Identification of Natural Ligands for the Orphan G Protein-coupled Receptors GPR7 and GPR8*

Stéphane Brezillon§§, Vincent Lannoy§§, Jean-Denis Franssen§§, Emmanuel Le Poul‡, Vincent Dupriez‡‡, Jean Lucchetti‡‡, Michel Detheux‡‡, and Marc Parmentier***

From ‡‡Euroscreen, 802 Route de Lennik, 1070 Brussels, Belgium, © Eurogentec, Parc Scientifique du Sart Tilman, 4102 Seraing, Belgium, ¶ Institute of Interdisciplinary Research, School of Medicine, Université Libre de Bruxelles, 808 Route de Lennik, 1070 Brussels, Belgium

GPR7 and GPR8 are two structurally related orphan G protein-coupled receptors, presenting high similarities with opioid and somatostatin receptors. Two peptides, L8 and L8C, derived from a larger precursor, were recently described as natural ligands for GPR8 (Mori, M., Shimomura, Y., Harada, M., Kurihara, M., Kitada, C., Asami, T., Matsumoto, Y., Adachi, Y., Watanabe, T., Sugo, T., and Abe, M. (December, 27, 2001) World Patent Cooperation Treaty, Patent Application WO 01/88494A1). L8 is a 23-amino acid peptide, whereas L8C is the same peptide with a C terminus extension of 7 amino acids, running through a dibasic motif of proteolytic processing. Using as a query the amino acid sequence of the L8 peptide, we have identified in DNA databases a human gene predicted to encode related peptides and its mouse ortholog. By analogy with L8 and L8C, two peptides, named L7 and L7C could result from the processing of a 123-amino acid human precursor through the alternative usage of a dibasic amino acid motif. The activity of these four peptides was investigated on GPR7 and GPR8. In binding assays, L7, L7C, L8, and L8C were found to bind with low nanomolar affinities to the GPR7 and GPR8 receptors expressed in Chinese hamster ovary (CHO)-K1 cells. They inhibited forskolin-stimulated cAMP accumulation through a pertussis toxin-sensitive mechanism. The tissue distribution of prepro-L7 (ppL7) and prepro-L8 (ppL8) was investigated by reverse transcription-PCR. Abundant ppL7 transcripts were found throughout the brain as well as in spinal cord, spleen, testis, and placenta; ppL8 transcripts displayed a more restricted distribution in brain, with high levels in substantia nigra, but were more abundant in peripheral tissues. The ppL7 and ppL8 genes therefore encode the precursors of a class of peptide ligands, active on two receptor subtypes, GPR7 and GPR8. The distinct tissue distribution of the receptor and peptide precursors suggest that each ligand and receptor has partially overlapping but also specific roles in this signaling system.

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§ These three authors contributed equally to this work.

** To whom correspondence should be addressed: Institute of Interdisciplinary Research, Campus Erasme, Bldg. C, 5th floor, 808 Route de Lennik, 1070 Brussels, Belgium. Tel.: 32-2-555-41-71; Fax: 32-2-555-46-55; E-mail: mparment@ulb.ac.be.

G protein-coupled receptors (GPCRs) constitute one of the largest gene families yet identified (2). Over the last decade, a growing number of GPCRs have been made available by various cloning procedures, among which PCR amplification using degenerate oligonucleotides, and more recently the systematic sequencing of cDNA libraries and genomes, have played prominent roles. In addition to about 160 characterized receptors, about 125 human genes encode proteins obviously belonging to this family of receptors, but their ligands and functions remain to be determined. These so far uncharacterized receptors are referred to as orphan GPCRs, but they are expected to play, by analogy with characterized members of the family, important roles in the regulation of physiological processes. From a structural viewpoint, orphan receptors are widely distributed throughout the GPCR superfamily, suggesting that they respond to a diverse range of ligands. Their similarity with well known receptors sometimes allows the construction of hypotheses regarding the chemical nature of their ligand (e.g. peptide, lipid derivative). The identification of their natural ligands can provide insight into new regulatory mechanisms and represent interesting opportunities for drug discovery (3).

Many of the presently characterized receptors have originally been cloned as orphan receptors before the identification of their ligand and the delineation of their function in vivo. As an example, the 5HT1A serotonin receptor was first cloned by cross-hybridization with a β2-adrenergic receptor probe (4), and its functional response to serotonin was identified afterward (5, 6). Orphan receptors led in a number of cases to the discovery of new molecules that were not recognized beforehand as extracellular mediators. Nociceptin was the first of these novel ligands to be described. It was purified from brain extracts with the help of functional assays constructed around the orphan receptor ORL1 (7, 8). This process has been referred to as reverse pharmacology. Other recent examples of new molecules identified by reverse pharmacology include orexins (9), prolatin-releasing peptide (10), MCH (11, 12), apelin (13), relaxin (14), ghrelin (15), kisspeptin (16, 17), and prokineticins (18). In addition to this approach based on the purification of an activity from a biological sample, some peptide ligands of orphan receptors have also been identified using bioinformatics and searches in human genomic databases (19).

GPR7 and GPR8 are two human orphan GPCRs that were originally cloned from genomic DNA by low stringency polymerase chain reaction (20). Their genes are both intronless, and
their amino acid sequences share 62% identity with each other (Fig. 1), suggesting that they could share common ligands. They display significant similarity to the opioid and somatostatin receptors (36–40% identity), but the ligands of these receptors do not activate cell lines expressing GPR7 or GPR8, despite the original description that bremazocin, a high affinity synthetic opioid ligand, could interact with GPR7 in a binding assay (20). GPR7 was mapped to the 10q11.2-q21.1 region of the human genome, and GPR8 to 20q13.3.

GPR7 transcripts were found by Northern blotting in brain regions including cerebellum and frontal cortex and with lower abundance in hypothalamus and pituitary, where two different transcripts are present. In situ hybridization revealed expression of GPR7 in the human anterior pituitary, and, using a partial mouse GPR7 sequence, in mouse brain regions, namely suprachiasmatic, arcuate, and ventromedial nuclei of hypothalamus, the dentate gyrus and ventral tegmental area (20). In situ hybridization studies were later performed on rat brain, and GPR7 transcripts were found in amygdala, hippocampus, hypothalamus, and cortex regions, suggesting a more widespread distribution of GPR7 in rat brain than in mouse brain (21). GPR8 was shown to have a different expression profile, as illustrated by Northern blot analysis of various regions of human brain, in which GPR8 transcripts were essentially restricted to the frontal cortex (20). Interestingly, GPR8 was not found so far in rodents but was cloned in several other species, including rabbit, shrew, and lemur, indicating that the GPR8 gene might have been lost in some mammalian branches (21).

Ligands of GPR8 have been recently identified by reverse pharmacology, using extracts from pig hypothalamus and a GTP-γ-S-based functional assay, as reported (1). Two peptides of 23 (L8) and 30 (L8c) amino acids were described, differing in their C-terminal extension (Fig. 2C). Injection of this ligand into rats was reported to induce an increase in prolactin release (1).

In the present study, we have identified, using bioinformatics, new peptides predicted to derive from a secreted protein precursor and structurally related to the L8 ligands. These peptides were shown to be high affinity agonists for GPR7 but also for GPR8. The pharmacology of these peptides was characterized, using binding and functional assays and recombinant CHO-K1 cell lines expressing GPR7 or GPR8. In addition, the expression profile of the genes expressing the two receptors and the two peptide precursors, prepro-L7 and prepro-L8, was established by RT-PCR for the first time in human tissues.

EXPERIMENTAL PROCEDURES

**Materials**—Culture media, antibiotics, fetal bovine serum, and trypsin were from BioWhittaker (Pertet Bechian, Belgium). Restriction and DNA-modifying enzymes were from Roche Diagnostics. Forskolin and IBMX were from Calbiochem. Pertussis toxin was from Sigma.

**Cloning of GPR7 and GPR8**—Oligonucleotide primers were synthesized on the basis of human GPR7 and GPR8 cDNA sequences (accession numbers U22401 and U24202, respectively) (20). For GPR7 cloning, sense primer 5'-CGGATTCACCATGGACAACGCCTCGTTCT CG-3' and antisense primer 5'-CTAGTCATTAGTCGCTGCGGGCGGGCGC-3' were used in a PCR experiment using human genomic DNA as template and Pfu DNA Polymerase (Stratagene) under the following conditions: 94 °C, 5 s; 50 °C, 1 min; 72 °C, 1 min, 35 cycles. A fragment of 0.98 kilobases containing the entire coding sequence of the GPR7 gene was amplified, digested by BamHI and XbaI, and cloned in the pEFIN3 bicistronic expression vector (22). For GPR8 cloning, sense primer 5'-ATCGGATTCACCATGGACAACGCCTCGTTCTCG-3' and antisense primer 5'-ATGGATTCGGCGCCCGGGCGCGCGCG-3' were used in a PCR experiment using human genomic DNA as template and Taq DNA polymerase (Eurogentec, Liège, Belgium) under the following conditions: 94 °C, 15 s; 56 °C, 30 s; 72 °C, 1 min, 25 cycles. The amplified 1-kb fragment was digested by EcoRI and Spel and cloned in the pEFIN3. The inserts of the resulting plasmids were sequenced on both strands, using the BigDye Terminator cycle sequencing kit (Applied Biosystems, Warrington, UK). Sequence alignments were performed using ClustalX version 1.8 software (23). Putative transmembrane domains were predicted by TMpred (available on the World Wide Web at www.ch.embnet.org/software/TMPRED_form.html).

**Cloning and Sequencing of Prepro-L7 (ppL7) cDNA**—Based on the sequence of the unique EST sequence containing a poly(A) tail (GenBank™ accession number BM978256), specific oligonucleotide primers were synthesized for L7 cDNA cloning (sense primer, 5'-gtaGAATTCCTCAAGATGCGATGAGCGGCG-3'; antisense primer, 5'-ctCTCAGAtgctggcgc-3'). Marathon-ready cDNAs from human adult and fetal brain (Clontech, Palo Alto, CA) were used as a template for PCR amplification using Goldstar DNA Polymerase (Eurogentec) under the following conditions: 94 °C for 4 min; three cycles of 94 °C for 1 min, 58 °C for 1 min,
A nucleotide sequence of the 0.5-kb PCR product cloned from brain and deduced amino acid sequence of the human L7 precursor gene. The dibasic motives are double underlined. The sequences of L7 (which stops at the first dibasic motif) and L7C (which stops at the second dibasic motives) are underlined. The dotted line indicates the in frame putative intronic sequence, which is absent from the 0.4-kb PCR product. The intronic sequence has been translated in a putative intronic sequence, which is absent from the 0.4-kb PCR product. The dibasic motif) are shaded in light gray and residues identical only in L7 precursors are shaded in black; residues identical only in L7 precursors are shaded in dark gray, and residues identical in L8 precursors are shaded in light gray. The arrow indicates the first residue after cleavage of the predicted signal peptide. The sharp indicate the localization of the two dibasic motives (only one for the mouse ppL7) that may serve as cleavage sites for prohormone convertase. The human L7 precursor (h-ppL7) sequence was deduced from the sequence of the cDNA we cloned by PCR. The mouse L7 precursor (m-ppL7) sequence was deduced from the sequence of an EST (BAA54998). The human ppL7 sequence (m-ppL7), rat (r-ppL7), and pig (p-ppL7) precursor sequences are from a published patent application (1). C, amino acid sequence of the human bioactive peptides used in this study.

**FIG. 2. Structure of L7 and L8 peptides, precursors, and genes.**

72 °C for 45 s; 30 cycles of 94 °C for 1 min, 63 °C for 1 min, 72 °C for 45 s; and 72 °C for 3 min. The amplified fragments were cloned in a PCR-Blunt TOPO vector (Invitrogen) and were sequenced on both strands using the BigDye Terminator cycle sequencing kit (Applied Biosystems).

**Identification and Synthesis of Putative GPR7 Ligands**—Using as a query the amino acid sequence of the L8 peptide, one of the GPR8 ligands described in the Patent Cooperation Treaty (1), we used TBLASTN and fasta software to search DNA databases. EST and genomic sequences presenting significant matches were collected, and proteins and peptides derived from these sequences were predicted. The peptides were synthesized using Fmoc (N-(9-fluorenylethoxycarbonyl)) synthesis protocols with double or triple coupling reactions using O-benzotriazol-1-yl-N,N,N′,N′-tetramethyl-uronium tetrafluoroborate (TBTU) as the activator on a Symphony (Rainin Instrument Co., Woburn, MA) synthesizer. Purifications were performed by reverse phase-HPLC on a Waters (Milford, MA) Delta-Pak C18 (15 μm, 100A, 25 × 100 mm) column using a Waters liquid chromatography system consisting of a model 600 solvent delivery pump, a Rheodean injector, and an automated gradient controller (solvent A, H2O plus 0.125% trifluoroacetic acid; solvent B, CH3CN plus 0.1% trifluoroacetic acid; gradient 15% B to 60% B in 20 min). Detection was carried out using a model M2487 variable wavelength UV detector connected to the Waters Millennium software control unit. The quality control was performed by analytical reverse phase-HPLC on a Waters Delta-Pak C18 (5 μm, 100A, 150 × 3.9 mm) column (solvent A, H2O plus 0.125% trifluoroacetic acid; solvent B, CH3CN plus 0.1% trifluoroacetic acid; gradient 100% A to 60% B in 20 min) using a Waters Alliance 2690 separation module equipped with a Waters 996 Photodiode Array Detector and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry using a PerSeptive Biosystems (Framingham, MA) Voyager-DE instrument.

**Cell Culture and Transfection**—The recombinant pEFIN3-GPR7 and pEFIN3-GPR8 plasmids and the empty pEFIN3 vector were transfected in CHO-K1 cells (CRL-9618; ATCC, Manassas, VA) or WTA11 cells (a CHO-K1 cell line coexpressing mitochondrial apoaerugin and Gαi1q), using Fugene 6 (Roche Diagnostics). The transfected cells were selected with 400 μg/ml G418 in nutritious Ham’s F-12 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 10 μg/ml streptomycin, from 2 days after transfection. The medium of WTA11 cells contained in addition 250 μg/ml zeocin. Only expressing high levels of GPR7 were selected from the mixture of transfected cells by using an anti-GPR7 antisera (see below) and magnetic beads, as recommended by the supplier (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, 2 million cells were reacted with 5 μl of anti-GPR7 serum, and GPR7-positive cells were recovered using goat anti-mouse IgG microbeads (Miltenyi Biotec) on a miniMACS column. Sorted cells were grown, and clonal cell lines were obtained by limiting dilution. Clones identified by fluorescence-activated cell sorting as expressing GPR7 at the cell surface, using the anti-GPR7 antisera, were further analyzed by Northern blotting and RT-PCR.

**Production of Antibodies Directed against GPR7—BALB/C mice were injected three times with 100 μg of the pEFIN3-GPR7 plasmid, as previously described (25). Sera were tested by fluorescence-activated cell sorting using GFP-transfected WTA11 cells and fluorescein isothiocyanate-labeled goat anti-mouse IgG antibodies (Sigma).**

**Phosphoinositide Accumulation Assays—Functional responses were assessed by recording the luminescence of aequorin in GPR7- and GPR8-expressing cells following the addition of (potential) agonists, as previously described (16). In brief, cells were collected from plates with PBS containing 5 mM EDTA, pelleted, resuspended at 5 × 10⁵ cells/ml in DMEM/F-12 medium containing 0.1% bovine serum albumin, incubated with 5 μM currentsensitive H (Molecular Probes, Inc., Eugene, OR) from room temperature, and diluted in DMEM/F-12 medium at a concentration of 5 × 10⁵ cells/ml. Cells where then mixed with the ligands, and the light emission was recorded over 30 s with a Microslam luminometer (PerkinElmer Life Sciences).**

**Phosphoinositide Accumulation Assays—COS-7 cells expressing GPR7 or GPR8 were labeled for 12 h with 3 Ci/ml [3H]inositol in isofomel-free DMEM containing 5% fetal bovine serum. Cells were washed two times with Krebs-Ringer Heps buffer (10 mM Heps, pH 7.4, 146 mM NaCl, 4.2 mM KCl, 0.5 mM MgCl₂, 1 mM CaCl₂, 55 mM glucose) prior to the incubation with agonists at 37 °C for 30 min in
Krebs-Ringer Hepes buffer containing 9.4 mM LiCl. The incubation was stopped by replacing the incubation medium with 1 ml of an ice-cold 5% perchloric acid solution. The medium was further neutralized with a 75 mM Hepes, 1.5 mM KOH solution. The total inositol phosphate (IP) content was then extracted and purified on Dowex columns as described (26). Total radioactivity remaining in the membrane fraction was counted after solubilization in 10% Triton, 0.1 M NaOH and used as a standard for each well. Results were expressed as the radioactivity associated to IP over the total radioactivity present in membranes.

**Cyclic AMP Assays**—CHO-K1 cell lines stably expressing GPR7 or GPR8 were cultured in Petri dishes at 37 °C in Ham’s F-12 medium containing or not 100 ng/ml pertussis toxin. Cells were recovered in PBS containing 5 mM EDTA, resuspended in Krebs-Ringer Hepes/IBMX buffer (1.25 mM KH2PO4, pH 7.4, 5 mM KCl, 124 mM NaCl, 1.25 mM MgSO4, 1.45 mM CaCl2, 25 mM Hepes, 0.5 mM/liter phenol red, 1 mM IBMX, and 13.3 mM glucose) and dispatched into 96-well plates at a density of 2.5 × 10^4 cells/well. Cells were further preincubated for 15 min in 1 ml Krebs-Ringer/IBMX buffer and incubated with various concentrations of agonists for GPR8. Briefly, 1–10 μg of membrane proteins were incubated in binding buffer (50 mM HEPES, pH 7.4, 5 mM MgCl2, 1 mM CaCl2, 0.5% protease-free bovine serum albumin) containing 0.1 mM [3H]-L7 or [3H]-L8 radioligand for 90 min at 27 °C. Bound tracer was separated by filtration through GF/B filters (Millipore Corp.) presoaked in 0.5% polyethyleneimine. Filters were then counted by scintillation counting.

Results were normalized for total binding in the absence of competitor (100%) and nonspecific binding (0%) in the presence of a 100-fold excess of unlabeled ligand and were analyzed by nonlinear regression, using a single site competition model (Graph-Pad Prism software).

**Tissue Distribution of Human GPR7 and GPR8 Receptors and ppL7 and Prepro-L8 (ppL8) Precursors**—Reverse transcription-polymerase chain reaction (RT-PCR) experiments were carried out using a panel of polyA+ RNA (Clontech and Ambion, Austin, TX). The GPR7 primers were 5′-CTTGGAGACTGGAACAGG-3′ (forward) and 5′-GGACA-CAGATGGTGACACG-3′ (reverse), with an expected product size of 746 bp. The GPR8 primers were 5′-GCCACTGCCGTTCCTCTAT-3′ (forward) and 5′-GGCCAT-CTCCACTGCGCGCCCAAAC-3′ (reverse), with an expected product size of 1251 bp. The PCR products amplified subsequently. It might therefore represent a minor allele in the population or result from a sequencing artifact and was therefore not considered further. The coding sequence of the ppL7 gene is interrupted by an intron, as shown in Fig. 2A. Two of the EST sequences (A1394469 and AW058203) contained this intron, suggesting inefficient splicing or contamination of the EST databases by genomic clones. Amplifications from adult brain resulted in two fragments of 0.4 and 0.5 kb, and amplification from fetal brain resulted in a single 0.4-kb fragment. The sequence of the 0.5-kb fragment corresponded to the genomic sequence, whereas 99 nucleotides were deleted in the middle of the 0.4-kb fragment sequence (Fig. 2A). The nucleotide sequence at the borders of the deleted fragment perfectly matches the splicing donor and acceptor sites, as described by Mount (27). We thus concluded that these 99 nucleotides correspond to a putative intron and that the 0.5-kb PCR fragment originated from nonsnapped RNA, whereas the 0.4-kb fragment originated from the spliced mRNA. Both fragments contained the entire coding sequence for ppL7. Since the intronic sequence is in-frame and contains no stop codons, it is possible that alternative splicing would result in a functional unsnapped transcript, encoding 31 additional amino acid residues downstream of the coding sequence of L7 (Fig. 2A).

Prepro-L7 and prepro-L8 displayed 23% amino acid identity (Fig. 2B), particularly within the peptides predicted to derive from them by proteolytic processing (66% identity between L7C and L8C; 61% identity between L7 and L8) (Fig. 2C). The peptides or their precursors did not exhibit any obvious structural relationships with other known peptide families, such as opiates or somatostatin. On the basis of the ppL7 precursor gene sequence, the full coding human cDNA sequence was isolated by RT-PCR from a pool of poly(A)+ RNA extracted from different tissues (kidney, fetal liver, and adrenal gland). The isolated cDNA encoded a prepropeptide of 125 amino acids (Fig. 2A), with a predicted signal peptide of 24 amino acids (28). A mouse EST exhibiting high sequence similarity with human ppL7 was found in the databases (AC BB655095) and is believed to encode the mouse ortholog of the human precursor. The overall amino acid identity between the mouse and human sequences is 58%. The prepropeptide contains two dibasic motifs, suggesting that three independent peptides could result from the full processing of the precursor by prohormone convertases. By analogy with the peptides derived from the L8 precursor, we designated as L7 the peptide located between the signal peptide and the first dibasic motif, whereas L7C designates a longer peptide extending through the first dibasic motif and ending before the second (Fig. 2C). The mouse sequence displayed only the second dibasic amino acid pair (Fig. 2B), and as other GPR7 ligands were considered for in DNA databases. The 23-amino acid sequence of the L6 peptide (Fig. 2C), the shortest GPR8 ligand described, was used as a query to screen, using tblastn and fasta software, the human genome and EST databases. Besides ppL8-encoding clones that mapped to 16q13.1, eight human genomic clones (accession numbers AC069004, AJ336655, AJ337187, AJ337377, AJ337373, AJ334434, AJ337491, and AJ337413), all corresponding to a single locus and belonging to the 17q25–26 region of the human genome, and eight overlapping human ESTs (accession numbers BM798236, AI934669, AW058203, AW167793, AW166253, BF841452, and W25219) were identified for their high similarity to the L8 sequence. The reconstruction of the coding sequences and their translation led to a predicted amino acid sequence, ppL7, encoded by these clones. The sequence derived from one of the genomic clones (AC069004) differed only by an alanine deletion and was not retrieved among the PCR products amplified subsequently. It might therefore represent a minor allele in the population or result from a sequencing artifact and was therefore not considered further.
a consequence, only the L7C peptide can be generated from this precursor.

Pharmacology of GPR7 and GPR8 Receptors—The ability of L7, L7C, L8, and L8C to activate GPR7 and GPR8 was tested using an aequorin-based functional assay. In this assay, L7C was the most potent agonist of GPR7 (EC50 = 20.9 ± 2.5 nM) (all values as mean ± S.E.) (Fig. 3A). Inhibition of cAMP accumulation was also observed in CHO-K1-GPR8 cells. L8 was more potent (IC50 = 0.98 ± 0.09 nM) than L8C (IC50 = 9.8 ± 2.0 nM), L7C (IC50 = 12.5 ± 2.3 nM), and L7 (IC50 = 20.9 ± 2.5 nM) (all values as mean ± S.E.) (Fig. 4B). Similar results were obtained with the CHO-WTA11 cells co-expressing Gs16 and GPR7 or GPR8. The effect of L7 and L8 peptides on each receptor was strongly inhibited by pertussis toxin (data not shown). No modification of phosphatidylinositol turnover was observed in COS-7 cells transiently expressing GPR7 or GPR8. However, COS-7 cells co-transfected with GPR7 or GPR8 and a Gs16 chimeric G protein exhibited inositol trisphosphate production in response to L7 or L8. The effect of L7 and L8 peptides on each receptor was significantly enhanced by pertussis toxin (data not shown).

Distribution of GPR7, GPR8, ppL7, and ppL8 Transcripts—Tissue distribution of the ligand and receptor transcripts was determined by RT-PCR in peripheral tissues and central nervous system regions (Fig. 5 and Table I). For the ppL7 precursor transcript, the expected size of the amplified band was 189 bp.
A band of this size was indeed obtained in several tissues, sequenced, and found to correspond to ppL7. Specific ppL7 transcripts were found at high levels in adult and fetal brain, substantia nigra, spinal cord, placenta, and colorectal adenocarcinoma (for extended distribution, see Table I). A second band of 289 bp was also observed following ppL7 amplification. This band was sequenced and found to correspond to an unspliced form of ppL7 transcript, similar to that found in the ESTs AI394669 and AW058203 of the public databases. This unspliced form of ppL7 transcripts was only detected at low levels (data not shown). Transcripts encoding the ppL8 precursors were detected at high levels by RT-PCR in the substantia nigra, lymphoblastic leukemia, fetal kidney, colorectal adenocarcinoma, and trachea (Table I).

Tissue distribution of the receptor transcripts was determined in parallel. Transcripts encoding GPR7 were detected at high levels by RT-PCR in the substantia nigra, lymphoblastic leukemia, fetal kidney, colorectal adenocarcinoma, and trachea (Table I).

FIG. 4. cAMP accumulation in CHO-K1 cells expressing GPR7 or GPR8. Cell lines expressing GPR7 (A) or GPR8 (B) were incubated in the presence of the various concentrations of L7, L7C, L8, and L8C, together with 5 μM forskolin. The displayed curves are representative of at least three independent experiments. The data represent the mean and S.E. of triplicate data points.

FIG. 5. Distribution of GPR7, GPR8, ppL7, and ppL8 transcripts in the human central nervous system. RT-PCR experiments were carried out using a panel of total and poly(A)+ RNA and specific primers for GPR7, GPR8, ppL7, and ppL8 sequences, as described under "Experimental Procedures." The expected sizes of the amplified bands were 746, 898, 189, and 377 bp for GPR7, GPR8, ppL7, and ppL8, respectively. Aliquots (10 μl) of the PCRs were analyzed by 1% agarose gel electrophoresis. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GapDH) (509 bp) transcripts was performed in parallel as a control.

DISCUSSION

GPR7 and GPR8 are two structurally related orphan G protein-coupled receptors sharing amino acid sequence similarity with a number of neuropeptide receptors, particularly with opioid and somatostatin receptor groups. As typically observed for G protein-coupled receptor subfamilies, GPR7 and GPR8 display similarities mostly within the transmembrane and intracellular loops, whereas the N terminus and extracellular loops are somehow more divergent (Fig. 1). The overall identity between the two sequences is 61%, whereas the most closely related receptors (somatostatin receptors) share no more than 41%. This makes of GPR7 and GPR8 a putative subfamily of G protein-coupled receptors that could share the same, or structurally similar, ligands. Partial sequences encoding GPR7 and GPR8 have been cloned from several nonhuman species, demonstrating the high conservation of the primary structure (on average 84% identity between mammalian orthologs across available sequences), although the GPR8 gene is apparently absent from the genome of rodents (21).

Using the sequence of the recently identified GPR8 ligands, L8 and L8C, we have identified, in the genome and EST databases, a gene encoding a protein precursor expected to generate, following proteolytic processing, new bioactive peptides related to L8, called L7 and L7C. The precursor of L7 and L7C peptides was predicted to contain a leader sequence of 24 amino acid sequence. The human precursor could generate both an analog of L8 and of the C-terminally extended form L8C. The mouse precursor lacks one of the dibasic cleavage motives, so that only the longer L7C peptide can be generated. By analogy, it is therefore possible that the actual peptide produced in humans is also the long L7C form, although this will have to be demonstrated following the design of adequate tools, such as antibodies directed at these peptides. The tetrapeptide SPYA, located between the first and second dibasic cleavage sites in the human precursor, is not expected to be generated in mice, and we have not tested so far whether the human peptide (which is inactive on GPR7 and GPR8) might have other biological activities by itself. The similarity between prepro-L7 and prepro-L8 is essentially concentrated within the...
L7C and L8C peptides themselves, suggesting that the C-terminal peptide resulting from proteolytic cleavage is probably devoid of biological activity. The structural similarity is higher in the N-terminal part of the peptides, which probably represents the bioactive domain, as suggested also by the activity of a L7 peptide variant lacking the last three amino acids. Additional structure-function analyses will however be necessary in order to determine more precisely the functional domain of the peptides. L7 and L8 represent a new family of bioactive peptides unrelated to other peptide families known so far.

GPR7 and GPR8 were shown to be coupled to the inhibition of adenylate cyclase in CHO-K1 cells, through pertussis toxin-sensitive G proteins. The presence of the chimeric Cα protein appeared necessary to couple GPR7 and GPR8 to the activation of phospholipase C in COS-7 cells.

Functional expression of GPR7 in CHO-K1 has been particularly difficult to establish, as compared with GPR8 or other G protein-coupled receptors in general. The selection of recombinant clones expressing significant GPR7 levels was indeed troublesome, requiring the use of magnetic cell sorting and the testing of a large number of clones. In addition, many of the established clones turned out to contain deletions in the GPR7 coding sequence, as shown by RT-PCR and sequencing, sometimes allowing the presence of a truncated receptor at the cell surface. Binding assays finally indicated that, on average, the GPR7 expression levels in the validated clones were lower than for GPR8. Altogether, it is therefore likely that CHO-K1 cells expressing high levels of GPR7 have been counterselected during the cloning procedure, although the nature of the selective pressure is presently not known.

Distribution studies of GPR7 in the rat and mouse central nervous systems by in situ hybridization have been reported previously (3, 7, 20, 21). These studies identified moderate to high GPR7 expression in various brain regions, but the distribution pattern appeared more restricted than that of the related opioid, somatostatin, and nociceptin receptors. The presence of GPR7 transcripts in neurons of limbic, hippocampal, and hypothalamic regions suggests a role in a number of central functions, including memory, learning, olfaction, and control of the endocrine system (21). Regulation of GPR7 gene expression has also been reported in human peripheral neuropathies (29). High levels of GPR7 transcripts were indeed found in biopsies of regenerating nerves showing efficient remyelination and perivascular infiltration by inflammatory cells. The regulation of GPR7 during the nerve repair process has led to the suggestion that it might contribute to the neuropolytic changes of sensory neurons that underlie neuropathic pain (29).

From the expression profile described here, it appears that the ppL7 gene is more widely expressed in the central nervous system than the ppL8 gene. Higher ppL7 signals are obtained by RT-PCR in adult and fetal brain, and ppL7 transcripts have been found in all brain regions investigated. In contrast, ppL8 transcripts were not detectable in many regions of the central nervous system, including thalamus, hypothalamus, caudate nucleus, pons, spinal cord, and dorsal root ganglia. In terms of receptors, GPR8 is more widely expressed than GPR7 in the human nervous system. Both GPR7 and GPR8 are highly expressed in hippocampus and amygdala, but additional sites are positive for GPR8. The high expression of ppL7 and ppL8 in substantia nigra and the expression of GPR8 in caudate nucleus suggest a role in locomotor control. GPR7 is poorly expressed in human hypothalamus, despite high expression reported previously in rat and mouse hypothalamus (21). The expression of ppL7 in hypothalamus and the presence of both GPR7 and GPR8 transcripts in pituitary suggest also a role of the peptides in the release of pituitary hormones. GPR7 transcripts have been described previously in the anterior lobe of human pituitary by Northern blot and in situ hybridization (20). We have also demonstrated the expression of GPR8 in the anterior lobe of human pituitary by immunohistochemistry and in situ hybridization (work in progress; data not shown).
context, it was reported that injection of L8 in rats increased prolactin release (1).

In contrast to the situation in brain, ppL8 expression was more widespread than that of ppL7 in peripheral tissues. Both ligand precursors are highly expressed in testis, ovary, uterus, and placenta, suggesting a regulatory role in reproductive functions. The expression of ligands in spleen and lymph nodes and of receptors in peripheral blood leukocytes could indicate a role in the immune system. High levels of GPR7 and ppL8 transcripts were also found in trachea, suggesting the possible existence of a local loop in the respiratory system.

GPR7 displays a relative preference for L7, and GPR8 displays a relative preference for L8. However, given the activity of both peptides on the two receptors in the low nanomolar range, it is difficult to postulate, on the basis of the presently available data, the respective physiological effects of L7 and L8 on each receptor. In rodents, in which the GPR8 gene has not been identified so far, it is likely that the effects of both peptides are mediated through their activity on GPR7.

In conclusion, we have identified by bioinformatics a protein precursor that is expected to generate a new peptide, called L7, which represents a high affinity natural ligand for the recently characterized GPR8 receptor. The tissue distribution of the GPR7 and GPR8 peptide precursors in humans suggests a broad range of potential activities in central and peripheral functions. Additional studies, including in vivo pharmacology and the generation of knockout models, will be necessary for specifying the most relevant functions of this new receptor and their potential applications in therapeutic areas. During the review process of the present manuscript, two publications (31, 32) describing partially overlapping data became available online.

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GPR7 and GPR8 Ligands

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