主論文の要約

N-glycans on EGF domain-specific *O*-GlcNAc transferase (EOGT) facilitate EOGT maturation and peripheral endoplasmic reticulum localization

EGFドメイン特異的*O*-GlcNAcトランスフェラーゼ(EOGT)上の*N*-グリカンはEOGTの成熟と小胞体辺縁への局在化を促進する

名古屋大学大学院医学系研究科　総合医学専攻
生物化学講座　分子細胞化学分野
（指導：岡島 徹也　教授）
Sayad Md. Didarul Alam

【Introduction】

Epidermal growth factor-like (EGF) domain-specific O-GlcNAc transferase (EOGT) is an endoplasmic reticulum (ER)-resident protein that modifies Ser/Thr residues on EGF repeats of Notch receptors and thereby regulates Delta-like ligand-mediated Notch signaling. Unlike OGT-mediated regulation of O-GlcNAcylated cytoplasmic, mitochondrial, and nuclear proteins, EOGT can modulate pericellular protein function directly by extracellular O-GlcNAc modifications of transmembrane or secreted proteins. Of particular interest are EOGT mutations at putative N-glycosylation sites. The biological significance of extracellular O-GlcNAc is further suggested by EOGT mutations found in patients with Adams-Oliver syndrome (AOS), a rare disorder characterized by congenital limb abnormalities and scalp defects. Genomic analysis of EOGT in AOS patients revealed several missense and nonsense mutations, including W206S, R377Q, and 359Dfs*28, all of which result in the loss of enzyme activity. However, the significance of N-glycans on Notch-modifying enzymes has not been extensively studied. Bovine O-fucosyltransferase 1 (POFUT1), another EGF-domain specific ER enzyme possesses two N-glycans that are required for its enzyme activity and solubility. However, the effect of POFUT1 N-glycans on its enzymatic products has not addressed directly yet and needs further investigation. In this study, we characterized mouse EOGT to determine all N-glycosylation sites, detailed N-glycan structures, and their effect on EOGT expression and localization. N-glycans on EOGT impacted protein maturation and O-GlcNAc stoichiometry, although a single N-glycan was sufficient to mediate these functions. Importantly, our results provided the first evidence of ER-resident proteins whose N-glycans facilitate their distribution to the peripheral ER.

【Methods】

To identify which *N*-glycosylation sequons on EOGT are occupied with *N*-glycans, three *Eogt* mutants were generated by site-directed mutagenesis where asparagine was replaced by glutamine **(Fig. 1A).** N-glycan structures on EOGT was analyzed by Q-Exactive mass spectrometer and reduction of O-GlcNAc stoichiometry in Notch1 was analyzed by Orbitrap Fusion mass spectrometer. Confocal microscopy has been used to examine the impaired subcellular localization of EOGT within the cell.

【Results】

The N263Q and N354Q EOGT isoforms expressed in HEK293T cells, but not the N493Q mutant, showed decreased molecular weight. Both the N263Q and N354Q mutants showed one additional band, whereas the N263Q/N354Q double mutant did not change its apparent molecular weight on the gels. These results demonstrated that mouse EOGT is modified with *N*-glycans at the N263 and N354 sites **(Fig. 1B)**. To address the effect of *N*-glycans on EOGT expression levels, wild-type or mutant EOGT were co-expressed with GFP, which was linked to the *Eogt* transgene via an internal ribosome entry site (IRES) sequence **(Fig. 1C)**. Quantification of EOGT level normalized to GFP revealed that the N263Q/N354Q mutant expression was significantly decreased by 66% compared to wild-type EOGT **(Fig. 1D)**. To determine the N-glycan structures on EOGT, purified FLAG-EOGT was subjected to tryptic or chymotryptic digestion followed by LC-MS analysis. MS/MS spectra showed that N263 and N354 sites are modified with oligomannose N-glycans **(Fig. 2A).** Endogenous EOGT,

upon Endo H digestion, exhibited mobility shift compared to untreated EOGT and co-migrated with the N-glycan deficient N263Q/N354Q FLAG- EOGT mutant **(Fig. 2B).** These results support the idea that endogenous EOGT is similarly modified with oligomannose N-glycans. To precisely investigate the decreased *O*-GlcNAc stoichiometry on Notch1 by loss of *N*-glycans on EOGT, purified FLAG-Notch1-TM was subjected to mass spectrometry. The semi-quantitative analysis revealed that *O*-GlcNAc stoichiometry is significantly decreased in EGF10, EGF21, and EGF23 in the absence of *N*-glycans (**Fig. 3A and B**). As previously described, the ER-resident chaperone calnexin was distributed in both the peripheral and perinuclear ER. To analyze the effect of *N*-glycans on the ER localization of EOGT, the alteration in the subcellular localization of each EOGT mutant was analyzed. As previously reported, wild type EOGT was detected throughout the ER in HEK293T cells. The single mutants (N263Q or N354Q), exhibited a similar staining intensity and pattern with wild-type EOGT. In contrast, the staining intensity of the double mutant (N263Q/N354Q) was apparently weaker than the wild type. Although we could readily observe the perinuclear EOGT staining, the signal at the ER periphery visibly decreased **(Fig. 4A)**. Quantification of peripheral to perinuclear staining ratio confirmed that EOGT distribution to the peripheral region is significantly decreased when *N*-glycans are removed from EOGT **(Fig. 4B)**. Similar result was also observed when trimming the N-glycans on endogenous EOGT by tunicamycin treatment. Taken together, these results demonstrate that *N*-glycans are generally required for the peripheral EOGT distribution **(Fig. 5A and B)**.

【Discussions】

In this study, we have performed in-depth analyses of *N*-glycan structure and function on the ER luminal *O*-GlcNAc transferase EOGT. Among the three putative *N*-glycosylation sites (N263, N354, and N493) in mouse EOGT, we determined that only N263 and N354, but not N493, are modified with oligomannose *N*-glycans. The lack of modification at N_{493}YS could be explained based on the fact that C-terminal sequons within the last 50 amino acids are modified post-translationally by STT3B isoform of the OST that prefer NXT sites over NXS sites. In our study, EOGT localized at both the perinuclear and peripheral ER as with calnexin. Importantly, the *N*-glycan-deficient FLAG-EOGT mutant was localized predominantly in the perinuclear ER due to the loss of staining in the peripheral ER. Similarly, tunicamycin treatment suppressed the peripheral ER localization of endogenous EOGT. Therefore, *N*-glycans *per se* or *N*-glycan-dependent structural aspects on EOGT may facilitate its diffusion from the perinuclear to peripheral ER. To the best of our knowledge, this is the first study to show that *N*-glycans mediate the correct distribution of ER-resident proteins within the ER. Thus, the decreased *O*-GlcNAc stoichiometry on Notch1, mediated by the absence of *N*-glycans on EOGT, could not only be attributed to the overall EOGT protein expression level but also the altered subcellular EOGT localization in the ER.

【Conclusions】

These results suggest critical roles of *N*-glycans in sustaining *O*-GlcNAc transferase function both by maintaining EOGT levels and by ensuring its proper subcellular localization in the ER.