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Roles for N-glycosylation in the dynamics of Edg-1/S1P1 in sphingosine 1-phosphate-stimulated cells

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Sphingosine 1-phosphate (Sph-1-P) is a bioactive lipid mediator released from activated platelets. To date, 5 seven-transmembrane-spanning receptors, Edg-1/S1P1, Edg-3/S1P3, Edg-5/S1P2, Edg-6/S1P4 and Edg-8/S1P5, have been identified as specific Sph-1-P receptors. Our recent novel studies established that Edg-1/S1P1 is glycosylated in its N-terminal extracellular portion and further identified the specific glycosylation site as asparagine 30. We also demonstrated that the structure of the N-terminal ectodomain of Edg-1/S1P1 affects both its transport to the cell surface and the N-glycosylation process. These studies revealed a possible regulatory role for the N-glycan on Edg-1/S1P1 in the dynamics of the receptor, such as its lateral and internal movements within the membrane, in ligand-stimulated mammalian cells.
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Introduction

The endothelial differentiation gene (Edg) products are seven-transmembrane-spanning, G protein-coupled receptors (GPCR) for the platelet-derived, bioactive lipid mediator sphingosine 1-phosphate (Sph-1-P) [1–3]. Sph-1-P is generated through phosphorylation by sphingosine kinases of its precursor sphingosine [4–6], an intermediary metabolite of sphingomyelin [7]. Sph-1-P is predominantly stored in human platelets and is released upon activation [8]. Studies have shown that, via the cell surface Edg family receptors, Sph-1-P regulates diverse signal transduction pathways implicated in cell processes such as endothelial cell motility, proliferation, NO production, and vascular smooth muscle cell contraction [9,10]. Northern blot analysis has demonstrated that Edg-1/S1P1, Edg-3/S1P3 and Edg-5/S1P2 are ubiquitously expressed in many mammalian tissues [11]. In contrast, Edg-6/S1P4 is specifically expressed in lymphocyte-containing tissues, such as spleen and lung [12], and Edg-8/S1P5 is expressed in neuronal cells [13].

Our recent studies have shown that the N-terminal ectodomains of Edg-1/S1P1 and Edg-3/S1P3 are truncated in overexpressing cells [14]. In those studies, we found that a deletion mutant lacking the N-terminal processing domain of Edg-1/S1P1 accumulated in the endoplasmic reticulum (ER) without undergoing N-glycosylation at the Golgi. Reasonably, this mutant receptor was not expressed on the cell surface. Conversely, though, when a basic amino acid residue was introduced at the cleavage site of Edg-1/S1P1, the molecular weight of the glycosylated form was greater for the mutant compared to the wild type, due to additional N-glycosylation. These results demonstrated that the structure of the N-terminal ectodomain of Edg-1/S1P1 affects both its transport to the cell surface and the N-glycosylation process.

Moreover, we found, for the first time, that Edg-1/S1P1 is glycosylated in its N-terminal extracellular portion. We further identified the specific glycosylation site as asparagine 30 [15]. In that report, we observed that the glycan of Edg-1/S1P1 is important for ligand-induced receptor internalization, based on experiments using a non-glycosylated mutant of Edg-1/S1P1 (N30D-Edg-1/S1P1). The absence or presence of an oligosaccharide on Edg-1/S1P1 did not affect the folding and stability of this protein, nor the ligand-binding affinity. Unlike the wild-type Edg-1/S1P1, which was associated with the caveolae, non-glycosylated N30D-Edg-1/S1P1 was dispersed broadly in the membrane microdomain, suggesting that internalization and microdomain localization of N-glycosylated Edg-1/S1P1 might be related. These observations strongly suggest that the presence of an N-linked glycan in the receptor may play a

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regulatory role in receptor dynamics, *i.e.* lateral and internal movements, in ligand-stimulated mammalian cells.

The N-terminal ectodomain of Edg-1/S1P1 affects its N-glycosylation

Reportedly, in some G protein-coupled receptors, which are generally activated by large peptides like glucagon or secretin, the ligand-binding affinity is regulated by the N-terminal ectodomain of the receptor [16]. The PARs (proteinase-activated receptors) are known to be activated when their ectodomains are truncated by endopeptidases [17]. In our studies overexpressed Edg-1/S1P1 localized to the cell surface, following N-glycosylation in the ER and further modification with complex-type oligosaccharides in the Golgi. We determined that the N-terminal ectodomain of Edg-1/S1P1 is truncated somewhere in the process of translocating to the cell surface from the ER membrane [14]. Additionally the deletion mutant lacking the N-terminal processing domain of Edg-1/S1P1 was not able to be glycosylated appropriately in the Golgi. Consequently, this mutant receptor accumulated in the ER and was not expressed on the cell surface. Sph-1-P, as an intracellular signaling molecule, engages with Edg family receptors on the cell surface. According to one report, a similar accumulation in the ER was observed for the V2-vasopressin receptor, a cell surface-expressed GPCR, after point mutations were introduced [18]. It is thought that the hereditary disease nephrogenic diabetes insipidus is caused by these same mutations. In the future, a pathologic phenomenon may be found in which Sph-1-P or its receptor is implicated as a causal factor, possibly due to aberrant localization of the receptor. If so, it will be especially meaningful to clarify the molecular mechanisms of the Edg-1/S1P1 mutant accumulating in the ER.

Interestingly, when a basic amino acid residue was introduced at the cleavage site in the N-terminal portion of Edg-1/S1P1, the molecular weight of the protein was increased, reflecting modification with oligosaccharides [14]. These findings suggest that the truncation of the N-terminal ectodomain of Edg-1/S1P1 affects its N-glycosylation. Therefore, we assume that a specific protein interacting with the N-terminal portion of Edg-1/S1P1 may regulate the N-glycosylation at the Golgi, but further studies are required to elucidate the molecular mechanisms.

Reportedly, alterations in the types of sugars in the prion protein (PrP) can reflect changes caused by developmental processes or by disease [19]. This change is consistent with a decrease in the activity of *N*-acetylglucosaminyltransferase III (GnTIII) toward normal PrP [20]. Moreover, structural changes in modifying oligosaccharides have been reported to be closely related to malignancy in cancer [21]. Edg-1/S1P1 expressed in endothelial cells induces cell migration and proliferation upon ligand stimulation [22,23]. Another report indicates that Edg-1/S1P1 has an important role in angiogenesis [24]. Therefore, it will be interesting to clarify the effects of structural changes in the oligosaccharides modifying Edg-1/S1P1 on the function of the receptor.

N-glycosylation among Sph-1-P receptors

G protein-coupled receptors (GPCR) are important molecules in transducing signals from various extracellular molecules such as neurotransmitters, peptide hormones, and bioactive lipids, as well as from stimuli such as odors, taste, light, and pain [25]. Many such GPCRs are modified by N-glycosylation at the extracellular Asn residue(s) in the consensus sequence (-N-X-T/S-) [26]. N-glycans are known to play a pivotal role in protein folding, oligomerization, quality control, sorting, and transport [27]. For opiate receptors, N-glycosylation is the rate-limiting step in their translocation to the cell surface [28], demonstrating that glycosylation can serve as a key regulatory event in protein expression.

Recently, we reported that Edg-1/S1P1 was modified with oligosaccharides at the 30th asparagine residue [15]. The region encompassing the N-glycosylation motif in Edg-1/S1P1 is highly conserved among the N-terminal portions of the Edg family Sph-1-P receptors; Edg-3/S1P3, Edg-5/S1P2, Edg-6/S1P4 and Edg-8/S1P5 (Figure 1). Therefore, these receptors may also be modified by N-glycosylation in this region. In fact, we have confirmed that Edg-6/S1P4 is glycosylated in this region [29].

Subcellular localization of a non-glycosylated Edg-1/S1P1 mutant

Because the N-glycosylation motif in the Edg family receptors is so conserved, we believe it may serve a common function in these receptors. Therefore, we examined the subcellular distribution of wild type- (WT) and N30D-Edg-1/S1P1 using our generated stable transfectants [15]. We found that the non-glycosylated N30D-Edg-1/S1P1 was not only stably expressed at the cell surface, but also that it folded appropriately, similar to its WT-control. Ligand-binding analysis revealed no apparent difference between the Kd values of WT- and N30D-Edg-1/S1P1. We concluded that the absence or presence of N-glycan in Edg-1/S1P1 has no influence on the subcellular localization of the receptor.

Roles of N-glycans in ligand-induced receptor internalization

In many GPCRs like Edg-1/S1P1, ligand stimulation induces receptor internalization [30]. Using stable CHO transfectants, we found a decrease in the internalization of receptors in ligand-stimulated cells expressing non-glycosylated N30D-Edg-1/S1P1, compared to the internalization in cells expressing WT-Edg-1/S1P1 [15]. This confirmed that the N-glycan of Edg-1/S1P1 have some function in ligand-induced receptor internalization. To date, many researchers have revealed information regarding the machinery of ligand-induced cell surface receptor internalization. First, upon ligand stimulation many GPCRs are phosphorylated by G protein-coupled receptor kinase (GRK) [31,32]. The phosphorylated GPCRs are then translocated from

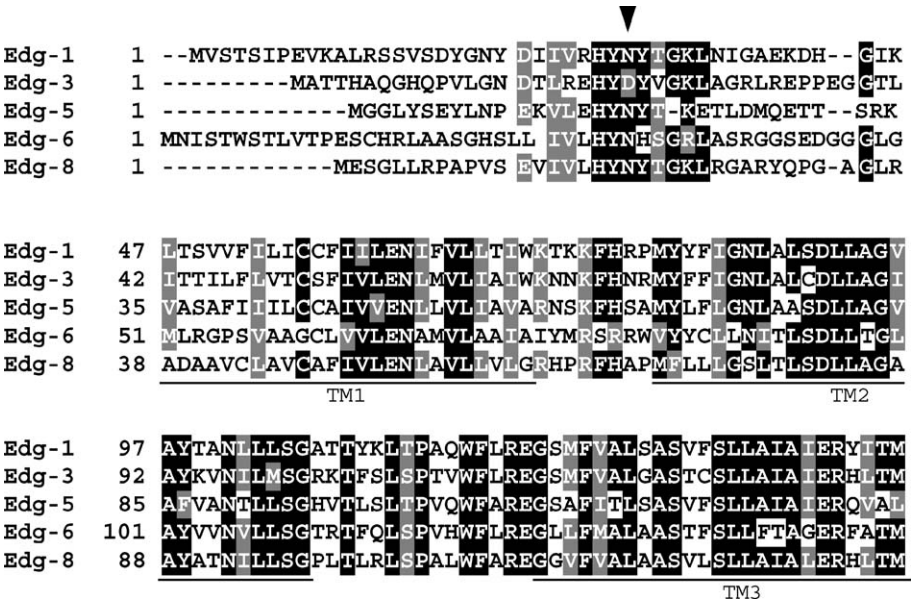

Figure 1. Comparison of amino acid sequences of the N-terminal regions among Sph-1-P receptors. Sequences were aligned using the CLUSTAL W 1.7 program and represented with the BOXSHADE program. The arrowhead indicates the N-glycosylation site of Edg-1/S1P1. Because the domain encompassing the N-glycosylation site in Edg-1/S1P1 is closely conserved in these receptors, the N-glycan may have important roles in these receptors.

the cell surface by forming a complex with β-arrestin, a process involving MAPK activation [33,34].

On the other hand, some GPCRs are internalized from the cell surface by the formation of clathrin-coated pits associated with the GTPase dynamin [35]. The internalized GPCRs are processed by lysosomal degradation or recycling pathways [36]. However, the roles of the N-glycan on Edg-1/S1P1 cannot be explained by this strategy. Some reports mention roles for N-glycans. One report indicates some cell surface proteins nonuniformly accumulate, with many signal transmitters, in

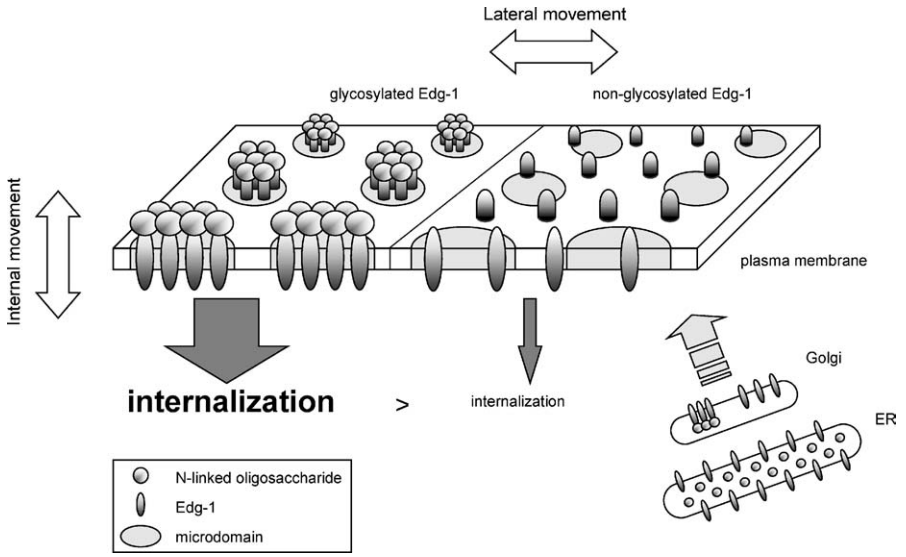

Figure 2. The roles of N-glycan in Edg-1/S1P1. (Left) Under normal conditions, following translation and synthesis in the ER, Edg-1/S1P1 is glycosylated in the Golgi with a complex-type oligosaccharide at Asn-30. Edg1 is then accumulated at the membrane microdomain by a specific interaction, for example, a glycan-glycan interaction (lateral movement). Since ligand-induced receptor internalization occurs at the membrane microdomain, N-glycosylated Edg-1/S1P1 is efficiently internalized (internal movement). (Right) Because the non-glycosylated N30D-Edg-1/S1P1 cannot accumulate in the membrane microdomain, the amount of N30D-Edg-1/S1P1 in the microdomain fraction is decreased. Hence, the loss of glycosylation is thought to be the reason for the loss of the ligand-induced receptor internalization.

a specific domain of the plasma membrane termed the lipid microdomain, rather than exhibiting a flat distribution in the membrane [37,38]. It is thought that extracellular signaling, such as ligand stimulation, is transduced efficiently from the membrane microdomain. Another report demonstrated that a glycan-glycan interaction regulates signal transduction pathways and suggested this as a role for glycans in some cell surface glycoproteins or glycolipids [39]. In this situation, a glycosylated receptor would be accumulated by glycan-glycan interactions at the membrane microdomain, and then the ligand-induced receptor internalization could efficiently occur from this structure.

According to our study using sucrose density gradient centrifugation and a hypertonic alkaline solution, WT-Edg-1/S1P1 accumulated in the membrane microdomain, whereas non-glycosylated N30D-Edg-1/S1P1 was evenly distributed in high-density fractions other than the microdomain fractions [15]. Hence, we confirmed that the N-glycan of Edg-1/S1P1 is in fact necessary for its accumulation in the membrane microdomain.

Conclusions

Now we propose a working hypothesis for the roles of the N-glycan on the sphingosine 1-phosphate receptor, Edg-1/S1P1 (Figure 2). WT-Edg-1/S1P1 is accumulated in the membrane microdomain by a specific interaction of the N-glycans. When the receptors are activated upon ligand stimulation, they are immediately internalized from the membrane microdomain. Conversely, because the non-glycosylated N30D-Edg-1/S1P1 mutant could not accumulate in the membrane microdomain, signaling from the receptor internalization was not transduced efficiently, even if the ligand was bound to the receptor. Hence, the loss of glycosylation is thought to be the reason for the loss of the ligand-induced receptor internalization. Since the non-glycosylated mutant generated by us was mostly expressed at the plasma membrane, the absence or presence of N-glycan in Edg-1/S1P1 may not affect receptor trafficking to the cell surface.

Then, in what fashion are N-glycosylated receptors accumulated in the membrane microdomain? We propose two mechanisms for the roles of N-glycan on the GPCR Edg-1/S1P1. One possible explanation is a glycan-glycan interaction between N-glycosylated proteins. Presumably, the N-glycosylated protein associates with other N-glycans on glycoproteins on the membrane; and, consequently, these proteins are stacked in the membrane and form a microdomain structure. On the other hand, it is possible that a lectin which recognizes an oligosaccharide on a glycoprotein may regulate the localization of glycoproteins. In the latter scenario, the glycoproteins accumulate toward the membrane microdomain.

Interestingly, in the non-glycosylated N30D-Edg-1/S1P1, a decrease in the number of internalized receptors was observed upon ligand stimulation. This finding demonstrates that receptor resensitization did not occur sufficiently. Because sphingosine 1-phosphate and its receptor Edg-1/S1P1 have been confirmed to be involved in Sph-1-P-induced vascular maturation, these molecules are implicated in angiogenesis [40]. Since the absence or presence of N-glycan in Edg-1/S1P1 affects its resensitization, it may be closely related to proliferation and malignancy in cancer cells. Although the precise molecular mechanism of the internalization of the Edg-1/S1P1 receptor localized in the microdomain remains to be examined, the studies discussed here strongly suggest that the N-linked glycan of the Edg-1/S1P1 receptor may play a regulatory role in the receptor dynamics in ligand-stimulated mammalian cells.

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