J. Biochem. **140**, 445–452 (2006)
doi:10.1093/jb/mvj170

Multiple Processing of Ig-Hepta/GPR116, a G Protein–Coupled Receptor with Immunoglobulin (Ig)-Like Repeats, and Generation of EGF2-Like Fragment

Taku Fukuzawa and Shigehisa Hirose*
Department of Biological Sciences, Tokyo Institute of Technology, 4259-B19, Nagatsuta-cho, Midori-ku, Yokohama 226-8501

Received July 9, 2006; accepted July 27, 2006

Ig-Hepta/GPR116 is a member of the LNB-TM7 subfamily of G protein–coupled receptors (GPCRs), also termed the adhesion GPCRs, whose members have EGF, cadherin, lectin, thrombospondin, or Ig repeats in their long N-terminus. In this study, we established that Ig-Hepta is processed at multiple sites yielding the following four fragments: (i) presequence (amino acid residues 1–24), (ii) proEGF2 (25–223, α-fragment), (iii) Ig repeats (224–993, β-chain), and (iv) TM7 (994–1349, γ-chain). The proEGF2 region is converted to EGF2 (52–223) by the processing enzyme furin and remains attached to the β- and γ-chains. Expression of some mRNA species was affected by the presence of α-fragment. These results suggest that the furin-processed α-fragment is involved in cellular signaling.

Key words: furin, G protein–coupled receptor, LNB-TM7, proteolytic processing, SEA module.

Abbreviations: EGF, epidermal growth factor; GPCRs, G protein–coupled receptors; GPS, GPCRs proteolytic site; LNB-TM7, long N-terminus family-B seven transmembrane; SEA, sea urchin sperm protein, enterokinase and agrin.

Ig-Hepta is a member of an emerging protein family known as the LNB-TM7 subgroup of the family-B G protein–coupled receptors (GPCRs) or heptahelical receptors with sequence similarity to the secretin receptor (*1*). Ig-Hepta is also known as the abbreviated serial name GPR116 (*2*). The human LNB-TM7 family comprises approximately 30 members (*3*). The members of this family have a long extracellular N-terminal tail attached to a 7-transmembrane domain, thus the name LNB-TM7. Although the functions of the LNB-TM7 family members, except those of Celsr1 (*4*) and BAI1 (*5*), are largely unknown, some members have been demonstrated to mediate cellular interactions through their large extracellular domains consisting of various protein modules implicated in protein–protein or protein–sugar residue interactions (*6*, *7*). Despite the sequence diversity in their membrane-distal region of the N-terminal extracellular domain, the LNB-TM7 family members share a high sequence similarity in their membrane-proximal stalk region. This highly conserved region is rich in cysteine (Cys-box) and is thought to be a proteolytic cleavage site as demonstrated in CL1 (*8*) and Ig-Hepta (*9*). The cleaved extracellular fragment remains linked to the membrane-associated TM7 fragment by non-covalent interactions (*9*).

Among the members of the LNB-TM7 family, Ig-Hepta is unique in having, in addition to the Ig-like repeats, a "SEA" module in its N-terminal extracellular domain. SEA modules, which were first identified in sea urchin sperm protein, enterokinase, and agrin (*10*), and are now found in a growing number of proteins (>200; http://smart.embl-heidelberg.de/), are defined as conserved motifs of 80–110 amino acid residues that occur in extracellular matrix proteins or intrinsic membrane proteins. SEA modules contain a highly conserved GSVVV or GSIVV sequence, which serves as a glycine↓serine cleavage site. As demonstrated in mucin MUC1 and MUC3 (*11*, *12*), the cleaved fragment is not released, but remains associated with the C-terminal membrane-tethered fragment. The functional significance of the cleavage and non-covalent association of the resultant two fragments is not clear, but this phenomenon appears to be important for later release of the soluble domain. Wreschner *et al*. (*13*) have proposed that SEA module–mediated cleavage permits a protein to function as both a ligand and a receptor to elicit a signaling cascade. Similar cleavage was also demonstrated in Ig-Hepta (*9*). In the case of Ig-Hepta, the cleaved N-terminal fragment contains a potential prohormone processing site ($RPKR^{51}$) and a repeat of two variant forms of the EGF-like domain signature 2 (EFG2). These structural features of Ig-Hepta make the ligand-receptor alliance hypothesis very likely. In the present study, we show that the potential prohormone processing site ($RPKK^{51}\downarrow A^{52}$) is indeed cleaved by furin, a calcium-dependent subtilisin-like serine endoprotease that is predominantly localized to the *trans*-Golgi network as a type I integral membrane protein and is known for its role in the processing of many precursor proteins. We further show, by microarray analysis, that the EGF2 fragment (termed here the α-fragment) upregulates certain mRNA species including that for ALS2CL, a guanine nucleotide exchange factor (GEF) for Rab5.

*To whom correspondence should be addressed. Tel: +81-45-924-5726, Fax: +81-45-924-5824, E-mail: shirose@bio.titech.ac.jp

MATERIALS AND METHODS

Reagents—Restriction enzymes were obtained from Takara, Kyoto, Japan; Protein G-Sepharose and ECL-plus reagent were from Amersham Pharmacia Biotech, Uppsala, Sweden; BL21(DE3)pLysS was from Stratagene, La Jolla, CA, USA; pcDNA3, pSecTag, pRSET, Zeocin, and LipofectAMINE 2000 were from Invitrogen, San Diego, CA, USA; anti-Myc monoclonal antibody (9E10), *N*-Glycosidase F and Endoglycosidase H were from Roche Molecular Biochemicals, Mannheim, Germany; Immobilon polyvinylidene difluoride membrane and Ultrafree-0.5 centrifugal filter device were from Millipore, Tokyo, Japan; Ni^{2+}-NTA resin was from Qiagen, Valencia, CA, USA; furin-deficient LoVo human colon adenocarcinoma cells were obtained from Japanese Collection of Research Bioresources; a plasmid expressing mouse furin was a gift from Dr. Kazuhisa Nakayama (Kyoto University); Isogen was from Nippon Gene, Tokyo, Japan; Hybond N^+ nylon membranes and Ready-To-Go DNA labeling kit were from GE Healthcare, Piscataway, NJ, USA.

Cell Culture—293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 5% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. LoVo cells were cultured in nutrient mixture F-12 Ham medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Plasmid Constructions—Expression vectors encoding wild type Ig-Hepta and Ig-Hepta-Myc were described previously (*9*). To generate expression vector for the α-fragment, the α-fragment of Ig-Hepta was amplified by PCR using *Pfu* DNA polymerase (Promega) with the primers α-F1 and α-R1 shown below, digested by *Kpn*I and *Apa*I restriction enzymes, and ligated into the *Kpn*I and *Apa*I sites of pcDNA3. DNA sequencing was used to verify the sequence. To generate expression vector for the soluble α-fragment–human immunoglobulin Fc chimera (α-hFc), the α-fragment of Ig-Hepta was amplified by PCR using *Pfu* DNA polymerase with the primers α-F2 and α-R2 shown below, digested by *Kpn*I and *Eco*RV restriction enzymes, and ligated into the *Kpn*I and *Eco*RV sites of pSecTagA. DNA sequencing was used to verify the sequence. A cDNA cassette encoding the Fc region of human IgG1 (*9*) was inserted between the *Eco*RV and *Apa*I sites of pSecTag-α-fragment, in-frame at the 3′ end of the α-fragment of Ig-Hepta. To generate the α-fragment-truncated Ig-Hepta constructs (Δα-Ig-Hepta), the N-terminal region of β-chain (amino acid residues 224–993) was amplified by PCR using KOD-plus DNA polymerase (Toyobo) with the primers TruncF and TruncR shown below, digested by *Kpn*I and *Xba*I restriction enzymes, and subcloned into pSecTag-Ig-Hepta-Myc to replace the target segment. DNA sequencing was used to verify the sequence. The primers used for constructing the expression vectors were as follows: α-F1 (*Kpn*I), 5′-GCGCGGTACCATGAAATCGTCAAGGACT-3′; α-R1 (*Apa*I), 5′-GCGCGGGCCCTAGCCCTTGGTGAACTG-3′; α-F2 (*Kpn*I), 5′-GCGCGGTACCAGGCCAGCAGAGCCCATT-′; α-R2 (*Eco*RV), 5′-ATCGCCCTTGGTGAACTG-3′; TruncF (*Kpn*I), 5′-GCGCGGTACCCAGCGTGGTTGTGGACTAC-3′; and TruncR (*Xba*I), 5′-GCAGGTGTTTCTAGAGCGC-3′.

Transient Transfections—Transient transfections were performed with the LipofectAMINE 2000 system according to the manufacture's protocol. In brief, 293T cells and LoVo cells at 80% confluence in 35-mm dishes were transfected with 1 μg of DNA and 4 μl of LipofectAMINE in Opti-MEM medium for 3 h. Cells were harvested 48 h after transfection.

Western Blot Analysis—The samples were dissolved in Laemmli buffer (60 mM Tris containing 2% SDS, 8% glycerol, and 0.005% bromphenol blue, pH 6.8) in the presence of 1% β-mercaptoethanol, heated at 95°C for 5 min, and electrophoresed through 12.5% SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% nonfat dry milk, 0.05% Tween 20 in Tris-buffered saline (TBS, 150 mM NaCl, 10 mM Tris-HCl, pH 8) (T-TBS) for 1 h at room temperature. The membrane was washed 3 times for 10 min each with T-TBS and subsequently treated over night at 4°C with the primary antibody (affinity purified anti–α-fragment antibody diluted 1:3,000) in T-TBS. After washing the blots 3 times for 10 min each with T-TBS, the membrane was incubated for 1 h at 25°C with a horseradish peroxidase-conjugated anti–rabbit IgG antibody diluted 1:30,000 in T-TBS. The blots were washed 3 times for 10 min each with T-TBS. The membrane was then developed using the ECL-Plus detection system.

Deglycosylation Experiments—Membrane proteins from rat lung and 293T cells transiently transfected with Ig-Hepta were solubilized with 1% Triton X-100, and the extracts were boiled for 3 min in 20 μl of 0.5% SDS (w/v), 0.1 M β-mercaptoethanol, and 50 mM sodium phosphate buffer, pH 7.2. After cooling to room temperature, Nonidet P-40 was added to give a final concentration of 1%. To the extracts, 2 milliunits of *N*-glycosidase F or Endoglycosidase H were added, and the reaction mixture was incubated at 37°C for 3 h. A control incubation was carried out in which 50 mM sodium phosphate buffer was added in place of the enzyme. The proteins were separated by SDS-PAGE, transferred to PVDF membrane, and detected by immunoblotting as described above.

Immunoprecipitation—At 48 h after transfection, the 293T cells were lysed in PBS containing 0.5% Triton X-100 and proteinase inhibitors. The cell lysates were centrifuged at 10,000 × *g* for 20 min to remove cellular debris and precleared with protein G-Sepharose beads. Proteins were immunoprecipitated by incubation with anti-Myc antibody for 2 h at 4°C and then with protein G-Sepharose beads for another 2 h at 4°C with rotation. After washing 5 times with PBS containing 0.5% Triton X-100, the bound materials were boiled for 5 min at 95°C in Laemmli sample buffer and analyzed by Western blotting.

Solubilization of Membrane Proteins with Alkali, Salt or Detergent—At 48 h post-transfection, the 293T cells were homogenized in PBS containing proteinase inhibitors. The homogenates were centrifuged for 20 min at 10,000 × *g* and the resulting pellet was extracted with 5 volumes of 1 M NaCl, 0.1 M Na_2CO_3 (pH 11) or 1% Triton X-100 at 4°C overnight. The extract was centrifuged at 100,000 × *g* for 30 min and supernatant fraction was then analyzed by Western blotting.

Antibody Production—For antibody production, the DNA fragment encoding the α-fragment of Ig-Hepta (residues 25–223) was amplified by PCR and cloned into

vector pRSET-A, and the construct was transformed into *Escherichia coli* BL21(DE3)pLysS and used for fusion protein production. Fusion protein was insoluble and purified under denaturing conditions on Ni^{2+}-NTA resin columns. 200 μg of His_6-Ig-Hepta α-fragment fusion proteins emulsified in TiterMax Gold adjuvant (CytRx, Atlanta, GA, USA) were injected four times into Japanese white rabbits. The antibodies in the antisera were purified against α-fragment coupled to HiTrap-NHS–activated affinity gel according to the manufacturer's instruction.

Stable Transfection of 293T Cells with α-hFc cDNA—To establish cell lines that express α-hFc, the expression vector encoding α-hFc was linearized by *Sca*I restriction enzyme and transfected into exponentially growing 293T cells using the LipofectAMINE 2000 method. Selection of stable transfectants was carried out by adding 300 μg/ml Zeocin into the medium. After 2 weeks, Zeocin-resistant cells were cloned by limiting dilution and expanded to obtain stable cell lines. Expression of α-hFc was determined by Western blotting of cell supernatants.

N-Terminal Amino Acid Sequence Analysis—For N-terminal sequencing, 1 μg of α-hFc purified with protein G-Sepharose was electrophoresed on 10% SDS-PAGE and transferred to PVDF membranes. The protein band of interest was excised from the membrane, and N-terminal sequence was determined by Apro Life Science Institute (Naruto, Japan).

Microarray Analysis—Hybridization probes for GeneChip analysis were prepared from total RNA isolated from 293T cells using Isogen. The GeneChip analysis was performed by Hitachi Life Science.

Northern Blot Analysis—Total RNA from 293T cells was electrophoresed in 1% formaldehyde-agarose gels and then transferred to Hybond N^+ nylon membranes by capillary blotting using 20× SSC as the transfer buffer. After transfer, the membranes were baked for 2 h at 80°C and prehybridized for 1 h at 68°C in PerfectHyb hybridization solution (Toyobo). The probes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) using Ready-To-Go DNA labeling kit. The membranes were then hybridized with 32P-labeled probe in the same buffer at 68°C for 16 h. After hybridization, the membranes were washed twice with 1× SSC and 0.1% SDS for 1 h at 60°C. The membranes were exposed to imaging plates (Fuji Film, Tokyo, Japan) in a cassette for 1 day. The results were analyzed using a Fuji BAS2000 Bio-image analyzer (Fuji Film).

RESULTS

Membrane-Associated Nature and Proteolytic Processing of α-Fragment of Ig-Hepta—We previously demonstrated the cleavage of Ig-Hepta at the SEA module near the N-terminus (*9*), which generates an ~200-residue fragment termed here "α-fragment." The α-fragment was further found to contain a consensus sequence for furin processing in its N-terminal region. To determine whether this potential cleavage site between residues 51 and 52 is actually used during processing of Ig-Hepta, we expressed wild type Ig-Hepta and the α-fragment in 293T cells and performed Western blot analysis using a purified antibody specific for the α-fragment (anti-$\alpha_{Ig\text{-}Hepta}$). To our surprise, the α-fragment was recovered only from membrane preparations and not from the culture medium, suggesting the possibility that the α-fragment is associated with the β/γ-chains or cell surface components (Fig. 1A, lanes 5 and 6). Multiple bands were observed, on Western blotting, in both cases of transfection of 293T cells with the α-fragment and wild type Ig-Hepta constructs. However, the multiple band pattern does not necessarily indicate the presence of proteolytic processing since there are three potential *N*-glycosylation sites in the α-fragment (Fig. 3C) and the difference in their sizes may represent the difference in the degree of glycosylation. We therefore performed deglycosylation of membrane preparations of rat lung and 293T cells expressing wild type Ig-Hepta to simplify the band pattern. *N*-glycosidase F and Endoglycosidase H treatment reduced the number and sizes of bands (Fig. 1B, lanes 2, 3, 5, and 6). Cell surface expression of the α-fragment was confirmed by biotinylation (data not shown).

Non-Covalent Association of α-Fragment, β-Chain, and γ-Chain—Since α-fragment was not released into the culture medium (Fig. 1A), we explored its possible interaction with the β/γ-chains using a Myc-tagging/immunoprecipitation approach previously employed for demonstrating

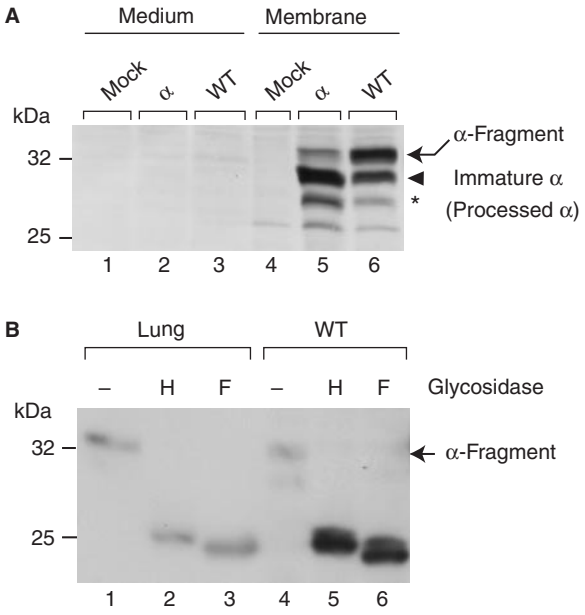

Fig. 1. **Expression of Ig-Hepta α-fragment in rat lung and 293T cells.** (A) Western blot analysis revealing the presence of processed Ig-Hepta α-fragment as a membrane component. 293T cells were transiently transfected with Ig-Hepta full length cDNA (WT), Ig-Hepta α-fragment cDNA, and control cDNA (Mock). Membrane fractions and conditioned medium concentrated with an Ultrafree-0.5 centrifugal filter device were analyzed by Western blotting with anti-$\alpha_{Ig\text{-}Hepta}$ polyclonal antibody. Fully glycosylated mature α-fragment and immature α-fragment are indicated by arrow and arrowhead, respectively. The band marked with an asterisk represents processed α-fragment. (B) Enzymatic deglycosylation of Ig-Hepta α-fragment expressed in lung and 293T cells. Crude membrane proteins isolated from rat lung and 293T cells transiently transfected with Ig-Hepta cDNA (WT) were digested with *N*-glycosidase F or Endoglycosidase H (lanes 2, 3, 5, and 6) and analyzed by immunoblotting. The size of fully glycosylated mature (32 kDa, arrow in lanes 1 and 4) α-fragment was reduced to ~25 kDa by the treatment (lanes 2, 3, 5, and 6). In the presence of *N*-glycosidase F or Endoglycosidase H, two bands were detected. Molecular mass markers are indicated on the left.

the non-covalent association of the β- and γ-chains (*9*). Ig-Hepta-Myc, a C-terminally Myc-tagged form of Ig-Hepta, was expressed in 293T cells, extracted with a dilute Triton X-100 solution, immunoprecipitated with anti-Myc antibody, and subjected to Western blot analysis using affinity-purified antibody specific for the α-fragment, β-chain, and anti-Myc monoclonal antibody. Figure 2A demonstrates non-covalent association of the three parts even after proteolytic cleavages.

To confirm the ability of α-fragment to interact with the β/γ-chains, we next expressed separately the α-fragment and N-terminally truncated Ig-Hepta lacking the α-fragment (Δα-Ig-Hepta). The α-fragment was indeed co-immunoprecipitated with Δα-Ig-Hepta (Fig. 2B, lane 3), demonstrating their association.

Determination of Processing Site on α-Fragment—A substantial amount of cleaved product is necessary for its N-terminal amino acid sequencing. For this purpose, we constructed a chimeric protein composed of the α-fragment of rat Ig-Hepta and the Fc domain of human IgG1 (α-hFc). hFc was introduced for affinity purification; it also helped to stimulate secretion of the chimera into the medium. When the chimeric construct was stably expressed in 293T cells, the expected proteolytic processing occurred, yielding a lower molecular weight species (Fig. 3A, arrow). The chimeric α-hFc was purified from the culture medium by affinity chromatography on protein G-Sepharose and found to have the following N-terminal sequence: AVAVGGPVAE (Fig. 3B). This result strongly indicates that the cleavage site is Arg^{51}-Ala^{52}.

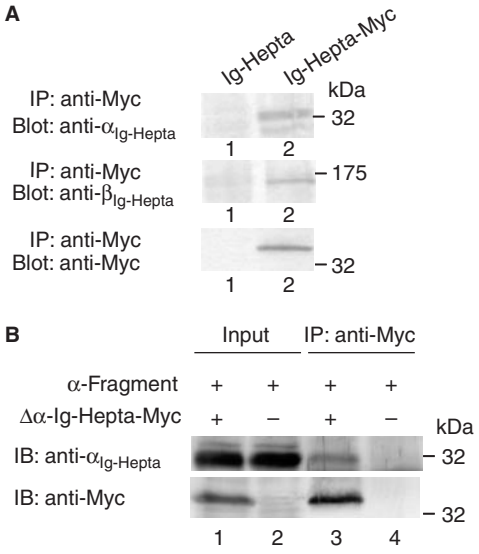

Fig. 2. **Non-covalent association of α-fragment and β/γ-chains of Ig-Hepta after cleavage.** (A) Immunoprecipitation analysis using Myc-tagged Ig-Hepta. Membranes of 293T cells transiently transfected with Ig-Hepta or C-terminally Myc-tagged full length Ig-Hepta (Ig-Hepta-Myc) were solubilized in 0.5% Triton X-100 and immunoprecipitated with anti-Myc monoclonal antibody 9E10. Immunoprecipitates were then run on 12.5% SDS-PAGE and analyzed by Western blotting with anti-$\alpha_{Ig\text{-}Hepta}$ polyclonal antibody (top panel), anti-$\beta_{Ig\text{-}Hepta}$ polyclonal antibody (middle panel), and anti-Myc monoclonal antibody (bottom panel). (B) Co-expression of α-fragment and truncated Ig-Hepta (Δα-Ig-Hepta) in 293T cells. 293T cells co-transfected with α-fragment and Δα-Ig-Hepta-Myc were solubilized and immunoprecipitated as indicated, followed by Western blotting with anti-$\alpha_{Ig\text{-}Hepta}$ and anti-Myc antibody. Molecular mass markers are indicated on the right.

Cleavage by Furin—The amino acid sequence upstream of the cleavage site (RPKR↓A^{52}) matches the consensus sequence known to serve as a cleavage site for the endoprotease furin, raising the possibility that the ~25-residue prosegment is cleaved from the α-fragment by furin. We tested this hypothesis using LoVo cells, a cell line that lacks active furin because of mutations in the furin gene (*14*). When the α-fragment was expressed in LoVo cells, no cleavage occurred (Fig. 4, lane 2) whereas significant levels of processing took place in 293T cells (Fig. 4, lane 3). However, cotransfection of LoVo cells with expression plasmids for the α-fragment and furin restored the processing of the α-fragment (Fig. 4, lane 1), indicating the involvement of furin in the processing.

Characterization of Associations of α-Fragment with Cell Surface and β/γ-Chains—As already mentioned, the results depicted in Figs. 1 and 2 imply that (i) the α-fragment, upon secretion, binds to cell surface components when it is expressed without the β- and γ-chains and (ii) a large proportion of it remains associated with the β/γ-chains when expressed as a part of wild type Ig-Hepta. To characterize binding properties of the α-fragment, we determined its extractability from cell surface using various solubilization conditions. Considerable amounts of α-fragment were released from cell surface by treatment with 1 M NaCl (Fig. 5A, lane 1), but its interaction with the β/γ-chains was not affected by the same high-salt condition (Fig. 5B, upper panel, lane 1). Interaction of the α-fragment with cell surface proteins and that with the β/γ-chains were both sensitive to Na_2CO_3 (pH 11) and Triton X-100 treatments. However, β-chain was not extracted with Na_2CO_3 (Fig. 5B, lower panel, lane 4), suggesting relatively strong interaction between the β- and γ-chains. Thus we used the term "chain" for β and γ, and "fragment" for α to indicate relatively strong and weak associations, respectively.

α-Fragment–induced Cellular Response Revealed by Microarray Analysis—The growth factor–like structure and processing of the α-fragment led us to examine its effect on the expression and phosphorylation levels of subcellular protein components. We first compared steady-state phosphorylation levels of proteins in 293T cells that were transfected with Ig-Hepta and Δα-Ig-Hepta and then incubated with [^{32}P]phosphate. SDS-PAGE analysis of their whole cell extracts, however, revealed no significant gross changes in the phosphorylation patterns (data not shown). We next performed microarray analysis by isolating total RNA from the Ig-Hepta- and Δα-Ig-Hepta–transfected 293T cells and by comparing the message levels using Hitachi microarrays. Several RNA species were up-regulated in the presence of the α-fragment including a noncoding RNA species (THC1808923, 26.7-fold increase), a guanine nucleotide exchange factor (GEF) for Rab5 (ALS2CL, 12.5-fold increase), and TC10-like Rho GTPase (2.3-fold increase). Among these, we chose ALS2CL for further characterization since its signal on the array was relatively strong and highly up-regulated (more than 10-fold) by the presence of the α-fragment; the upregulation of mRNA levels of the other two species were not confirmed by Northen blotting.

Northern blot analysis confirmed the differential expression of ALS2CL mRNA in the Ig-Hepta- and

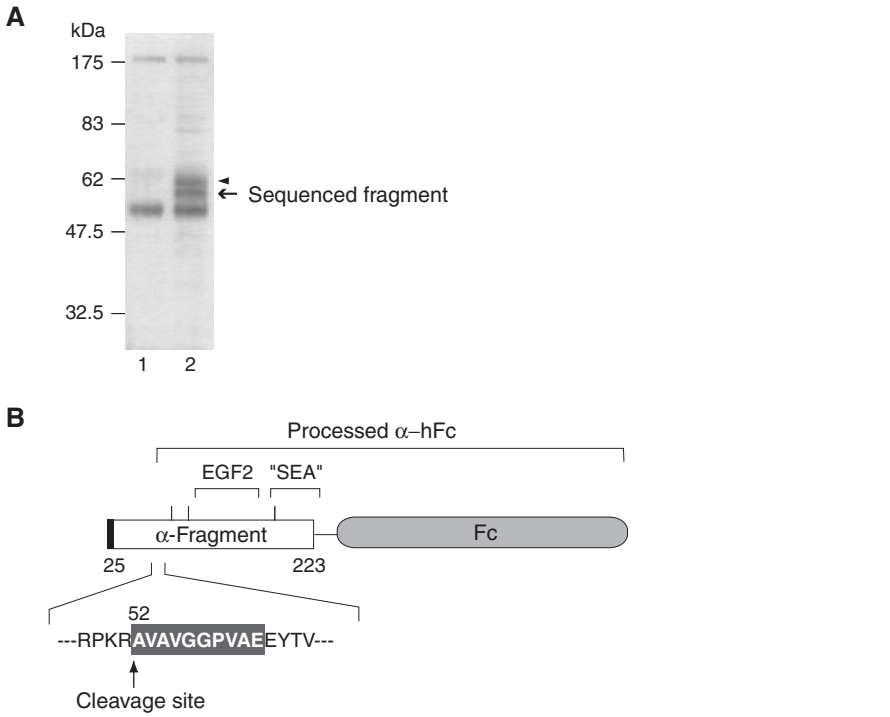

Fig. 3. **Identification of the cleavage site within Ig-Hepta α-fragment.** (A) SDS-PAGE analysis of α-fragment-hFc (α-hFc) produced in 293T cells. The α-hFc chimeric protein, purified using protein G-Sepharose beads from the culture medium of 293T cells stably transfected with α-hFc expression plasmid, was subjected to 10% SDS-PAGE and visualized by silver staining. Lane 1, 20 μl of cell supernant; lane 2, 1 μg of purified protein. Positions of the full-length α-hFc and proteolytically cleaved α-hFc are indicated by arrowhead and arrow, respectively. Molecular mass markers are indicated on the left. (B) Amino acid sequence surrounding the proteolytic cleavage site contained in α-hFc. The arrow designates the point of cleavage in α-hFc. The N-terminal 10 amino acid residues of α-hFc, determined as described under "MATERIALS AND METHODS," are shaded. The dark box at the N-terminus represents a presequence of about 24 residues that was removed cotranslationally; vertical bars indicate potential *N*-linked glycosylation sites; EGF2, EGF-like domain signature 2; "SEA," SEA module; 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 three non-covalently associated chains generated by proteolytic processing of the precursor.

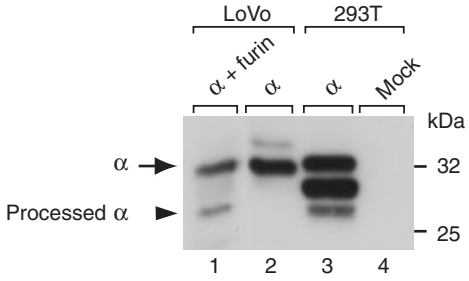

Fig. 4. **Processing of α-fragment of Ig-Hepta in transfected 293T cells and LoVo cells.** 293T cells were transfected with α-fragment cDNA and control cDNA (Mock). LoVo cells were transfected with α-fragment cDNA together with a plasmid encoding mouse furin, as described under "MATERIALS AND METHODS." Cell lysates were analyzed by SDS-PAGE and Western blotting with anti-$\alpha_{\text{Ig-Hepta}}$ antibody. Mature α-fragment and processed α-fragment are indicated by arrow and arrowhead, respectively. Molecular mass markers are indicated on the right.

Δα-Ig-Hepta–transfected 293T cells (Fig. 6). Since ALS2CL is a GEF specific for Rab5 (*15*), a small GTPase playing a key regulatory role in endocytosis, we therefore determined the subcellular locations of Rab5 and early endosome antigen 1 (EEA1) by immunocytochemistry, but no significant difference was observed in their distribution patterns in the Ig-Hepta- and Δα-Ig-Hepta–transfected 293T cells. We also determined transferrin uptake according to the published method (*16*) using fluorescent transferrin, but no difference was observed either in the rate of uptake or subcellular distribution of the endocytosed transferrin (data not shown).

DISCUSSION

Our previous and current studies revealed that Ig-Hepta is processed at multiple sites yielding the following four fragments: (i) presequence (residues 1–24), (ii) proEGF2 (residues 25–223, α-fragment), (iii) Ig repeats (residues 224–993, β-chain), and (iv) TM7 (residues 994–1349, γ-chain). The proEGF2 region is converted to EGF2 (residues 52–223) by the processing enzyme furin and remains attached to the β-chain, which in turn is non-covalently linked to the γ-chain (Fig. 7). Although physiological significance of the cleavage and reassociation of the EGF2 domain (α-fragment) is not clear, it may represent a mechanism by which the functional domain is primed for its later role as a ligand for a receptor as suggested

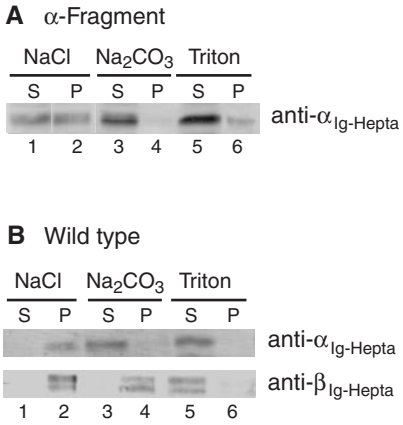

Fig. 5. **Extraction of Ig-Hepta α-fragment with alkali or salt.** Membranes of 293T cells transiently transfected with either wild type Ig-Hepta or its α-fragment were subjected to differential extraction protocols using the following buffers: 1 M NaCl, 0.1 M Na_2CO_3 (pH 11), or 1% Triton X-100. Pellets and supernatants were analyzed by Western blotting with anti-$\alpha_{Ig\text{-}Hepta}$ and anti-$\beta_{Ig\text{-}Hepta}$ polyclonal antibody. P, pellet; S, supernatant.

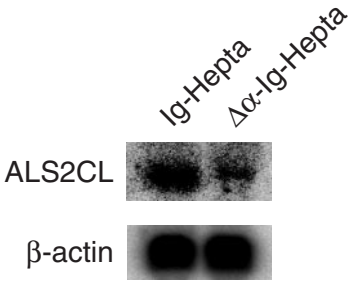

Fig. 6. **Up-regulation of the ALS2CL gene by Ig-Hepta.** ALS2CL gene expression was enhanced by the presence of Ig-Hepta. 293T cells were transiently transfected with either wild type Ig-Hepta or Δα-Ig-Hepta. 20 αg of total RNA were used for Northern blot analysis to measure ALS2CL mRNA level. β-actin was used as an internal control.

by earlier studies by others (*13*, *17–20*). Considering the highly restricted tissue expression of Ig-Hepta revealed by Northern blot analysis and immunohistochemistry (*21*), the Ig-Hepta–derived EGF2 is expected to act as an autocrine/paracrine regulator in the lung, kidney (around the intercalated cells), and heart. Relatively low levels of EGF2 (processed α-fragment) were found in lung extracts compared to those in 293T cells exogenously overexpressing Ig-Hepta (Fig. 1B). This is probably due to rapid clearance of mature EGF2 *in vivo*.

The cleavage at the GPS (Cys-box) in the juxtamembrane stalk region and non-covalent association of the cleaved fragments are common features of LNB-TM7 family members. The cleavage has recently been shown to occur autocatalytically (*22*). Although the significance of this processing common to the LNB-TM7 members is not clear, the cleavage is likely to be involved in trafficking to the cell surface (*23*). In the case of Polycystin-1, which plays an essential role in renal tubular morphogenesis, it has been demonstrated that prevention of cleavage at the GPS by mutaions leads to impairment of renal function (*24*). These observations imply that the self-cleavage at the juxtamembrane region is essential for the receptors to be targeted to plasma membrane and function there properly.

SEA modules are thought to serve a dual role of cleavage and reassociation (*13*). A characteristic denominator of SEA modules is their location in heavily glycosylated regions, suggesting the involvement of sugar residues in the dual role. Khatri *et al*. (*12*) have, however, demonstrated, by analyzing rMuc3, that reassociation of the cleaved fragments requires the SEA module, but is independent of *N*-linked oligosaccharides. The mechanism of cleavage has been demonstrated to be autocatalytic as in the case of the GPS site (*25*, *26*). However, major questions still remain to be answered: (i) clarification of the mechanism of reassociation and (ii) the signal that induces later release of the active component preceding the SEA module.

In this study, using LoVo cells, we showed that the α-fragment of Ig-Hepta is cleaved by furin, a member of the subtilisin-like serine endoprotease family. This family includes PC1/PC3, PC2, PC4, PACE4, PC5/PC6, and PC7,

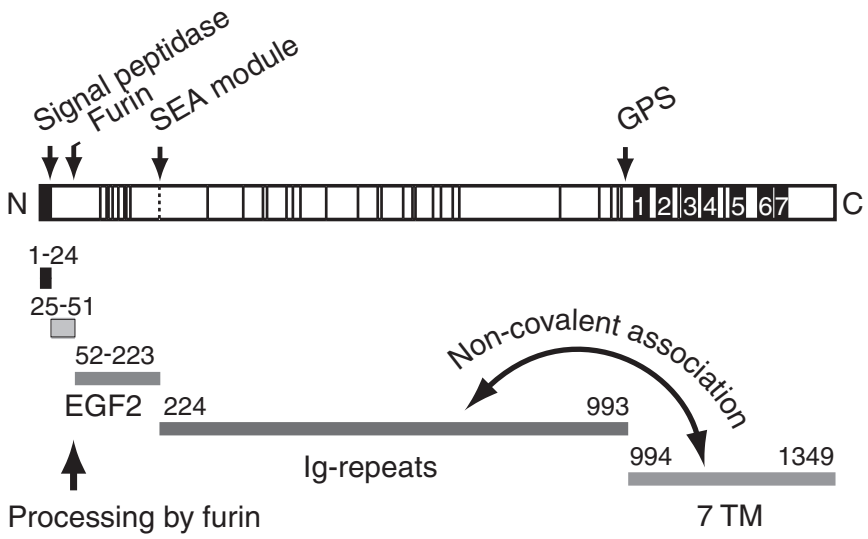

Fig. 7. **Schematic representation of processing of Ig-Hepta generating growth factor–like α-fragment.** Schematic diagram summarizing the domain organization of full-length Ig-Hepta. Putative cleavage site for signal peptidase, proteolytic cleavage site for furin, and those at the SEA module and GPS are indicated by arrows. Positions of the cysteine residues are indicated by vertical lines. The dark box represents presequence, and numbered dark boxes indicate transmembrane spans. EGF2, EGF2 domain; Ig-repeats, C2-type immunoglobulin–like domain; 7TM, 7 transmembrane region. Ig-repeats and 7TM are bound by non-covalent association after proteolytic cleavage at GPS.

which recognize the consensus sequence R-X-K/R-R. The possibility, therefore, remains that members other than furin may be involved in the α-fragment processing *in vivo*. This possibility, however, seems to be low because (i) the processing did not occur in LoVo cells that were deficient in furin activity (*14*), (ii) PC1/3 and PC2 are convertases mediating mainly the processing of neuropeptides (*27*), (iii) PC4 is expressed predominantly in testis (*28*), and (iv) we confirmed that PACE4 did not promote the cleavage of the α-fragment in cultured cells (data not shown).

Until recently, Ig-Hepta is considered to be present only in mammals. By a combination of a database search and PCR amplification of cDNA, however, we have identified the fugu ortholog in *Takifugu rubripes*, which consists of 1674 amino acid residues (accession number AB246172). Although the size of the fugu ortholog is larger than the mammalian Ig-Hepta, the domain structure is highly conserved among the fish and mammalian Ig-Hepta including the presence of the furin-processing site (-REKR-) in the α-fragment. Fugu Ig-Hepta has a tandem repeat of six EGF2 sequences and a long stretch of mucin-like domain in its α-fragment, making it much longer than the mammalian counterpart. Determination of expression of fugu Ig-Hepta at the tissue and cellular levels may help elucidate the function of Ig-Hepta especially that of the furin-processed α-fragment.

Microarray analysis suggested a functional link between Ig-Hepta and Rab5, a key regulator of endocytosis. So far, however, direct evidence for their link has not been obtained despite the intensive immunofluorescence monitoring of the subcellular locations of Rab5 and EEA1 and the endocytosis of transferrin in the presence and absence of the α-fragment of Ig-Hepta. Further studies including targeted disruption of the Ig-Hepta gene will be necessary to clarify the physiological role and mechanism of signaling of Ig-Hepta that is cleaved at multiple sites.

We thank Jumpei Abe for his helpful suggestions and Setuko Satoh for secretarial assistance. This work was supported by COE21 Program from Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), JSPS Reserach Fellowships for Young Scientists and JSPS Grants-in-Aid for Scientific Research (S, 14104002).

REFERENCES

1. Stacey, M., Lin, H.H., Gordon, S., and McKnight, A.J. (2000) LNB-TM7, a group of seven-transmembrane proteins related to family-B G-protein-coupled receptors. *Trends Biochem Sci.* **25**, 284–289
2. Fredriksson, R., Lagerstrom, M.C., Hoglund, P.J., and Schioth, H.B. (2002) Novel human G protein-coupled receptors with long N-terminals containing GPS domains and Ser/Thr-rich regions. *FEBS Lett.* **531**, 407–414
3. Fredriksson, R., Gloriam, D.E., Hoglund, P.J., Lagerstrom, M.C., and Schioth, H.B. (2003) There exist at least 30 human G-protein-coupled receptors with long Ser/Thr-rich N-termini. *Biochem Biophys. Res. Commun.* **301**, 725–734
4. Curtin, J.A., Quint, E., Tsipouri, V., Arkell, R.M., Cattanach, B., Copp, A.J., Henderson, D.J., Spurr, N., Stanier, P., Fisher, E.M., Nolan, P.M., Steel, K.P., Brown, S.D., Gray, I.C., and Murdoch, J.N. (2003) Mutation of Celsr1 disrupts planar polarity of inner ear hair cells and causes severe neural tube defects in the mouse. *Curr. Biol.* **13**, 1129–1133
5. Kaur, B., Brat, D.J., Devi, N.S., and Van Meir, E.G. (2005) Vasculostatin, a proteolytic fragment of brain angiogenesis inhibitor 1, is an antiangiogenic and antitumorigenic factor. *Oncogene* **24**, 3632–3642
6. Stacey, M., Chang, G.W., Davies, J.Q., Kwakkenbos, M.J., Sanderson, R.D., Hamann, J., Gordon, S., and Lin, H.H. (2003) The epidermal growth factor-like domains of the human EMR2 receptor mediate cell attachment through chondroitin sulfate glycosaminoglycans. *Blood* **102**, 2916–2924
7. Leemans, J.C., te Velde, A.A., Florquin, S., Bennink, R.J., de Bruin, K., van Lier, R.A., van der, P.T., and Hamann, J. (2004) The epidermal growth factor-seven transmembrane (EGF-TM7) receptor CD97 is required for neutrophil migration and host defense. *J Immunol.* **172**, 1125–1131
8. Sugita, S., Ichtchenko, K., Khvotchev, M., and Sudhof, T.C. (1998) alpha-Latrotoxin receptor CIRL/latrophilin 1 (CL1) defines an unusual family of ubiquitous G-protein-linked receptors. G-protein coupling not required for triggering exocytosis. *J. Biol. Chem.* **273**, 32715–32724
9. Abe, J., Fukuzawa, T., and Hirose, S. (2002) Cleavage of Ig-Hepta at a "SEA" module and at a conserved G protein-coupled receptor proteolytic site. *J. Biol. Chem.* **277**, 23391–23398
10. Bork, P. and Patthy, L. (1995) The SEA module: a new extracellular domain associated with O-glycosylation. *Protein Sci.* **4**, 1421–1425
11. Baruch, A., Hartmann, M., Yoeli, M., Adereth, Y., Greenstein, S., Stadler, Y., Skornik, Y., Zaretsky, J., Smorodinsky, N.I., Keydar, I., and Wreschner, D.H. (1999) The breast cancer-associated MUC1 gene generates both a receptor and its cognate binding protein. *Cancer Res.* **59**, 1552–1561
12. Khatri, I.A., Wang, R., and Forstner, J.F. (2004) Evidence for a second peptide cleavage in the C-terminal domain of rodent intestinal mucin Muc3. *Biochem. J.* **378**, 207–212
13. Wreschner, D.H., McGuckin, M.A., Williams, S.J., Baruch, A., Yoeli, M., Ziv, R., Okun, L., Zaretsky, J., Smorodinsky, N., Keydar, I., Neophytou, P., Stacey, M., Lin, H.H., and Gordon, S. (2002) Generation of ligand-receptor alliances by "SEA" module-mediated cleavage of membrane-associated mucin proteins. *Protein Sci.* **11**, 698–706
14. Nakayama, K. (1997) Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. *Biochem. J.* **327 (Pt 3)**, 625–635
15. Hadano, S., Otomo, A., Suzuki-Utsunomiya, K., Kunita, R., Yanagisawa, Y., Showguchi-Miyata, J., Mizumura, H., and Ikeda, J.E. (2004) ALS2CL, the novel protein highly homologous to the carboxy-terminal half of ALS2, binds to Rab5 and modulates endosome dynamics. *FEBS Lett.* **575**, 64–70
16. Nakamura, N., Fukuda, H., Kato, A., and Hirose, S. (2005) MARCH-II is a syntaxin-6-binding protein involved in endosomal trafficking. *Mol. Biol. Cell* **16**, 1696–1710
17. Carraway, K.L., III, Rossi, E.A., Komatsu, M., Price-Schiavi, S.A., Huang, D., Guy, P.M., Carvajal, M.E., Fregien, N., Carraway, C.A., and Carraway, K.L. (1999) An intramembrane modulator of the ErbB2 receptor tyrosine kinase that potentiates neuregulin signaling. *J. Biol. Chem.* **274**, 5263–5266
18. Julian, J. and Carson, D.D. (2002) Formation of MUC1 metabolic complex is conserved in tumor-derived and normal epithelial cells. *Biochem. Biophys. Res. Commun.* **293**, 1183–1190
19. McNeer, R.R., Huang, D., Fregien, N.L., and Carraway, K.L. (1998) Sialomucin complex in the rat respiratory tract: a model for its role in epithelial protection. *Biochem. J.* **330 (Pt 2)**, 737–744
20. Schroeder, J.A., Thompson, M.C., Gardner, M.M., and Gendler, S.J. (2001) Transgenic MUC1 interacts with epidermal growth factor receptor and correlates with mitogen-activated protein kinase activation in the mouse mammary gland. *J. Biol. Chem.* **276**, 13057–13064

21. Abe, J., Suzuki, H., Notoya, M., Yamamoto, T., and Hirose, S. (1999) Ig-Hepta, a novel member of the G protein-coupled hepta-helical receptor (GPCR) family that has immunoglobulin-like repeats in a long N-terminal extracellular domain and defines a new subfamily of GPCRs. *J. Biol. Chem.* **274**, 19957–19964
22. Lin, H.H., Chang, G.W., Davies, J.Q., Stacey, M., Harris, J., and Gordon, S. (2004) Autocatalytic cleavage of the EMR2 receptor occurs at a conserved G protein-coupled receptor proteolytic site motif. *J. Biol. Chem.* **279**, 31823–31832
23. Krasnoperov, V., Lu, Y., Buryanovsky, L., Neubert, T.A., Ichtchenko, K., and Petrenko, A.G. (2002) Post-translational proteolytic processing of the calcium-independent receptor of alpha-latrotoxin (CIRL), a natural chimera of the cell adhesion protein and the G protein-coupled receptor. Role of the G protein-coupled receptor proteolysis site (GPS) motif. *J. Biol. Chem.* **277**, 46518–46526
24. Qian, F., Boletta, A., Bhunia, A.K., Xu, H., Liu, L., Ahrabi, A.K., Watnick, T.J., Zhou, F., and Germino, G.G. (2002) Cleavage of polycystin-1 requires the receptor for egg jelly domain and is disrupted by human autosomal-dominant polycystic kidney disease 1-associated mutations. *Proc. Natl. Acad. Sci. USA* **99**, 16981–16986
25. Levitin, F., Stern, O., Weiss, M., Gil-Henn, C., Ziv, R., Prokocimer, Z., Smorodinsky, N.I., Rubinstein, D.B., and Wreschner, D.H. (2005) The MUC1 SEA module is a self-cleaving domain. *J. Biol. Chem.* **280**, 33374–33386
26. Macao, B., Johansson, D.G., Hansson, G.C., and Hard, T. (2006) Autoproteolysis coupled to protein folding in the SEA domain of the membrane-bound MUC1 mucin. *Nat. Struct. Mol. Biol.* **13**, 71–76
27. Steiner, D.F., Smeekens, S.P., Ohagi, S., and Chan, S.J. (1992) The new enzymology of precursor processing endoproteases. *J. Biol. Chem.* **267**, 23435–23438
28. Nakayama, K., Kim, W.S., Torii, S., Hosaka, M., Nakagawa, T., Ikemizu, J., Baba, T., and Murakami, K. (1992) Identification of the fourth member of the mammalian endoprotease family homologous to the yeast Kex2 protease. Its testis-specific expression. *J. Biol. Chem.* **267**, 5897–5900