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GALNT6 Stabilizes GRP78 Protein by O-glycosylation and Enhances its Activity to Suppress Apoptosis Under Stress Condition

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Abstract

We previously reported that overexpression of an O-type glycosyltransferase, GALNT6 (polypeptide *N*-acetylgalactosaminyltransferase 6) played critical roles in mammary carcinogenesis. To further investigate the biological function of GALNT6, we screened a substrate protein(s) of GALNT6 using a VVA (*Vicia villosa agglutinin*) lectin (specific to GalNAc-Ser/Thr) pull-down method followed by mass spectrometry analysis. Here we report GRP78 (glucose-regulated protein 78, also known as HSPA5, heat shock 70 kDa protein 5), which is highly expressed in cancer cells and indicated to play important roles in various cellular processes including ER (endoplasmic reticulum) stress and autophagy, as a novel substrate of GALNT6. We found that GALNT6-induced O-glycosylation is critical for the stability of GRP78, its subcellular localization in ER, and its anti-apoptotic function. Furthermore, we demonstrated that overexpression of GRP78 could be important for Golgi-to-ER relocation of GALNT6. Collectively, our study revealed biological significances of O-glycosylation of GRP78 protein, which might play significant roles in the survival of cancer cells, and thus provided a new insight in cancer cell death and useful information for development of anti-cancer treatment targeting the GALNT6-GRP78 pathway.

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Introduction

O-glycosylation is a protein modification at serine or threonine residue of protein, and enhanced O-glycosylation is often observed in malignant cells [1–3]. The mechanism to cause such aberrant O-glycosylation in cancers is not well understood, but emerging evidence has suggested that the expression of polypeptide GalNAc (N-acetylgalactosamine)-transferases (GalNAc-Ts), which control the initiation of O-glycosylation, was markedly up-regulated in cancer cells [4–7]. We previously indicated that GALNT6 (polypeptide N-acetylgalactosaminyltransferase 6, GalNAc-T6) was involved in O-glycosylation of mucins and could be a promising molecular target for development of a new class of anti-cancer drugs for breast and pancreatic cancers [7–9]. Studies from other groups also indicated that GALNT6 could be a potential biomarker in breast cancer progression and metastasis [5,6]. However, it is still not well understood how GALNT6 promotes carcinogenesis since its O-glycan substrates are not fully clarified. Therefore, identification and functional analysis of GALNT6 substrates should be important for better understanding of the oncogenic pathways medicated by GALNT6 and for obtaining the fundamental information for development of GALNT6-targeted and/or its substrate-targeted therapies.

Abbreviations: GALNT6, polypeptide N-acetylgalactosaminyltransferase 6; VVA, *Vicia villosa agglutinin*; GalNAc, *N*-acetylgalactosamine; GRP78, glucose-regulated protein 78; ER, endoplasmic reticulum

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GRP78 (glucose-regulated protein 78, also known as HSPA5, heat shock 70 kDa protein 5) is highly expressed in various types of human cancer, and was shown to be important for tumor formation and progression *in vitro* and *in vivo* [10]. GRP78 is involved in many cellular processes including the endoplasmic reticulum (ER) stress which activates the unfolded protein response (UPR) to alleviate this stress and restore ER homeostasis [11,12]. GRP78 controls a cross-talk between apoptosis and autophagy [13,14], and exhibits oncogenic activities by promoting cell proliferation, survival of cancer cells, angiogenesis, metastasis, and drug resistance [15,16].

In this study, we report that GALNT6 is involved in O-glycosylation and stabilization of GRP78 protein and that O-glycosylation was essential for proper subcellular localization and anti-apoptotic function of GRP78. In addition, we demonstrated that overexpression of GRP78 could enhance Golgi-to-ER relocation of GALNT6. Our findings revealed biological significances of O-glycosylation in GRP78 protein, which seemed to be important for the survival of cancer cells, and thus provided a new insight in efficient induction of cancer cell death by targeting the GALNT6-GRP78 pathway.

Materials and Methods

Cell Culture

Human cancer cell lines MCF7, T47D, MDA-MB-435S, HeLa, and human embryonic kidney 293T cell were purchased from American Type Culture Collection (ATCC) and cultured according to the manufacturer's protocols. Three HeLa cell-derived cell lines stably expressing HA-tagged wild-type GALNT6 protein (HeLa-GALNT6-WT), HA-tagged enzyme-dead H271D-substituted GALNT6 protein (HeLa-GALNT6-H271D), and empty vector (HeLa-Mock) were established as previously described [7]. MCF7 stable cells of mock, wild-type GRP78 (GRP78-WT), and GRP78 with alanine-substitutions at O-glycosylation sites (T85A, T151A, T166A, T184A, and T203A) were newly generated. Briefly, no insert (mock), GRP78-WT, or alanine-substituted GRP78 in pCAGGS-3xFlag expression vectors were transfected into MCF7 cells by using TransIT-BrCa Transfection Reagent (Mirus). Forty-eight hours after transfection, cells were selected under incubation with culture medium containing 0.5 mg/ml of G418 (Geneticin). Two or 3 weeks later, single clones were picked up for further study. Similarly, no insert (mock) and GALNT6-WT in pCAGGS-HA expression vectors were transfected into MDA-MB-435S, and polyclone stable cells were generated by selection of positively transfected cells using 0.8 mg/ml of G418. Transfection of plasmids in the study was done by using TransIT-BrCa Transfection Reagent (Mirus), Lipofectamine 2000 (Life Technologies), or FuGENE 6 (Roche) reagents according to the manufacturer's protocols.

Identification of GALNT6's Substrates

To detect proteins harboring the Tn antigen (GalNAc-Ser/Thr), we did western blot using biotinylated *Vicia villosa agglutinin* (VVA) lectin (1:1000, Vector Laboratories) and Streptavidin-HRP (1:10,000, Thermo Scientific) as described previously [7]. For screening candidate O-glycan subtracts of GLANT6, total proteins from HeLa-Mock, –GALNT6-WT, and -H271D stable cells were extracted and then performed pull-down assay using biotin-conjugated VVA-lectin (specific to GalNAc-Ser/Thr) and streptavidin-conjugated agarose (Invitrogen), followed by elution with 100 mM of GalNAc, according to manufacturer's protocol. Considering a possibility of incomplete elution of O-glycan proteins, we examined both eluted protein fraction as well as finally precipitated proteins with the Streptavidin-conjugated agarose beads. The isolated proteins were visualized by the SilverQuest Staining Kit (Invitrogen). Protein bands that were specifically observed in the HeLa-GALNT6-WT lane were excised with a clean, sharp scalpel and the extracted proteins were applied for PMF (Peptide Mass Fingerprint) analysis using MALDI-TOF MS (Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry). Expression levels of the genes that encode identified proteins were examined by semi-quantitative RT-PCR as described previously [7]. Primer sets and PCR conditions are provided in Supplementary Table 1.

Gene Cloning and Mutagenesis

Flag-tagged full-length wild type GRP78 expression vector (pCAGGS-GRP78-WT-3xFlag) was constructed according to the protocol described previously [7,17]. Primer sets are described in Supplementary Table 2. To generate expression vectors for alanine-substituted GRP78 (T85A, T151A, T166A, T184A, and T203A) that correspond to the candidate O-glycosylation sites, we performed two-step mutagenesis PCR [7], using four primers: a primer set for GRP78 wild-type cloning and another set for mutant (harboring a mutated nucleotide in the middle of the primer) as shown in Supplementary Table 2. For construction of GRP78 fragments (GRP78–1-280, GRP78–125-500 and GRP78–281-654), we performed PCR by using GRP78 wild-type plasmid as a template DNA. The primer sets were described in Supplementary Table 2. Sequences of all constructs were confirmed by BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and ABI3500XL (Life Technologies), and protein expression of these plasmids was also confirmed by western blot.

Western Blot

Western blot was performed as described previously [17]. Finally, protein bands were visualized by ECL or ECL prime detection reagents (GE Healthcare). The primary antibodies used in this study were: anti-human GRP78 polyclonal antibody (1:1000, Santa Cruz), anti-human GALNT6 polyclonal antibody (1:1000, Sigma-Aldrich), anti-Flag M2 monoclonal antibody (1:1000, Sigma-Aldrich), anti-HA monoclonal antibody (1:1000, Roche), anti-PARP-1 antibody (1:1000, Santa Cruz), anti-caspase 7 (1:1000, Cell signaling), anti-PERK (1:1000, Cell signaling), anti-IRE1α (1:1000, Cell signaling), anti-ATF6 (1:1000, Cell signaling), and anti-β-actin monoclonal antibody (1:10,000, Sigma-Aldrich). The secondary antibodies were goat anti-rabbit, anti-rat, and anti-mouse IgG-HRP secondary antibodies (1:10,000~ 1:30,000, Santa Cruz). Intensity of protein band was quantified by ImageJ software as previously described [8].

Immunoprecipitation (IP)

Cell extracts were prepared by adding CelLytic M reagent (Sigma-Aldrich) with 1% of Protease Inhibitor Cocktail Set III (Calbiochem) according to the manufacturer's protocols. Extracts were pre-cleared by incubation with 40 μl of rec-Protein A- or G-Sepharose 4B Conjugate (Invitrogen) and 2 μg of rabbit or mouse IgG (Santa Cruz) at 4°C for 1 h. For pulling down endogenous GRP78 or Flag-tagged GRP78 proteins, pre-cleared cell extracts were then incubated with 2 μg of anti-GRP78 (Proteintech) or anti-Flag