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*

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A novel member of the G protein-coupled receptor (GPCR) family was cloned and characterized, which is unique, among the members, in its long extracellular domain comprising Ig-like repeats and in its high expression predominantly in the lung. The clone (Ig-Hepta) was first identified as a polymerase chain reaction product generated with primers designed to amplify secretin receptor family members including the parathyroid hormone-related peptide receptors. Analysis of the open reading frame of cDNAs isolated from a rat lung cDNA library indicated that Ig-Hepta is a protein of 1389 amino acid residues and has two Ig-like repeats in the N-terminal extracellular domain (exodomain) of 1053 amino acid residues and 7 transmembrane spans in the C-terminal region. Northern blot analysis revealed very high expression of its mRNA in the lung and low but detectable levels in the kidney and heart. The mRNA expression in the lung was found to be strongly induced postnatally. Biochemical analysis indicated that Ig-Hepta is a highly glycosylated protein and exists as a disulfide-linked dimer. Immunohistochemistry on rat lung and kidney sections revealed dense localization of Ig-Hepta in alveolar walls and intercalated cells in the collecting duct, respectively, suggesting a role in the regulation of acid-base balance. Ig-Hepta defines a new subfamily of GPCRs.

Since the cloning of rhodopsin (1) and β-adrenergic receptor (2), more than 1000 G protein-coupled receptors (GPCRs) have been cloned and characterized (3–5). They constitute one of the largest family of proteins, which have in common seven transmembrane domains, and are involved in broad spectrum of biological processes by mediiating the signal of a wide variety of stimuli such as hormones, neurotransmitters, cytokines, light, and odorants. GPCRs can be grouped into various subfamilies based on their amino acid sequences. Recently a subfamily has emerged that shares the seven-transmembrane topology but has a low overall amino acid sequence similarity with other members of the GPCR superfamily. This subfamily, now referred to as the class II GPCR family (Figs. 2 and 3), comprises receptors for secretin, glucagon, VIP, calcitonin, PTH, PTHrP, glucagon-like peptide 1, gastrin inhibitory polypeptide, growth hormone-releasing hormone, corticosteroid-releasing factor, pituitary adenylate cyclase-activating peptide, and an insect diuretic hormone and is therefore also called the secretin receptor family or the glucagon/VIP/calcitonin receptor family (for review see Refs. 6–8). The class II receptors are characterized not only by the lack of the structural signature sequences present in the class I rhodopsin/β-adrenergic receptor family but also by the presence of a large N-terminal extracellular domain (exodomain).

Recently Baud et al. (9), Hamann et al. (10), and McKnight et al. (11) have identified a novel subtype of the class II receptors through the structural analyses of a cDNA clone (EMR1) of neuroectodermal origin, of a leukocyte activation antigen (CD97), and of a macrophage-restricted cell-surface glycoprotein (F4/80), respectively, that has an extraordinarily long N-terminal domain containing EGF-like repeats (for review see Refs. 12 and 13). In addition, Hadjantonakis et al. (14, 15) have reported the presence of a developmentally regulated gene, Celsr1, that encodes an orphan receptor of the class II GPCR family with an extended exodomain containing a block of cadherin repeats. Although the exact physiological functions are unknown, these receptors are gaining a great deal of attention because of their unusual structural properties; they are composed of two well characterized protein motifs usually seen in distinct protein superfamilies as follows: one is the motifs (EGF and cadherin repeats) found in many cell-surface molecules with a single membrane-spanning domain, and the other is the transmembrane bundle that is composed of seven helices. Here we report another unusual member of the class II GPCR family whose hepta-helical transmembrane region is similar to those of the secretin receptor family, but its large exodomain (1053 amino acid residues) is unique in having immunoglobulin-like repeats, a motif characteristic of the members of the immunoglobulin superfamilies of cell-surface proteins (16, 17). Based on this chimeric structural feature, we term the protein “Ig-
Hepta.” Its localization and regulation of expression are also very unique; Ig-Hepta is predominantly expressed in the lung, and its expression was found to be markedly induced postnatally. Immunostaining of the lung and kidney, which exhibited, by Northern analysis, strongly and weakly positive signals, respectively, revealed specific staining in the alveolar walls of the lung and intercalated cells in the collecting duct of the kidney, suggesting that Ig-Hepta may be involved in pH-sensing or pH regulation.

EXPERIMENTAL PROCEDURES

Materials—Wistar rats were obtained from Tokyo Laboratory Animals Science, Tokyo, Japan; Expand Long Template PCR System was from Roche Molecular Biochemicals, Mannheim, Germany; pBluescript II SK+, EcoRI/SalI-strain XL1-Blue MRF², αZAP II, Gigapack II Gold in vitro packaging kits were from Stratagene, La Jolla, CA; mRNA purification kits and Ready-To-Go DNA labeling kit were from Amersham Pharmacia Biotech, Uppsala, Sweden; pcDNA3, pTrcHis B, and pSecTag were from Invitrogen, San Diego, CA; SequiTherm Long-read-LC cycle sequencing kit was from Epicentre Technologies, Madison, WI; [α-32P]dCTP was from Amersham Pharmacia Biotech, Buckinghamshire, UK; Superscript II reverse transcriptase and DTT were from Invitrogen, San Diego, CA; modified Eagle’s medium were from Life Technologies, Inc.; alkaline phosphatase-conjugated mouse anti-rabbit IgG antibody was from Sigma, Munich, Germany; immobilon polyvinylidene difluoride membrane was from Millipore, Tokyo, Japan; Protran BA85 nitrocellulose membranes were from Schleicher & Schuell, Dassel, Germany; nito blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate were from Wako Pure Chemicals, Osaka, Japan; BCA Protein Assay Reagent Kit was from Pierce.

Preparation of Rat Ig-Hepta cDNA Probe by PCR—Based on the amino acid sequence alignment of the rat secretin receptor family, two degenerate oligonucleotides corresponding to conserved elements found in the transmembrane helices III and VII were synthesized. These oligonucleotides were used as primers for PCR amplification of the PCR products with a single-stranded cDNA template derived from rat hypothalamus poly(A)⁺ RNA. The sequence of the sense primer was 5′-AATTTAYTGGTATCCCTGTTAGGG-3’ (where I is inosine; Y is C, T; R is A, G), and the sequence of the antisense primer was 5′-TGIACTCCTCAGCGCCGACCT-3′ (I is inosine; R is A, G, W is A, T). The reactions were performed in a MJ Research thermal cycler for 35 cycles of denaturation/PCR of the synthesis product (92 °C, 30 s), annealing (68 °C, 2 min) with an additional 10-min primer extension after the final cycle using Expand Long Template PCR System (Roche Molecular Biochemicals). The PCR products of expected size (about 500 base pairs) were isolated by agarose gel electrophoresis, purified using the QIAquick Gel Extraction Kit (Qiagen), and ligated into the pBluescript II SK+ vector (Stratagene) to a density of approximately 3×10⁷ plaque-forming units of the library were plated on E. coli XL1-Blue. When the A₀ₕ of purified recombinant His6-Ig-Hepta protein emulsified in Complete Freund’s adjuvant reached 0.8, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1.5 mM, and incubation was continued for an additional 6 h at 37 °C. The cells were harvested by centrifugation and resuspended in 10 mM Tris-HCl, pH 7.5, 0.1% (v/v) Triton X-100, disrupted by sonication, and centrifuged at 10,000 × g for 20 min. The pellet was resuspended and mixing in 8 M urea, 0.1 M NaH₂PO₄, 50 mM Tris-HCl, pH 8.0 at 4 °C with continuous temperature modulation. The mixtures were then centrifuged at 10,000 × g for 30 min to remove any insoluble material. Urea-solubilized recombinant His₆-Ig-Hepta protein was purified in a denatured state using a Ni⁺⁺-nitrilotriacetic acid agrose column (Qiagen) and dialyzed against phosphate-buffered saline (PBS; 20 mM phosphate, pH 7.4, containing 140 mM NaCl). Protein concentration was determined with a BCA Protein Assay Reagent kit (Pierce). Polyclonal antibodies to Ig-Hepta were prepared in Japanese White rabbits by immunizing subcutaneously with 200 µg of purified recombinant His₆-Ig-Hepta protein emulsified in complete Freund’s adjuvant (1:1) at multiple sites. Rabbits were boosted three times, each time with 100 µg of the purified protein in incomplete Freund’s adjuvant (1:1) at 2-week intervals. The rabbits were bled 10 days after the fourth immunization.

Expression of cDNA Encoding Rat Ig-Hepta in COS-7 Cells—Full-length Ig-Hepta cDNA was ligated into the NotI/ApaI sites of pcDNA3 (Invitrogen). COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO₂. COS-7 cells grown to 70–80% confluence in 100-mm diameter were transfected with pcDNA3 (30 µg) containing 200 µg of purified recombinant His₆-Ig-Hepta protein emulsified in complete Freund’s adjuvant (1:1) at multiple sites. Rabbits were boosted three times, each time with 100 µg of the purified protein in incomplete Freund’s adjuvant (1:1) at 2-week intervals. The rabbits were bled 10 days after the fourth immunization.

DNA Sequencing and Analysis—Nucleotide sequences were determined by the denaturing gradient gel electrophoresis method with an automated sequencer (LI-COR model 4000L) using a SequiTherm Long-read Cycle Sequencing Kit-LC (Epicentre Technologies). The DNA sequences were compiled and analyzed using the Genetyx-Mac computer program (Software Development). Data base searches were performed using the BLAST program of National Center for Biotechnology Information. Multiple protein sequence alignments were carried out using the program ClustalW, and final adjustments were made manually. Primary sequence motifs were identified using the PROSITE data base of ExPaSy.

Northern Blotting—Total RNA was isolated from various tissues of 6-week-old male Wistar rats and lungs of 3-day-old and 13-week-old Wistar rats by the acid guanidinium thiocyanate/phenol/chloroform method. Total RNA (0.1 µg) was denatured in 1.0% agarose gel containing formaldehyde and transferred to Magna nylon membranes (Micron Separations) by capillary blotting overnight using 10× SSC as the transfer buffer. After transfer, membranes were baked for 2 h at 80 °C and covalently immobilized on the membrane by exposure to UV light using a UV Stratalinker 2400 (Stratagene) and probed with the same 3²P-labeled cDNA probe as that used for the cDNA library screening under moderate and high stringency conditions. Briefly, the membranes were prehybridized for 1 h in prehybridization buffer consisting of 50% formamide, 5× SSPE, 2× Denhardt’s solution, and 0.5% SDS and hybridized at 42 °C (moderate stringency) or 37 °C (mild stringency) for 16 h. After hybridization, the membranes were washed twice with 2× SSC and 0.1% SDS for 15 min at 65 °C. Membranes were then washed in 1× SSC: 0.15 mM NaCl, 0.15 mM sodium citrate, pH 7.0, 0.1% SDS for 15 min at room temperature, washed in 1× SSC, 0.1% SDS for 30 min at 55 °C, and then washed in 0.5× SSC, 0.1% SDS at 55 °C and exposed to Kodak X-Omat AR film for 48 h at 80 °C with an intensifying screen. Thirty-two positive clones were identified, and the clones were purified in three rounds under the same conditions as the primary screening and transformed into pBluescript II SK⁺ by in vivo excision using ExAssist helper phage and XL1-Blue MRF² E. coli.
25–1056), was produced using the leader sequence in the pSecTag A expression vector designed for secreted proteins. 

**Preparation of Membrane Proteins from Transfected COS-7 Cells and Rat Lung**—At 72 h post-transfection, the COS-7 cells were rinsed in PBS to remove remaining serum, scraped from dishes, and homogenized on ice with a Dounce homogenizer in 1 ml of homogenization buffer (50 mM sodium phosphate, 25 mM NaCl, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μM peptstatin A, pH 7.5). The homogenate was centrifuged for 30 min at 10,000 × g, and the resulting pellet was extracted with 5 volumes of 1% Triton X-100 at 4°C overnight. Insoluble material was removed by centrifugation at 10,000 × g for 60 min. Rat lungs were first cut into small pieces and disrupted using a Dounce homogenizer with 5 volumes of ice-cold homogenization buffer and then further disrupted by 20 strokes in a Dounce homogenizer. Crude plasma membranes were isolated from the homogenate as described above.

**Preparation of His-tagged Exodomain by Ni²⁺ Chelate Chromatography**—COS-7 cells were transfected with the Ig-Hepta-EC/SecTag A expression vector described above. After 5 days, culture medium was collected from 5 dishes (10-cm diameter) and dialyzed three times against 2 liters of 50 mM NaH₂PO₄, pH 8.0, containing 300 mM NaCl for 12 h, and the Ig-Hepta-EC was purified by nickel chelate chromatography. Briefly, a column with 2 ml of bed volume was washed with 15 ml of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0), loaded with 50 ml of the dialyzed culture medium containing 20 mM imidazole, washed with 30 ml of elution buffer, bound was eluted with 500 μl of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). Unsecreted Ig-Hepta-EC was recovered as Triton extracts from membrane preparations as described above.

**Western Blotting**—The samples (30 μg of protein/lane) were dissolved in Laemmli buffer (50 mM Tris containing 2% SDS, 10% glycerol, and 0.1% bromophenol blue, pH 6.8) in the presence or absence of 1% β-mercaptoethanol, heated at 95°C for 5 min, electrophoresed through 7.5% SDS-polyacrylamide gel. Proteins were then transferred electrophoretically to Immobilon polyvinylidene difluoride membrane (Millipore) with a semi-dry blotting apparatus (Atto) using Bjerrum and Schafer-Nielsen buffer (48 mM Tris, 39 mM glycine, 20% methanol, 0.1% SDS) for 75 min at 120 mA. 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 7.6) (T-TBS) for 1 h at room temperature. Membrane was washed 3 times for 10 min each with T-TBS and subsequently treated for 1 h at 25°C with the primary antibody (anti-Ig-Hepta diluted 1:1000) 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 an alkaline phosphatase-conjugated mouse anti-rabbit IgG antibody (Takara), 50 milliunits of neuraminidase (sialidase A) (EC 3.2.1.18, Roche Molecular Biochemicals), and 2 milliunits of O-glycosidase (EC 3.2.1.97, Roche Molecular Biochemicals) were added, and the reaction mixture was incubated at 37°C for 18 h. A control incubation was carried out in which 50 mM sodium phosphate buffer was added in place of the enzyme(s). The proteins were separated on SDS-PAGE and transferred to a polyvinylidene difluoride membrane and were detected using the enzyme(s). Pieces of rat lungs and kidneys were fixed in 4% paraformaldehyde in 0.1 M 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 glycopeptidase F (EC 3.5.1.52, Takara), 50 milliunits of neuraminidase (sialidase A) (EC 3.2.1.18, Roche Molecular Biochemicals), and 2 milliunits of O-glycosidase (EC 3.2.1.97, Roche Molecular Biochemicals) were added, and the reaction mixture was incubated at 37°C for 18 h. A control incubation was carried out in which 50 mM sodium phosphate buffer was added in place of the enzyme(s). The proteins were separated on SDS-PAGE and transferred to a polyvinylidene difluoride membrane and were detected by immunoblotting as described above.

**Immunohistochemistry**—Pieces of rat lungs and kidneys were fixed in methyl-Carney fixative overnight and embedded in paraffin. Sections of the paraffin-embedded tissues were dewaxed in xylene, hydrated through graded ethanol, and incubated with the antibody, normal rabbit serum, or anti-rat H-ATPase antibody (18) (1:1000 dilution) overnight. After washing in PBS, the sections were incubated with peroxidase- and goat anti-rabbit IgG-conjugated dextran polymer (EnVision, Dako Japan, Kyoto, Japan) for 1 h and colored with diaminobenzidine and hydrogen peroxide.

**RESULTS**

**Isolation and Sequence Analysis of a cDNA Encoding Rat Ig-Hepta**—The clone to be described was obtained as an unex-pected by-product of the following attempt to isolate a receptor selective for PTHrP. The presence of such a PTHrP receptor has been suggested in the rat supraoptic nucleus of the hypothalamus (19, 20). We therefore isolated poly(A)+ RNA from the rat supraoptic nucleus, synthesized cDNA, and amplified PTH receptor-related sequences by PCR. Analysis of the PCR products generated by a combination of primers revealed a total of 183 clones possessing features of the class II GPCR family: 182 clones identical to the known sequences and a single clone with a novel sequence. The known species included the receptors for PTH/PTHrP (66 clones), PTH (56 clones), pituitary adenylate cyclase-activating peptide (26 clones), calcitonin (22 clones), VIP (3 clones), calcitonin gene-related peptide (3 clones), glucagon (3 clones), glucagon-like peptide 1 (2 clones), secretin (1 clone). We therefore decided to isolate full-length cDNA corresponding to the novel clone. A preliminary search, using the PCR product as a probe, for the tissues that contain relatively large amounts of the message indicated that the rat lung is such a tissue and is suitable for use as a source of cDNA library construction.

A full-length cDNA clone was obtained by screening 3 × 10⁵ plagues of a rat lung cDNA library with the above novel PCR product as a probe under stringent conditions. The nucleotide sequence of the clone (5 kb) has been deposited in the DDBJ/EMBL/GenBank™ data base (accession number AB019120), and the amino acid sequence deduced from its open reading frame is shown in Fig. 1A together with the structural features (Fig. 1, B and C). The novel sequence codes for a protein of 1389 amino acid residues. Hydropathy and motif analyses indicated that the protein is a member of the class II GPCR family and is composed of two major domains as follows: a large N-terminal domain containing two immunoglobulin-like repeats and a sev-en-transmembrane domain. We therefore named the protein Ig-Hepta. At the N terminus, there is a typical signal peptide sequence, indicating that the large N-terminal region is located extracellularly. In addition to the two immunoglobulin-like repeats, the exodomain contains a Cys-rich domain of ~50 amino acid residues (residues 107–160) and multiple potential N-linked and O-linked glycosylation sites (Fig. 1C). The heptahelical domain in the C-terminal region is distantly related to the secretin family members including the PTH receptor (Figs. 2 and 3). Two cysteine residues that are located in exolopes 1 and 2 and are known to be highly conserved among GPCRs are also present in Ig-Hepta: Cys-1129 in exoloop 1 between transmembrane segments 2 and 3 and Cys-1205 in exoloop 2 connecting transmembrane segments 4 and 5. These conserved cysteine residues are expected to be disulfide-linked as has been generally considered for other members of the GPCR family based on the structural analyses of rhodopsin (21) and the receptors for thyrotropin-releasing hormone (22), thromboxane (23), and gonadotropin-releasing hormone (24). There are two consensus sequences for phosphorylation by protein kinase C as follows: TQK (residues 1169–1171) in cytoloop 2 and TQ (residues 1176–1178) in cytoloop 4. Another structural feature is the presence of a relatively long stretch of Ser/Thr-rich sequence at the C terminus; more than 40% of the C-terminal 66 residues are Ser or Thr (Fig. 1, A and C).

**Predominant Expression of Rat Ig-Hepta mRNA in Lung and Its Developmental Changes**—Northern analysis of 10 rat tissues revealed a very restricted expression pattern of Ig-Hepta (Fig. 4A). Ig-Hepta mRNA was highly expressed, as a 7.8-kb transcript, in the lung and to a much lesser extent in the kidney and heart. The expression in the lung was so abundant as to be easily detectable using total RNA preparations. During the postnatal development of rat, expression of Ig-Hepta...
Cys-rich domain contains a variation of an EGF-like motif. Potential transmembrane spans, and a Ser/Thr-rich domain of amino acid residues, two immunoglobulin-like domains (C2), seven shown. From N to C terminus, there are a Cys-rich domain of variant, identified by cDNA cloning and partial gene analysis, is also isolated over a window of 19 residues by the method of Kyte and Doolittle data bases using the accession number AB019120.

Fig. 1. Primary structure and hydrophy profile of rat Ig-Hepta deduced from its cDNA sequence. A, the deduced amino acid sequence of Ig-Hepta. The putative N-terminal signal sequence and transmembrane domains are shown in reversed contrast. The amino acid residues comprising the potential N-linked glycosylation sites are boxed. The residues of the C2 type immunoglobulin-like domains are underlined. The alternately spliced sequence (residues 469–508) is in italic type and confirmed by PCR amplification. The entire nucleotide sequence of the Ig-Hepta cDNA is available from the DDBJ/EMBL/GenBank TM nucleotide sequence data bases using the accession number AB019120. B, hydrophy index profile of Ig-Hepta. The residue-specific hydrophy index was calculated over a window of 19 residues by the method of Kyte and Doolittle (51) using the Genetyx-MAC computer program, version 9.0. C, schematic representation of the domain structures of rat Ig-Hepta. A splice variant, identified by cDNA cloning and partial gene analysis, is also shown. From N to C terminus, there are a Cys-rich domain of ~50 amino acid residues, two immunoglobulin-like domains (C2), seven transmembrane spans, and a Ser/Thr-rich domain of ~60 residues. The Cys-rich domain contains a variation of an EGF-like motif. Potential N-linked glycosylation sites are shown as bars.

mRNA in the lung increased markedly (Fig. 4B). Control hybridization of the same blot, used for the above determination of the developmental expression of Ig-Hepta mRNA, with a glyceraldehyde-3-phosphate dehydrogenase probe revealed no significant difference in the signal intensity (Fig. 4B). A weak band at 4.8 kb in Fig. 4A is likely to represent a homologous mRNA species since its relative intensity increased greatly under conditions giving recognition of Ig-Hepta mRNA (Fig. 4B), and total Southern blot analysis of the rat genomic DNA indicated the presence of a closely related gene (data not shown).

Disulfide-linked Dimeric Structure of Ig-Hepta—For characterization of biochemical properties of Ig-Hepta, we raised an antiserum and used it as an analytical reagent for detecting the Ig-Hepta molecule. The antiserum stained a single band of ~160 kDa on Western blots of detergent extracts of rat lung membrane preparations, demonstrating its specificity (Fig. 5B, lane 1). Under nonreducing conditions, however, significant amounts of Ig-Hepta migrated as an ~260-kDa species on SDS-PAGE (Fig. 5C, lane 1). Similarly, recombinant Ig-Hepta expressed in COS-7 cells behaved as an ~130- and ~260-kDa species under reducing and nonreducing conditions, respectively (Fig. 5, B and C, lane 2). These results suggest that considerable amounts of Ig-Hepta exist as a dimer linked by disulfide bond(s).

We further determined whether the large exodomain of Ig-Hepta has by itself the ability to form the disulfide-linked dimer or not. The exodomain (Fig. 5A, Ig-Hepta ECD) was expressed as a His-tagged soluble protein in COS-7 cells, purified from culture medium by metal chelate chromatography on a Ni2+ column, and analyzed by SDS-PAGE. The exodomain existed as an ~125-kDa species even under nonreducing conditions (Fig. 5C, lane 6). If we assume that the truncated exodomain has the tertiary structure identical to that of the exodomain of the wild type receptor, this result indicates that the exodomain lacks the ability to dimerize by forming intermolecular disulfide bond(s). However, it is not clear whether the disulfide-linked dimer is formed through the cysteine residues in the 7-transmembrane domain or whether the transmembrane domain is necessary for noncovalent association of the nascent monomeric Ig-Hepta, which is then covalently linked at the site of contact in the exodomain. To clarify this point, we designed a construct that encodes a C-terminally truncated single-transmembrane version of Ig-Hepta (Ig-Hepta TM1, Fig. 5A), expressed it in COS-7 cells, and determined its size. The truncated form migrated as a dimer (Fig. 5C, lane 5), supporting the possibility that although the dimer form is formed through intermolecular disulfide bond(s) in the exodomain, the transmembrane region is necessary for the molecular association that is a prerequisite for the covalent dimer formation. The above interpretation is based on the assumption that the truncated exodomain (Ig-Hepta ECD) adopts a conformation similar to the exodomain of the wild type receptor. If this is not the case and the truncated exodomain has a structure different from the exodomain of the wild type receptor, which could prevent dimer formation, the above results may indicate that the transmembrane region is required for a proper conformation of the exodomain and for such the presence of the first transmembrane span is sufficient.

Highly Glycosylated Nature of Ig-Hepta—As mentioned above, sequence analysis of Ig-Hepta revealed numerous potential glycosylation sites in the exodomain (Fig. 1) as follows: 20 N-glycosylation sites in the exodomain, one N-glycosylation site in exolop 1, and several potential O-glycosylation sites in the juxtamembrane region (rich in Ser and Thr) of the exodomain. Furthermore, Ig-Hepta migrated as a diffuse band on SDS-PAGE (Fig. 6), a characteristic feature of highly glycosylated proteins. Therefore, to determine roughly the degree of glycosylation, we digested, with various glycosidases, membrane extracts from the rat lung or COS-7 cells transfected with the expression plasmid pcDNA3/Ig-Hepta prepared under “Experimental Procedures.” Treatment of the lung Ig-Hepta preparation with sialidase A caused a slight reduction in the apparent molecular mass from ~160 to ~110 kDa (Fig. 6, lane 2). Ig-Hepta was also sensitive to glycopeptidase F which cleaves N-linked oligosaccharides; glycopeptidase F treatment led to a dramatic shift in the electrophoretic mobility of Ig-Hepta that corresponds to a reduction in the apparent molecular mass from ~160 to ~110 kDa (Fig. 6, lane 3). We next examined the O-glycosylation status by digesting the glycopeptidase F-treated Ig-Hepta with sialidase A and O-glycosidase. No significant difference in the size was observed when
glycopeptidase F-treated Ig-Hepta was further digested with sialidase A (Fig. 6, lane 4), suggesting that the sialyl residues are carried on the N-linked oligosaccharides. O-Glycosidase treatment did not affect the electrophoretic migration pattern of Ig-Hepta (Fig. 6, lane 5). Essentially identical results were obtained with the recombinant Ig-Hepta produced in COS-7 cells. These results suggest that Ig-Hepta is highly glycosylated primarily at Asn.

Immunohistochemical Localization of Ig-Hepta in Rat Lung and Kidney—The localization of Ig-Hepta in the rat lung and kidney were examined by immunohistochemistry using the antiserum characterized above. In the lung, alveolar walls were apparently stained with the antibody (Fig. 7A), although no staining was observed in the alveolar walls when normal rabbit serum, absorbed antiserum, or unrelated anti-rat H1-ATPase antibody was used instead of the immune serum (Fig. 7B). Alveolar capillaries or alveolar basement membranes were often intensely stained in the alveolar walls. In the kidney, the antibody staining was exclusively observed in parts of tubular epithelia in the cortex (Fig. 7, C and E). The staining was comparable to that of anti-H1-ATPase antibody which defines the intercalated cells in cortical collecting tubules (Fig. 7D). These cells were not stained with normal rabbit serum or absorbed antiserum (Fig. 7F).

DISCUSSION

In this study, we have isolated and characterized a cDNA clone encoding a new member, termed Ig-Hepta, of the class II GPCR family. Ig-Hepta has a chimeric structure consisting of two major parts as follows: a very large and highly glycosylated exodomain and a hepta-helical membrane-spanning region that is distinct from but most closely related to the members of the secretin receptor family. The exodomain is unique in containing two immunoglobulin-like repeats, a feature reminiscent...
cent of single-span, integral membrane proteins, known as the immunoglobulin superfamily, including growth factor receptors (25) and cell adhesion molecules (26). The unusual structure of Ig-Hepta is therefore expected to have evolved from a remote common ancestor gene of the class II GPCR family as a consequence of exon shuffling leading to the acquisition of N-terminal immunoglobulin-like repeats. As suggested by these structural features and confirmed by the phylogenetic analysis (Fig. 3), Ig-Hepta represents a new subfamily of class II GPCRs. In the future, additional members of this subfamily will be identified, which should contribute to clarifying the evolutionary relationship among the members and elucidating the physiological functions of the subfamily. We hope that the discovery of Ig-Hepta may open a new avenue of research where two major fields concerning the immunoglobulin and GPCR superfamilies meet and merge into one. As briefly mentioned in the Introduction and shown in Figs. 2 and 3, it is noteworthy that several similar categories of proteins have recently been identified that have a long characteristic N-terminal domain and a class II hepta-helical transmembrane domain, which include the following: 1) the leukocyte cell-surface molecules EMR1, CD97, and F4/80 containing EGF-like repeats (9–11); 2) the epididymis-specific protein human epididymal gene product 6 with a mucin-like domain (27); 3) the Celsr1 gene product containing cadherin repeats, EGF-like repeats, and laminin A G-type repeats (14, 15); 4) the brain-
specific angiogenesis inhibitors BA11, BA12, and BA13 containing thrombospondin type 1 repeats (28, 29); and 5) the calcium-independent receptor of α-latrotoxin with lectin-like, olfactomedin-like, and mucin-like domains (30, 31).

Recently, a growing number of GPCRs have been shown to exist as an oligomer, and such an oligomeric structure is essential for their agonist-induced activation (for review see Ref. 32). For example, dimers or tetramers or both of the following receptor proteins have been demonstrated: β2-adrenergic receptor (33), muscarinic M2 receptor (34), dopamine receptors (35–37), histamine H2 receptor (38), vasopressin V2 receptor (39, 40), δ-opioid receptor (41), angiotensin AT1 receptor (42), serotonin 5 m-HT1b receptor (43), metabotropic glutamate receptor 5 (44), substance P receptor (45), C5α anaphylatoxin receptor (46), calcium/polyvalent cation-sensing receptor (47), and GABAB receptor (48–50). Among these, the metabotropic glutamate receptors and the calcium/polyvalent cation-sensing receptor have been shown to be disulfide-linked dimers and are attaining a great deal of interest. In this context, Ig-Hepta, which has been shown in the present study to be a disulfide-linked dimer, will be useful for clarifying the functional consequences of GPCR oligomerization.

Although the physiological functions of Ig-Hepta remain unknown, its highly restricted expression may deserve comment in relation to its functions. In addition to the principal role in the exchange of O2 and CO2 in the lung, the alveolar walls of the lung play an important role in the maintenance of acid-base balance in the body by expiration of CO2. In the kidney, two types of intercalated cells have been identified in the collecting tubules, which secrete acid. One type (A cell) secretes H+ into the urine by H+-ATPase present on the apical membrane, and the other (B cell) secretes H+ into the peritubular capillary by H+-ATPase present on the basolateral membrane or secretes HCO3- into the urine by HCO3-/Cl− exchanger in the apical membrane. These cells have also been shown to play a crucial role in regulation of acid-base balance in the body. The localization of Ig-Hepta in the alveolar walls and in the intercalated cells of the collecting duct (Fig. 7) may therefore indicate that Ig-Hepta is a molecule that is involved in the regulation of acid-base balance at least in the lung and kidney. Future studies aimed at defining the natural ligand and physiological function of Ig-Hepta will answer the most relevant question: What novel functional properties have been acquired by the fusion of the immunoglobulin superfamily domain and heptahelical domain?

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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

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