain patterns, both forms of ATF6 showed similar interaction with GRP78, which has been shown previously to bind ectopically expressed ATF6 in a constitutive manner (6). We note that the size of CNX is nearly identical to HA-ATF6(p90), therefore the two proteins would not be separable in the silver stain analysis. The relative binding of CRT, CNX, and GRP78 to wild type ATF6 and T645I, after normalization to the amount of HA-tagged ATF6 immunoprecipitated in each sample, is summarized in Fig. 5D. The selective association of endogenous CRT with wild type p90ATF6 but not ATF6(f) was confirmed by performing the reciprocal experiments, using anti-CRT as the immunoprecipitating antibody, followed by Western blot with the anti-HA antibody. The microsomal fractions were prepared from control cells and Tg-treated cells where the ATF6 doublet formation was evident; nonetheless, for both nontreated and Tg-treated cells, only the fully glycosylated form was able to complex with CRT (Fig. 5E). Because CRT is a resident ER luminal protein, the reduced binding with the T645I mutant could be one contributing factor for its higher rate of constitutive transport to the Golgi.

**T645I Mutation Converts ATF6 into a More Potent Constitutive Activator of the Grp78 Promoter**—To test whether the T645I mutation that results in higher constitutive transport to the Golgi translates to a higher transactivating activity in nonstressed cells, COS-7 cells were cotransfected with a luciferase reporter gene driven by the Grp78 promoter and equal amounts of the expression vector for HA-ATF6 wild type and T645I mutant. The reporter gene used was H11002169/Luc, which spans from 11002 to 29 of the rat Grp78 promoter containing all three endoplasmic reticulum stress elements and is a well characterized target for ATF6 (3, 11). The quality and quantity of the ATF6 expression plasmids were confirmed by agarose gel (data not shown). Immunoblots of whole cell lysates with the HA antibody revealed that the expression level of the wild type p90ATF6 was equivalent to T645I in the transfected cells (Fig. 6A). As reported previously, ectopically expressed wild type ATF6 is able to activate the Grp78 promoter in nonstressed

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**Fig. 5.** **T645I mutation specifically reduces interaction with CRT.** A, 293T cells were transfected with either pCGN-ATF6 or the T645I mutant. The transfection efficiency is about 70%. Microsome extracts were isolated from transfected cells. After immunoprecipitation with the anti-HA antibody, the immunoprecipitates were subjected to silver staining. The positions of HA-ATF6(p90), p78, p65, and IgG are indicated; molecular size markers are shown on the left. B, microsome extracts were isolated from 293T cells transfected with pCGN-ATF6 or the T645I mutant. The experiments were performed in duplicate. After immunoprecipitation (IP) with the anti-HA antibody, the immunoprecipitates were subjected to Western blotting (WB) using the anti-CRT antibody, followed by the anti-HA antibody. Western blotting performed with the microsome extracts showed equivalent amounts of endogenous CRT in transfected with the wild type or T645I. C, the filter used in B was reprobed with the anti-CNX antibody. The immunoprecipitates were also immunoblotted with the anti-KDEL antibody to detect GRP78. Western blotting performed with the microsome extracts showed equivalent amounts of endogenous calnexin and GRP78 in cells transfected with the wild type or T645I. D, results from four transfection experiments are summarized. The band intensities for the immunoprecipitated CRT, CNX, and GRP78 were normalized against HA-ATF6 in each sample. The level of each protein binding to the wild type form was set as 1.0 and compared with the level binding to T645I. E, 293T cells transfected with pCGN-ATF6 were either not treated (−) or treated with Tg for 16 h. Microsome extracts were isolated, immunoprecipitated with the anti-CRT antibody, and immunoblotted with the anti-HA antibody. Western blot analysis of the cell extracts using the anti-HA antibody is shown in the bottom panel. The experiments were performed in duplicate. The positions of HA-ATF6 and HA-ATF6(f) are indicated.
cells. The T645I mutant exhibited a 2.5-fold higher transactivating activity than wild type ATF6 in a plasmid dosage-dependent manner. Upon Tg treatment, the activity of wild type ATF6 was increased further by about 5-fold, whereas the T645I mutant increased by about 2-fold, with the final activity attained by the wild type ATF6 only slightly lower than T645I (Fig. 6A).

The enhancing effect of T645I mutation was confirmed using vectors expressing physiologic levels of ATF6. This was achieved by introducing the T645I mutation into pTK-HSV-ATF6 by site-directed mutagenesis. In this expression vector, human p90ATF6 was tagged with the HSV epitope and was expressed under control of the HSV thymidine kinase promoter, which gives a low but detectable level of expression (4). Western blots confirmed equivalent amounts of HSV-ATF6 derived from wild type and T645I mutant being expressed in the transfected cells (Fig. 6B). Similar to the HA-ATF6 expression system, the T645I mutation resulted in a 2.5-fold increase in its transactivating activity toward the Grp78 promoter in nonstressed cells, and the increase was dependent on the dosage of the expression vectors. Further, through the higher sensitivity and specificity of the HSV antibody, we confirmed that the T645I mutant produces a higher level of the p60ATF6, the nuclear form of ATF6. Immunoblot analysis using β-actin as a loading control for the whole cell extracts showed that equivalent amounts of HSV-ATF6(p90) were expressed in cells transfected with the wild type or the T645I mutant (Fig. 6C, lanes 1 and 3). A higher level of the nuclear p60ATF6 was detected in nuclear extracts for the T645I mutant compared with the trace amounts observed for wild type ATF6 (Fig. 6C, lanes 1 and 3). Equal loading for the nuclear fractions was shown by equivalent amounts of the nuclear transcription factor YY1 in all the samples. Upon Tg treatment, similar to endogenous ATF6 (Fig. 1C), both forms of ATF6 showed an increase in total protein level, and as expected, more accumulation of p60ATF6 in the nucleus (Fig. 6C, lanes 2 and 4). Quantitation of the protein bands after normalization for loading revealed about a 4-fold increase of p60ATF6 for the T645I mutant in nonstressed cells and only a slightly higher level in Tg-treated cells (Fig. 6D).

Thus, two independent expression systems for ATF6 showed that mutation of a single amino acid that destroys the glycosylation site at amino acid 643 converts it into a more potent activator of the Grp78 promoter in the absence of ER stress, and this effect could in part be caused by a higher level of the nuclear form of ATF6 through a higher rate of constitutive S1P/S2P processing.

The Enhancing Property of T645I Is Dependent on S2P Cleavage of ATF6.—To test directly whether the higher activity of T645I is dependent on formation of the cleaved form of the ATF6 through the regulated intramembrane proteolysis pathway, wild type HSV-ATF6 and the T645I mutant were transfected under identical conditions into either CHO-7 (S2P<sup>+/−</sup>) or M19 cells (S2P<sup>−/−</sup>). We expected T645I-mediated enhancement of the Grp78 promoter activity over the level of wild type ATF6 to be preserved in CHO-7 cells but lost in the S2P-defective M19 cells. In both CHO-7 and M19 cells, the expression level of HSV-ATF6(p90) derived from wild type and T645I expression vector was equivalent in the transfected cells (Fig. 7). In agreement with 293T transfection results (Fig. 6C), the level of the ectopically expressed HSV-ATF6(p90) was also elevated in Tg-treated CHO-7 and M19 cells. Also in agreement with results observed in 293T cells, T645I conferred increased activity to the Grp78 promoter compared with the wild type ATF6 in nonstressed CHO-7 cells (Fig. 7A). In Tg-treated cells, the activity of the two forms of ATF6 was further elevated but reached similarly high levels. Transfections performed in parallel with M19 cells revealed that the enhanced activity of T645I over the wild type ATF6 was lost, confirming that the stronger constitutive activity conferred by the T645I mutation on the Grp78 promoter requires S2P function (Fig. 7B).
Single or Multiple Mutation of the N-Glycosylation Sites Results in Higher Constitutive Transactivating Activity—To determine whether the other N-glycosylation sites also affect p90ATF6 activity, additional single and multiple site mutations were created. As shown in Fig. 8A, mutants T645I, T586I, and T474I are single site mutations that destroy one of the three glycosylation sites; DoubleM contains both the T645I and T586I mutations, thus destroying two of the three N-glycosylation sites. TripleM contains all three mutations (T645I, T586I and T474I), thus all three N-glycosylation sites are destroyed. To compare their activity, COS-7 cells were cotransfected with −169/Luc and equal amounts of expression vector for wild type HA-ATF6 or the mutant constructs. Immunoblots of whole cell lysates with the HA antibody revealed that in the transfected cells, the expression level of each p90ATF6 mutant construct was equivalent to wild type HA-ATF6, confirming that the site mutations did not affect the stability of the protein (Fig. 8D). The T645I, T586I, and T474I mutants exhibited a 3.0-, 4.5-, and 4.7-fold higher transactivating activity than wild type ATF6, respectively. The highest activity was observed for the double site mutation mutant, about 8.2-fold, whereas the triple site mutation showed a 3.3-fold enhancement over the wild type ATF6 (Fig. 8C).

DISCUSSION

The UPR is central to maintaining ER homeostasis and has been implicated in health and diseases including cancer, tissue ischemia, neurodegeneration, diabetes, and hyperhomocysteinemia (13, 19–22). A central transcription factor that regulates ER homeostasis is the N-linked Golgi enzyme GRP78 (30), also becomes unfolded in response to stress treatment. Through multiple approaches, in particular, that blockage of N-linked glycosylation eliminates the formation of the altered form, we established that this new form of p90ATF6 is caused by partial glycosylation of newly synthesized ATF6. Further, because the new form of p90ATF6 is sensitive to endoglycosidase H digestion, altered glycosylation in the trans-Golgi is ruled out. We did not detect alternative splicing of ATF6 mRNA, and phosphatase treatment has no effect on the p90ATF6 doublet (data not shown). Proteolytic trimming at the amino terminus is also ruled out because of integrity of the amino end in tagged constructs after Tg treatment. How might ER Ca^{2+} depletion lead to protein underglycosylation? Because perturbation of ER Ca^{2+} homeostasis can affect protein folding and protein glycosylation in the ER lumen (29), Tg treatment could affect folding of p90ATF6 directly or enzymes required for N-glycosylation such that some fraction of p90ATF6 becomes only partially glycosylated. Interestingly, GRP94, a major glycoprotein in the ER which is associated with a kinase activity modulated by GRP78 (30), also becomes underglycosylated in Tg-treated cells (data not shown).

To investigate the functional consequence of partial glycosylation of p90ATF6, we first generated mutant T645I that specifically destroyed the glycosylation site at amino acid 643. The choice of this site for mutation is based on progressive deletions.

**Fig. 7.** The enhancing effect of T645I mutation is S2P-dependent. A: upper panel, immunoblot analysis of the HSV-ATF6(p90) levels in CHO-7/S2P^{+/+} cells transfected with pTK-HSV-ATF6 (wild type, WT) or the T645I mutant in the presence or absence of Tg treatment. Lower panel, the −169/Luc reporter gene was cotransfected with either the empty pCGN vector (V), pTK-HSV-ATF6 (WT), or the T645I mutant into CHO-7 cells. The transfected cells were either grown under normal culture conditions (Ctrl) or treated with Tg for 16 h. The luciferase activity in cells transfected with the empty pCGN vector was set at 1. The relative promoter activities are shown ± S.D. B, same as A except the plasmids were transfected into M19 (S2P^{+/+}) cells.
of the carboxyl end of p90ATF6 showing that the N-linked glycosylation site at amino acid 643, which is closest to the carboxyl terminus is most critical for the doublet formation (data not shown). Further, a previous statistical study shows that nonglycosylated sites tend to be found more frequently toward the carboxyl termini of glycoproteins especially when this site is within ~60 amino acid residues of the carboxyl terminus (31, 32). The site at amino acid 643 is 27 amino acids residues away from carboxyl terminus of p90ATF6 and is therefore the most likely candidate for nonglycosylation. However, we note that although the glycosylation site at amino acid 643 is the major site affected by Tg, partial glycosylation of the other two remaining sites could also be observed occasionally. We further generated other p90ATF6 constructs with single or multiple N-glycosylation site mutations. All three single site mutations showed enhancing activity. The highest activity was observed when both N-glycosylation sites at amino acids 584 and 643 were simultaneously mutated, resulting in a substantial (8-fold) increase over the wild type ATF6 level. However, the triple site mutant was less effective, suggesting that the additional mutation of amino acid 472 in the background of the double mutation may negatively affect the protein topology or protein-protein interaction required for its processing. Interestingly, amino acid 472 lies within one of the two Golgi localization signals of ATF6 spanning amino acids 431–475 (6). Future investigations are required to resolve the mechanism.

How might a partially glycosylated p90ATF6 activate the UPR? One straightforward explanation is that the partially glycosylated ATF6 may act as a malfolded protein by itself. As such, it titrates away GRP78, therefore releasing UPR signaling molecules such as IRE1, PERK, and endogenous p90ATF6 that are kept in an inactive form through interaction with GRP78 (6, 33, 34). Although this is plausible, the mechanism for p90ATF6 dissociation from GRP78 is currently not known; further, several lines of evidence suggest that the mechanisms could be more complex and involve novel pathways. First, wild type p90ATF6 is known to bind to GRP78 constitutively. In immunoprecipitation assays, we did not detect any increase in the amount of GRP78 binding to the partially glycosylated form of p90ATF6 compared with wild type protein. Here we discover that although the glycosylated form of p90ATF6 is an interactive partner of CRT, the T645I mutant sharply reduces association with CRT but has no effect on CNX binding. CRT and CNX are known to bind transiently to newly synthesized glycoproteins in the ER as part of the folding process known as the CRT/CNX cycle, and their function is affected by the concentration of Ca2+ in the ER (8, 10). During the maturation of glycoproteins, transfer from the GRP78/GRP94 chaperone system to the CRT/CNX system can occur (35). It has been reported that selective removal of the N-linked glycans from the α1 domain of the H chain ablates association of major histocompatibility complex class I with CRT but not CNX (36). Thus, the T645I mutant provides another example that CRT and CNX interact with different glycan determinants and further reveals that the glycosylation site at amino acid 643 is uniquely required for optimal interaction of ATF6 with CRT but not CNX. Mutation of N-linked glycosylation sites of factor VIII, an ER glycoprotein, facilitates its transport out of the ER through dissociation from GRP78 (37). CRT contains the ER retention signature Lys-Asp-Glu-Leu sequence also found in GRP78. Thus, one possibility is that CRT could be part of the retention system of ATF6 in the ER, and depletion of ER Ca2+ store results in partial glycosylation of a fraction of p90ATF6 which shows reduced affinity for CRT. This in turn results in a higher rate of traffic to the Golgi where ATF6 is processed, leading to generation of the nuclear form of ATF6 and trigger the transcriptional response arm of the UPR, as exemplified by Grp78 promoter induction. In support of this mechanism, the enhanced activity of the T645I mutation requires S2P function. Even though the enhancement over wild type ATF6 is in the range of 2–3-fold, this increase is achieved through mutation of a single amino acid destroying one glycan moiety, and through this mutation, the association with CRT is substantially reduced.

Grp78 is a major target of the UPR and is an anti-apoptotic protein that can interfere with caspase activation (13). The up-regulation of Grp78 in the UPR can be achieved by ATF6-dependent and -independent pathways (2, 3, 38, 39). Although Grp78 up-regulation can contribute to cell viability, and this response could be critical for organ homeostasis in a variety of
pathological states that threaten the survival of the host, for precancerous cells, the onset of UPR could lead to resistance to immune surveillance, cancer progression, and drug resistance (13, 20). Recently, through fingerprinting the circulating repertoires of antibodies from cancer patients, GRP78 has been identified as a major protein eliciting the immune response (40). A strong and specific positive correlation is detected between serum reactivity to GRP78 and development of metastatic androgen-independent disease and shorter overall survival. Thus, understanding the multiple pathways that can modulate ATF6 activation not only adds to the basic knowledge of the UPR but will also lead to better therapeutic approaches toward metabolic diseases and cancers that invoke the UPR.

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Underglycosylation of ATF6 as a Novel Sensing Mechanism for Activation of the Unfolded Protein Response

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