Structure and function of extracellular O-GlcNAc

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Abstract

Extracellular O-GlcNAc is a unique modification restricted to the epidermal growth factor (EGF) domain-containing glycoproteins. This O-GlcNAcylation is catalyzed by the EGF-domain specific O-GlcNAc transferase (EOGT), which is localized in the lumen of endoplasmic reticulum. In humans, *EOGT* is one of the causative genes of a congenital disease, Adams-Oliver syndrome. EOGT is highly expressed in endothelial cells and regulates vascular development and integrity by potentiating Delta-like ligand-mediated Notch signaling. In *Drosophila*, Eogt modifies Dumpy, an apical extracellular matrix glycoprotein, and affects Dumpy-dependent cell-matrix interaction. In this review, we summarize the current findings of the structure and functions of extracellular O-GlcNAc in animals.

25 **Introduction**

26 Since Dr. Hart discovered O-GlcNAc in 1984 [1], O-GlcNAc modification has been
27 considered to occur only in proteins localized to the nucleus, cytosol, and mitochondria in animals
28 [2-4]. However, O-glycan analysis of Notch receptors unexpectedly revealed the presence of an
29 O-GlcNAc modification on the extracellular domain of Notch receptors [5] (Figure 1). Unlike O-
30 GlcNAc modification by OGT, extracellular O-GlcNAc modification is catalyzed by the
31 epidermal growth factor (EGF)-domain specific O-GlcNAc transferase (EOGT) in the lumen of
32 the endoplasmic reticulum (ER) [6]. In *Drosophila*, O-GlcNAc has been detected as a
33 monosaccharide. In contrast, extended O-GlcNAc structures have been observed in mammals
34 [7,8]. Regardless of the structural difference, extracellular O-GlcNAc is pivotal for
35 developmental processes in animals as evidenced from the phenotype of the mutant deficient in
36 *Drosophila* or mice Eogt [6,9], and *EOGT* mutations found in the patients of Adams-Oliver
37 syndrome (AOS) [10-14].

38

39 **Extracellular O-GlcNAc and EOGT**

40 Notch receptors are membrane glycoproteins that initiate a signal transduction pathway
41 important for intercellular communication and thereby mediate a variety of cell fate determining
42 processes [15]. Notch receptors have more than thirty consecutive EGF domains in the
43 extracellular region, each of which contains six cysteine residues that form three disulfide bridges
44 and thus the unique tertiary structure. O-GlcNAc on EGF occurs simultaneously with O-fucose
45 or O-glucose, the other regulators of Notch receptor (see also chapters by Holdener and
46 Haltiwanger and by Yu and Takeuchi in this issue) [9,16-19].

47 O-GlcNAc was initially found on the 20th EGF domain (EGF20) fragment of *Drosophila*
48 Notch that was expressed in S2 cells [5]. As with intracellular O-GlcNAc, the O-GlcNAc on EGF
49 can be detected with anti-O-GlcNAc antibody such as CTD 110.6 [5,20,21] and removed by β-
50 hexosaminidase digestion [5,22]. As with POFUT1 and POGLUT1, EOGT is an ER-localized
51 protein and exerts a KDEL-like ER retrieval signal [6]. Although no information is available
52 regarding the tertiary structure of EOGT, the primary sequence predicts N-terminal signal

53 peptides and putative DXD motifs [23]. In vitro enzyme assays have revealed that the optimal pH
54 of mouse EOGT is pH 7.0, and the Km value for UDP-GlcNAc is 25 μM [10].

55 To date, more than 10 proteins have been modified by extracellular O-GlcNAc. In addition
56 to Notch receptors, Notch ligands—Delta and Serrate—contain EGF repeats with putative
57 consensus sites and are modified with O-GlcNAc when expressed in S2 cells [6,23]. In
58 *Drosophila*, Dumpy, a membrane-tethered extracellular matrix (ECM) protein, was identified as
59 a prominent O-GlcNAcylated protein [6]. In mammals, Notch1 and TSP-1 (thrombospondin-1)
60 are found to be O-GlcNAcylated as expected from the presence of putative consensus sequence
61 [24]. Further comprehensive mass spectrometry-based analysis of the mouse cerebral cortex has
62 revealed that HSPG2 (Perlecan), NELL1 (neural EGFL like 1), LAMA5 (laminin subunit alpha
63 5), PAMR1 (peptidase domain containing associated with muscle regeneration 1), and NOTCH2
64 are modified with O-GlcNAc in their EGF domains [25].

65

66 **Biological function of O-GlcNAc in *Drosophila***

67 Although extracellular O-GlcNAc was initially found on Notch receptors, expected Notch
68 mutant phenotypes were not evident in the *eogt* mutant [6]. Instead, loss of Eogt caused wing
69 blister and detachment of the dorsal and ventral wing surfaces. The wing blister phenotype in the
70 *eogt* mutant is further enhanced by removing one allele of *dumpy*. Furthermore, Dumpy was
71 identified as a major O-GlcNAcylated protein in the apical ECM. These data suggest that Eogt
72 regulates the integrity of apical ECM by modifying Dumpy. Interestingly, an independent study
73 revealed that the mutant phenotype is suppressed by the removal of one allele of a gene
74 corresponding to Notch signaling or pyrimidine synthesis [23]. Since Notch signaling also affects
75 pyrimidine synthesis, EOGT may regulate cellular metabolism by affecting pyrimidine synthesis,
76 which is linked to UDP-GlcNAc biosynthesis via the hexosamine biosynthetic pathway (Figure
77 2).

78

79 **Biological function of O-GlcNAc in the mammals**

80 AOS is a rare congenital disorder characterized by terminal transverse limb defects
81 (absence or hypoplasia of distal structures of limbs) combined with aplasia cutis congenita, a
82 condition characterized by defects of the scalp and skull. AOS is often accompanied with vascular
83 abnormalities and heart defects [26]. Although the pathological mechanism remains elusive,
84 several causative genes were identified from the genome sequencing of patients with AOS. These
85 genes can be classified into two groups: actin-cytoskeleton regulatory components (*ARHGAP31*
86 and *DOCK6*) and Notch signaling components (*NOTCH1*, *DLL4*, *RBPJ*, and *EOGT*) [27-38].

87 *EOGT* mutations in AOS indicate a direct link between the EOGT and Notch signaling
88 pathways and also provide insights into the structure and function of EOGT. All *EOGT* mutations
89 including W206S, R377Q, and 359Dfs*28 [12,13] result in the loss of enzyme activity, as
90 evidenced by the absence of elevation in the O-GlcNAc level when co-expressed with their
91 substrates [10]. W206S mutation induces misfolding of EOGT as suggested from altered
92 subcellular localization and degradation through the ubiquitin-proteasome system. In contrast,
93 R377Q mutation shows normal protein stability and localization in spite of the impaired ability
94 to bind UDP-GlcNAc. Thus, R377 appears to mediate binding to the donor substrate of EOGT.

95 Unlike AOS patients, no gross abnormalities are found in mice deficient for *Eogt [9]*.
96 However, detailed phenotypic analysis has revealed vascular defects in *Eogt* mutant mice [9].
97 Retinal angiogenesis starts after birth and serves as an ideal model of vascular development. In
98 situ hybridization revealed elevated *Eogt* expression in endothelial cells. As predicted from the
99 roles of Notch signaling in vascular development, *Eogt* mutant mice showed increased vascular
100 branching and filopodia formation at the vascular front. EOGT is also required for vascular
101 integrity as demonstrated by local extravasation of plasma fibrinogen in the mutant mice. These
102 phenotypes are similar with those of *Notch1* and *Rbpj* heterozygous mutant mice. Importantly,
103 enhanced phenotypes were observed in $Eogt^{-/-}$ $Notch1^{+/-}$ or $Eogt^{-/-}$ $Rbpj^{+/-}$ double mutant mice.
104 These results suggested that EOGT is required for precise regulation of Notch signaling activity
105 in endothelial cells.

106 EOGT is dispensable for cell surface expression of Notch1 [9]. However, signaling
107 analysis in endothelial cells revealed that Notch signaling induced by Delta-like ligands is
108 potentiated by EOGT in signal-receiving cells. Accordingly, EOGT increases Notch1 binding to
109 Delta-like ligand 4 (DLL4), but not Jagged 1. The effect of EOGT to enhance DLL4-NOTCH1

110 binding was abolished by alanine substitution of O-GlcNAcylated sites at EGF2, EGF10, EGF17,
111 and EGF20. These observations showed that O-GlcNAcylation of Notch1 enhances Notch
112 signaling induced by DLL ligands.

113

114 **Structure of O-GlcNAc glycan**

115 Comprehensive glycoproteomic analysis of O-GlcNAcylated EGF domains was first
116 reported in *Drosophila* Notch expressed in S2 cells, in which 18 O-GlcNAc sites were detected
117 [39] (Figure 3A). Among those, high stoichiometry was observed in 5 domains (EGF4, 11, 12,
118 14, 20), whereas low stoichiometry was observed in 13 domains (EGF3, 5, 9, 13, 15, 16, 17, 19,
119 22, 25, 26, 27, 28). Although O-GlcNAc appears to remain as a monosaccharide in *Drosophila*,
120 elongated forms of O-GlcNAc glycans were observed on Notch1 expressed in HEK293T cells
121 [8,40]. Similar to O-fucose glycans, O-GlcNAc is modified with β1,4-linked galactose to generate
122 an O-linked lactosamine structure, which can be further modified by a sialic acid. Recent findings
123 in O-GlcNAc glycoproteomics have revealed the majority of the potential O-GlcNAcylation sites
124 on Notch1 [7] (Figure 3B). Interestingly, O-GlcNAc glycan structures vary depending on the
125 position of EGF domains within the EGF repeats. O-GlcNAc elongation to the oligosaccharide
126 was observed in EGF2, EGF10, and EGF20. However, in the majority of Notch1 EGF repeats,
127 including EGF11, EGF15, EGF21, EGF23, and EGF35, only a small percentage of O-GlcNAc is
128 modified with galactose. In the case of EGF14, EGF27, and EGF28, these domains are refractory
129 to further elongation and remain as the O-GlcNAc monosaccharide. These results provide an
130 unexpected result that majority of O-GlcNAc moieties remain unmodified by additional sugars
131 with the exception of a few EGF domains. The stoichiometry of O-GlcNAc modification on EGF
132 repeats is not apparently conserved between *Drosophila* and mammals: O-GlcNAc exhibits low
133 stoichiometry in 5 domains of mouse Notch1 (EGF10, 11, 23, 27, 28), whereas it exhibits high
134 stoichiometry in three domains (EGF14, 23, 35) when expressed in HEK293T cells.

135

136 **Consensus sequence for O-GlcNAcylation on the EGF domains**

The putative consensus sequence for O-GlcNAcylation can be proposed by comparing the modified sequences of EGF domains in previous studies. Based on the sequences between the 5th and 6th conserved cysteines, $C^{5}XXG(Y/F/W/T/K)(T/S)GXXC^{6}$ (Figure 4A) and $C^{5}XX(G/P/S)(Y/F/W/L)(T/S)GXXC^{6}$ are the common sequences in *Drosophila* and mammals, respectively [7,39] (Figure 4B). Considering Dumpy EGF repeats that contain an extra amino acid between the modifiable T/S and 6th cysteine, the putative consensus sequence of $C^{5}XXXX(T/S)GX_{2\text{-}3}C^{6}$ could be proposed based on the unified information. However, this consensus may be too broad, and requirements of glycine, proline, or serine at position 2 in mammals and variable amino acids at position 1 may especially require further investigation to precisely establish the consensus sequence. It should be noted that the difference in O-GlcNAc stoichiometry cannot be explained only based on the variable amino acids within the putative consensus sequence for O-GlcNAc modification. Amino acid sequences distant from the modified residue likely affect physical interactions with EOGT.

Future perspective

The biological importance of extracellular O-GlcNAc has been established from genetic analysis of *Eogt/eogt* mutant animals [9]. However, molecular mechanisms as to how O-GlcNAc glycans affects protein function remains largely unknown. Recent findings of the contribution of four EGF domains (i.e. EGF2, 10, 17, and 20) for EOGT to enhance NOTCH1-DLL4 binding indicate that these O-GlcNAc glycans may create additional binding site(s) for the Delta-like ligand [9]. However, Notch1 carries many additional O-GlcNAcylation sites, most of which are displayed as a monosaccharide. Given that O-fucose and O-glucose monosaccharides stabilize the structure of individual EGF domains [18,19], arrays of O-GlcNAc monosaccharides may impose additional layers of regulation by affecting the structure of the Notch EGF repeats.

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169

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171

172 **Figure legends**

173 **Figure 1. O-GlcNAc glycan on Notch receptor in mammals**

174 The extracellular O-GlcNAc modification is catalyzed by EOGT that is localized to the ER. UDP-
175 GlcNAc is synthesized from glucose via the hexosamine biosynthetic pathway (HBP). Specific
176 domains can be elongated into the O-GlcNAc-Gal-Sia trisaccharide by β1,4-
177 galactosyltransferases (β4-GalT) and α2,3/6-sialyltransferases (ST3/6GalT). The O-GlcNAc
178 glycans on Notch 1 enhance the interaction between Delta-like ligand and Notch1. NICD, Notch
179 intracellular domain; Su(H), Suppressor of Hairless; *Blue square*, GlcNAc; *yellow circle*,
180 galactose; *purple diamond*, sialic acid.

181

182 **Figure 2. O-GlcNAc glycan on Notch and Dumpy in *Drosophila***

183 In *Drosophila*, the loss of *eogt* causes wing blistering, which is enhanced by removing a single
184 allele of Dumpy, whereas it is suppressed by removing a single allele of genes involving the
185 Notch signaling pathway. Based on the finding that Notch signaling promotes pyrimidine
186 metabolism, it is proposed that the increased metabolic products in the pyrimidine synthesis
187 pathway, caused by decreased hexosamine biosynthetic pathway (HBP) flux in *eogt* mutation,
188 may impact the integrity of ECM to maintain flat wing morphology. Alternatively, O-
189 GlcNAcylation of Dumpy directly affects ECM integrity. Rudimentary, Dhod (dihydroorotate

dehydrogenase), and R-I (Rudimentary-like) are enzymes in pyrimidine metabolism. Su(H), suppressor of hairless; *blue square*, GlcNAc.

Figure 3. Comparison of O-GlcNAc glycan on EGF repeats between *Drosophila* Notch and mouse Notch1

The schematic representation of the modification sites and stoichiometry of O-GlcNAcylation in *Drosophila* Notch expressed in S2 cells (A) and mouse Notch1 expressed in HEK293T cells (B). Color code indicates no O-GlcNAc (*black*), O-GlcNAc (*blue*), O-GlcNAc-Gal (*orange*), and O-GlcNAc-Gal-NeuAc (*purple*). EGF domains with potential O-GlcNAcylation sites are shown by blue squares. Uncharacterized EGF domains are shown by the question mark.

Figure 4. Putative consensus sequence for extracellular O-GlcNAcylation

Alignment of amino acid sequences between the 5th and 6th conserved cysteines of EGF repeats modifiable with O-GlcNAc. (A) The common sequence for O-GlcNAcylation in *Drosophila* is C^5XXG(Y/F/W/T/K)(T/S)G$X_{3\text{-}4}C^6$. It should be noted that Dumpy EGF repeats contain an extra amino acid between the modifiable T/S and 6th cysteine although the exact modification sites have not been defined. (B) The common sequence for O-GlcNAcylation in mammals is C^5XX(G/P/S)(Y/F/W)(T/S)GXXC^6.

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