Biochemical and Biophysical Research Communications 419 (2012) 14–19

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

O-linked-*N*-acetylglucosamine modification of mammalian Notch receptors by an atypical O-GlcNAc transferase Eogt1

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ARTICLE INFO

Article history:
Received 16 January 2012
Available online 28 January 2012

Keywords:
O-GlcNAc
Notch
Eogt

ABSTRACT

O-linked-β-*N*-acetylglucosamine (O-GlcNAc) modification is a unique cytoplasmic and nuclear protein modification that is common in nearly all eukaryotes, including filamentous fungi, plants, and animals. We had recently reported that epidermal growth factor (EGF) repeats of Notch and Dumpy are O-GlcNAcylated by an atypical O-GlcNAc transferase, EOGT, in *Drosophila*. However, no study has yet shown whether O-GlcNAcylation of extracellular proteins is limited to insects such as *Drosophila* or whether it occurs in other organisms, including mammals. Here, we report the characterization of *A130022J15Rik*, a mouse gene homolog of *Drosophila Eogt* (*Eogt* 1). Enzymatic analysis revealed that Eogt1 has a substrate specificity similar to that of *Drosophila* EOGT, wherein the Thr residue located between the fifth and sixth conserved cysteines of the folded EGF-like domains is modified. This observation is supported by the fact that the expression of *Eogt1* in *Drosophila* rescued the cell-adhesion defect caused by *Eogt* downregulation. In HEK293T cells, *Eogt1* expression promoted modification of Notch1 EGF repeats by O-GlcNAc, which was further modified, at least in part, by galactose to generate a novel O-linked-*N*-acetyllactosamine structure. These results suggest that *Eogt1* encodes EGF domain O-GlcNAc transferase and that O-GlcNAcylation reaction in the secretory pathway is a fundamental biochemical process conserved through evolution.

1. Introduction

O-linked-*N*-acetylglucosamine (O-GlcNAc) modification is a unique post-translational modification that regulates a wide range of cellular processes such as intracellular signaling, endocytosis, transcription and protein stability, and also serves as a modulator of nutrient sensing and is implicated in human diseases including type-2 diabetes [1,2]. The modification and cellular functions of O-GlcNAc have been studied in various cytoplasmic and nuclear proteins and in many species, including mammals, insects, nematodes, and plants [3–6]. Recently, O-GlcNAc was unexpectedly identified on the EGF repeats of Notch [7] and subsequently on Dumpy—a giant membrane-anchored apical extracellular matrix (aECM) protein [8,9].

In *Drosophila*, O-GlcNAcylation of EGF domains is catalyzed by an atypical O-GlcNAc transferase—EOGT [9]. EOGT does not show recognizable sequence similarity to the intracellular O-GlcNAc transferase (OGT), but rather is phylogenetically related to the plant xylosyltransferase. Genetic analysis of *Drosophila Eogt* mutants revealed that *Eogt* is not required for the activity of Notch receptors but is critical for the function of Dumpy in mediating the interaction of epithelial cells with the aECM [9]. However, whether O-GlcNAcylation of EGF repeats is limited to *Drosophila* or is present in higher vertebrates, including mammals, has not been understood.

In this study, we have characterized *A130022J15Rik,* a mouse gene homolog of *Drosophila Eogt (Eogt1). Eogt1* is a candidate gene in mice that contributes to the predisposition to acute functional tolerance—a measure of alcohol sensitivity [10]. *Eogt1* was also reported as a novel gene expressed in the presomitic mesoderm [11]. However, the molecular functions of Eogt1 had not been investigated previously.

2. Materials and methods

2.1. Materials

A plasmid encoding Notch EGF repeat-FLAG fusion proteins (pRmHa/ECN: FLAG) was as described previously [12]. pSectag2/mNotch1-EGF: mycHis-IRES-GFP encoding Myc-His epitope-

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doi:10.1016/j.bbrc.2012.01.098

tagged mouse Notch1 EGF repeats (mN1-EGF) was provided by Pamela Stanley [13]. A plasmid encoding mouse Eogt-like (pCMV-Sport6-AGO61) was obtained from Invitrogen. *Drosophila* Notch EGF repeat 20 (EGF20) was produced using the *Kluyveromyces lactis* expression system as described previously [14]. Unfolded EGF20: V5His was prepared by reduction of disulfide bonds with dithiothreitol. The following antibodies were used: mouse anti-FLAG M2 (Sigma), mouse anti-6xHis (Genscript), rabbit anti-myc (Santa Cruz), and mouse anti-O-GlcNAc (CTD110.6; Abcam). A rabbit polyclonal antibody raised against the bacterially expressed recombinant Eogt1 will be described elsewhere. *Drosophila* RNAi lines (*UAS-Eogt*^{IR1} and *UAS-Eogt*^{IR3}) were obtained from the National Institute of Genetics (Mishima, Japan).

2.2. Plasmid constructs

The plasmids containing mouse *Eogt1* cDNA (Invitrogen) were amplified by PCR to obtain the coding region, which was cloned into the *Bgl*II and *Xho*I sites of the pEF expression vector (pEF-Eogt1) or *Nhe*I and *Xho*I sites of the pSectag2-IRES-GFP expression vector. To produce a FLAG-tagged Eogt1 construct (pEF-FLAG: Eogt1), the FLAG tag was inserted after Lys21 by PCR with the KOD-Plus-Mutagenesis Kit (Toyobo), so that the FLAG tag remained on Eogt1 after cleavage of the signal peptide. FLAG-Eogt1$^{\Delta HDEL}$, which lacks the carboxyl-terminal RNEL sequence was generated by PCR as described above using pEF-FLAG: Eogt1 as a template.

For creating the pMT-Bip-FLAG: Eogt1 construct (for the expression of FLAG-Eogt1 in *Drosophila* cell lines), FLAG: Eogt1 fragment was cloned into pMT/BiP/V5HisA (Invitrogen). The flanking sequence between Bip and FLAG, and FLAG and *Eogt1* is as follows: FVGLSLGRSDYKDDDDK^{21}AHSEADDAPGKALYDYSS (FLAG tag underlined). For *pUASTattB-Bip-FLAG: Eogt1* construct, the *Bip-FLAG: Eogt1* fragment generated by PCR was transferred to pUASTattB. Germline transformation of *Drosophila* was performed using BestGene.

2.3. Northern blotting and RT-PCR

Total RNA was prepared from tissue samples obtained from adult C57BL/6 mice by using TRIZOL® reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Total RNA (10 μg) was separated on 1.2% agarose–formaldehyde gel, and then transferred onto a GeneScreen Plus membrane (NEN). The blots were probed with a gel-purified, [α-^{32}P]dCTP-labeled *Eogt1* or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [15]. The following primers were used to generate the template for the cDNA probe of *Eogt1*: Eogt1-404F: 5′-GCCAGCCAGAGAGAGCAAGC-3′; Eogt1–1260R: 5′-GTCCGTGTTGTGCGTGATCC-3′. Reverse transcription PCR (RT-PCR) analysis was performed as described [15] using Eogt1-404Fw (5′-GCCAGCCAGAGAGAGCAAGC-3′) and Eogt1-1069Rv (5′-GAGTGATGTTCAGCCTATGC-3′) primers.

2.4. Transfection and RNAi

HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 7.5% fetal bovine serum; and transfection was performed using Lipofectamine 2000 (Invitrogen). S2 cells were cultured in Sf-900 II medium (Invitrogen) supplemented with 5% fetal bovine serum; and transfection was performed using Cellfectin II (Invitrogen). RNAi in S2 cells was performed using dsRNA for *Eogt* as described previously [9,16].

2.5. Production and purification of Eogt1

S2 cells expressing FLAG-Eogt1 were established by transfecting pMT-Bip-FLAG-Eogt1 together with pMK33 vectors into S2 cells followed by the drug selection using 600 μg/mL hygromycin for 4 weeks. Protein expression was under the control of an inducible metallothionein promoter and was induced in Sf-900 II supplemented with 0.7 mM $CuSO_4$. For the purification of FLAG-EOGT1, cells were harvested and lysed in the buffer containing 0.5% Triton X-100, 150 mM NaCl, and 100 mM HEPES, pH 7.5. After removing cell debris by centrifugation, the supernatant was incubated with anti-FLAG M2 affinity gel beads (Sigma). After washing three times with the lysis buffer, the bound enzymes were eluted with 100 μg/mL 3 × FLAG peptides (Sigma) in TBS (50 mM Tris–HCl, pH 7.4, and 150 mM NaCl). Eluted proteins were concentrated and buffer-exchanged into 25 mM Hepes-NaOH, pH 7.0 by using Ultracel-3K centrifugal filter units (Millipore).

2.6. Enzyme activity assay

GlcNAc transferase assay was performed in 20 μL of the glycosylation buffer (25 mM HEPES-NaOH, pH 7.0, 1 mM $MnCl_2$, 0.5 mg/mL bovine serum albumin) containing 1 μM UDP-[^{3}H]GlcNAc (60 Ci mmol-1; ARC), 0.1 μg of EGF20-V5His, and FLAG-tagged EOGT1 as an enzyme source. After incubation at 37 °C for 60 min, the reaction was applied to a LC-18 SPE tube (Supelco), washed, and eluted with 1 mL of 80% acetonitrile and 0.052% trifluoroacetic acid as described previously [14].

2.7. Glycosidase treatment

mN1-EGF was purifed from culture medium with anti-His antibody-conjugated beads (Genscript). After elution and denaturation of the bound protein with 1× denaturing buffer (NEB), glycosidase digestion was performed for 16 h at 37 °C in 50 mM sodium citrate, pH 6.0, containing 1% Nonidet P-40, protease inhibitors (Complete; Roche), and 8 units/μL β1-4 galactosidase (NEB; P0730) or 50 units/μL neuraminidase (NEB; P0720).

3. Results

3.1. Characterization of a mouse Eogt1 gene

A homology search of *Drosophila Eogt* successfully identified a previously uncharacterized mouse gene, *Eogt1* (*A130022J15Rik*), which encodes 527 amino acids. The amino acid sequence of Eogt1 shows 45% homology with that of *Drosophila* EOGT. The mammalian genome encodes another uncharacterized Eogt-like protein, AGO61 (Fig. 1A). To determine whether Eogt1 and/or Eogt-like mediate O-GlcNAcylation of mammalian Notch receptors, mN1-EGF was co-expressed with *Eogt1* or *Eogt-like* in HEK293T cells. Immunoblotting of mN1-EGF isolated from cell lysates or culture media indicated that Eogt1, but not Eogt-like, is responsible for O-GlcNAcylation of EGF repeats of Notch1 (Fig. 1B).

3.2. Primary structure, expression, and genomic organization of Eogt1

Eogt1 homologous genes are evolutionarily conserved from *Caenorhabditis elegans* to humans, and all contain a hydrophobic region corresponding to a signal peptide and a KDEL-like sequence at the carboxy terminus (Supplementary Fig. S1). As with *Drosophila* EOGT, deletion of the HDEL sequence impairs the intracellular retention of Eogt1 (Fig. 1C), suggesting that the sequence serves as an endoplasmic reticulum retention signal.

The expression of *Eogt1* was confirmed by Northern blot analysis in adult mouse tissues including brain, heart, kidney, lung, skeletal muscles, and thymus. Major transcripts of 4.6 kb were detected in all tissues, with the highest expression in lung and the lowest in skeletal muscles (Fig. 1D). Similarly, the NCBI

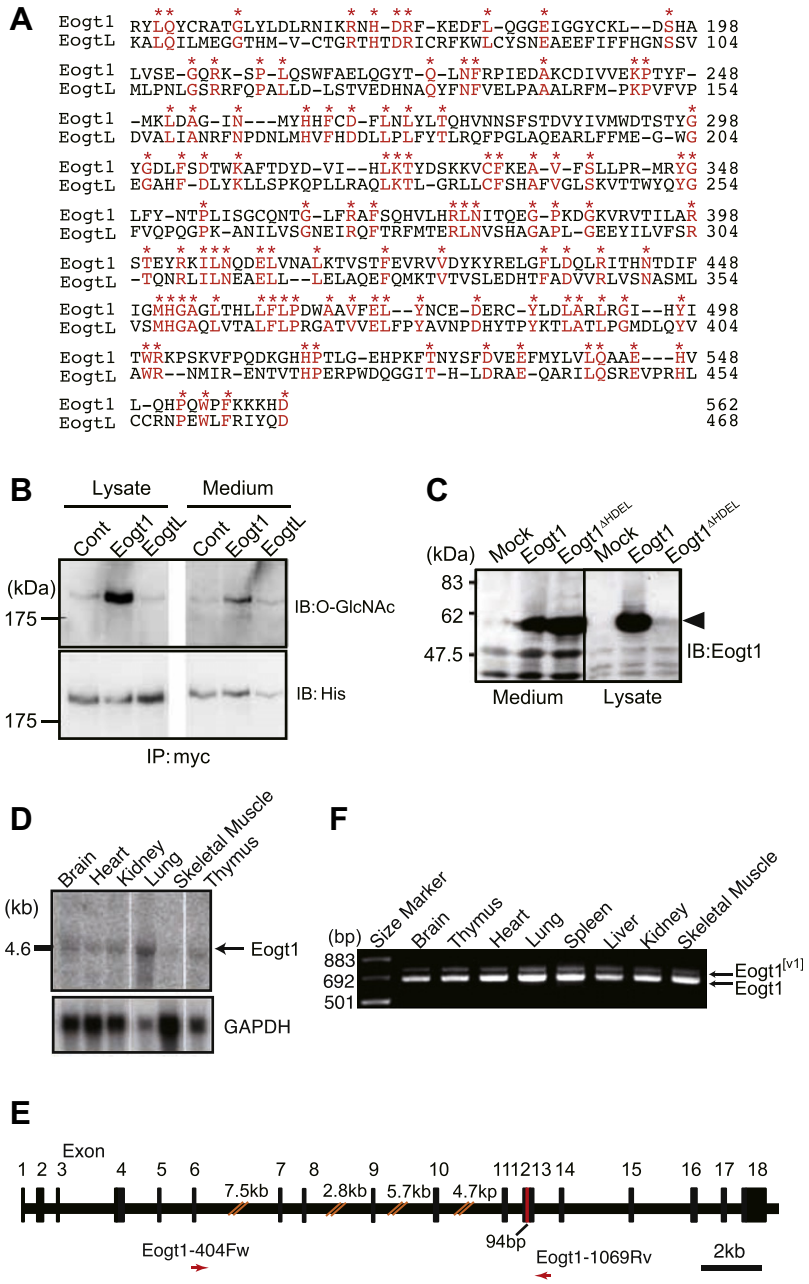

Fig. 1. *Eogt1* is responsible for O-GlcNAcylation of Notch EGF repeats. (A) The amino acid sequence alignment of Eogt1 (uncharacterized glycosyltransferase AER61; GeneBank ID: NP_780522) and Eogt-like protein (uncharacterized glycosyltransferase AGO61; UniProt ID: Q8BW41). Identical amino acids are shown by asterisks. (B) Immunoblot analysis of mN1-EGF isolated from cell lysates or culture supernatants of HEK293T cells co-transfected with *Eogt1*, *Eogt-like*, or vector control. (C) Immunoblot analysis of HEK293T cells expressing FLAG-Eogt1 or FLAG-Eogt1$^{\Delta HDEL}$. Ability of the cells to retain each construct was analyzed by immunoblotting of cell lysates and culture supernatants with anti-Eogt1 antibody. (D) Northern blotting analysis of *Eogt1 and GAPDH*. (E) Schematics showing the genomic structure. Black boxes represent exons and red boxes represent alternative exons; lines indicate introns. (F) RT-PCR analysis of *Eogt1* splice isoforms in mouse tissues. The position of primers used for PCR amplification is shown in (E). The splicing variant of *Eogt1* (*Eogt1*$^{[v1]}$), which includes 94 bp of the intron 12, was observed in all tissues examined.

GEO Profiles database confirmed that *Eogt1* is ubiquitously expressed, but at a higher level in lung and a lower level in skeletal muscles, based on gene expression studies in 22 and 15 adult mouse tissues (GEO DataSets ID: GDS3142 and GDS3052, respectively).

The genomic organization of *Eogt1* is composed of 18 exons spanning at least 40 kb of genomic DNA (Fig. 1E). RT-PCR analysis revealed the occurrence of a minor splice isoform of *Eogt* that contains an extra 94 bp corresponding to intron 12, which results in a frame shift and a premature stop codon, producing a truncated and presumably inactive form of *Eogt1* (Fig. 1E and F).

3.3. Eogt1 exhibits O-GlcNAc transferase activity in vitro

To characterize the O-GlcNAc transferase activity of Eogt1 *in vitro*, we initially attempted to utilize Eogt1: V5His produced from *Drosophila* S2. Unexpectedly, unlike *Drosophila* EOGT: V5His enzyme, Eogt1: V5His did not exhibit detectable enzyme activity. We reasoned that carboxy-terminal V5His tag may cause the steric hindrance because Eogt1 lacks a stretch of peptide sequence located upstream of KDEL-like sequence compared with *Drosophila* EOGT (i.e., ENPSKTQ sequence in *Drosophila*). To circumvent this problem, the signal peptide of Eogt1 was replaced with Bip-FLAG,

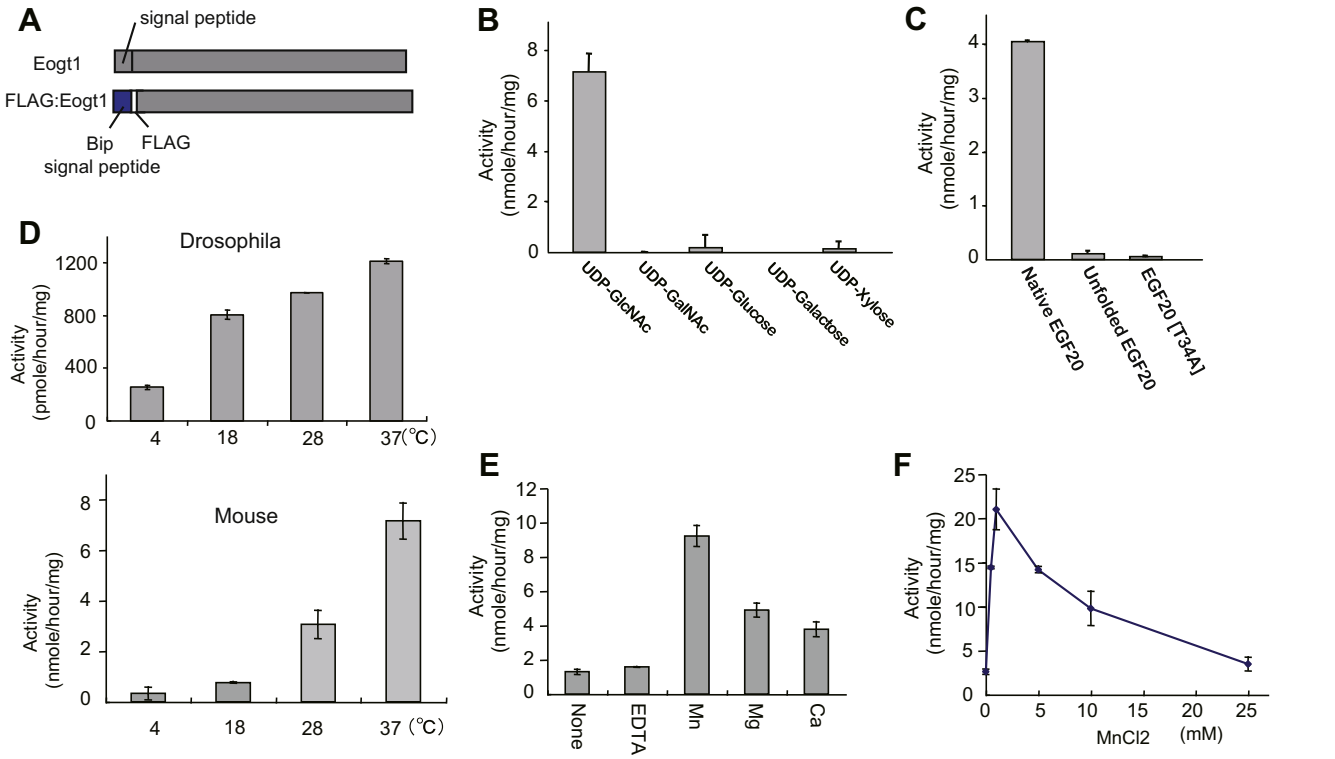

Fig. 2. In vitro enzymatic activity of Eogt1. (A) Schematic representation of mouse Eogt1 constructs used in this study. In the Bip: Eogt1 construct, the signal peptide was replaced with Bip-FLAG sequence that is functional in *Drosophila* cells. (B and C) Donor and acceptor specificity of Eogt1. Various radiolabeled UDP-donor substrates (B) and various EGF domains or its derivatives (C) were used to measure enzymatic activity. Vertical bars represent a range of values obtained from duplicate samples. Unfolded EGF20: V5His was prepared by reduction of disulfide bonds by DTT. (D) The temperature dependence of the O-GlcNAc transferase activity of *Drosophila* EOGT and Eogt1. (E) Metal requirements of the O-GlcNAc transferase activity of Eogt1. Enzyme activity was measured using EGF20 and radiolabeled UDP-GlcNAc as substrates. (F) Effect of increasing concentration of $MnCl_2$ on the O-GlcNAc transferase activity of Eogt1.

so that the FLAG tag remained on the amino-terminus of Eogt1 after cleavage of the Bip signal peptide (Fig. 2A).

To detect the enzyme activity of Eogt1, FLAG-Eogt1 was expressed and affinity-purified from the cell lysates prepared from the stable transfectants. Using various donor substrates, we found that Eogt1 specifically utilizes uridine diphosphate (UDP)-GlcNAc as a sugar donor when EGF20 was used for the assay (Fig. 2B). In terms of acceptor specificity, the O-GlcNAc transferase activity of FLAG-Eogt1 requires the folded structure of EGF20 because unfolded EGF domains were not served as acceptable substrates (Fig. 2C). Moreover, Thr to Ala substitution at Thr34 of EGF20 was not detectably O-GlcNAcylated by FLAG-Eogt1, suggesting that the Thr/Ser residue located between the fifth and sixth conserved cysteines ($C^5XXXX\underline{T/S}G$) is the site for the modification (Fig. 2C). In contrast, unlike the *Drosophila* enzyme, FLAG-Eogt1 showed markedly elevated enzyme activity at 37 °C than at 18 °C and 25 °C (Fig. 2D). Similar to that of *Drosophila* EOGT, the enzyme activity of Eogt1 is enhanced in the presence of divalent cations, especially by Mn^{2+}, and it exhibits the maximal activity at the concentration of 1 mM (Fig. 2E and F). Thus, except for the temperature dependence, the enzymatic property of Eogt1 is indistinguishable from that of the *Drosophila* enzyme.

3.4. O-GlcNAc transferase activity is evolutionarily conserved

Next, we asked whether the expression of *Eogt1* substitutes the glycosyltransferase activity of EOGT in S2 cells. To this end, FLAG-tagged Notch EGF repeats (N-EGF: FLAG) were expressed and isolated from the culture medium and O-GlcNAc modification was detected with the specific antibody, CTD110.6. Overexpression of FLAG-Eogt1 in wild-type S2 cells markedly increased the O-GlcNAc level of N-EGF: FLAG (Fig. 3A). In contrast, upon depletion of endogenous *Eogt* by RNAi, O-GlcNAcylation of N-EGF: FLAG was decreased (Fig. 3B). The decreased O-GlcNAc level of N-EGF: FLAG produced from *Eogt* RNAi cells was restored when cells were co-expressed with FLAG-Eogt1 (Fig. 3B). These results suggested that Eogt1 exhibits comparable enzymatic activity to *Drosophila* EOGT.

We further investigated whether Eogt1 has the ability to substitute the biological requirement of EOGT during development. To this end, we generated transgenic lines expressing the *FLAG-Eogt1* construct under Gal4-UAS control. RNAi-mediated down regulation of *Eogt* in developing wing discs using en-Gal4 driver caused a cell adhesion defect, exhibiting wing blistering in the posterior compartment (Fig. 3C) [9]. This phenotype is completely rescued by expressing FLAG-Eogt1 (Fig. 3C and D). These results demonstrated that O-GlcNAc transferase activity modifying EGF domains is conserved between mouse and *Drosophila*.

3.5. Eogt1 mediates the biosynthesis of O-GlcNAc glycan in HEK293T cells

A previous *in vitro* study suggested that O-GlcNAc is readily modified with galactose by an β1,4-galactosyltransferase enzyme [7,17]. Since β1,4-galactosyltransferase genes are ubiquitously expressed in various mammalian cells, the O-GlcNAc moiety could be further modified to form elongated glycan structures [7,18]. Interestingly, mN1-EGF isolated from the culture medium of Eogt1-transfected cells was less reactive to O-GlcNAc antibody than mN1-EGF purified from the cell lysate (Fig. 1B). This observation can be explained if O-GlcNAc moieties on mN1-EGF are capped by the subsequent modification during secretion, which precludes detection of the epitope by O-GlcNAc antibody. To test this idea,

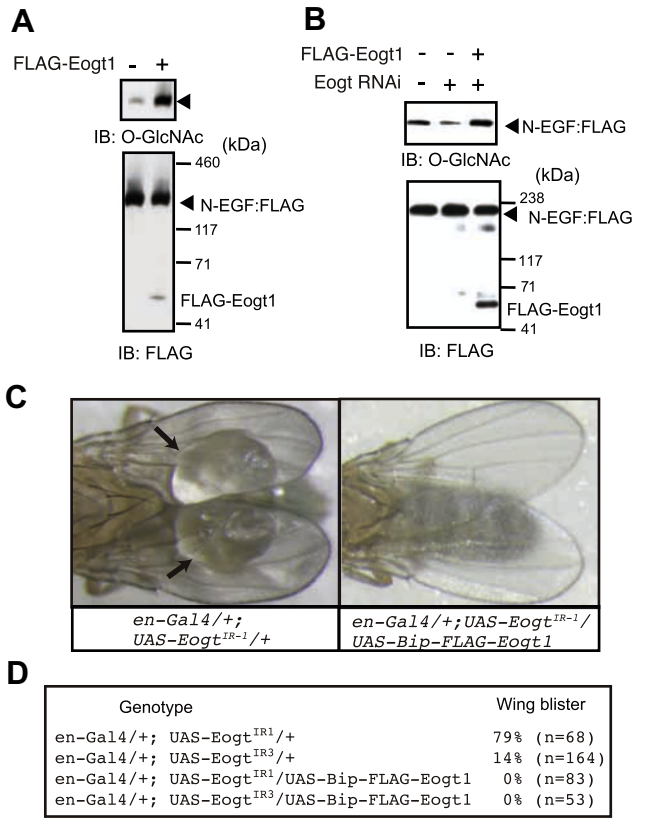

Fig. 3. O-GlcNAc transferase activity is evolutionarily conserved. (A) Expression of *Eogt1* elevated O-GlcNAc level of N-EGF: FLAG. S2 cells were transfected to express N-EGF: FLAG with or without FLAG: Eogt1. N-EGF: FLAG was affinity-purified from the culture media and subjected to immunoblotting with O-GlcNAc antibody. (B) Expression of *Eogt1* substitutes endogenous O-GlcNAc transferase activity in S2 cells. Wild type S2 cells or *Eogt* RNAi cells are transfected to express N-EGF: FLAG with or without FLAG: Eogt1 and analyzed as mentioned above. (C) RNAi-mediated knockdown of *Eogt* caused blistering in the posterior wing where RNAi was induced (arrow). Crosses were conducted at 25 °C. Expression of *Bip-FLAG-Eogt1*, encoding the mouse homolog of *Eogt* rescued the wing blister phenotype. (D) The frequency of wing blisters counted in animals of indicated genotypes.

mN1-EGF isolated from culture medium of *Eogt1*-transfected cells was treated with β1,4-galactosidase alone or together with α2,3/2,6-neuraminidase. As expected, galactosidase treatment of mN1-EGF increased the reactivity of O-GlcNAc antibody. Interestingly, the simultaneous digestion with α2,3/2,6-neuraminidase had no apparent effect on the reactivity with O-GlcNAc antibody (Fig. 4A). These results suggest the presence of the elongated O-GlcNAc glycan structure, namely O-linked *N*-acetyl-lactosamine (O-LacNAc) (Fig. 4B).

4. Discussion

In this study, we investigated the previously uncharacterized *Eogt1* and showed that it encodes an O-GlcNAc transferase, specifically modifying the EGF domains. *In vitro* enzyme assay revealed that Eogt1 selectively utilizes UDP-GlcNAc as a donor substrate and the enzyme activity is enhanced by Mn^{2+}. Thus, except for the slight difference in temperature dependence, most of the enzymatic activity of Eogt1 is indistinguishable from that of the *Drosophila* enzyme, supporting the data obtained from the genetic rescue experiments in *Drosophila*. This is the first report describing the novel O-GlcNAcylation pathway in mammals.

Although O-GlcNAc modification was detected in mN1-EGF isolated from cell lysates and culture media, the O-GlcNAc moiety was subject to additional modifications during secretion, resulting in the generation of unique structures such as O-LacNAc in HEK293T cells. However, the terminal structures of O-GlcNAc glycans require further investigation because they may vary among individual EGF domains depending on the substrate specificity and accessibility of subsequently acting glycosyltransferases. Interestingly, the O-GlcNAcylation site of Notch1 EGF15 is modified with O-HexNAc monosaccharide [19]. Thus, if this modification is O-GlcNAc, EGF15 appears to be refractory to further glycosylation.

We have shown that Dumpy, a membrane-anchored extracellular matrix protein, is a functionally important substrate for *Drosophila Eogt*, on the basis of the observations that loss of *Eogt* causes defects in Dumpy-dependent cell–matrix interactions, resulting in wing blistering and cuticle detachment [9]. The ability of Eogt1 to rescue wing blister phenotype indicates that Eogt1 can glycosylate Dumpy, thereby conferring the biological activity regulating the cell–matrix interactions. However, the mammalian genome does not encode apparent Dumpy homologs or structurally related proteins, and thus the biological function of Eogt1 in mice, if any, would rely on other EGF domain-containing substrates. Indeed, one such candidate substrate is the Notch receptor. Although the phenotypic analysis of *Drosophila Eogt* mutant suggested that *Eogt* appears to be dispensable for most Notch-dependent processes, we cannot officially exclude the possibility that *Drosophila Eogt* functions only in a subset of Notch-dependent developmental processes that have not been investigated in previous studies. Alternatively, O-GlcNAcylation is functionally redundant with other O-glycosylations. Thus, the roles of atypical O-GlcNAcylation in mammalian Notch signaling and their functional relationship with other O-glycosylations such as O-fucose [13,20,21], O-xylose [22], and O-glucose [19,23–27] glycosylations need to be investigated. Lastly, it should be noted that O-GlcNAc modification by *Eogt1* could act on yet unidentified substrates only present in mammals. Future studies, including phenotypic analyses of Eogt1-deficient mice, will be necessary to address the roles of atypical O-GlcNAcylation in mammals. Nonetheless, these

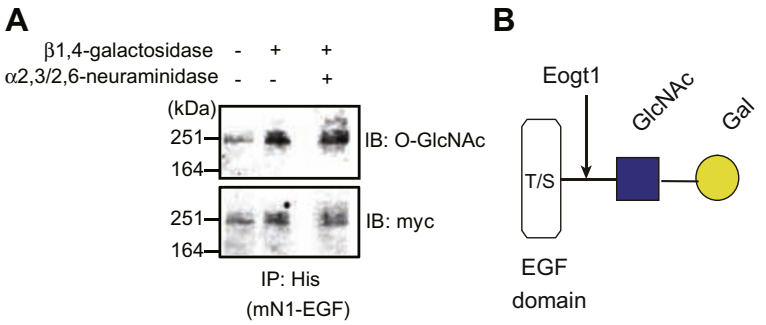

Fig. 4. Eogt1 initiates synthesis of O-GlcNAc glycans. (A) mN1-EGF secreted from HEK293T cells expressing *Eogt1* was untreated or treated with β1,4-galactosidase alone or together with α2,3/2.6-neuraminidase, and analyzed by immunoblotting with O-GlcNAc antibody. (B) The proposed structure for O-GlcNAc glycans.

considerations raise the intriguing possibility that the O-GlcNAcylation reaction in the secretory pathway is evolutionarily conserved, whereas the significance of O-GlcNAcylated proteins in the biological processes had changed during the evolution.

Acknowledgments

We thank P. Stanley for sharing unpublished results, N. Nakamura for comments on the manuscript, K. Basler for gifting us pUASTattB, Y. Funakoshi and P. Stanley for the reagents, and A. Matsuura and K. Ochiai for their support during the initial phase of the project. This work was supported by grants from the Japanese Ministry of Education, Science, Sports and Culture and the 21st Century COE Program (to T.O., D.N., and T.M.), the Takeda Foundation, the Sumitomo Foundation, the Uehara Foundation, and the Mizutani Foundation (to T.O.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.01.098.

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