Cleavage of Ig-Hepta at a “SEA” Module and at a Conserved G Protein-coupled Receptor Proteolytic Site*

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Ig-Hepta is a member of a new subfamily of the heptahelical receptors and has an unusually long N terminus extending toward the extracellular side of the plasma membrane. Pulse-chase experiments in 293T cells using antisera specifically recognizing its N- and C-terminal regions demonstrated that Ig-Hepta is core-glycosylated cotranslationally and proteolytically processed into a two-chain form in the endoplasmic reticulum, followed by maturation of oligosaccharide chains and dimerization. The cleavage occurs at two highly conserved sites: one in a “SEA” module (a module first identified in sperm protein, enterokinase, and agrin) near the N terminus and the other in the stalk region preceding the first transmembrane span, generating ~20-, 130-, and 32-kDa fragments. The latter two remain tightly associated non-covalently even after cleavage as revealed by immunoprecipitation of native and myc-tagged Ig-Hepta constructs that were transiently expressed in 293T cells. The dimer consisting of four chains, (130 kDa + 32 kDa)₂, is linked by disulfide bonds. A fusion protein of the extracellular domain of Ig-Hepta and the Fc domain of immunoglobulin was found to be a good substrate of the processing enzymes and used for determining the exact cleavage sites in the SEA module and juxtamembrane stalk region.

G protein-coupled receptors (GPCRs)¹ comprise a large superfamily of proteins in the body and are involved in the recognition and transduction of a variety of extracellular signals. They share a common basic structure of seven transmembrane spans (TM7) with extracellular N and intracellular C termini. Mammalian GPCRs have been classified into three major groups on the basis of their sequence similarity to rhodopsin (class I or type A), secretin receptor (class II or type B), and metabotropic receptor (class III or type C) (1). During the past few years, a subgroup of the class II GPCRs has emerged whose members have unusually large N-terminal extracellular domains that contain a number of well-defined protein modules (2, 36). Identification of cell types expressing these molecules and their potential roles in cell adhesion and signaling have become a focus of research in immunology, neuroscience, and developmental biology. This subgroup is referred to as LNB-TM7, where LNB means long N-terminal and type B (2). The members of the LNB-TM7 family include (i) EMR1 (3), CD97 (4), F4/80 (5), and ETL (6), which contain EGF modules; (ii) HE6 (7) and GPR56 (8) with mucin-like regions; (iii) α-latrotoxin receptors CL1, CL2, and CL3, which have a galactose-binding lectin homologous region and an olfactomedin homologous region (9); (iv) BA11, BA12, and BA13 with thrombospondin type-1 repeats (10, 11); (v) Celsr1–3 (12, 13, and Flamingo (14) with cadherin repeats; and (vi) Ig-Hepta with immunoglobulin repeats (15). Despite these variations in the membrane-distal region of the extracellular domain, their membrane-proximal regions or stalks are highly conserved. Namely, they contain a characteristic Cys-box motif close to the extracellular face of the membrane, suggesting a common role. For the α-latrotoxin receptors, it has been shown that proteolytic cleavage takes place immediately downstream to the conserved Cys-box (9), and similar processing has been suggested for other members (2).

Ig-Hepta cDNA was cloned from a rat lung cDNA library in our laboratory. It was shown to contain two C2-type immunoglobulin-like domains and to be highly glycosylated (15). Northern blot analysis subsequently demonstrated that mRNA transcripts are expressed abundantly in the lung and significantly in the kidney (15). Immunohistochemistry demonstrated alveolar wall and intercalated cell localizations in the rat lung and kidney, respectively (15). One striking and unusual feature of the Ig-Hepta molecule is that it exists as a disulfide-linked dimer; although there are a growing number of GPCRs that can be detected as homo- or heterodimers, many of them are non-covalently associated except the disulfide-linked dimers between calcium-sensing and metabotropic glutamate receptors (15, 17) and between κ and δ opioid receptors (18) (for review, see Refs. 19 and 20). While performing Western blot analysis of mature Ig-Hepta to characterize the nature of the dimer, we noticed that N- and C-terminal-directed antisera stained distinct bands of ~130 and ~32 kDa, respectively, suggesting that Ig-Hepta undergoes proteolytic processing during the biosynthetic process. In the present study, therefore, we performed pulse-chase experiments to confirm this possibility. We further determined the site of cleavage and found it is located in the highly conserved stalk region mentioned above, implying that similar processing also occurs in other members of the LNB-TM7 family.

* This work was supported by grant-in-aid for Scientific Research 0910200 from the Ministry of Education, Culture, Sport, Science and Technology of Japan, by Research Grant for Cardiovascular Diseases 11C-1 from the Ministry of Health, Labour and Welfare of Japan, and by an SRF Grant for Biomedical Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: GPCR, G protein-coupled receptor; BA1, brain-specific angiogenesis inhibitor; BFA, brefeldin A; Celsr, cadherin EGF LAG seven-pass G-type receptor; CIRL, calcium-independent receptor of α-latrotoxin; EMR1, epidermal growth factor module-containing mucin-like receptor; ECD, extracellular domain; EGF, epidermal growth factor; GFP, green fluorescent protein; GPS, GPCR proteolytic site; HE6, human epididymal gene product 6; LNB, long N-terminal type B; PNGase F, peptidyl N-glycanase F; SEA, a module first identified in sperm protein, enterokinase, and agrin; TM, transmembrane domain; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBD, phosphate-buffered saline; ER, endoplasmic reticulum.
N-terminal sequencing of Ig-Hepta also revealed another proteolytic processing occurring at a “SEA” module that is located close to the N terminus. The SEA module was first described as a motif present in an ectodomain of a number of mucin-like membrane proteins and so named after the first three proteins in which it was identified (gripin protein, entokerin, and agrin). The number of SEA module-containing proteins now exceeds 73 (available at dylan.embl-heidelberg.de/). The function of the SEA module is not clear, but it serves as a site for proteolytic cleavage (21, 22). Based on this fact, Wreschner et al. (22) have proposed a mechanism whereby a combination of ligand and receptor is generated from a single precursor molecule by its SEA module-mediated cleavage. The significance of the cleavage of Ig-Hepta at its SEA module is discussed in relation to this hypothesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes were from Takara, Kyoto, Japan; Pro-mix I-35S in vitro cell-labeling mix and protein G-Sepharose were obtained from Amersham Biosciences, Upsalla, Sweden; Complete protease inhibitor mixture was from Roche Molecular Biochemicals, Mannheim, Germany; BL21(DE3)plyS was from Stratagene, La Jolla, CA; pCDNA3, pSecTag, pRSET, pZEO-0, Zep, and LipofectAMINE Plus were from Invitrogen, San Diego, CA; anti-myc monoclonal antibody (9E10) was from Santa Cruz Biotechnology, Santa Cruz, CA; Brefeldin A (BFA) was from Sigma Chemical Co., Munich, Germany; brefeldin A (BFA) was from Sigma Chemical Co., Munich, Germany; Immobilon polyvinylidene difluoride membrane was from Millipore, Tokyo, Japan; nickel-nitrilotriacetic acid resin was from Qiagen, Valen-

**Plasmid Constructions**—Expression vectors encoding wild type Ig-Hepta and Ig-Hepta-C were described previously (15). To generate the C-terminally myc-His-tagged full-length Ig-Hepta constructs, the transmembrane sequence of Ig-Hepta without the stop codon was amplified by PCR using Pfu DNA polymerase with the primers IgH7TMF and IgH7TMR shown below, digested by Apat restriction enzyme, and ligated into the EcoRV and Apat sites of pSecTag-Ig-Hepta-ECD, in-frame at the 3′-end of the extracellular domain (residue 25–246 for Ig-Hepta and 1412–246 for Ig-Hepta-C). The expression vector encoding an Ig-Hepta-GFP fusion protein (pEGFP-N3-Ig-Hepta) was generated by ligating a 4.0-kb SpeI/KpnI fragment of pcDNA3-Ig-Hepta-myc comprising the full-length Ig-Hepta into the NheI/KpnI site of pEGFP-N3. To generate expression vector for the soluble rat Ig-Hepta-human immunoglobulin Fc chimera (sIg-Hepta-Fc), the C region of human IgG1 was amplified from human spleen mRNA with the primers hIGF1-F (EcoRV) and hIGF1-R (ApaI) shown below, and cloned into pZeo-R-2 cloning vector and verified by sequencing. A cDNA cassette encoding the Fc region of human IgG1 was inserted between the EcoRV and Apat sites of pSecTag-Ig-Hepta-ECD, in-frame at the 3′-end of the extracellular domain (residues 25–1016 of Ig-Hepta). The primers used for constructing the expression vectors were as follows: IgH7TMF (EcoRV), S′–ATCATTTCTTACATCG

**Transient Transfections**—Transient transfections were performed with the LipofectAMINE Plus system according to the manufacturer’s protocol. In brief, 293T cells at 80% confluence in 35-mm dishes were transfected with 1 μg of DNA, 6 μl of Plus reagent, and 4 μl of LipofectAMINE in Opti-MEM medium for 3 h. Cells were harvested 48 h after transfection.

**Pulse-Chase Analysis and Immunoprecipitation**—For pulse-chase analysis, transiently transfected 293T cells were starved in cysteine/methionine-free DMEM with dialyzed 5% FBS for 30 min, labeled for 15 min with 35S/methionine/cysteine and chased for 1 h with cold media in the continued presence of BFA. Cells were then washed with BFA-free medium three times and chased for additional 6 h. The medium was lysed at each time point and immunoprecipitated with a mixture of anti-Nε-Ig-Hepta and anti-Cα-Ig-Hepta polyclonal antibody. Immune complexes were analyzed by SDS-PAGE in the presence of 2-mercaptoethanol, and the radioactive bands were visualized.

**Brefeldin A Treatment**—293T cells transiently transfected with Ig-Hepta cDNA were pretreated with BFA (5 μg/ml) for 1 h and metabolically labeled for 15 min with [35S]methionine/cysteine and chased for 1 h with radiolabeled medium in the continued presence of BFA. Cells were then washed with BFA-free medium three times and chased for additional 6 h. The cell lysates were immunoprecipitated with a mixture of anti-Nε-Ig-Hepta and anti-Cα-Ig-Hepta polyclonal antibody. Immune complexes were analyzed by SDS-PAGE in the presence of 2-mercaptoethanol, and the radioactive bands were visualized.

**Enzymatic Deglycosylation of Immunoprecipitants**—Following immunoprecipitation, samples were washed with radiolabeled precipitation buffer without protease inhibitors for three times and eluted from the beads by incubation in 30 μl of denaturing buffer (0.5% SDS, 1% 2-mercaptoethanol). Treatment with PNGase F was performed for 3 h at 37 °C as described above. Samples were electrophoresed on polyacrylamide.

**Antibody Production**—For antibody production, DNA fragments encoding the C-terminal cytosolic domain of Ig-Hepta (residues 1265–1349) and the N-terminal extracellular domain of Ig-Hepta (residues 571–1016) were amplified by PCR and cloned into the vector pRSSET, and the constructs were transformed into Escherichia coli BL21 (DE3)pLyS and used for fusion protein production. Fusion proteins were insoluble and purified under denaturing conditions on nickel-nitrilotriacetic acid resin columns. Rabbit polyclonal antibodies using the denatured fusion proteins were produced as previously described (15).

**Stable Transfection of 293T Cells with sIg-Hepta-Fc cDNA**—To establish cell lines that express sIg-Hepta-Fc, the expression coding sIg-Hepta-Fc was linearized by ScaI restriction enzyme and transfected into exponentially growing 293T cells using the LipofectAMINE Plus method. Selection of stable transfecants was carried out by adding 500 μg/ml Zeocin into the medium. After 3 weeks, Zeocin-resistant cell lines were cloned by limiting dilution and expanded to obtain stable cell lines. Expression of sIg-Hepta-Fc was determined by Western blotting of cell supernatants.

**Purification of sIg-Hepta-Fc**—Stably transfected cell lines were grown for 7 days in serum-free Opti-MEM medium, and the supernatants were harvested, centrifuged (10,000 × g, 1 h), filtered through a 0.22-μm filter membrane to remove cell debris and membranes, and loaded as 40-ml aliquots into 1-ml columns of protein G-Sepharose equilibrated in PBS. The columns were washed with 10 ml of PBS and eluted with 2.5 ml of 50 mm citric acid. The eluates were neutralized with 1 M Tris-base and concentrated using Centriprep-10 concentrators. Protein concentration was determined by the bicinchoninic acid (BCA) method using bovine serum albumin as the standard, and the purity of the samples was assessed by SDS-PAGE and Coomassie Blue staining.

**N-terminal Sequence Analysis**—For N-terminal sequencing, 5 μg of purified sIg-Hepta-Fc was electrophoresed on 10% SDS-polyacrylamide gel and electrotransferred onto polyvinylidene difluoride membranes. The protein band of interest was excised from the membrane, and N-terminal sequence was determined by automated Edman degradation using a PPSQ-21 sequencer (Shibadzu, Kyoto, Japan). Following the rad Mutagenesis of Ig-Hepta-Fc, a single Thr (ACG) to Ala (GCG) mutation at proteolytic cleavage site (P1′, residue 994) was introduced into pEGFP-N3-Ig-Hepta by PCR-based mutagenesis using mutated synthetic oligonucleotides. Briefly, a DNA fragment was amplified using the forward primer T994A-Xho-F shown below and the antisense mutant primer T994A-Nru-R, and an overlapping fragment was amplified using the sense mutant primer T994A-Nru-F and the
RESULTS

Evidence for Proteolytic Processing of Ig-Hepta—While characterizing antisera, anti-N\textsubscript{Ig-Hepta} and anti-C\textsubscript{Ig-Hepta} raised against the N- and C-terminal domains of rat Ig-Hepta, respectively, we noticed that they recognize distinct bands on Western blot analysis of Ig-Hepta (Fig. 1A). When extracts of 293T cells transiently expressing Ig-Hepta were analyzed, anti-N\textsubscript{Ig-Hepta} antiserum reacted with broad bands of 30–160 kDa and their dimers (lanes 1 and 2), whereas anti-C\textsubscript{Ig-Hepta} antiserum detected a band of 32-kDa and its dimer (lanes 3 and 4). In both cases, the dimer bands disappeared upon reduction (data not shown). The broad bands of 130–160 kDa can be explained by glycosylation; treatment with glycosidase PNGase F, which cleaves both high mannose and complex N-linked oligosaccharides, reduced their sizes to ~95 kDa (1B, lane 3). These results suggest that Ig-Hepta is proteolytically processed into a mature two-chain form composed of the fully glycosylated 150-kDa N-terminal fragment and the 32-kDa C-terminal fragment.

To see whether this proteolytic processing is a general property of Ig-Hepta, we next performed Western blot analysis using whole detergent extracts of the rat lung. Although anti-N\textsubscript{Ig-Hepta} detected 130- to 160-kDa bands corresponding to the N-terminal fragment, anti-C\textsubscript{Ig-Hepta} yielded only a faint band of 32 kDa that was difficult to distinguish from nonspecific staining (data not shown). We therefore enriched the C-terminal fragment by immunoprecipitation and subjected it to Western blot analysis (Fig. 1C), which demonstrated the presence of the processed C-terminal fragment of 32 kDa in the lung (lane 5) as well as in 293T cells (lane 4).

Processing of Ig-Hepta Monitored by Pulse-Chase Experiments—To determine the time course of the post-translational modification of Ig-Hepta, we performed pulse-chase experiments. 293T cells expressing rat Ig-Hepta were pulse-labeled with \[^{35}\text{S}\]methionine/cysteine for 15 min and chased for 0–120 min (Fig. 2). Immunoprecipitation of labeled products with either N- or C-terminal domain-directed antiserum revealed that rat Ig-Hepta is synthesized as a 170-kDa precursor and is rapidly cleaved into two chains: a 130-kDa N-terminal fragment (Fig. 2A, upper panel, arrowhead) and a 32-kDa C-terminal fragment (Fig. 2A, lower panel). The size of the C-terminal fragment remained unchanged during the 2-h chase time. However, the size of the N-terminal fragment increased from 130 to 150 kDa after 1 h of chase (Fig. 2A, arrow) because of modification of oligosaccharide chains as demonstrated by deglycosylation of the pulse-chased products (Fig. 2B). Deglycosylation experiments also showed that the initial glycosylation (attachment of a common N-linked oligosaccharides or core-glycosylation) occurs almost cotranslationally as seen by a marked reduction (~20 kDa) in size of the 170-kDa band as well as the band of 130 kDa (Fig. 2A and B). The time courses of processing

Fig. 1. Proteolytic processing of Ig-Hepta in rat lung and 293T cells. A, Western blot analysis revealing the presence of processed N- and C-terminal domains of Ig-Hepta and their dimers. Cell extracts from 293T cells transiently transfected with Ig-Hepta cDNA (WT) or control cDNA (Mock) were analyzed by Western blotting under non-reducing conditions with anti-N\textsubscript{Ig-Hepta} (left panel) and anti-C\textsubscript{Ig-Hepta} (right panel) polyclonal antibodies. Under the non-reducing conditions where disulfide bonds remain intact, disulfide-linked species tended to give smaller values for their molecular sizes: N\textsubscript{Ig-Hepta} (135 kDa), N\textsubscript{Ig-Hepta} dimer (230 kDa), and C\textsubscript{Ig-Hepta} dimer (60 kDa) except C\textsubscript{Ig-Hepta} monomer (32 kDa). B, enzymatic deglycosylation of Ig-Hepta expressed in 293T cells with PNGase F. Crude membrane proteins isolated from 293T cells transiently transfected with Ig-Hepta were digested with PNGase F (lane 3) and analyzed by immunoblotting. The sizes of both fully glycosylated mature (150 kDa, arrow in lane 2) and core-glycosylated immature (130 kDa, arrowhead in lane 2) N-terminal chains of Ig-Hepta, were reduced to ~95 kDa by the treatment (lane 3). C, demonstration of the presence of the processed C-terminal fragment by immunoprecipitation. Lysates from rat lung and 293T cells transiently transfected with Ig-Hepta were analyzed by immunoprecipitation with anti-C\textsubscript{Ig-Hepta} antibody and Western blotted using anti-C\textsubscript{Ig-Hepta} antibody. 2ME, 2-mercaptoethanol; anti-C, anti-C\textsubscript{Ig-Hepta} antiserum; anti-N, anti-N\textsubscript{Ig-Hepta} antiserum; IP, immunoprecipitation; and WT, wild type Ig-Hepta.
this speculation, we performed the following experiment using BFA, an inhibitor that prevents the ER-to-Golgi vesicular trafficking. The identity of an ~150-kDa band maximally seen at 20 min of chase (Fig. 2A, double arrowhead on the left) will be addressed later.

**Proteolytic Cleavage of Ig-Hepta in ER—** 293T cells were transiently transfected with an Ig-Hepta expression vector, preincubated for 1 h with BFA to achieve complete inhibition, pulse labeled for 15 min in the presence of BFA, and chased for 1 h in a medium containing BFA. BFA was then removed, the chase was further continued for 6 h, and cell lysates were assayed for the molecular species of Ig-Hepta accumulated during the chase by immunoprecipitation, SDS-PAGE, and autoradiography. As shown in Fig. 3, Ig-Hepta that accumulated in ER in the presence of BFA was the proteolytically processed form consisting of the 130-kDa N-terminal fragment (Fig. 3, lanes 2–4, upper panel) and 32-kDa C-terminal fragment (Fig. 3, lower panel). However, the possibility cannot be excluded that the cleavage could be occurring due to the presence of enzymes normally found in the cis-Golgi, because some of the enzymes normally found in the early part of the Golgi are known to be present in ER through retrograde transport. On removal of BFA, the 130-kDa N-terminal extracellular domain was gradually converted to the fully glycosylated higher molecular weight species (lanes 5 and 6), suggesting that the complete maturation of the sugar chains occurs at a later stage in the Golgi. From the sizes of the cleaved fragments, the site of cleavage is predicted to be located in a juxtamembrane region of the N-terminal extracellular domain.

**Efficient Cleavage of Ig-Hepta—** The degree of cleavage was monitored by constructing a fluorescent derivative of Ig-Hepta termed Ig-Hepta-GFP that has a GFP tag at its C terminus. Because GFP is stable, if not heated, in the Laemmli sample buffer for SDS-PAGE, this construct allowed us to detect the GFP-tagged C-terminal fragment by using a fluorescence gel scanner (Fig. 4). A single band of ~50 kDa was seen when extracts of 293T cells were transiently transfected with the Ig-Hepta-GFP construct (lanes 2 and 5, WT-GFP). The absence of higher molecular weight unprocessed species indicates that the processing occurs highly efficiently.

**Non-covalent Association of N- and C-terminal Fragments—** Despite the efficient cleavage of the N-terminal extracellular domain of Ig-Hepta during its post-translational modification, the cleaved extracellular domain was not released into the culture medium (Fig. 5A, lane 1) and was rather recovered from membrane fractions (lane 3). As expected, when a truncated form covering only the extracellular domain of Ig-Hepta (Ig-Hepta-ECD) was expressed in the same expression system, it was secreted and recovered from the culture medium (Fig. 5A, lane 2). These pieces of experimental evidence suggest that the N-terminal fragment is tightly associated with certain membrane component(s), most likely with its C-terminal fragment. To explore this possibility, we prepared Ig-Hepta that has an myc tag at its C terminus (Ig-Hepta-myc) and performed immunoprecipitation analysis using a commercially available anti-myc monoclonal antibody. The anti-myc antibody precipitated the myc-tagged C-terminal domain together with the N-terminal fragment (Fig. 5B). These results clearly demonstrate non-covalent association of the cleaved N- and C-terminal fragments.

**Determination of the Cleavage Site in Membrane Proximal Region—** In an attempt to determine the proteolytic cleavage site of Ig-Hepta, we first tried immunoadfinity purification of the 32-kDa C-terminal fragment. Such studies, however, have been hampered because of its membrane-bound nature, and hence, difficulties result in obtaining sufficient amounts for sequencing. As an alternative approach, we constructed a chimeric protein that contains a candidate cleavage site sequence and is easy to purify. The chimera was composed of the N-terminal extracellular domain of rat Ig-Hepta and the Fc domain of human IgG1 and named s Ig-Hepta-Fc (s for soluble). When the chimeric construct was expressed in 293T cells, sim-
ilar proteolytic processing occurred, yielding a 38-kDa fragment corresponding to the C-terminal Fc portion (Fig. 6A). The C-terminal fragment was purified from the culture medium by affinity chromatography on protein G-Sepharose and found to have the following N-terminal sequence by amino acid sequencing: TSFSILMSPD (Fig. 6B, upper sequence). This result strongly indicates that the cleavage site is Leu 993-Thr994, which is located in a juxtamembrane region of the N-terminal extracellular domain (23 amino acid residues N-terminal to the first transmembrane span; Figs. 6B and 7).

To confirm the site of cleavage, we constructed a mutant Ig-Hepta-GFP molecule whose cleavage site sequence is changed by site-directed mutagenesis. Mutation of Thr994 to Ala (T994A-GFP) at the P1/H11032 position completely abolished the cleavage (Fig. 4, lane 3). This result strongly supports the above

FIG. 4. Efficient cleavage and confirmation of cleavage site by site-directed mutagenesis. Cell lysates from 293T cells transiently transfected with Ig-Hepta-GFP or its mutant in which Thr994 at the P1 position was replaced by Ala (T994A) were equilibrated with Laemmli sample buffer for 5 min at room temperature and resolved by SDS-PAGE at 4°C under reducing (left panel) or non-reducing conditions (right panel). Fluorescent bands of GFP were detected by exposing the gel to Image reader FLA2000.

FIG. 5. Non-covalent association of N- and C-terminal chains of Ig-Hepta after proteolytic processing. For secretion of Ig-Hepta-ECD, the leader sequence in the pSecTag A expression vector was used (15). A, recovery of the cleaved N-terminal chain from membrane fractions (lane 3) but not from culture medium (lane 1). In the upper panel, media from 293T cells transfected with either wild type Ig-Hepta cDNA or soluble Ig-Hepta lacking the membrane-spanning domain (Ig-Hepta-ECD) were analyzed by a combination of immunoprecipitation and Western blotting with anti-N Ig-Hepta under reducing conditions. In the bottom panel, membranes of 293T cells transfected with either wild type Ig-Hepta or Ig-Hepta-ECD cDNA were analyzed by Western blotting under reducing conditions using anti-N Ig-Hepta. B, immunoprecipitation analysis using myc-tagged Ig-Hepta. Membranes of 293T cells transiently transfected with either wild type Ig-Hepta or Ig-Hepta-ECD cDNA were analyzed by Western blotting under reducing conditions using anti-Myc monoclonal antibody 9E10. Immunoprecipitates were then run on 10% SDS-PAGE and subsequently probed with anti-Myc monoclonal antibody (top panel), and the same membrane was reprobed with anti-myv monoclonal antibody 9E10 (bottom panel). Arrows indicate immature core-glycosylated form of the N-terminal chain (N Ig-Hepta).

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FIG. 6. Identification of the cleavage site within Ig-Hepta. A, SDS-PAGE analysis of sIg-Hepta-Fc produced in 293T cells. The sIg-Hepta-Fc chimeric protein purified using protein G-Sepharose beads from the culture medium of 293T cells stably transfected with sIg-Hepta-Fc expression plasmid was subjected to 12.5% SDS-PAGE and visualized by Coomassie Brilliant Blue staining. Lane 1, molecular weight marker; lane 2, 10 µg of cell supernatant; lane 3, 5 µg of purified protein. Positions of the 150-kDa full-length sIg-Hepta-Fc and 38-kDa proteolytically cleaved Fc domain are indicated by arrow and arrowhead, respectively. B, amino acid sequences surrounding the proteolytic cleavage sites contained in sIg-Hepta-Fc. The arrows designate the points of cleavage in the sIg-Hepta-Fc. The N-terminal 10 amino acid residues of the 150-kDa N-terminal and 38-kDa Fc fragments, determined by automated Edman degradation, are shaded. C2, C2-type immunoglobulin-like domain; the dark box at the N terminus represents a prescence of about 24 residues that was removed cotranslationally; vertical bars indicate potential N-linked glycosylation sites; Fc, Fc domain of human immunoglobulin. C, schematic model of mature Ig-Hepta that is a disulfide-linked homodimer whose monomeric unit is composed of two non-covalently associated chains generated by proteolytic processing of the precursor at the SEA module (site 1) and the GPCR proteolytic site (GPS, site 2) near the juxtamembrane region. The N-terminal fragment generated by site 1 cleavage appears to be released. Dark boxes represent transmembrane spans.
conclusion that the peptide bond between Leu\(^{993}\) and Thr\(^{994}\) in the juxtamembrane region is the authentic cleavage site. Although the juxtamembrane nature of the cleavage site is reminiscent of that of membrane protein secretases, the relatively strict sequence specificity and the ability to cleave even a soluble substrate, sIg-Hepta-Fc, indicate that the proteinase involved in the processing of Ig-Hepta may be different from the secretases.

**Another Cleavage within a “SEA” Module Near the N Terminal**—A surprising result was obtained when the N-terminal sequence of sIg-Hepta-Fc (Fig. 6A, lane 3, upper band) was determined. Our expectation was that it begins just after the signal sequence, but it yielded a sequence beginning from a residue far beyond the signal sequence cleavage site, namely from Ser\(^{224}\): VVVDYIVEV (Fig. 6B, lower sequence). A database search (available at dylan.embl-heidelberg.de/) indicated that the sequence around this cleavage site conforms well to the consensus sequence of the SEA module (23), which has been established as a proteolytic cleavage site of membrane-associated mucin proteins, including MUC1 (22). After cleavage, the relatively short N-terminal fragment appears to be released from sIg-Hepta-Fc, because no corresponding band of \(~20–30\) kDa was observed on SDS-PAGE of the affinity-purified sIg-Hepta-Fc (Fig. 6A, lane 3).

This cleavage between Gly\(^{223}\) and Ser\(^{224}\) explains the intermediate band of \(~150\) kDa seen in an early phase of the pulse-chase experiment (Fig. 2A, double arrow). The N-terminal cleavage fragment cleaved off at the SEA module, however, could not be chased, because the anti-N\(^{222}\)Ig-Hepta, used for immunoprecipitation of the labeled products, are directed to the Ig-like repeat region and therefore its epitope does not cover the most N-terminal short fragment.

**DISCUSSION**

The LNB-TM7 subfamily has recently emerged in the class II or type B GPCR family (2, 36). Typical members include (i) leukocyte activation antigen CD97, which is associated with inflammation (4); (ii) epithelial cell-restricted cell-surface glycoprotein HE6 of the epididymis (7); (iii) receptors for \(\alpha\)-latrotoxin, a potent excitatory neurotoxin in the black widow spider venom (9); (iv) brain-specific angiogenesis inhibitors BAI1–3 (10, 11); (v) catherin/EGF/laminin A G-type repeat/seven-pass receptor Celsr1 (12) and its close relative, Flamingo, whose Drosophila relative is localized at cell-cell boundaries in the wing and involved in the regulation of planar cell polarity (14); (vi) ETL with EGF-like repeats whose expression is developmentally regulated (6); and (vii) Ig-Hepta studied here. These members are considered to be putative GPCRs based on their structural features; in the case of the \(\alpha\)-latrotoxin receptors, there is direct evidence for their interaction with \(G\) proteins (24). Attention is now focused on their roles in cell-cell recognition, cell adhesion, and signaling. Concerning the processing during maturation, only limited information is available. For example, although most LNB-TM7 family members have been suggested to be proteolytically cleaved during biosynthesis (6, 9, 14, 25), their processing events have not been studied in detail except the determination of the cleavage site in the calcium-independent receptor of \(\alpha\)-latrotoxin (24). In the present study, we monitored the biosynthesis of Ig-Hepta by pulse-chase experiments and clarified its proteolytic processing and glycosylation.

Interestingly, two cleavage sites were identified by using a chimeric construct of Ig-Hepta containing Fc as a tag for purification. One site is located close to the extracellular surface of the membrane (Figs. 6B and 7, site 2). The amino acid sequences surrounding this cleavage site are highly conserved among the family members (Fig. 7), suggesting that similar processing occurs in other members; in fact, the processing site of CL1 determined by Krasnoperov et al. (9) is indeed identical to that of Ig-Hepta determined here. This explains why the stalk regions are highly conserved among the family members despite striking differences in the membrane-distal regions. It is also noteworthy that, in addition to the family members mentioned above, there are many protein sequences deposited in the DDBJ/GenBank\(^{TM}\)/EBI database that contain the conserved Cys-box and cleavage site sequences (Fig. 7 and accession numbers listed in the legend), many of which are members of the LNB-TM7 family, but there are examples of the following: (i) Type 1 membrane proteins with a single transmembrane span, including suREJ1, a sea urchin sperm glycoprotein considered to be acting as a receptor for egg jelly (26, 27) and

![Fig. 7. Protein sequence alignment of the juxtamembrane region of LNB-TM7 family members.](image-url)
channel-like proteins with 11 putative transmembrane
spans, including suREJ3 (28) and its human homolog (29), a
member of the polycystin-1 family whose last six transmem-
brane spans are similar to those of voltage-activated calcium
channels (30–32). Until recently, the cleavage has been
thought to be a phenomenon specifically seen in the GPCR
processing, and the cleavage site has been called “GPCR pro-
eteolytic site” (GPS). But the motif name GPS becomes inap-
propriate, because similar proteolytic cleavages have been
demonstrated in suREJ3 (28) and here in soluble, secreted sIg-
Hepta-Fc (Fig. 6A). The recognition of the cleavage site,
therefore, appears to be solely dependent on the sequence sur-
rounding the processing site. The Cys-box motif present imme-
diately before the cleavage site is characterized by four invari-
ant Cys residues (Fig. 7). The presence of a large number of
proteins containing the Cys box followed by the cleavage site
sequence suggests that the proteolytic processing seen in Ig-
Hepta is a general event in the maturation of precursors of a
certain group of membrane proteins. The conserved juxta-
membrane location of the cleavage site suggests that the processing
enzyme responsible is a membrane protein, and such a jux-
tamembrane location allows an efficient encounter between the
substrate and the active site of the enzyme. Indeed, efficiency
of the processing was significantly reduced in a soluble secreted
form (Ig-Hepta-Fc) compared with the membrane-bound form
(Figs. 4 versus 6A). The nature of the non-covalent association
of the cleaved fragments is also interesting but remains to be
clarified.

The other cleavage site identified by N-terminal sequencing
is located in the SEA module (Fig. 8A). The SEA module is an
extracellular domain found in a number of highly O-glycosyl-
ated membrane proteins (for a list, see the Single Modular
Architecture Research Tool (SMART) site, dylan.embl-heidel-
berg.de/) and serves as a proteolytic cleavage site (22, 23). The
C-terminal sequence of the module present in Ig-Hepta (223GS-
VVV227), and its cleavage site (Gly223–Ser224) match perfectly
those found in MUC1, a mucin-like transmembrane protein
(21). Wreschener et al. (22) have recently proposed a mechanism
whereby one and the same gene can encode both a receptor
protein and its specific ligand. It is postulated that generation
of such receptor-ligand partnership is effected by SEA module-
mediated proteolytic cleavage of transmembrane proteins such as
MUC1. They further suggested that Ig-Hepta might be a
typical example of the ligand-receptor alliances based on the
presence of a perfect SEA module in Ig-Hepta. In the present
study, we established that the module actually functions as a
site for proteolytic cleavage. Furthermore, in the case of Ig-
Hepta, the cleaved N-terminal fragment contains a potential
prohormone processing site (48RPKR51, Fig. 8, A and C) and
a repeat of two variant forms of the EGF-like domain signature 2
(Fig. 8D) (33), making the hypothesis and hence the functional
characterization of the N-terminal fragment very attractive.

Several groups of proteinases, including the following two
major groups, have been identified that are involved in post-
translational proteolysis of membrane proteins and secreted
proteins: (i) membrane protein secretases that are involved in
the processing of the Alzheimer’s amyloid precursor protein,
angiotensin-converting enzyme, certain cytokine receptors,
and others (for review, see Ref. 34) and (ii) subtilisin/Kex2p-
like endoproteinases such as furin and prohormone convertases
(35). The majority of secretases are metalloproteinases, located
at the cell surface or in vesicles close to the plasma membrane,
and cleave precursors at a site located a fixed distance from the
membrane; the cleavage is not strictly dependent on the
sequence. Members of the prohormone convertase family are
mainly localized in the trans-Golgi network and cleave prohor-
mones at sites marked by paired or multiple basic amino acid
residues. Although the identity of the processing enzyme for
Ig-Hepta remains to be clarified, there is a possibility that it
may represent a new family of processing proteinases that are
present in the endoplasmic reticulum and recognize the highly
conserved HLTXF(S/A/I/V/L/M/L) sequence following the Cys-
box motif (Fig. 7). The facts that proper processing of Ig-Hepta
occurs in cultured 293T cells and COS-7 cells and that the
potential recognition sequences are found in a variety of pro-
teins in the Expressed Sequence Tag data base suggest that
one or more of the processing enzymes are ubiquitously distrib-
uted and involved in a novel mechanism of proteolytic process-
ing common to the Cys box-containing proteins. Our finding
that sIg-Hepta-Fc, a soluble chimeric construct of Ig-Hepta, can
serve as a good substrate for the processing enzyme may stim-
ulate the purification of the enzyme, because availability of
soluble substrates is essential for purification.

Acknowledgments—We thank Drs. Hiroshi Okazaki, Mika Miyake,
and Norihiko Misawa for discussion and encouragement, and Seiji
Kawashima and Setsuko Satoh for technical and secretarial assistance.

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