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Mammalian GFRα-4, a Divergent Member of the GFRα Family of Coreceptors for Glial Cell Line-derived Neurotrophic Factor Family Ligands, Is a Receptor for the Neurotrophic Factor Persephin*

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Four members of the glial cell line-derived neurotrophic factor family have been identified (GDNF, neurturin, persephin, and enovin/artemin). They bind to a specific membrane-anchored GDNF family receptor as follows: GFRα-1 for GDNF, GFRα-2 for neurturin, GFRα-3 for enovin/artemin, and (chicken) GFRα-4 for persephin. Subsequent signaling occurs through activation of a common transmembrane tyrosine kinase, cRET. GFRα-4, the coreceptor for persephin, was previously identified in chicken only. We describe the cloning and characterization of a mammalian persephin receptor GFRα-4. The novel GFRα receptor is substantially different in sequence from all known GFRαs, including chicken GFRα-4, and lacks the first cysteine-rich domain present in all previously characterized GFRαs. At least two different GFRα-4 splice variants exist in rat tissues, differing at their respective COOH termini. GFRα-4 mRNA is expressed at low levels in different brain areas in the adult as well as in some peripheral tissues including testis and heart. Recombinant rat GFRα-4 variants were expressed in mammalian cells and shown to be at least partially secreted from the cells. Persephin binds specifically and with high affinity (K_D = 6 nM) to the rat GFRα-4 receptor, but no cRET activation could be demonstrated. Although the newly characterized mammalian GFRα-4 receptor is structurally divergent from previously characterized GFRα family members, we suggest that it is a mammalian orthologue of the chicken persephin receptor. This discovery will allow a more detailed investigation of the biological targets of persephin action and its potential involvement in diseases of the nervous system.

Neurotrophic factors are involved in the differentiation, development, and maintenance of neurons. These proteins can prevent degeneration and promote survival of different types of neuronal cells and are thus potential therapeutic agents for neurodegenerative diseases. The ligands of the glial cell line-derived neurotrophic factor (GDNF)[1] family and their receptors have been extensively reviewed (1, 2). In the GDNF family, four different members have been identified as follows: GDNF (3), neurturin (NTN, Ref. 4), persephin (PSP, Ref. 5), and enovin/artemin (EVN/ART, Refs. 6 and 7). All four growth factors require a heterodimeric receptor complex in order to carry out downstream intracellular signal transduction. GDNF binds to the GDNF family receptor α-1 (GFRα-1, Ref. 8) subunit, a glycosylphosphatidylinositol (GPI)-anchored membrane protein (9, 10). Several other members of the GFRα family of ligand-binding receptors have been characterized. GFRα-2 (11–14) and GFRα-3 (15–18) have been identified in different species by several groups. A fourth family member, GFRα-4, was cloned from chicken cDNA (19). According to one signaling model (9), the ligand-GFRα complex binds to and activates the cRET proto-oncogene, a transmembrane tyrosine kinase (10, 20), resulting in phosphorylation of tyrosine residues in cRET and subsequent activation of downstream signal transduction pathways. However, it has also been shown that GFRα-1 to GFRα-3 subunits are able to modulate cRET tyrosine phosphorylation, thus suggesting that GFRα and cRET can interact in the absence of ligand (21–24). Recently, other signaling mechanisms have been proposed, wherein GFRα coreceptors function to present GDNF family ligands in *trans* to cRET-expressing cells (25, 26) or wherein ligand-GFRα complexes can activate intracellular signaling pathways independently from cRET (27, 28). *In vitro*, GFRα-1 is the preferred receptor for GDNF, whereas GFRα-2 preferentially binds NTN (9, 13, 29). EVN/ART has been shown to signal through cRET using GFRα-3 as the preferred ligand-binding receptor (6, 7), whereas chicken GFRα-4 forms a functional receptor complex for PSP in combination with cRET (30). Cross-talk between the neurotrophic factors and GFRα receptors is possible *in vitro*, as GDNF can bind to GFRα-2 or GFRα-3 in the presence of cRET (11, 21, 24), and NTN can bind to GFRα-1 with low affinity (13). The observed *in vitro* binding preferences of GDNF ligands to their respective GFRαs have been confirmed *in vivo* by the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ294475 (for rat GFRα-4 variant A) and AJ294476 (for rat GFRα-4 variant B).

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[1] The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; BLAST, basic local alignment search tool; bp, base pairs; CHO, Chinese hamster ovary; EST, expressed sequence tag; EVN/ART, enovin/artemin; FISH, fluorescent *in situ* hybridization; GFRα, GDNF family receptor α; GPI, glycosylphosphatidylinositol; NTN, neurturin; PSP, persephin; RACE, rapid amplification of cDNA ends; SPR, surface plasmon resonance; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MES, 4-morpholineethanesulfonic acid; PCR, polymerase chain reaction; kb, kilobase pair.

corresponding phenotypes of ligand and/or receptor mouse knockouts (31, 32). In summary, GDNF, NTN, EVN/ART, and PSP are part of a neurotrophic signaling system whereby specific high affinity ligand-binding subunits (GFRα-1 to GFRα-4) can interact with the same tyrosine kinase subunit (cRET) to elicit functional responses in neuronal cells.

Persephin shares many of the neurotrophic effects previously described for GDNF and NTN (5). It supports the survival of motor neurons in culture and *in vivo* after sciatic nerve axotomy. It also promotes the survival of ventral midbrain dopaminergic neurons in culture and prevents their degeneration after *in vivo* 6-hydroxydopamine treatment. PSP further affects kidney morphogenesis, since it is able to promote ureteric bud branching. However, PSP does not support peripheral neurons from the superior cervical, dorsal root, nodose, trigeminal, or enteric ganglia. Due to the extremely low expression levels of PSP, its tissue distribution has been studied by reverse transcription PCR only (5, 33). Low level PSP mRNA expression is found throughout the embryonic and adult central nervous system and in all peripheral tissues examined (including heart, kidney, liver, skin, and muscle).

The GFRα-4 coreceptor has been identified in chicken only (19) and has been shown to mediate signaling of (mammalian) persephin via cRET *in vitro* (30). However, a functional mammalian persephin receptor has not been identified. In this paper, we describe the identification and characterization of a novel mammalian GDNF family receptor substantially different from previously described GFRαs, and we show that it is a mammalian persephin receptor GFRα-4.

EXPERIMENTAL PROCEDURES

Data Base Homology Searching, Sequence Comparison, and Gene Prediction—Similarity searching using cDNA or cDNA-derived protein sequences was carried out on the EMBL human expressed sequence tag (EST) and genomic data bases or on the proprietary LifeSeq™ and ZooSeq™ data bases (Incyte Pharmaceuticals, Palo Alto, CA) using the Basic Local Alignment Search Tool (BLAST version 1.4, see Ref. 34). The percentage identity and percentage similarity between members of the GFRα family were calculated by pairwise comparison of the sequences using the BESTFIT program (Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, WI). Prediction of signal peptide cleavage was done using the SPScan program (SeqWeb version 1.1 with the Wisconsin Package Version 10.0). Gene prediction analysis on a genomic mouse GFRα-like sequence was performed with GeneHunt (Compugen, Tel Aviv, Israel) and with HMMgene (version 1.1, Center for Biological Sequence Analysis, Denmark).

Oligonucleotide Synthesis and DNA Sequencing—All oligonucleotide primers were purchased from Eurogentec (Seraing, Belgium). Insert-specific sequencing primers (15- and 16-mers) and primers for use in PCRs were designed manually. Sequencing reactions were done on both DNA strands using the ABI prism BigDye Terminator Cycle sequencing kit and were run on an Applied Biosystems 377XL sequencer (PerkinElmer Life Sciences). The Sequencher™ software was used for sequence assembly and manual editing (GeneCodes, Ann Arbor, MI).

Cloning of Rat GFRα-4—Based on novel rat GFRα EST sequences identified in a proprietary data base, oligonucleotide primers were developed for PCR amplification and 5′- and 3′-rapid amplification of cDNA ends (RACE). PCRs were performed on MarathonReady™ cDNA (CLONTECH Laboratories, Palo Alto, CA) derived from rat brain, heart, or kidney. Since the coding sequence of the novel rat GFRα turned out to have a high GC content, a GC-rich PCR kit (CLONTECH Laboratories) was used for all amplification reactions according to manufacturer's instructions. PCR fragments were cloned (TOPO-TA cloning, Invitrogen BV, Leek, The Netherlands) and the cloned inserts sequenced. A consensus coding sequence was derived by assembling all partial sequences obtained and by excluding putative intron sequences still present in some PCR fragments but absent in others. A DNA fragment containing the full-length rat GFRα-4 coding sequence was then amplified from cDNA using primers designed at the 5′ and 3′ ends of the consensus sequence.

GFRα Expression Constructs—The rat GFRα-4 coding sequence of variant A was subcloned in the mammalian expression vector pcDNA-3 (Invitrogen). A FLAG tag (DYKDDDDK) was inserted by recombinant PCR between the predicted signal sequence and the mature coding sequence (positions 33/34 of the amino acid sequence in Fig. 1*B*) for expression of a FLAG-tagged rat GFRα-4. Since both these rat GFRα-4 constructs could not be expressed at detectable levels, constructs with an improved signal sequence were made for variants A and B by recombinant PCR. Construct rGFRa4AhumsigFLAG/pcDNA3 encodes a protein containing the predicted human GFRα-4 signal sequence (amino acid residues 1–22 of the human sequence in Fig. 1*B*) followed by a FLAG tag and the rat GFRα-4 variant A mature protein sequence (amino acid residues 34–273 of sequence rGFRα-4(A) in Fig. 1*B*). Construct rGFRa4BhumsigFLAG/pcDNA3 encodes a protein with the same signal sequence, FLAG tag and rat GFRα-4 variant B mature protein sequence (amino acid residues 34–258 of sequence rGFRα-4(B) in Fig. 1*B*).

Constructs for the expression of soluble GFRα-IgGFc fusion proteins were made as follows. cDNA regions of human GFRα-1, GFRα-2, and GFRα-3, chicken GFRα-4, and rat GFRα-4 variant A (coding for amino acid residues 27–427, 20–431, 28–371, 20–399, and 29–252, respectively), excluding the sequences coding for the signal peptide and for the COOH-terminal hydrophobic region involved in GPI anchoring, were cloned in-frame in the expression vector Signal pIg plus (R & D Systems Europe Ltd, Abingdon, UK). The inserts of all constructs were confirmed by DNA sequence analysis. The resulting proteins expressed from these constructs contain a 17-amino acid residue NH_2-terminal CD33 signal peptide, the respective GFRα protein region, and a 243-amino acid residue COOH-terminal human IgG_1-Fc fusion domain. Fusion proteins were expressed in Chinese hamster ovary (CHO) cells and purified as described (24).

Northern Blot Analysis—Northern blots containing 2 μg of poly(A)-rich RNA derived from different rodent tissues (mouse MTN™ blot, mouse embryo MTN™ blot, and rat MTN™ blot; CLONTECH Laboratories) were hybridized according to the manufacturer's instructions with an α-[^{32}P]dCTP random priming labeled (HighPrime kit, Roche Molecular Biochemicals) 948-base pair (bp) fragment derived from the rat GFRα-4 coding sequence. Stringency washes were performed in 0.1× SSC, 0.1% SDS at 50 °C (for the mouse blots) or at 55 °C (for the rat blot). In order to check for mRNA quality and equal sample loading, blots were rehybridized with a 2-kb human β-actin cDNA control probe. The blots were exposed to Biomax-MR Scientific Imaging Film (Eastman Kodak Co.) for 1 month (GFRα-4) or for 16 h (β-actin).

In Situ Hybridization on Adult Brain Sections—A 0.45-kb DNA fragment from mouse GFRα-4 (common to all splice variants) was subcloned in vector pBS-SK (Stratagene, La Jolla, CA), and the resulting vector was linearized with *Eco*RI. An antisense riboprobe was synthesized by *in vitro* transcription with T3 polymerase in the presence of ^{35}S-UTP (Amersham Pharmacia Biotech). *In situ* hybridization was performed on fresh-frozen 14-μm transverse sections of adult mouse (Balb/c) brain. Sections were made using a cryostat and stored at −20 °C until used. Sections were thawed at room temperature, fixed for 5 min in 4% (v/v) paraformaldehyde in phosphate-buffered saline, rinsed twice in phosphate-buffered saline and twice in distilled water, delipidated with 0.2 M HCl for 10 min, acetylated for 20 min with 0.25% acetic anhydride in 0.1 M ethanolamine, and dehydrated with ethanol. After drying, the sections were prehybridized for 5 h in buffer A (50% formamide, 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, pH 8.0, 0.3 M NaCl, 0.1 M dithiothreitol, 0.5 mg/ml yeast tRNA, 0.1 mg/ml poly(A)-rich RNA, 1× Denhardt's solution, and 10% dextran sulfate) and then incubated overnight in a humidified chamber with 180 μl of buffer A containing 2.25×10^6 cpm/ml of mouse GFRα-4 antisense riboprobe or an unrelated control riboprobe. After hybridization, the sections were washed once in 1× SSC at 48 °C for 40 min, treated with RNase (10 mg/ml) in 0.5 M NaCl, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA at 37 °C for 30 min, and washed twice with 0.5× SSC and twice with 0.1× SSC for 10 min each at 37 °C. Finally, sections were dehydrated with ethanol, dried, and then dipped in Kodak NTB2 emulsion (Kodak). After a 6-week exposure at −20 °C, the slides were developed, stained lightly with cresyl violet, and mounted in Permount.

Chromosomal Localization of Rat GFRα-4—A 0.95-kb rat GFRα-4 cDNA fragment (containing the full rat GFRα-4 coding sequence of variant A) and a 2.3-kb genomic fragment were used as probes in fluorescent *in situ* hybridization (FISH) analysis on rat chromosomes. A mixture of the two probes (1 μg of total DNA) was labeled with digoxigenin-11-dUTP (Roche Molecular Biochemicals) by nick translation (Life Technologies, Inc.) producing a final probe fragment size of 200–400 bp. The labeled probe was mixed with hybridization buffer (50% formamide, 2× SSC, 10% dextran sulfate). After denaturation, the mixture was placed on metaphase rat chromosome slides (35, 36) denatured at 72 °C for 2 min in 70% formamide, 2× SSC. After hybrid-

TABLE I
Intron-exon structure of the rat GFRα-4 gene

The intron-exon boundaries in the DNA sequence of rat GFRα-4 together with the sizes of identified introns and exons (in bp) are shown. Exonic sequence is shown in capital letters, and introns are in lowercase. Exon numbering is according to the diagrams in Fig. 1*A*. The right column shows the sizes of the corresponding exons in the genomic sequence of human GFRα-1 (40).

Exon	Exon size	Intron size	Splice acceptor	Splice donor	GFRα-1 exon
	bp				*bp*
1	>124	560		GAGgtaaggaggt	
2	355	49	ccctcaccagGGT	CCGgtgcgtgcgg	337
3	110	80	gcgcgcgcagGCC	TAGgtacgctggg	110
4	135	741	gtccctgcagGCA	TGGgtgagggggc	135
5a	92	139	cactccatagATG	TGGgtaggtatgg	182
5b	76	84		TGGgtgctgtttc	
6a	>137		ttgtcccaagGTG		753
6b	>208		cccttctcagGCA		

ization for 48 h at 37 °C, preparations were washed for 15 min in 50% formamide, 2× SSC. Detection of labeled chromosomes was done by standard fluorescein isothiocyanate anti-digoxigenin. Chromosome spreads were counterstained with 4′,6-diamidino-2-phenylindole. Results were derived from micrographs of 100 different cells.

Binding of Persephin to GFRα Receptors—Surface plasmon resonance (SPR) experiments were performed at 25 °C using a BIACORE 3000 instrument (Biacore AB, Uppsala, Sweden). Sensor chip CM5, the amine coupling kit, and buffers used were also obtained from Biacore AB. Recombinant PSP, NTN, EVN/ART, and GDNF were used as immobilized ligands. Recombinant human GDNF was obtained from R & D Systems Europe Ltd. (Abingdon, UK). NH_2-terminally 6His-tagged recombinant human NTN, rat PSP, and human EVN/ART were produced in *Escherichia coli* as described previously (6, 24, 37). The carboxylated matrix of a CM5 sensor chip was first activated with a 1:1 mixture of 400 mM *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide and 100 mM *N*-hydroxysuccinimide for 10 min. Recombinant neurotrophic factors were applied onto the activated surface in 10 mM sodium acetate buffer, pH 4.5 at a flow rate of 5 μl/min. Unreacted carboxyl groups were blocked with 1 M ethanolamine HCl. For binding experiments, soluble GFRα-IgGFc fusion proteins were superfused using the kinject program at 30 μl/min. Concentrations of GFRα-IgGFc used in kinetic experiments were between 1 and 120 nM in Hepes-buffered saline (150 mM NaCl, 3.5 mM EDTA sodium salt, 0.005% polysorbate 20, 10 mM Hepes, pH 7.4). The association of the GFRα receptors to the immobilized ligands was monitored for 3 min and the dissociation for 1 min, followed by regeneration with 10 mM glycine buffer. Dissociation was initiated by superfusion with Hepes-buffered saline. To improve the quality of sensor data, double referencing was used (38). Data were analyzed using a global analysis with the BIACORE evaluation software (version 3.0.1). Global analysis calculates the association rate (k_a) and dissociation rate (k_d) simultaneously, and the apparent equilibrium dissociation constant (K_D) is then calculated as k_d/k_a. A simple 1:1 Langmuir model was used to fit the data.

Transient Transfections and Western Blot Analysis—CHO cells were routinely cultured in Dulbecco's modified Eagle's medium/Nutrient Mix F-12 supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Inc.). Cells were transfected with GFRα-4 expression constructs rGFRa4AhumsigFLAG/pcDNA3 or rGFRa4BhumsigFLAG/pcDNA3 using an optimized LipofectAMINE Plus method (24). One day post-transfection, cells were subcultured onto poly-L-lysine-coated 96-multiwell plates in 10% fetal calf serum and incubated for 48 h. Cell culture supernatants were collected and concentrated 3-fold using chloroform/methanol precipitation. Cells were collected by scraping directly into Laemmli buffer and boiled for 5 min. Concentrated supernatants and cell pellets were subjected to SDS-polyacrylamide gel electrophoresis on 4–12% Bis-Tris acrylamide mini-gels under reducing conditions in MES-SDS running buffer (NOVEX, San Diego, CA).

After electrophoresis, proteins were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech). The blots were blocked overnight at 4 °C in NaCl/P_i containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dried milk. FLAG-tagged proteins were detected with a primary mouse anti-FLAG IgG M2 monoclonal antibody (5 μg/ml; Kodak), and a secondary anti-mouse horseradish peroxidase-conjugated $F(ab)_2$ fragment (dilution 1:2000; Amersham Pharmacia Biotech). The signals were visualized using the enhanced chemiluminescence (ECL) Western blotting system according to the manufacturer's instructions (Amersham Pharmacia Biotech).

RESULTS

Identification and Cloning of Mammalian GFRα-4—Similarity searching of the EMBL data base using the protein sequences of known GFRαs as query sequences yielded two mouse ESTs derived from mouse brain and mouse mammary gland (GenBank™ accession numbers AU035938 and AA823200, respectively), coding for a putative novel member of the GFRα family, which we called GFRα-5, but later renamed GFRα-4 (see "Discussion"). These EST sequences were used as the query sequence in BLAST searches on the proprietary ZooSeq™ data base. Two rat ESTs of 270 and 250 bp with high similarity to these mouse sequences were identified. DNA sequencing of the inserts of the corresponding clones revealed the rat GFRα-4 partial 3′-coding sequence, a stop codon and a 3′-untranslated region of 549 bp followed by a poly(A)-tail. Based on these ESTs, primers for PCR and for 5′- and 3′-RACE experiments were designed. Cloning and sequence analysis of PCR and RACE fragments derived from rat brain, heart, or kidney cDNA showed the existence in cDNA of several variants of rat GFRα-4. Alignment and comparison of tens of sequences indicated that most of these variants could be explained by the retention of unspliced introns in the cDNA. In some RACE products, an ATG translation start codon could be identified preceded by an in-frame stop codon at position −16 to −18 relative to the start ATG. The open reading frame following this start codon codes for a weak signal peptide of 29 amino acid residues (SPScan score 7.0, probability 1.17e-02). Other RACE fragments also included an in-frame start codon preceded by an in-frame upstream stop codon, but the resulting open reading frame did not code for a signal peptide. To determine which of the possible variant rat GFRα-4 reading frames exist in cDNA, PCRs were performed on rat brain, heart, or kidney cDNA with forward primers designed 5′ of the two possible translation start codons in combination with reverse primers designed 3′ of the translation stop codon of the obtained GFRα-4 consensus sequence. The sequences of resulting cloned PCR fragments were then aligned and compared. This allowed the rat GFRα-4 DNA sequence to be divided into six sequence stretches common to all identified variants, with five intervening sequence stretches present or absent depending on the variant. All five intervening sequences contain 5′ and 3′ splice consensus sites (GT at the 5′ end and AG at the 3′ end (39)) and could thus potentially represent unspliced introns (Table I). In order to strengthen the hypothesis that the majority of the identified variants result from the retention of unspliced introns in certain mRNA transcripts, the rat GFRα-4 sequence was compared with the genomic sequence of human GFRα-1 (40). This analysis revealed that the GFRα-4 sequence parts common to all transcripts coincided with exons in GFRα-1, whereas the intervening sequences absent in some

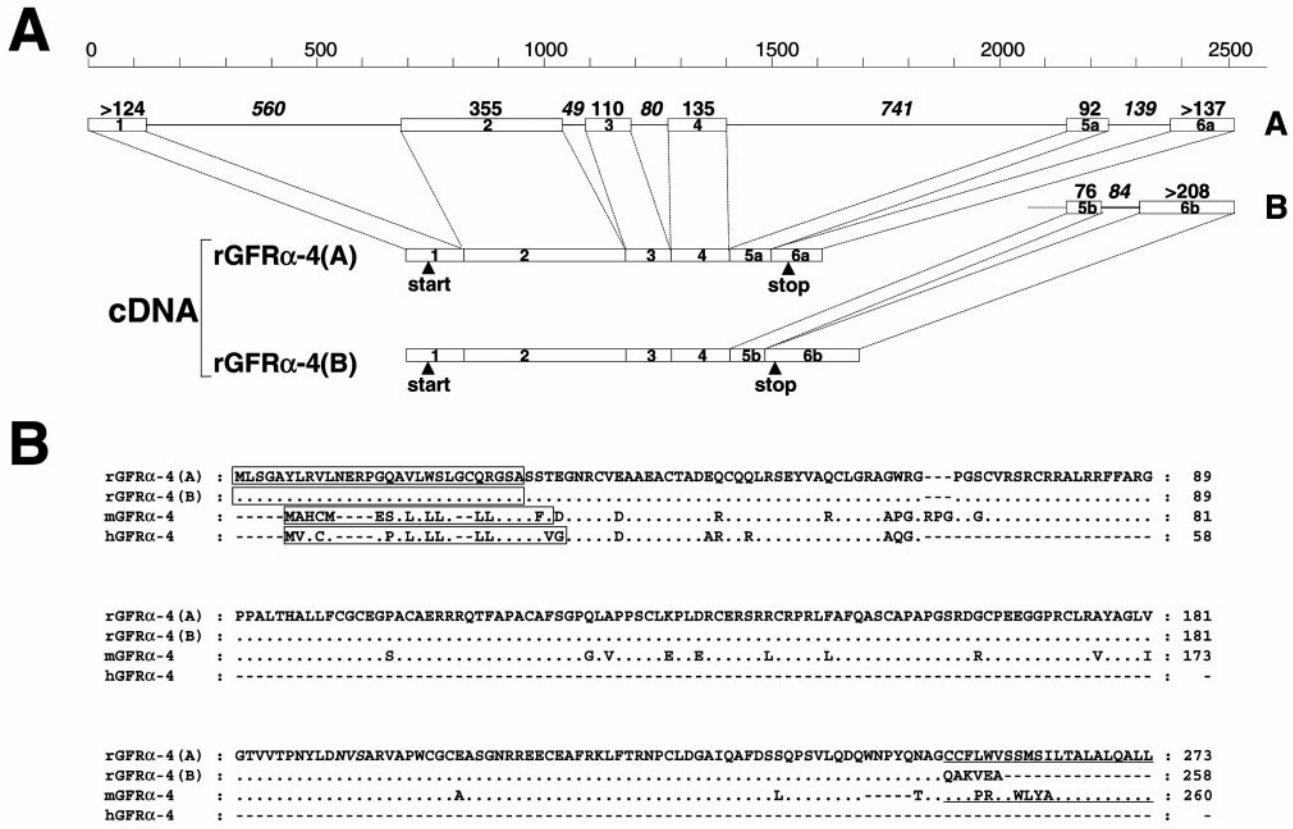

FIG. 1. **Structure of the rat *gfra4* gene.** *A,* structural diagram of rat GFRα-4. The *top line* shows a scale in bp. The *line below* shows the deduced genomic structure of the rat *gfra4* gene. Exons are represented by *boxes* and *numbered*, and intron sequences are depicted as *lines.* The sizes (in bp) of the respective introns and exons are indicated *above* the diagram (intron sizes in *italics*). Different splicing of intron 5 as shown in the diagram leads to the existence of the two different cDNA species rGFRα-4(A) and rGFRα-4(B) shown *below* the genomic sequence. The positions of the putative translation start and stop codons are indicated by *arrowheads. B,* sequence comparison of predicted mammalian GFRα-4 proteins. Alignment of the predicted protein sequences of rat GFRα-4 splice variants *A* and *B* (rGFRα-4(A) and rGFRα-4(B)) and of the mouse (mGFRα-4) and (partial) human (hGFRα-4) protein sequences derived from the analysis of genomic DNA (see text). Amino acid residues conserved between the different proteins are indicated by *dots,* and *dashes* indicate gaps introduced into the sequence to optimize the alignment. Amino acid residues are numbered to the *right*. The predicted signal peptides are *boxed*, the putative *N*-glycosylation site is *italicized*, and the hydrophobic COOH-terminal region possibly involved in GPI anchoring is *underlined.*

transcripts coincided with intron sequences in human GFRα-1 (Table I).

The putative intron present between exon 5 and exon 6 in rat GFRα-4 can be spliced out in two different ways, resulting in the presence in cDNA of two different splice variants, which we have called A and B. This alternative splicing event results in a different COOH terminus of the translated protein. Fig. 1*A* schematically shows the structure of the rat *gfra4* gene together with the derived cDNA for splice variants A and B after removal of the intron sequences.

A single nucleotide polymorphism was detected in the cDNA sequence with T found in 50% of the sequenced clones and C in the other 50%. This polymorphism leads to an amino acid change in the protein sequence of variant A (Trp to Arg at position 257 in the hydrophobic region involved in GPI anchoring).

The consensus sequence obtained by removing introns 1–4 and intron 5A (variant A) translates into a predicted protein of 273 amino acid residues with a calculated molecular mass of 29.7 kDa. The consensus sequence obtained by removing introns 1–4 and intron 5B (variant B) translates into a protein of 258 amino acid residues with a calculated molecular mass of 28.0 kDa. The alignment of variants A and B of rat GFRα-4 together with partial mouse and human GFRα-4 sequences derived from genomic DNA (see below) is shown in Fig. 1*B*. GFRα-1 to GFRα-3 and chicken GFRα-4 are characterized by a COOH-terminal sequence typical of a GPI-anchored membrane protein, consisting of a hydrophobic region of 17–31 amino acid residues preceded by a hydrophilic sequence containing a stretch of three small amino acid residues such as Asp, Cys, Ala, Ser, Gly, or Asn (41). The rat GFRα-4 variant A protein sequence has a hydrophobic carboxyl terminus of 21 amino acid residues (position 253–273) preceded by two possible GPI cleavage sites (DSS at position 234–236 or NAG at position 250–252). Variant B has a shorter hydrophilic carboxyl terminus and could represent a soluble GFRα-4 isoform. One possible site for *N*-linked glycosylation (NVS at residues 192–194 in the protein) is present in both variants.

Within both variants, a weak signal peptide of 29 amino acid residues is predicted (SPScan score 7.0, probability 1.2e-02). Two genomic sequence entries containing mouse and human GFRα-4 coding sequences, respectively, can be found in the EMBL data base using BLAST analysis as follows: a fully sequenced mouse genomic sequence of 48 kb (GenBank™ accession number AF155960; see ref. 42) and a human genomic sequence contig of 11.5 kb (GenBank™ accession number AC017113; unfinished sequence). A comparison of the obtained rat GFRα-4 sequences with these genomic entries yielded the predicted protein sequences for mouse and human (partial) GFRα-4 included in the alignment of Fig. 1*B*. A DNA sequence coding for a signal peptide similar to the one identified for rat GFRα-4 could not be found in these genomic sequences. However, a sequence coding for a high probability ("prototype") signal peptide could be identified in the mouse genomic se-

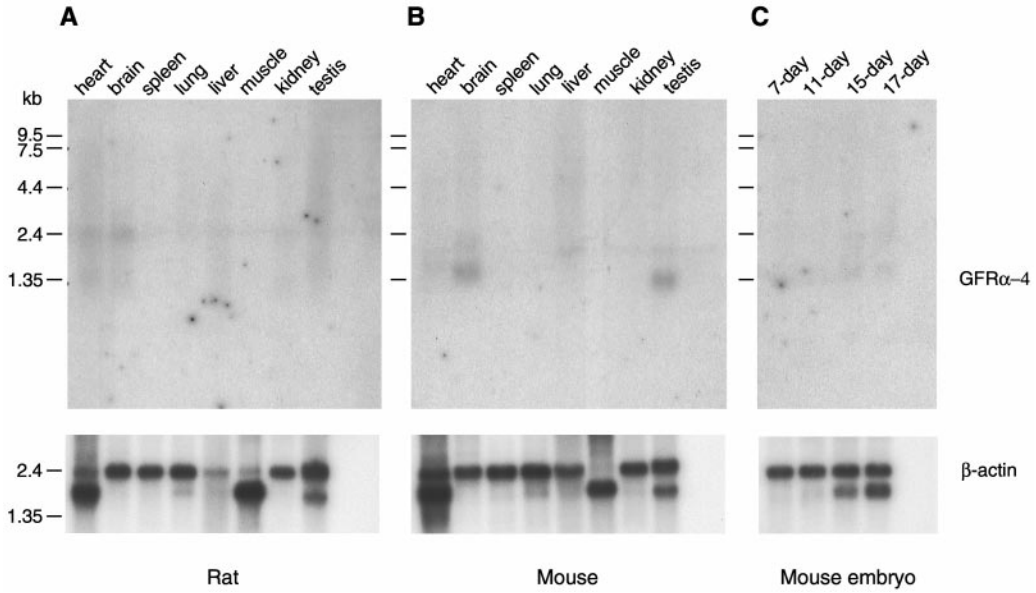

FIG. 2. **Northern blot analysis of rodent GFRα-4 mRNA expression.** The expression of rat and mouse GFRα-4 mRNA in different tissues was assessed using a probe corresponding to the coding sequence of rat GFRα-4 to analyze blots of poly(A)-rich RNA (*top panels*). After exposure for 1 month, blots were rehybridized with a 2-kb human β-actin probe and exposed for 16 h (*bottom panels*). *A,* rat multiple tissue Northern blot; *B,* mouse multiple tissue Northern blot; and *C,* mouse embryo multiple tissue Northern blot. Apparent sizes are indicated (in kilobase pairs) by *horizontal lines* to the *left* of each panel.

quence using two different gene prediction programs. The predicted protein sequence obtained from this analysis contains the 21 amino acid residue signal peptide (SPScan score 10.7, probability 1.6e-05) shown in the alignment of Fig. 1*B*. A similar sequence can be found in the human genomic sequence (22 amino acid residues, score 12.0, probability 3.7e-05).

Tissue Distribution of Mammalian GFRα-4—Northern blots of mRNA derived from rat and mouse tissues were hybridized with the rat GFRα-4 coding sequence (Fig. 2). GFRα-4 mRNA was expressed at very low levels as evidenced by the weak signals obtained in several tissues. Rehybridization of the blots with the β-actin housekeeping gene under similar conditions showed that the low GFRα-4 levels detected were not due to low mRNA quality or quantity. In rat, a very weak GFRα-4 signal could be detected around 2.3 kb in heart, brain, and testis. An even weaker second transcript was present around 1.4 kb in the same tissues. In mouse, a 1.35-kb transcript was most intense in brain and testis, with a much weaker signal present around 2 kb. Very low mRNA expression of the 1.35-kb transcript was also present in 15-day and 17-day mouse embryo. The size of the smaller transcript is in agreement with the predicted size of the rat GFRα-4 coding sequence (± 880 bp + the 3′-untranslated region of 570 bp).

In *in situ* hybridization experiments on adult mouse brain (Figs. 3 and 4), expression of GFRα-4 was detected in the cortex, including the cingulate cortex, in hippocampus, including CA1–CA3 areas and in the dentate gyrus. Weak labeling is observed in the habenulae. At the posterior parts of the brain, labeling is found in cells of the pars substantia nigra (predominantly dopaminergic cells of the pars compacta), in the ventral tegmental area and in the area of the red nuclei (Fig. 3).

GFRα-4 mRNA is also expressed in the glomerular layer, between the glomeruli and in the mitral cell layer of the olfactory bulb (Fig. 4). Weak labeling is seen in the granular cell layer. GFRα-4 labeling is also observed in the facial nucleus. In the cerebellum, GFRα-4 expression is predominantly found in the Purkinje cell layer, with weak labeling in the granular cell layer.

An unrelated control probe hybridized in parallel to comparable tissue sections did yield an expression pattern completely different from the one observed for GFRα-4 (data not shown), confirming specificity of the GFRα-4 signal.

Chromosomal Localization of Rat, Mouse, and Human GFRα-4—Two partially overlapping probes of 0.9 and 2.3 kb, respectively, were used for FISH analysis on rat chromosomes. Due to the small size of the probes there was considerable background. However, most studied cells showed label at position RNO3q36 (Fig. 5). About 35% of the metaphase studies showed "double spot" label at both homologues of RNO3, whereas about 50% had double spots on only one of the homologues or "single spot" label on both homologues. No other chromosomal site showed label in more than one cell.

Based on comparative mapping, the corresponding mouse locus would be expected to be located at MMU2 (band F), whereas a possible human location for the gene would be HSA2, HSA15, or HSA20. In agreement with this, the genomic GFRα-4 sequences identified in the EMBL data base are derived from mouse chromosome 2 (BAC clone 389B9) and from human chromosome 2 (GenBank™ accession number AC013324; BAC388_K_24map2), respectively.

Binding of Rat and Chicken GFRα4-IgGFc Receptors to Persephin—SPR was used to measure the binding of soluble rat or chicken GFRα4-IgGFc receptors to immobilized PSP. Specific binding to PSP could be detected with both proteins. The observed binding of GFRα4-IgGFc was specific as there was no binding to GDNF, NTN, or EVN/ART. Control experiments confirmed binding of GFRα-1 to GDNF, of GFRα-2 to NTN, and of GFRα-3 to EVN/ART (data not shown). From the binding curves obtained using three determinations at differing concentrations of rat and chicken GFRα4-IgGFc, the binding constants k_a (association rate) and k_d (dissociation rate) were derived (Table II).

Although apparent K_D values were very similar for both fusion proteins, R_{max} values were significantly different. Binding levels of ~1000 relative units were routinely obtained with chicken GFRα-4-IgGFc, whereas binding levels of rat GFRα4-IgGFc were approximately 20 times lower, around 50–60 relative units. This could be due to differences in the concentration of active chicken and rat GFRα-4-IgGFc fusion protein. The calculated equilibrium dissociation constant, K_D, of 5.9 ±

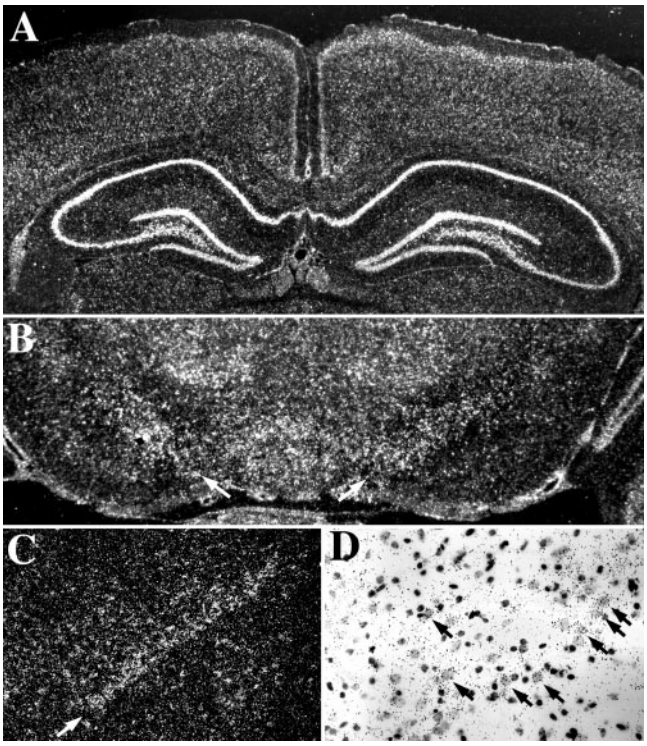

FIG. 3. **GFRα-4 mRNA is expressed in the cortex, hippocampus, and substantia nigra.** Transverse sections of the adult mouse brain were labeled by radioactive *in situ* hybridization with a mouse GFRα-4-specific riboprobe. Sections were exposed for 6 weeks to photographic emulsion in order to increase the signal level. Sections were photographed under dark field (*A–C*) and bright field (*D*) illumination. *A,* transverse section of the adult mouse brain visualizing parts of the cortex, including the cingulate cortex, hippocampus, and habenulae. Labeling is seen in the cortex, including the cingulate cortex, in hippocampus, including CA1–CA3 areas and in the dentate gyrus. Weak labeling is also observed in the habenulae. *B,* at posterior parts of the brain, labeling is found in cells of the substantia nigra (predominantly in the pars compacta, *arrows*), in the ventral tegmental area (area between the *arrows*), and in the area of the red nuclei (area above the compact part of the substantia nigra). *C,* dark field, higher magnification of the substantia nigra. Labeled cells are mainly located in the compact part of the structure (*arrow*); however, a few labeled cells are also found in the reticular part. *D,* bright field higher magnification of the pars compacta area in *C*. GFRα-4 labeling (*arrows*) is clearly associated with large cells in this area. These cells are generally dopaminergic cells.

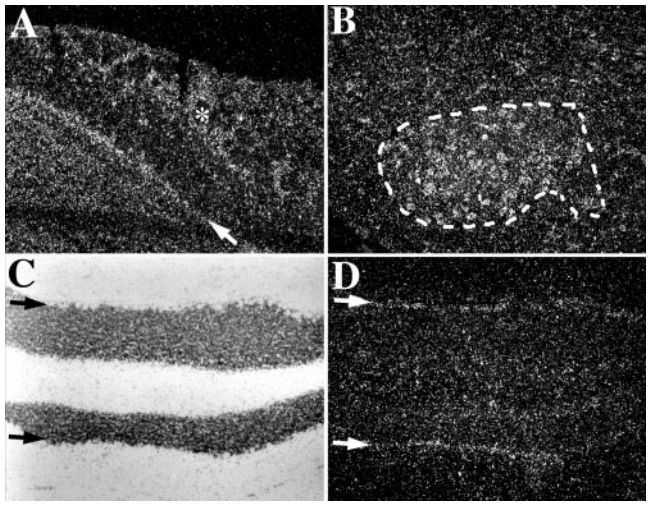

FIG. 4. **GFRα-4 mRNA is expressed in the olfactory bulb, facial nucleus, and cerebellum.** Transverse sections of the adult mouse brain were labeled by radioactive *in situ* hybridization as in Fig. 3. Sections were photographed under dark field (*A, B,* and *D*) and bright field (*C*) illumination. *A,* high magnification of a transverse section of the olfactory bulb. GFRα-4 labeling is seen in the glomerular layer, between the glomeruli (a glomerulus is marked by an *asterisk*). Labeling is also observed in the mitral cell layer (*arrow*). Weak labeling is also observed in the granular cell layer. *B,* GFRα-4 labeling in the facial nucleus (demarcated by the *dotted line*). *C* and *D,* bright field and dark field photomicrographs of an adult mouse cerebellar tissue section. GFRα-4 labeling is predominantly found in the Purkinje cell layer. In addition, weak labeling can be seen in the granular cell layer.

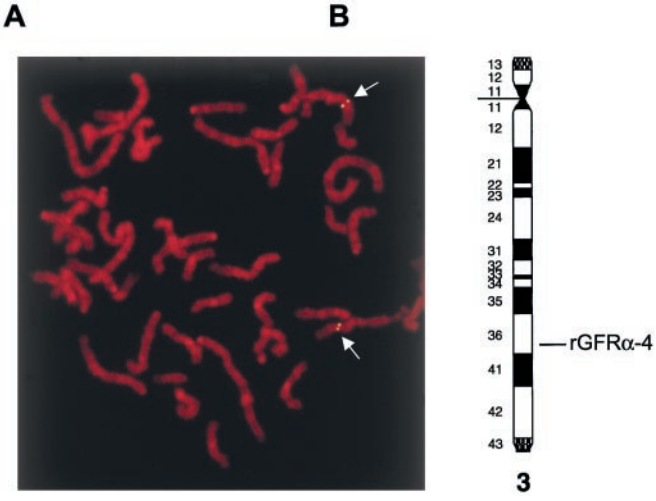

FIG. 5. **The rat *gfra4* gene is localized on chromosome 3q36.** A mixture of two rat GFRα-4 probes was used for FISH analysis. *A,* double-spot FISH signals on the middle-distal part of rat chromosome 3 (*arrows*). *B,* position of the *gfrα-4* gene locus on rat chromosome 3q36.

2.8 nM ($n = 3$) suggests that rat GFRα-4 is a receptor specific for persephin.

Recombinant Expression of Rat GFRα-4 in CHO Cells—Transient transfection of rat GFRα-4 constructs in CHO or NIH 3T3 cells did not yield detectable expression of the GFRα-4 receptor protein (data not shown). When the putative rat signal peptide was replaced by a human GFRα-4 signal peptide identified by the use of two different gene prediction programs, both GFRα-4 variants were expressed at detectable levels (Fig. 6).

A Western blot probed with an anti-FLAG antibody showed GFRα-4-specific bands migrating at approximately 29, 33, and 52 kDa, respectively, in cell lysates and concentrated supernatants from CHO cells transfected with constructs for FLAG-tagged rat GFRα-4 variants A or B (*lanes 2, 3, 5*, and *6*). These bands were absent in untransfected control cells (*lanes 1* and *4*). The 29- and 33-kDa bands presumably correspond to the unglycosylated and a glycosylated form, respectively, of the GFRα-4 receptor. The 33-kDa band obviously comigrates with a nonspecific band also present in the control (*lanes 1* and *4*). The 52-kDa band probably represents a GFRα-4 dimer. In supernatants, the nonspecific bands migrating at 70 kDa provide an internal control for equal sample loading. The specific bands migrating around 90 kDa in the cell pellets of *lanes 5* and *6* probably represent GFRα-4 multimers or aggregates. The expression of variant A is elevated in the cell lysate fraction when compared with variant B, whereas variant B expression in the supernatant is higher than that of variant A.

DISCUSSION

Four members of the GDNF family of neurotrophic factors have been identified so far (GDNF, NTN, PSP, and EVN/ART). All four signal through binding to a specific GPI-linked GFRα receptor (GFRα-1 for GDNF, GFRα-2 for NTN, GFRα-3 for EVN/ART, and (chicken) GFRα-4 for PSP) in combination with a common transmembrane tyrosine kinase, cRET. GFRα-4, the specificity-conferring coreceptor for PSP, had been identified in chicken only, and no mammalian counterpart had been found

TABLE II
Persephin binding to rat and chicken GFRα-4

Binding constants for chicken GFRα-4-IgGFc and rat GFRα-4-IgGFc binding to immobilized persephin as determined by SPR. The mean association rate (k_a), dissociation rate (k_d), and apparent equilibrium dissociation constant (K_D) ± S.E. were derived from the binding curves obtained using three determinations at differing concentrations of the respective soluble receptors. No significant binding of GDNF, NTN, or EVN/ART to GFRα-4 could be detected.

	k_a (1/Ms)	k_d (1/s)	K_D (M)
Chicken GFRα-4	$2.3 \pm 2.6 \times 10^4$	$8.8 \pm 5.1 \times 10^{-4}$	$5.4 \pm 2.9 \times 10^{-9}$
Rat GFRα-4	$2.7 \pm 1.6 \times 10^4$	$1.1 \pm 0.2 \times 10^{-3}$	$5.9 \pm 2.8 \times 10^{-9}$

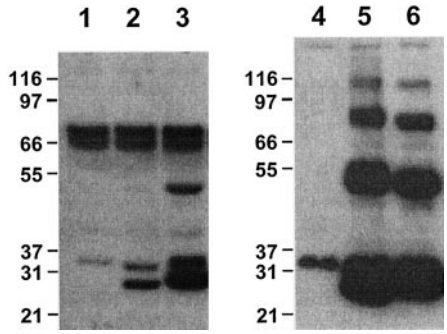

FIG. 6. **Recombinant expression of rat GFRα-4 in CHO cells.** CHO cells were transfected with rGFRa4AhumsigFLAG/pcDNA3 (*lanes 2* and *5*), rGFRa4BhumsigFLAG/pcDNA3 (*lanes 3* and *6*), or left untreated (*lanes 1* and *4*). After 48 h, cell culture supernatants were collected and concentrated, and cell lysates were prepared. Concentrated supernatants (*lanes 1–3*) and cell lysates (*lanes 4–6*) were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting. The blot was probed with an anti-FLAG M2 monoclonal antibody as described under "Experimental Procedures." Molecular mass standards are indicated in kDa.

yet. We describe the cloning and characterization of a mammalian persephin receptor GFRα-4. The similarity between the newly discovered rat GFRα sequence described in this paper and the chicken GFRα-4 (19) is only 37% (27% identity). In fact, homology of the novel receptor with the chicken GFRα-4 is not significantly greater than homology with (mammalian) GFRα-1, GFRα-2, or GFRα-3. This suggested to us that we had cloned a novel member of the GFRα family, GFRα-5, that could be a coreceptor for an as yet unknown member of the GDNF family but, alternatively, could be the mammalian persephin receptor. After we discovered that this novel GFRα was a high affinity coreceptor for persephin, we renamed it GFRα-4 in order to comply with established GFRα nomenclature (8). During the preparation of this manuscript, a mouse GFRα-4 receptor equivalent to the one described in this paper was reported (43). The high divergence of the mammalian GFRα-4 from the avian orthologue is remarkable, since the similarity of mammalian GFRα-1 and GFRα-2 to their chicken counterparts is much higher (*e.g.* 84.5 and 82% for rat GFRα-1 to chicken GFRα-1 and rat GFRα-2 to chicken GFRα-2, respectively). The possibility therefore remains that there exists another mammalian GFRα receptor that is more similar to chicken GFRα-4, and that the mammalian GFRα-4 we discovered and this unidentified receptor have overlapping binding properties for persephin and/or other GDNF family ligands.

Although many different variant GFRα-4 sequences could be found in cDNA derived from rat heart, brain, or kidney mRNA, only few of these sequences seemed to represent fully spliced mRNA transcripts. The occurrence of unspliced mRNA species has also been reported for the GFRα ligands enovin/artemin (6) and persephin (5). It remains to be determined whether this observation underlies a complex transcriptional regulation mechanism or whether it is an artifact due to the low expression levels of the GFRαs and their ligands, necessitating PCR to be forced to its limits.

The predicted protein sequences of the fully spliced rat GFRα-4 variants A and B are both similar to the known GFRα sequences and only differ from each other in a small amino acid stretch (21 amino acid residues) at the COOH terminus. These two sequences could represent biologically active GFRα-4 variants. Variant A is possibly attached to the membrane via a GPI anchor. Rat GFRα-4 variant B is different from all previously described GFRαs in that it has a hydrophilic COOH terminus, implying that GPI-anchoring is not possible for this variant. This could mean that variant B is a soluble form of the rat GFRα-4 receptor. We have indeed found that variant B is probably secreted by transfected cells at higher levels than variant A. The occurrence of soluble GFRα-4 receptor isoforms could function to present PSP to cRET in *trans* to cells expressing cRET but no GFRα-4, as hypothesized for GDNF/GFRα-1 (25, 26).

Recently, a model has been proposed for the domain structure of GFRαs based on the comparison of the sequences of mouse GFRα-1 to GFRα-3 and chicken GFRα-4 (1). The model distinguishes three conserved cysteine-rich domains joined together by less conserved adaptor sequences. The molecules are anchored to the membrane by a GPI anchor. Rat GFRα-4 conforms partly to this model, since it also contains the second and third cysteine-rich region and a possible GPI anchor (at least for variant A). However, it differs significantly from the other GFRαs in that the first cysteine-rich region is absent. This is not due to the incompleteness of the 5′ end of the obtained GFRα-4 cDNA sequence, since translation in three reading frames of 25 kb of the mouse genomic sequence upstream of the GFRα-4 coding sequence does not show any possible amino acid regions with homology to the first GFRα domain. Several short peptide motifs present in the first domain and highly conserved within known GFRαs (SPYE, CL*X*IYW, and C*X*C*X*R*X*MK) cannot be detected in any of the three reading frames in this 25-kb upstream genomic region. Moreover, this upstream region contains the known sialoadhesin and *cdc25b* genes and sequences related to the ADAM (a disintegrin and metalloproteinase) family of genes (42). This makes it unlikely that additional GFRα-4 exons are present upstream of the sequenced 25-kb genomic region. Thus, the mammalian GFRα-4 receptor is NH_2-terminally "truncated" compared with the other GFRα family members. Since we have observed persephin binding to this truncated GFRα-4 receptor, the first cysteine-rich domain seems not directly involved in ligand binding.

Signal peptide analysis shows that the signal peptide predicted for human and mouse GFRα-4 is a much better signal sequence than the one derived from our rat GFRα-4 cDNA consensus sequence. The 5′-coding sequence of the rat GFRα-4 derived from our cloned cDNAs encodes a poor signal peptide. Alternatively spliced mRNA transcripts with better signal peptides (*e.g.* corresponding to the predicted signal peptide in mouse and human GFRα-4) might be expressed in specific tissues, at specific stages in development, or under specific conditions.

In the central nervous system, GFRα-4 was shown by *in situ* hybridization to be expressed in the cortex, hippocampus, cerebellum, substantia nigra, olfactory bulb, and facial nucleus. It is not clear whether its ligand, persephin, is also expressed at significant levels in these areas, since persephin mRNA expression has been studied by reverse transcription PCR only and shown to be ubiquitous, but probably at extremely low levels (5, 33). Expression of GFRα-4 and persephin mRNA (again by

reverse transcription PCR) was observed in several peripheral tissues including heart and kidney.

The binding of GDNF family members to GFRα-4 was characterized using SPR. We have already shown that SPR can be used to characterize the binding of GFRα-IgGFc fusion proteins to GDNF, NTN, and EVN/ART (6, 24). Here we show that both rat- and chicken-soluble GFRα-4 receptors bind specifically to immobilized PSP. No other known ligand of the GDNF family (GDNF, neurturin, or enovin/artemin) showed significant binding to soluble GFRα-4. This is in agreement with the results of Enokido *et al.* (30), who showed that persephin is a ligand for chicken GFRα-4. In addition, we show that the novel rat GFRα-4 receptor binds to persephin with similar kinetics and therefore represents a mammalian orthologue of chicken GFRα-4. We did not obtain significant evidence for activation of cRET by PSP binding to GFRα-4, although we have tried to demonstrate cRET activation with several different phosphorylation assays and with a luciferase assay (data not shown). This could mean that the activation levels of cRET are too low to be detected by the used assays or, alternatively, that mammalian GFRα-4 signals through a different as yet unidentified membrane tyrosine kinase.

Limited information on the biological functions of persephin is currently available. The discovery of a mammalian persephin receptor structurally divergent from other GFRα family members together with the possible existence of membrane-anchored and soluble forms of this receptor may have important implications on the regulation of persephin function and will allow a more detailed investigation of the targets of persephin action.

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