A human serotonin 1D receptor variant (5HT1D β) encoded by an intronless gene on chromosome 6

(polymerase chain reaction/sumatriptan/guanine nucleotide-binding protein/limbic system)

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ABSTRACT An intronless gene encoding a serotonin receptor (5HT1D β) has been cloned and functionally expressed in mammalian fibroblast cultures. Based on the deduced amino acid sequence, the gene encodes a 390-amino acid protein displaying considerable homology, within putative transmembrane domains ($\approx 75\%$ identity) to the canine and human 5HT1D receptors. Membranes prepared from CHO cells stably expressing the receptor bound [3 H]serotonin with high affinity (K_d 4 nM) and displayed a pharmacological profile consistent, but not identical, with that of the characterized serotonin 5HT1D receptor. Most notably, metergoline and serotonergic piperazine derivatives, as a group, display 3- to 8-fold lower affinity for the 5HT1D β receptor than for the 5HT1D receptor, whereas both receptors display similar affinities for tryptamine derivatives, including the antimigraine drug sumatriptan. Northern blot analysis revealed an mRNA of ≈ 5.5 kilobases expressed in human and monkey frontal cortex, medulla, striatum, hippocampus and amygdala but not in cerebellum, olfactory tubercle, and pituitary. The 5HT1D β gene maps to human chromosome 6. The existence of multiple neuronal 5HT1D-like receptors may help account for some of the complexities associated with [3 H]serotonin binding patterns in native membranes.

Radioligand binding studies originally provided evidence for two distinct serotonin (5-hydroxytryptamine, 5-HT) receptor subtypes in the brain, 5HT1 and 5HT2 (1). Based on more recent pharmacological and biochemical criteria, four major 5-HT receptor families, termed 5HT1, 5HT2, 5HT3, and 5HT4 (2, 3) have been identified, with each family possibly comprising numerous receptor subtypes (4). In particular, the 5HT1 receptor family has been subdivided into three receptor populations, 5HT1A, 5HT1B, and 5HT1D (3, 5–8), primarily on the basis of their distinct pharmacological profiles and tissue distribution within the central nervous system. Of the numerous 5-HT receptors identified, five have been cloned and pharmacologically characterized: 5HT1A (9–11), 5HT1B (12), 5HT1C (13), 5HT1D (14), and 5HT2 (15, 16). These belong to a large group of neurotransmitter and peptide hormone receptors whose biological effects are mediated via guanine nucleotide-binding proteins (G proteins; see refs. 17–21).

Native membrane-bound neuronal 5HT1D and 5HT1B receptors are defined on both pharmacological and biochemical grounds by their ability to inhibit adenylate cyclase activity (22) and bind [3 H]5-HT and related analogues, such as 5-carboxamidotryptamine, with high affinity (23) and, as such, they appear more closely related to the 5HT1A receptor. The deduced amino acid sequence and pharmacological

profile of the expressed human 5HT1D receptor (14) confirm these observations. The neuronal 5HT1D receptor subtype has been identified in various mammalian species, including humans (24–26), whereas binding sites consistent with a 5HT1B pharmacological profile were originally thought to have been present only in rodent species such as rats, mice, and hamsters (7, 23). The observed species 5HT1D/1B receptor dichotomy may not be absolute, since there have been reports of 5HT1D-like receptor binding characteristics in rat brain (27–29), and a gene encoding a 5HT1D-like receptor has been isolated from rat tissues (30).

Using the polymerase chain reaction (PCR) and a strategy based on the strong sequence homology between genes encoding members of the G-protein-linked receptor family (31, 32), we report here on the cloning of an intronless gene located on chromosome 6 that encodes a protein, termed 5HT1D β , sharing amino acid homology with the recently cloned human (14) and canine (ref. 31; previously named RDC4) 5HT1D receptor. The expressed 5HT1D β receptor^{††} displays a pharmacological profile consistent with some of the observed multiple 5HT1D-like binding sites in native membranes (27, 28). The 5HT1D β receptor may possibly be involved in the attenuation of migraine, since the affinity of sumatriptan (10 nM) for this receptor correlates well with clinical plasma levels of the drug at dosages effective in producing anti-migraine relief (33). Given the high degree of structural and pharmacological homology between these receptors, we suggest that the cloned 5HT1D receptor (14) be referred to as 5HT1D α .

EXPERIMENTAL PROCEDURES

Cloning of the 5HT1D β Receptor Gene. Five microliters of a human genomic λ EMBL3-SP6-T7 library (Clontech) was denatured (95°C for 5 min) and submitted to 30 cycles of PCR with 2.5 units of *Taq* DNA polymerase (Perkin-Elmer/Cetus) and 5 μ g each of degenerate oligonucleotide primers (Biotechnology Service Center, Toronto) encoding highly conserved sequences of transmembrane domains 3 and 6 of the human dopamine receptor family (D1–D5) under conditions previously described (32). A 10- μ l aliquot of amplified DNA was removed and resubmitted for an additional 30 cycles of PCR with 2 μ g each of degenerate primer, under identical conditions. Following reamplification, DNA from each reaction was subjected to electrophoresis, and contiguous gel slices containing PCR products ranging in size from 300 to 700 base pairs (bp) were subcloned into the *Sma* I site of the

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Abbreviation: 5-HT, serotonin (5-hydroxytryptamine).

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††The nucleotide sequence reported in this paper has been deposited in the GenBank data base (accession no. M75128).

plasmid pSP73 (Promega), transformed into AG-1 cells, and selected on ampicillin plates.

Bacterial colonies were lifted onto nitrocellulose filters (Millipore) and were hybridized at 42°C for 24 hr under low-stringency conditions (37) with an [α -³²P]dCTP (NEN)-labeled fragment encoding transmembrane domains 1–5 of the dopamine D5 receptor (34). Filters were subsequently washed for 1 hr in 2× standard saline citrate (SSC)/1% SDS at 48°C. Hybridizing clones were characterized by sequence analysis with the dideoxynucleotide chain-termination method using Sequenase version 2.0 (United States Biochemical) and 7-deaza-dGTP. One isolated clone, GA5-25, revealed the presence of a 700-bp insert and displayed a deduced amino acid sequence with strong homology to the 5HT1A receptor gene family.

To obtain a full-length clone, GA5-25 was radiolabeled with [α -³²P]dCTP by nick-translation (Amersham) and used to probe a human genomic λ EMBL3 library. Three hundred thousand independent clones were lifted in duplicate on nylon filters (Colony/Plaque Screen, DuPont) and prehybridized for 16 hr at 42°C in prehybridization buffer (as above) containing 50% (vol/vol) formamide. The filters were then incubated for 24 hr at 42°C in hybridization buffer (as above). Filters were washed for 1 hr in 1× SSC/1% SDS buffer at 50°C. Four clones were isolated and analyzed by restriction endonuclease and Southern blot analysis. A strongly hybridizing 2-kilobase (kb) *Sac* I fragment (LD1) was identified, subcloned into pSP73, and sequenced.

Transfection and Ligand Binding Analysis. The 2-kb *Sac* I genomic fragment was subcloned into the expression vector pCD-PS and cotransfected with pSVneo into CHO K1 cells by using calcium phosphate (35). Positive transfectants were selected by G418 resistance. Individual colonies of positive clones were isolated and tested for receptor expression by [³H]5-HT binding. Of 10 isolated colonies, 4 were found to express [³H]5-HT binding. One cell line, KM6, was chosen and used for all pharmacological studies.

CHO-KM6 cells were harvested from plates into 50 mM Tris-HCl, pH 7.4/0.5 mM EDTA/10 mM MgSO₄ and homogenized (Kinematica Polytron). The homogenate was centrifuged at 11,000 × *g* for 20 min, resuspended in the above buffer, incubated at 37°C for 15 min, and recentrifuged for 20 min. The resulting pellets were resuspended in buffer at a protein concentration of ≈130 μg/ml. For saturation experiments, 1-ml samples of tissue homogenate were incubated in triplicate with 0.25–10 nM [³H]5-HT (23.4 Ci/mmol, NEN; 1 Ci = 37 GBq) for 30 min at 37°C in a total volume of 2 ml. For competition binding experiments, assays were initiated by the addition of 1 ml of membranes and incubated with the indicated concentrations of competing agents (10⁻¹⁰–10⁻⁵ M) and [³H]5-HT (2 nM) for 30 min at 37°C. Assays were terminated by rapid filtration and filters were monitored for ³H (26). For all experiments, nonspecific binding was defined in the presence of 10 μM 5-HT. Data were analyzed with the EBDA software (36). Protein was determined with bicinchoninic acid (BCA; Pierce).

Northern Blot Analysis. Poly(A)⁺ mRNA was isolated from various monkey brain areas (37). Samples (2 μg) were denatured in glyoxal and dimethyl sulfoxide (37), electrophoresed in a 1% agarose gel, transferred to nylon membranes (Hybond, Amersham), and probed with an [α -³²P]dCTP-labeled 2-kb *Sac* I fragment encoding the 5HT1D β receptor. Filters were hybridized under the same conditions as described for genomic library screening (see above). Filters were washed twice for 15 min in 2× SSC/1% SDS at 50°C and then twice for 15 min in 0.1× SSC/1% SDS at 50°C. The blots were exposed for 3 days at –80°C to XAR-5 film (Kodak) in the presence of one intensifying screen.

Chromosomal Location of the 5HT1D β Receptor Gene. The PCR was used to ascertain the chromosomal location of the

5HT1D β receptor gene. To ensure reaction specificity, a 300-bp region flanking both 3' translated and untranslated nucleotides was chosen for amplification. Oligonucleotide primers 5'-AAAGATGCCTGGTGGTCCACCTAGCC-3' and 5'-AGGACTATTCTGGCTTCTCAGGAT-3', 0.2 μM each, encompassing nucleotides 1021–1324 of LD1 and target DNA (250 ng) from panels of hybrid human–hamster somatic cell lines (Bios, New Haven, CT) were subjected to 30 cycles of PCR (Perkin-Elmer/Cetus GeneAMP 9600) in 1× PCR buffer (Cetus) containing 2.5 units of *Taq* polymerase and 200 μM dNTPs in a total volume of 25 μl. Each PCR cycle consisted of denaturation for 45 sec at 94°C, annealing for 45 sec at 58°C, and extension for 45 sec at 72°C. Aliquots (20 μl) of amplified DNA samples were electrophoresed in a 1.5% agarose gel containing 0.1% ethidium bromide and analyzed for the presence of a 300-bp fragment. Under the conditions used, only human genomic DNA was amplified.

RESULTS AND DISCUSSION

During our search for additional members of the dopamine receptor gene family, PCR amplification of human genomic DNA with degenerate oligonucleotide primers corresponding to conserved segments of transmembrane domains 3 and 6 of dopamine D1-like receptors generated numerous positive clones when screened under low-stringency conditions with a dopamine D5 receptor probe. These clones included the human adrenergic $\alpha_{1B,1C}$, 5HT1A, dopamine D1 and D5, histamine H2, vasoactive intestinal polypeptide (VIP), and adenosine A1 receptors. In addition, an ≈700-bp DNA fragment (GA5-25) was isolated that encoded an amino acid sequence homologous to members of the G-protein-linked 5-HT receptor family. Thus, within transmembrane domains, the amplified clone exhibited sequence homology of ≈55% with the cloned human and rat serotonin 5HT1A receptor and ≈75% with the canine and human 5HT1D receptor. Homologies of only 35% were seen with the 5HT1C and 5HT2 receptors.

The high sequence homology between clone GA5-25 and 5HT1A and 5HT1D receptors, which unlike the 5HT1C and 5HT2 receptors contain no introns in their coding sequence (5, 18), suggested that the gene encoding this novel serotonergic receptor might be intronless as well. A human genomic library in λ EMBL3 was probed with GA5-25 for a full-length clone, and following restriction endonuclease and Southern blot analysis a 2-kb *Sac* I fragment (LD1) was isolated and found to contain sequence (99.4%) identical to that of the GA5-25 clone. Complete nucleotide sequencing of the LD1 clone revealed consensus sequences for a putative initiator methionine (38) followed by a long open reading frame of 1170 nucleotides encoding a 390-amino acid protein with an estimated relative molecular mass of 43,539.

Hydrophobicity analysis of the deduced amino acid sequence of LD1 indicates the presence of seven transmembrane domains characteristic of all members of the G-protein-linked superfamily of receptors (data not shown). A comparison of the deduced amino acid sequence of LD1 (the 5HT1D β receptor) with that of other cloned 5-HT receptors (Fig. 1) shows that the greatest similarities exist between putative transmembrane domains, particularly with the human 5HT1D receptor and the canine homologue of the 5HT1D α receptor (RDC4; ref. 31), where the homology is 75%. The overall amino acid homology between the 5HT1D β receptor and other serotonergic receptors is 54% for the human 5HT1A, 38% for the rat 5HT1C and 39% for the rat 5HT2 receptor. Overall, the 5HT1D β receptor shares 55% identity with the human 5HT1D receptor.

The 5HT1D β receptor contains only one consensus site for N-linked glycosylation, at Asn²⁴, unlike the 5HT1D receptor, which contains three potential N-linked glycosylation sites in

FIG. 1. Alignment of deduced amino acid sequence of the human 5HT1D β (S1D β) receptor with other members of the 5-HT receptor family. Boxed and colored areas (pink, transmembrane; yellow, extra- and intracellular loops) denote the amino acid residues conserved between the human 5HT1D β receptor and human (14) and canine RDC4 (31) 5HT1D receptor, human (10) and rat (11) 5HT1A receptors, a cAMP-stimulatory 5-HT receptor from *Drosophila* (39), and the rat 5HT1C (13) and 5HT2 (15, 16) receptors. Single-letter amino acid code is used.

the amino terminus alone, with an additional site found in the second extracellular loop. There are several putative phosphorylation sites for protein kinase C and cAMP-dependent protein kinase A in the second and third cytoplasmic loops of the 5HT1D β receptor. The deduced amino acid sequence of the 5HT1D β receptor reveals a conserved Ser²¹² residue in transmembrane domain 5 that has been found in all members of the mammalian 5-HT receptor family and has been postulated to mediate high-affinity binding of 5-HT (40). Also conserved in the 5HT1D β receptor is Cys³⁸⁸, which is located in the carboxyl-terminal tail and has been proposed to function as a site for palmitoylation (17). This cysteine residue is not conserved in either the human or the canine 5HT1D α receptor. Further, the size and sequence of the third cytoplasmic loop of 5HT1D β , as well as the size of the carboxyl-terminal tail, are similar to those of the 5HT1D and dopaminergic D2-like receptors (37), suggesting that the 5HT1D β receptor might be coupled to G_i and inhibit the activity of adenylate cyclase.

To identify the pharmacological nature of this clone, the 2-kb *Sac* I fragment (LD1) was subcloned into the eukaryotic expression vector pCD-PS (34) and stably transfected into CHO cell lines. Four stable cell lines were isolated, of which

one (CHO-KM6) was selected for detailed pharmacological examination due to the relatively high level of receptor expression. Membranes prepared from CHO-KM6 expressing the pCD-LD1 gene product bound [³H]5-HT in a saturable manner and with high affinity, with an estimated K_d of 4 nM and a receptor density of 770 fmol/mg of protein (Fig. 2A). The rank order of potency for the inhibition of [³H]5-HT binding to membranes of CHO-KM6 by various serotonergic agonists and antagonists is listed in Table 1 and illustrated in Fig. 2B. Tryptamine derivatives including 5-HT, 5-carboxamidotryptamine, and sumatriptan displayed high affinity for the transfected receptor, similar to the affinities reported for the 5HT1D receptor expressed in CHO cells (14). Significant differences were seen in the affinity of metergoline and piperazine derivatives such as spiperone, 1-(*m*-trifluoromethylphenyl)piperazine, and 1-(*m*-chlorophenyl)piperazine, with estimated dissociation constants $\approx$ 4-fold lower than those observed for the 5HT1D receptor. Further, the expressed receptor can interact with G proteins: a concentration-dependent inhibition of high-affinity [³H]5-HT binding was observed in these cells in the presence of guanine nucleotides, but not their adenylyl analogues (Fig. 2C). Based on these considerations the LD1 gene product appears to be a 5HT1D receptor variant, which we term 5HT1D β .

FIG. 2. Pharmacological profile of the 5HT1D β receptor. (A) Saturation isotherm of [3 H]5-HT binding to membranes prepared from CHO cells transfected with pCD-LD1. Results represent an experiment conducted in triplicate. Estimated $B_{\max}$ (770 fmol/mg of protein) and K_d (4 nM) values were determined by computer-assisted analysis (8). (Inset) Scatchard plot of the same data. (B) Pharmacological specificity of [3 H]5-HT binding to pCD-LD1-transformed CHO cells. Representative curves are illustrated for the concentration-dependent inhibition of [3 H]5-HT binding (≈ 2 nM) with various serotonergic agonists and antagonists. 5-CT, 5-carboxamidotryptamine; Methy, methysergide; mCPP, 1-(*m*-chlorophenyl)piperazine; TFMPP, 1-(*m*-trifluoromethylphenyl)piperazine. Estimated inhibitory constants (K_i) for each compound, listed in Table 1, were determined by EBDA (36) and the results shown are means of triplicate determinations and represent two independent experiments. (C) Guanine nucleotide regulation of [3 H]5-HT binding to membranes of CHO cells expressing the 5HT1D β receptor. Inhibition of [3 H]5-HT (2 nM) binding to membranes prepared from cells transformed with the LD1 gene is dependent on the concentration of

Table 1. Drug affinities for [3 H]5-HT binding to cloned 5HT1D β and 5HT1D receptors

Drug*	K_i , nM		1D β /1D
	5HT1D β	5HT1D	
5-CT	0.9 $\pm$ 0.1	0.99	0.9
Methiothepin	3.1 $\pm$ 1.0	5.80	0.53
5-HT	4.0 $\pm$ 1.9	8.90	0.45
Metergoline	5.2 $\pm$ 1.5	0.61	8.52
Methysergide	7.6 $\pm$ 0.6		
Sumatriptan	11.0 $\pm$ 0.7	3.7	2.97
Yohimbine	41.2 $\pm$ 0.2	59.0	0.69
TFMPP	247 $\pm$ 36	85.0	2.90
mCPP	501 $\pm$ 53	210	2.38
8-OH-DPAT	634 $\pm$ 11	300	2.11
Mesulergine	1284 $\pm$ 275	1700	0.75
Ketanserin	1908 $\pm$ 372		
DOI	3200 $\pm$ 964		
Sipiperone	>10,000	3100	
Spiroxastrine	>10,000		
Dopamine	>10,000		
(-)-Propranolol	>10,000		

Inhibitory constants of various serotonergic agonists and antagonists are listed in order of their potency for the human 5HT1D β receptor. Values represent the means of experiments conducted in triplicate. K_i values for the 5HT1D receptor were taken from ref. 14. *5-CT, 5-carboxamidotryptamine; TFMPP, 1-(*m*-trifluoromethylphenyl)piperazine; mCPP, 1-(*m*-chlorophenyl)piperazine; 8-OH-DPAT, ($\pm$)-8-hydroxy-*N,N*-dipropyl-2-aminotetralin; DOI, ($\pm$)-1-(2,5-dimethoxy-4-phenyl)-2-aminopropane.

Northern blot analysis of poly(A) $^+$ RNA from various regions of monkey brain, using the 2-kb *Sac* I fragment of LD1 as probe, revealed mRNA transcripts in frontal cortex, striatum, medulla, and amygdala (Fig. 3). Similarly, poly(A) $^+$ mRNA isolated from human striatum, hippocampus, and frontal cortex indicated the presence of 5HT1D β receptor transcripts, with no detectable signal observed with RNA from cerebellum or pituitary (data not shown). The distribution of 5HT1D β receptor mRNA correlates well with the known localization of 5HT1D-like receptors in mammalian brain, with the highest levels of receptor expression observed in limbic regions and the basal ganglia (7, 23). It is unclear whether the 5HT1D and the 5HT1D β receptor mRNA have similar or distinct expression patterns within the central nervous system. To gain some understanding of the possible functional significance of the 5HT1D and 5HT1D β receptors, the relative distribution of these mRNAs in the human brain could be determined by *in situ* hybridization histochemistry.

PCR analysis of two panels of hybrid human-hamster somatic cell lines for the 5HT1D β receptor showed chromosome 6 to be the only human chromosome to segregate perfectly with the gene. Moreover, the presence of a restriction fragment length polymorphism for the 5HT1D β receptor (D. Sidenberg, L.D., H.B.L., and J.L.K., unpublished data) may aid in mapping the exact chromosomal loci of the 5HT1D β gene and in identifying the role this receptor gene plays in the maintenance and expression of various human disease states involving the serotonergic system.

While this manuscript was under review, two groups have reported on the cloning and expression of the human 5-HT receptor variant described here (41, 42) and of the rat 5HT1B receptor (43, 44). Although the rat 5HT1B and human 5HT1D β receptor (also termed 5HT1B; ref. 41) share an amino acid sequence homology of >93%, we concur with arguments based on both molecular and pharmacological

the nonhydrolyzable GTP analogues guanosine 5'-[γ -thio]triphosphate (GTP γ S) and guanosine 5'-[β , γ -imido]triphosphate (GPPNHP) but not on the concentration of the adenyl analogues.

FIG. 3. Northern blot analysis of 5HT1D β mRNA in monkey brain areas. Poly(A)⁺ RNAs were prepared and samples (2 μ g per lane) were denatured, electrophoresed through agarose, transferred to nylon membranes, and hybridized with a 2-kb nick-translated *Sac* I restriction fragment encoding the entire 5HT1D β receptor gene (7 $\times$ 10⁶ cpm/ml). Nylon membranes were exposed for 3 days at -80°C with an intensifying screen. Sizes of RNA markers (kb) are shown at left.

grounds (42, 44), that the human receptor be classified as a member of the 5HT1D-like family.

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