J. Biochem. **134**, 667–673 (2003)
DOI: 10.1093/jb/mvg191

Truncation of the N-Terminal Ectodomain Has Implications in the *N*-Glycosylation and Transport to the Cell Surface of Edg-1/S1P1 Receptor

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Received June 28, 2003; accepted August 25, 2003

The endothelial cell-expressed sphingosine 1-phosphate receptors Edg-1/S1P1 and Edg-3/S1P3 have been implicated in various physiological events such as the regulation of angiogenesis. Since there is an excess of a ligand constitutively in blood, these receptors may have some mechanism(s) avoiding overstimulation. In this study, we found that the N-terminal ectodomains of Edg-1/S1P1 and Edg-3/S1P3 were truncated in overexpressing cells. The truncated form of Edg-1/S1P1 expressed on the cell surface had undergone complex-type oligosaccharide modification at the Golgi. A deletion mutant lacking the N-terminal processing domain of Edg-1/S1P1 accumulated in the endoplasmic reticulum, and was not expressed on the cell surface. When a basic amino acid residue was introduced at the cleavage site of Edg-1/S1P1, the molecular weight of the glycosylated protein was greater in the mutant compared to the wild type, due to the bound oligosaccharide. 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. Ectodomain shedding of many membrane proteins has been implicated in various diseases. Therefore, N-terminal processing of Edg-1/S1P1 and Edg-3/S1P3 might play roles in endothelial cell functions.

Key words: ectodomain shedding, Edg-1/S1P1, glycosylation, GPCR, sphingosine 1-phosphate.

Abbreviations: CHO, Chinese hamster ovary; Edg, endothelial differentiation gene; Endo H, endoglycosidase H; GPCR, G protein-coupled receptor; PNGase F, peptide *N*-glycosidase F; Sph-1-P, sphingosine 1-phosphate.

Sphingosine1-phosphate (Sph-1-P) is a bioactive lipid generated through phosphorylation of its precursor, sphingosine, by sphingosine kinase (*1–4*). Studies have shown that Sph-1-P, which can be released from activated platelets, is also constitutively present in human blood at levels of approximately 100 nM (*5–7*). The Sph-1-P receptors Edg-1/S1P1, Edg-3/S1P3, Edg-5/S1P2, Edg-6/S1P4, and Edg-8/S1P5 belong to the G protein–coupled receptor (GPCR) family, and share with other members a common structural feature of seven transmembrane-spanning domains (*8–12*). At physiological concentrations, Sph-1-P can bind to its receptor, which regulates diverse signal transduction pathways implicated in cell motility, differentiation, proliferation and development via intracellular signaling molecules (*13*).

Modification with oligosaccharides is a potential factor in the regulation of protein folding, stability, sorting and secretion (*14*). Many GPCRs are modified through *N*-glycosylation at the ER and Golgi (*15*). For opiate receptors, this *N*-glycosylation is a rate-limiting step in their translocation to the cell surface (*16*), demonstrating that glycosylation can serve as a key regulatory event.

Recently, we reported that Edg-1/S1P1 was modified with an oligosaccharide at the 30th asparagine residue, and that this oligosaccharide was indispensable for the accumulation of the receptor in the membrane microdomain (*17*). Now, we report that the N-terminal portion of Edg-1/S1P1 is truncated independently of oligosaccharide modification. Many GPCRs do not have a signaling peptide at the N-terminus. Therefore, we examined the significance and function of N-terminal ectodomain processing of Edg-1/S1P1.

MATERIALS AND METHODS

DNA Constructs—A DNA construct encoding Edg-1/S1P1 was prepared as previously described (*17*). Murine Edg-5/S1P2 cDNA was amplified from mRNA of NIH3T3 cells using the reverse transcriptase-polymerase chain reaction (RT-PCR) with primers (sense, 5′-GGATCCACCACCATGGGCGGTTTATACTC-3′; antisense, 5′-CGGGATCCAGACCACTGTGTTGCCCTCCA-3′). The amplified cDNA was subcloned into the *Bam*HI site of expression vector pcDNA3-FLAG1-HA4 (*17*). Murine Edg-3/S1P3-coding sequences were amplified from an EST clone, AI115954, by PCR using KOD polymerase (TOYOBO, Tokyo) with primers (sense, 5′-CGGGTACCATGGCAACCACGCATGCG-3′; antisense, 5′-CGGGATCCTTGCAGAGGACCCCGTTC-3′). Full-length murine Edg-6/S1P4 coding sequences were amplified from EST clone AI158066, which lacks two base pairs at the initiation codon, using Ex Taq polymerase (Takara, Shiga) and primers (sense,

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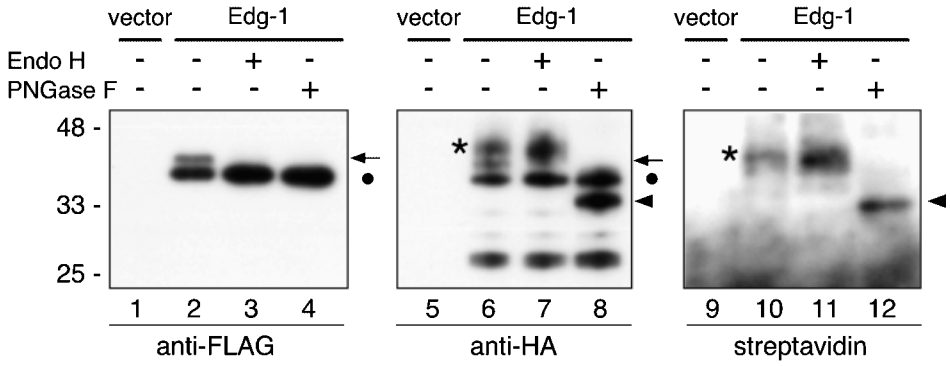

Fig. 1. **Ectodomain of Edg-1/S1P1 on the cell surface is truncated.** Edg-1/S1P1tagged with both N-terminal FLAG and C-terminal HA was transiently expressed in CHO cells. Cell surface proteins were biotinylated with membrane impermeable sulfo-NHS-LC-biotin. Cells were lysed, and the lysates were immunoprecipitated with an anti-HA antibody. Samples, untreated or treated with endoglycosidase Endo H or PNGase F, were separated by SDS-PAGE and then analyzed by Western blotting using an anti-FLAG (left panel) or an anti-HA (middle panel) antibody, or, to detect biotinylated cell surface proteins, streptavidin-HRP (right panel). The asterisk indicates the PNGase F-sensitive, complex-type oligosaccharide-conjugated Edg-1/S1P1; the arrow, the immature Edg-1/S1P1 with Endo H-sensitive, high mannose-type oligosaccharide; the closed circle, the intact form of non-glycosylated Edg-1/S1P1; and the arrowhead, the N-terminal-truncated Edg-1/S1P1. Protein molecular weight standards (in kDa) are indicated.

5′-GAAGATCTATGAACATCAGTACCTGGTCC-3′; antisense, 5′-AGCGTCCGCAGCACCAGATCTTC-3′), the sense primer containing the initiation codon. PCR introduced a *Bam*HI site or a *Bgl*II site at both the 3′ and 5′ ends of the Edg-3/S1P3 or Edg-6/S1P4 cDNA. The amplified Edg-3/S1P3 or Edg-6/S1P4 cDNA was subcloned into the *Bam*HI site of an expression vector, pcDNA3-FLAG1-HA4.

Mutagenesis—Edg-1/S1P1 N-terminal–truncated mutants and the mutants carrying point mutations were constructed by PCR mutagenesis. PCR was performed with 0.4 ng of DNA template and primers encompassing the entire coding region, except for the truncated region (NΔ06 sense, 5′-CCGGAGGTTAAAGCTCTCCGCAGC-3′; NΔ07 sense, 5′-GAGGTTAAAGCTCTCCGCAGCTCA-3′; NΔ08 sense, 5′-GTTAAAGCTCTCCGCAGCTCAGTC-3′; NΔ09 sense, 5′-AAAGCTCTCCGCAGCTCAGTCTCT-3′; NΔ10 sense, 5′-GCTCTCCGCAGCTCAGTCTCTGAC-3′; NΔ15 sense, 5′-GTCTCTGACTATGGGAACTATGATATCAT-3′; NΔ25 sense, 5′-GTCCGGCATTACAACTACACAGGCAAGTT-3′; antisense primer used for all deletion mutants, 5′-CTTATCGTCGTCATCCTTGTAATCCATGG-3′; E8R, sense, 5′-CCGCGGGTTAAAGCTCTCCGCAGC-3′; V9R sense, 5′-CCGGAGCGCAAAGCTCTCCGCAGC-3′; K10A sense, 5′-CCGGAGGTTGCAGCTCTCCGCAGC-3′; and antisense primer used for all point mutations, 5′-GATGCTAGTGGACACCATGGATCCCTT-3′). The antisense primers were treated with T4 polynucleotide kinase (Takara). All genes were completely sequenced after mutagenesis.

Cell Culture and Transfection—Chinese hamster ovary (CHO) cells were cultured in Ham's F-12 medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (Iwaki, Chiba), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma) at 37°C under a humidified 5% CO_2 atmosphere. To generate stable transfectants, the cDNA corresponding to each receptor, subcloned into the pcDNA3-FLAG1-HA4 vector, was transfected into CHO cells using a LipofectAMINE Plus kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Stably transfected clones were selected with 600 μg/ml Geneticin (G-418 sulfate, Invitrogen).

Biotinylation and Analysis of Cell Surface Proteins—The transfected CHO cells were washed twice with cold phosphate-buffered saline (PBS), and then incubated with 1 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL) at 4°C. After 60 min, the biotinylation reagent was removed, and the cells were washed three times with 50 mM Tris-buffered saline (pH 8.5) to quench the remaining reagent. These cells were lysed with extraction buffer [100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 5 mM sodium orthovanadate, 1% NP-40, and protease inhibitors] and then immunoprecipitated with an anti-HA antibody (Y-11; Santa Cruz Biotechnology, Santa Cruz, CA). Samples were then resuspended in Laemmli's buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol) and analyzed by Western blotting using anti-FLAG antibody M2 or anti-HA clone 7 antibody (both from Sigma). The blots were then washed with TBS-T [20 mM Tris-HCl (pH 7.5), 137 mM NaCl, and 0.05% Tween-20] and incubated with the secondary antibody for 1 h at room temperature. The blots were washed again with TBS-T and then developed with ECL-plus. The biotinylated proteins were visualized using streptavidin-HRP (Sigma) and developed with ECL plus. When necessary, the antibodies were stripped off the membranes by incubation in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS) for 30 min at 50°C with constant agitation, rinsed twice in TBS-T, and then reprobed with other antibodies, as indicated.

Preparation and Endoglycosidase Digestion of Edg-1/S1P1—Edg-1/S1P1-expressing CHO cells were washed twice with cold PBS and then lysed with extraction buffer [50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10 mM $MgCl_2$, protease inhibitor cocktail (Complete, EDTA-free; Roche, Mannheim, Germany), and 5 mM EDTA]. The cell lysates were immunoprecipitated with an anti-HA antibody (Y-11) at 4°C for 2 h. Then, Sepharose beads were collected by centrifugation at 8,000 ×*g* for 40 seconds at 4°C and washed three times with lubrol buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA and 0.1% Lubrol). After removal of the final wash, the pellets were resuspended in Laemmli's sample buffer. For endoglycosidase digestion, the pellets were resuspended in denaturing buffer (50 mM Tris-HCl, pH7.5, 1% SDS and 1 mM EDTA). Endo H (Cell Signaling, Beverly, MA) or peptide N-glycosidase F (PNGase F, Cell Signaling) digestion was

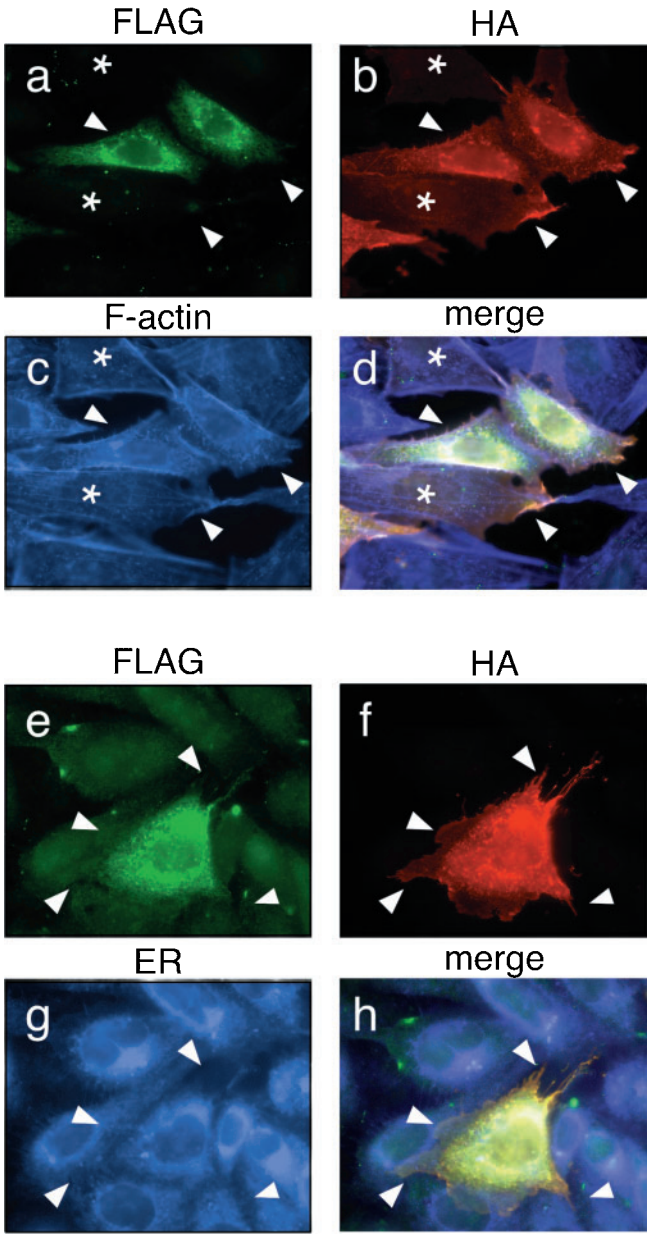

Fig. 2. **Edg-1/S1P1 in the ER avoids N-terminal truncation.** Epitope-tagged Edg-1/S1P1 was transiently expressed into CHO cells. After transfection for 24 h, the cells were fixed, permeabilized, and immunostained with an anti-FLAG (a, e, green fluorescence) or anti-HA (b, f, red fluorescence) antibody. Cells were co-stained with phalloidin to visualize F-actin (c, blue stain), or with ER-tracker blue to visualize the ER (g, blue stain). Merged images are shown (d, h). The arrowhead indicates the edge of the plasma membrane; the asterisk, low-level expression of Edg-1/S1P1.

performed on lysates at 37°C for 3 h, by the manufacturer's recommended procedures. The reactions were terminated by adding 4 × Laemmli sample buffer (250 mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol and 20% 2-mercaptoethanol). Samples were then separated by SDS-PAGE, and immunoblotting was performed as described previously (*17*).

Immunofluorescence Microscopy—CHO cells were seeded onto sterile glass coverslips (Matsunami, Kyoto) in 12-well cell culture cluster plates. Following attachment for 24 h, the cells were transfected as described above. Twenty-four hour after transfection, the coverslips were fixed with 3.7% formaldehyde in PBS for 20 min at room temperature, and then left intact or permeabilized with 0.5% saponin in PBS containing 0.1% BSA for 5 min. Immunostaining was performed with an anti-HA antibody (Y-11) and the anti-FLAG M5 antibody, and detected using the AlexaFluor 488 anti-mouse IgG conjugate or AlexaFluor 594 anti-rabbit IgG conjugate (Molecular Probes, Eugene, OR). Primary and secondary antibodies were incubated for 1 h at room temperature each. F-actin was stained with Alexa 594-phalloidin (Molecular Probes), and the endoplasmic reticulum (ER) was stained with ER-Tracker Blue-White DPX (Molecular Probes). After the reaction, the coverslips were rinsed in water and then mounted on glass slides with Mowiol 4-88 (Calbiochem, San Diego, CA). Cell images were digitally captured under a Zeiss Axioskop 2 Plus microscope with an AxioCam CCD camera (Carl Zeiss, Thornwood, NJ).

RESULTS

The Ectodomain of Cell Surface Edg-1/S1P1 Is Truncated—We examined the subcellular localization of Edg-1/S1P1, tagged with both N-terminal FLAG and C-terminal HA, transiently expressed in CHO cells. In order to distinguish the Edg-1/S1P1 expressed on the cell surface from the intracellular form, cell-surface proteins were biotinylated with membrane-impermeable sulfo-NHS-LC-biotin. As shown in Fig. 1 (lanes 6 and 10, asterisks), a 46 kDa form of Edg-1/S1P1 was expressed on the cell surface. This molecule could not be detected with the anti-FLAG antibody, indicating that the cell surface-expressed Edg-1/S1P1 was truncated at the N-terminal ectodomain (Fig. 1, lane 2). Digestion with an Endo H revealed an Edg-1/S1P1 modified with high mannose-type oligosaccharide, which was retained in the ER (Fig. 1, lanes 2, 3, 6, and 7, arrows). On the other hand, a PNGase F-sensitive oligosaccharide-modified Edg-1/S1P1 (34 kDa) was expressed on the cell surface (Fig. 1, lanes 8 and 12, arrowheads). Endoglycosidase-resistant Edg-1/S1P1 (38 kDa) represents a non-glycosylated, intact form of the protein (Fig. 1, closed circles). Based on these results, we determined that Edg-1/S1P1 was localized to the cell surface after *N*-glycosylation at the ER and additional modification with complex-type oligosaccharide at the Golgi complex. The molecular weight of PNGase F-digested Edg-1/S1P1 (34 kDa) was less than that of the intact form of Edg-1/S1P1 (38 kDa), and it was not detected on immunoblotting with an anti-FLAG antibody (Fig. 1, lanes 4 and 8). Taken together, these results strongly suggest that a mature form of Edg-1/S1P1 was truncated at its N-terminal ectodomain before complex-type glycan modification and after high mannose-type glycan modification.

Edg-1/S1P1 in the ER Avoids N-Terminal Truncation—To further analyze the subcellular localization of Edg-1/S1P1, immunofluorescence was examined for transfected cells. First, Edg-1/S1P1 on the cell surface was examined with an anti-FLAG antibody in intact cells, however, no expression was detected (data not shown). Then, cells were peremabilized with saponin,

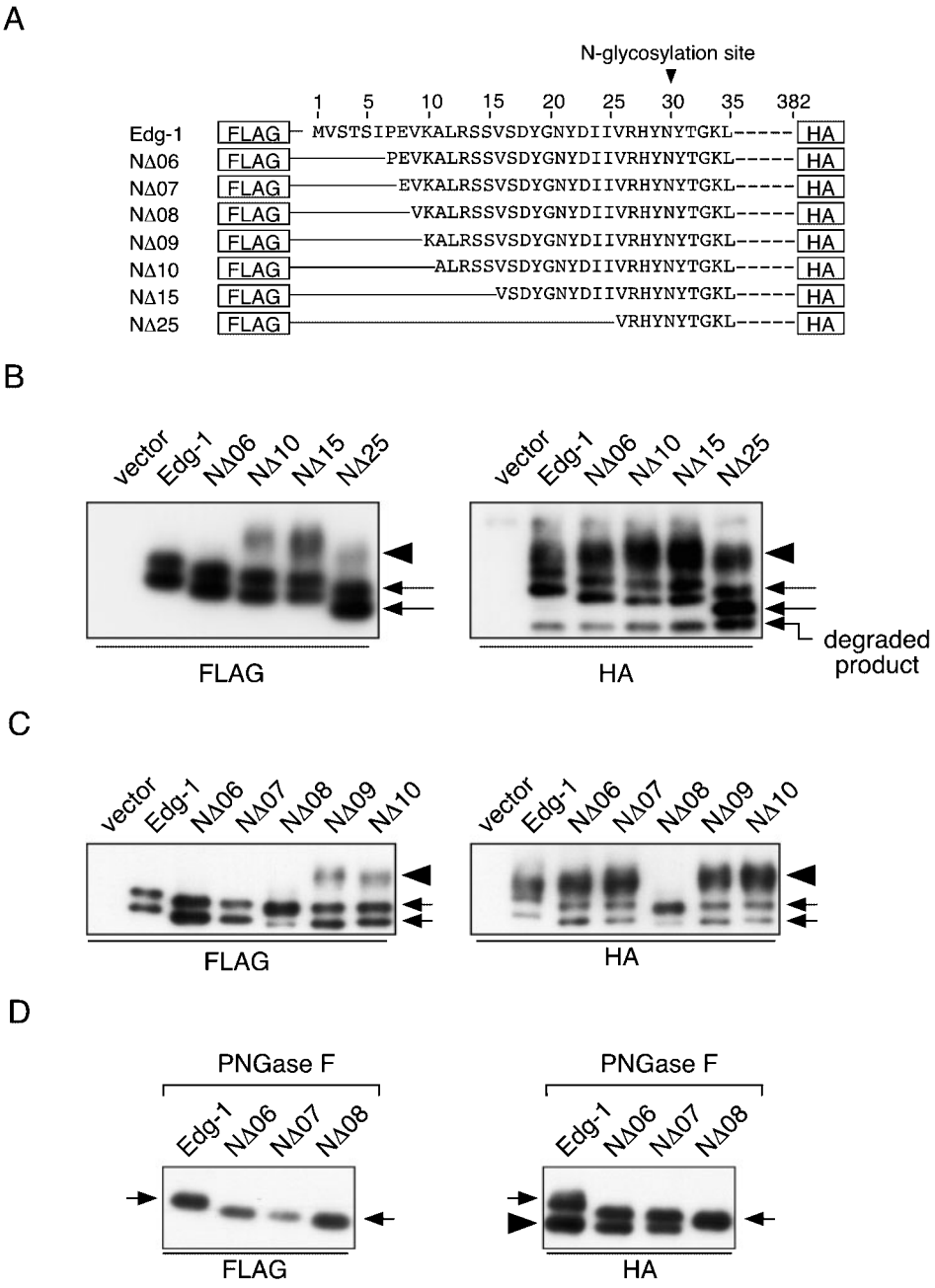

Fig. 3. **Prediction of the cleavage site by deletional analysis.** A: Schematic representation of the amino acid sequences of N-terminal deletion mutants of Edg-1/S1P1 used in this study. The 30th asparagine residue is the glycosylation site in Edg-1/S1P1 (arrowhead). B: The expression of epitope-tagged Edg-1/S1P1, NΔ06, NΔ10, NΔ15, or NΔ25 was analyzed by Western blotting using an anti-FLAG antibody (left panel) or anti-HA antibody (right panel). The arrowheads indicate the Edg-1/S1P1 modified with PNGase F-sensitive, complex-type oligosaccharide. The upper arrows indicate the immature form of Edg-1/S1P1 bearing Endo H sensitive, high mannose-type oligosaccharide, and the lower arrows the intact form of non-glycosylated Edg-1/S1P1. C: The expression of epitope tagged Edg-1/S1P1, NΔ06, NΔ07, NΔ08, NΔ09 or NΔ10 was analyzed as in B. D: Cell lysates of CHO cells expressing Edg-1/S1P1, NΔ07, NΔ08 and NΔ09 were prepared as in B. Samples were then treated with PNGase F in order to remove the asparagine-linked oligosaccharide. After separation by SDS-PAGE, Western blotting was performed with an anti-FLAG antibody (left panel). The PVDF membrane was then reprobed with an anti-HA antibody (right panel). The arrowhead indicates the lower molecular weight Edg-1/S1P1 fragment, which is smaller than the intact form as a result of the N-terminal truncation. The arrows indicate the intact form of non-glycosylated Edg-1/S1P1.

and the localization of Edg-1/S1P1 was examined using anti-FLAG and anti-HA antibodies. As shown in Fig. 2 (b and f), the cell surface-expressed Edg-1/S1P1 was detected with the anti-HA antibody, whereas the anti-FLAG stained Edg-1/S1P1 was only apparent in the intracellular compartment (Fig. 2, a and e). In contrast, co-staining with anti-FLAG antibodies and ER-tracker blue-white DPX revealed that the Edg-1/S1P1 in the ER avoided N-terminal truncation (Fig. 2, g and h). These results suggest that the N-terminal ectodomain of Edg-1/S1P1 was cleaved somewhere in the process of translocation to the cell surface from the ER membrane.

Prediction of the Cleavage Site Using Deletion Mutants—In order to identify the cleavage site in its N-terminal ectodomain, deletion mutants of Edg-1/S1P1 were prepared (Fig. 3A). First, to begin narrowing down the position of the cleavage site, Edg-1/S1P1, NΔ06, NΔ10, NΔ15, or NΔ25 was transiently expressed into CHO cells. As shown in Fig. 3B, the mature form of NΔ06 was truncated, as was the wild type protein. On the other hand, the FLAG-positive mature form was observed in all the NΔ10, NΔ15, and NΔ25 transfectants (Fig 3B, arrowhead). These results demonstrated that the cleavage site in Edg-1/S1P1 exists between the 6th and 10th amino acid residues.

Next, in order to determine the exact cleavage site, Edg-1/S1P1, NΔ06, NΔ07, NΔ08, NΔ09, or NΔ10 was transiently expressed into CHO cells. As shown in Fig. 3C, the FLAG-positive, mature form of Edg-1/S1P1 was observed only in NΔ09 and NΔ10, whereas the HA-posi-

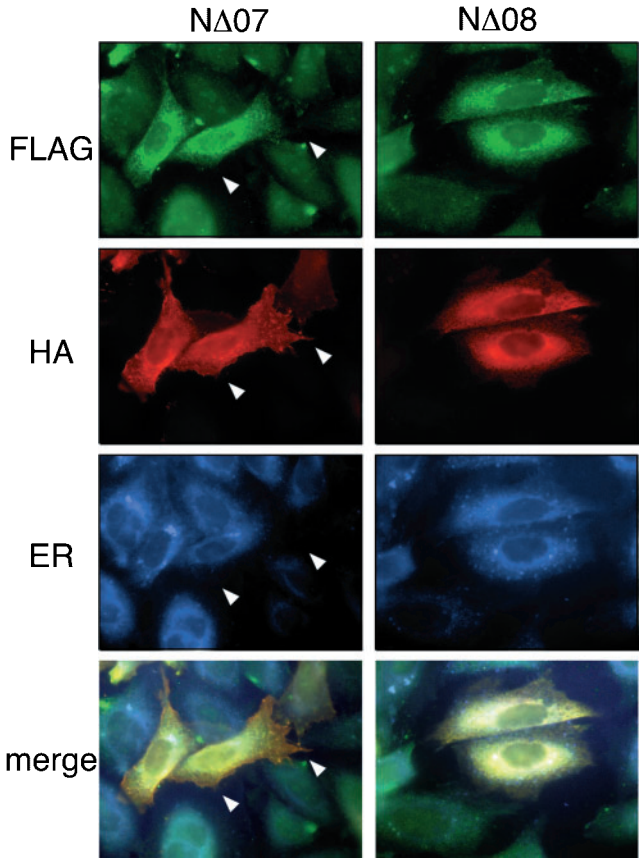

Fig. 4. **NΔ08 is localized in the ER.** NΔ07 or NΔ08 was transiently expressed in CHO cells. Twenty-four hour after transfection, the cells were fixed, permeabilized, and then immunostained with an anti-FLAG or anti-HA antibody, and costained with ER-tracker blue. Merged images stained for FLAG (green) or HA (red) and ER (blue) are also shown (lower panels). Arrowheads indicate the edge of the plasma membrane.

tive, mature form appeared not to be different among these mutants except for NΔ08. Assuming that the N-terminal ectodomain of Edg-1/S1P1 is truncated, the molecular weight of the deglycosylated form is smaller than that of its intact form. With this in mind, we examined PNGase F-digested samples of NΔ06, NΔ07, and NΔ08. The deglycosylated products of NΔ06 and NΔ07 were observed as smaller molecules accompanied by their intact forms (Fig. 3, right panel, arrowhead). On the other hand, the presence of only a single band indicated that NΔ08 avoided N-terminal truncation. Based upon these findings, we concluded that Glu-8 is likely the cleavage site in the N-terminal ectodomain of Edg-1/S1P1. Interestingly, the accumulation of high mannose-type oligosaccharide-conjugated Edg-1/S1P1 was observed in the NΔ08 mutant (Fig. 3C). These results suggest that the NΔ08 mutant may accumulate in the ER.

NΔ08 Is Localized in the ER—High mannose-type oligosaccharide modification occurs in the ER. Therefore, we examined the cellular localization of transfected NΔ07 and NΔ08 in CHO cells. The NΔ07 on the cell surface was truncated, as it was not stained by anti-FLAG antibodies, in the N-terminal ectodomain (Fig. 4, left panels) like the wild type protein (Fig. 2). On the other hand, most of the

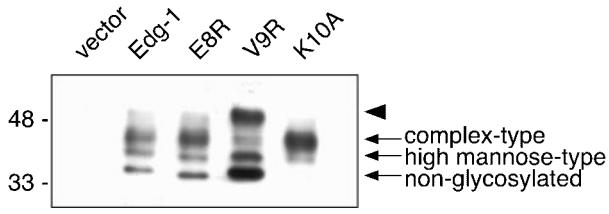

Fig. 5. **The charge at the cleavage site in Edg-1/S1P1 is important for its *N*-glycosylation.** The epitope-tagged wild type, E8R, V9R, K10A, or pcDNA3 vector was transiently transfected into CHO cells. Twenty-four hours after transfection, expression was analyzed by Western blotting with an anti-HA antibody. The arrowhead indicates the mature form of V9R-Edg-1/S1P1, which is modified with a larger PNGase F-sensitive oligosaccharide. Protein molecular weight standards (in kDa) are indicated.

NΔ08 was found to be expressed at the ER by co-staining with ER-tracker blue-white DPX, and the cell surface-expressed Edg-1/S1P1 protein could not be detected (Fig. 4, right panels). NΔ08 was only modified with high mannose-type oligosaccharide (Fig. 3C). These results demonstrate that the NΔ08 mutant accumulated in the ER. Taken together, these findings suggest that the position of the cleavage site in the N-terminal ectodomain of Edg-1/S1P1 plays an important role in the translocation of Edg-1/S1P1 from the ER to the Golgi.

The Charge at the Cleavage Site in Edg-1/S1P1 Is Important for N-Glycosylation of the Protein—To further analyze the role of the N-terminal ectodomain of Edg-1/S1P1, we prepared three Edg-1/S1P1 mutants, in which Glu-8 was replaced by Arg (E8R), Val-9 was replaced by Arg (V9R), or Lys-10 was replaced by Ala (K10A). As shown in Fig. 5, the mature forms of E8R and K10A exhibited the same expression pattern as the wild-type Edg-1/S1P1. On the other hand, the mature form of the protein from the V9R transfectant had a markedly increased molecular weight compared to the mature form of the wild-type Edg-1/S1P1 (Fig. 5, arrowhead). When these mutants were analyzed by Western blotting using an anti-FLAG antibody, the N-terminal portion of each mutant was found to be truncated (data not shown). The immunostaining study indicated that these mutants were expressed on the cell surface like the wild-type Edg-1/S1P1 (data not shown). Whereas the high mannose-type oligosaccharide-modified form or the non-glycosylated form of core protein itself did not differ much in molecular weight compared to the wild type, V9R might be modified with a larger oligosaccharide (Fig. 5, arrows). These results suggest that the charge at the cleavage site in Edg-1/S1P1 has some important functions in the *N*-glycosylation process.

The N-Terminal Ectodomain of Edg-3/S1P3 Is Truncated Similar to That of Edg-1/S1P1—Sph-1-P receptor Edg-1/S1P1 contains a seven transmembrane-spanning domain, its N-terminus is extracellular, and its C-terminus lies in the cytosol (Fig. 6A). To date, five 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. We examined whether or not the N-terminal ectodomain was also truncated in Edg-3/S1P3, Edg-5/S1P2, and Edg-6/S1P4. In order to detect the expression of the full-length receptor, we designed N-terminal

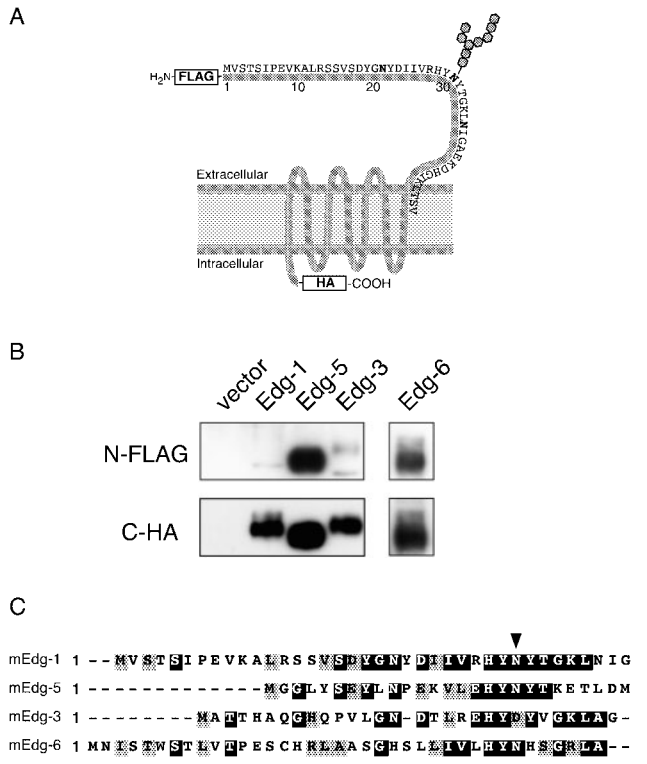

Fig. 6. **The N-terminal ectodomain of Edg-3/S1P3 is truncated similar to that of Edg-1/S1P1.** A. Schematic representation of the amino acid sequence of the N-terminal extracellular region of Edg-1/S1P1. In this study, we used Edg-1/S1P1 tagged with both N-terminal FLAG- and C-terminal HA, as described. Asparagine 30 is the *N*-glycosylation site. B. CHO cells were stably transfected with the pcDNA3 vector or constructs for the Sph-1-P receptors Edg-1/S1P1, Edg-3/S1P3, Edg-5/S1P2, and Edg-6/S1P4, in which the N- and C-termini were tagged with FLAG and HA, respectively. The Edg receptors were purified from these cell lysates, separated by SDS-PAGE, and analyzed by Western blotting with an anti-FLAG (upper panels) or anti-HA (lower panels) antibody. C. Comparison of the amino acid sequences of the N-terminal regions of Sph-1-P receptors. Each sequence was 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.

FLAG- and C-terminal HA-tagged forms of these receptors. Then, we generated stable transfectants of each in CHO cells. As shown in Fig. 6B, neither Edg-1/S1P1 nor Edg-3/S1P3 was detected on Western blotting with an anti-FLAG antibody, whereas the expression of all the Sph-1-P receptors was demonstrated by the anti-HA antibody. These results suggest that the N-terminal ectodomains of Edg-1/S1P1 and Edg-3/S1P3 are truncated, but those of Edg-5/S1P2 and Edg-6/S1P4 are not.

To determine the cleavage site in Edg-3/S1P3, we compared the amino acid sequences of the N-terminal regions, which in Edg-1/S1P1 contains the *N*-glycosylation site, of these four receptors (Fig. 6C). Consequently, we found that the domain encompassing the 30th asparagine residue of Edg-1/S1P1, which is modified through *N*-glycosylation, was highly conserved. On the other hand, there was little homology in the rest of the N-terminal region. Therefore, we had difficulty in determining the N-terminal cleavage site in Edg-3/S1P3 from its amino acid sequence.

DISCUSSION

In this study, we used Edg-1/S1P1 and mutant constructs of it tagged with N-terminal FLAG and C-terminal HA. In order to avoid the influence of a certain epitope tag, we designed two other cDNA constructs, N-Myc/C-HA tagged Edg-1/S1P1 and N-HA/C-FLAG tagged Edg-1/S1P1. We analyzed the expression of Edg-1/S1P1 and confirmed the N-terminal truncation in both (data not shown). Therefore, it can be assumed that the N-terminal ectodomain was fundamentally cleaved in overexpressed Edg-1/S1P1 in cultured cells. Since there was no antibody that recognizes Edg-1/S1P1 or Edg-3/S1P3 specifically in our experiments, it was difficult to consider the truncation of these ectodomains without tags or in endogenously expressed proteins.

Reportedly, in some G protein-coupled receptors that are activated by large peptides like glucagon or secretin, ligand binding affinity is regulated by the N-terminal ectodomain (*18*). The PARs (proteinase-activated receptors) are known to be activated when their ectodomains are truncated by endopeptidases (*19*). In this study, we demonstrated that the N-terminal ectodomain of Edg-1/S1P1 was truncated during cell-surface expression. Because the eight amino acid residues of the N-terminal portion of Edg-1/S1P1 were removed, we might regard this domain as a signaling peptide. However, the mutants, which were truncated by nine or more amino acids in the ectodomain of Edg-1/S1P1, were also translocated to the cell surface (Fig. 3C). Therefore, we theorized that the N-terminal ectodomain of Edg-1/S1P1 is not merely a signaling peptide for cell surface localization. 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, reflecting modification with oligosaccharide, increased (Fig. 5). These results suggest that some protein, which interacts with the N-terminal portion of Edg-1/S1P1, may regulate the *N*-glycosylation at the Golgi. Structural changes of modifying oligosaccharides have been reported to be closely related to malignancy in cancer (*20*). Edg-1/S1P1 expressed in endothelial cells induced cell migration and proliferation upon ligand stimulation (*21*). Another report revealed that Edg-1/S1P1 plays an important role in angiogenesis (*22*). Therefore, it will be interesting to clarify the effect of a structural change in the modifying oligosaccharide on the function of Edg-1/S1P1. Moreover, considering that *N*-glycosylation in a GPCR is a limiting step in its translocation to the cell surface (*16*), it will be meaningful to clarify the regulatory mechanism in this process.

Many membrane proteins are truncated proteolytically in their extracellular domains (*23*). Some metalloproteases such as ADAM (a disintegrin and metalloprotease domain) are implicated in ectodomain shedding (*24*). Ectodomain shedding from membrane proteins affects biological activities such as proliferation, inflammation and pathogenesis (*25*). In the current study, we did not identify the protease that cleaved the ectodomain of Edg-1/S1P1. Further study is required, with a protease-resistant mutant of Edg-1/S1P1, to determine whether or not the ectodomain of Edg-1/S1P1 plays some role in its cellular functions. Such studies should reveal the receptor function of the N-terminal truncated form of Edg-1/S1P1.

According to one report, when point mutations were introduced into the V2-vasopressin receptor, accumulation of the receptor in the ER was observed (*26*). It is thought that the hereditary disease nephrogenic diabetes insipidus is caused by such mutations. Interestingly, our NΔ08 mutant, in which eight amino acid residues of the N-terminal portion of Edg-1/S1P1 were deleted, accumulated in the ER and was not translocated to the plasma membrane. 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 meaningful to clarify the molecular mechanisms of the Edg-1/S1P1 mutant that accumulated in the ER.

We wish to thank Drs. Akio Kihara and Yu Fukuda for the helpful discussions. We also thank Drs. Atsushi Wada and Katsuhiko Ohta (this laboratory) for providing the cDNAs. This work was supported in part by a grant-in-aid for Scientific Research on Priority Areas (B) (12140201) from the Ministry of Education, Culture, Sports, Science and Technology. A nomenclature subcommittee of the International Union of Pharmacologists has renamed the Sph-1-P receptors for S1P1 (Edg-1), S1P2 (Edg-5), S1P3 (Edg-3), S1P4 (Edg-6), and S1P5 (Edg-8) (*27*).

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