Cloning of the cDNA and gene for a human D₂ dopamine receptor

(schizophrenia/alternative splicing/DRD2/introns/receptor evolution)

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ABSTRACT A clone encoding a human D₂ dopamine receptor was isolated from a pituitary cDNA library and sequenced. The deduced protein sequence is 96% identical with that of the cloned rat receptor with one major difference: the human receptor contains an additional 29 amino acids in its putative third cytoplasmic loop. Southern blotting demonstrated the presence of only one human D₂ receptor gene. Two overlapping phage containing the gene were isolated and characterized. DNA sequence analysis of these clones showed that the coding sequence is interrupted by six introns and that the additional amino acids present in the human pituitary receptor are encoded by a single exon of 87 base pairs. The involvement of this sequence in alternative splicing and its biological significance are discussed.

Dopamine neurons in the vertebrate central nervous system are involved in the initiation and execution of movement, the maintenance of emotional stability, and the regulation of pituitary function. Several human neurological diseases, including Parkinson disease (1) and schizophrenia (2), are thought to be manifestations of imbalances between dopamine receptors and dopamine. The receptors that mediate dopamine's effects have been divided into D₁ and D₂ subtypes, which are distinguished by their guanine nucleotide-binding protein (G-protein) coupling (3, 4), ligand specificities, anatomical distribution, and physiological effects (5). The D₂ dopamine receptors have been of particular clinical interest due to their regulation of prolactin secretion (6) and their affinity for antipsychotic drugs (7, 8).

The D₂ dopamine receptors belong to the family of G-protein-coupled receptors. The sequence similarity shared by members of this family enabled Bunzow *et al.* (9) to clone a rat brain D₂ dopamine receptor cDNA. We have used that clone to isolate the human pituitary D₂ dopamine receptor cDNA described here.[§] We have found that the deduced amino acid sequences of these two receptors are very similar, with one notable difference. The human receptor contains an additional 29 amino acids in its putative third cytoplasmic loop that are encoded by one of the gene's exons. This genomic organization suggests that the existence of two D₂ receptor mRNAs is the result of an alternative splicing event.

MATERIALS AND METHODS

Cloning of the Human Pituitary cDNA. Human pituitary tissue was a generous gift from N. Seidah and M. Chretien (Clinical Research Institute of Montreal). Poly(A)⁺ mRNA and cDNA were prepared as described (9). The cDNA was size-selected [1–6 kilobases (kb)] in agarose gels, isolated using GeneClean (Bio 101, La Jolla, CA), ligated to *Eco*RI

adaptors, cloned into λ gt10 arms (Stratagene), and packaged (Gigapack Gold, Stratagene). Recombinants (1.5×10^6) were screened on replica nylon filters (DuPont Colony/Plaque Screen hybridization filters) with ³²P-labeled hybridization probes. Prehybridization and hybridization were performed in 50% formamide/1% SDS/2× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7) at 37°C. The complete sequences of both strands of DNA were determined in M13mp19 by using Sequenase (United States Biochemical) primed with synthetic oligonucleotides.

Expression and Pharmacology. The 2.5-kb human pituitary cDNA (hPitD₂) was cloned into pZem3 (a gift from E. Mulvihill, Zymogenetics, Seattle) and cotransfected with pRSVneo into thymidine kinase-deficient mouse cells (Ltk⁻ cells) by calcium phosphate precipitation (10). A stable transfectant (L-hPitD₂Zem) was selected and maintained in medium containing G418 sulfate (Geneticin; GIBCO) at 750 μ g/ml. Twenty hours prior to the harvesting of membranes, these cells were incubated with 70 μ M zinc sulfate. Membranes were prepared from L-hPitD₂Zem, from the Ltk⁻ cell line expressing the cloned rat D₂ dopamine receptor (L-RGB2Zem-1), and from freshly dissected rat striata (Taconic Farm, Germantown, NY) as described (11, 12). For the binding assays, membrane protein was used at 10–15 μ g from L-hPitD₂Zem, 50–75 μ g from L-RGB2Zem-1, and 200–250 μ g from rat striatum. The binding assays were incubated at 37°C for 60 min in 50 mM Tris-HCl/120 mM NaCl/5 mM KCl/2 mM CaCl₂/1 mM MgCl₂, pH 7.4, and then stopped by rapid filtration over glass-fiber filters (no. 32; Schleicher & Schuell) that had been presoaked in 0.5% polyethyleneimine. The filters were washed twice in ice-cold 50 mM Tris-HCl, pH 7.4. Saturation curves were generated using increasing concentrations of the highly D₂-specific antagonist [³H]domperidone (13). Antagonist drugs were evaluated for their ability to inhibit specific binding of [³H]domperidone (1 nM). The $B_{\max}$, K_d , and IC_{50} values were determined as described (14).

Southern Blotting. Human genomic DNA prepared from a normal male donor was a gift from M. Litt. Three micrograms of DNA was digested with restriction enzymes and the fragments were electrophoresed in 0.7% agarose and blotted onto nitrocellulose filters (Schleicher & Schuell). Prehybridization and hybridization were performed at 37°C in 50% formamide as described (15).

Genomic Sequencing. Genomic bacteriophage λ libraries, prepared from normal human male DNA, were purchased from Stratagene and Clontech and screened with portions of the cloned rat D₂ dopamine receptor cDNA. DNA sequence was determined by a genomic sequencing approach (16, 17). Briefly, for each restriction enzyme used, cloned genomic

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Abbreviation: G protein, guanine nucleotide-binding protein.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M29066).

phage DNA (50 μ g) was digested and subjected to chemical cleavage, and the resulting fragments were resolved in a denaturing polyacrylamide gel. The DNA was then transferred from the gel and immobilized onto nylon filters (Gene-Screen; Plasco, Woburn, MA). Using 32 P-end-labeled synthetic oligomers, ladders of sequence were visualized within exons and read into neighboring introns. Then either the filters were

reprobed with a different oligomer or a new filter was made in order to read the complementary sequence back across the exon. Both strands of the coding region were sequenced.

RESULTS

Cloning and Sequence Analysis of the Human Pituitary cDNA. By using the rat brain D₂ dopamine receptor cDNA as

FIG. 1. Nucleotide sequence of the human pituitary D₂ receptor cDNA. The deduced amino acid sequence is indicated above the human cDNA sequence. Below is the nucleotide sequence of the cloned rat cDNA (9) and the amino acids that differ between the two clones. Shaded regions, numbered I-VII, represent the putative transmembrane domains. Triangles indicate the exon/intron splice junctions; a period is one missing base pair; asterisks identify potential N-linked glycosylation sites; targets of protein kinase A phosphorylation are underlined. The polyadenylation signal is double underlined.

FIG. 2. Competition curves of [3 H]domperidone binding to L-hPitD₂Zem membranes. The radioligand was used at a concentration of 1 nM and specific binding was defined using 1 μ M (+)-butaclamol. Data are shown for one of three experiments.

probe, three partial cDNAs were isolated from a human pituitary library and sequenced. Two oligonucleotide probes based on these sequences were used to isolate a fourth cDNA, hPitD₂, which encoded a full-length receptor protein (Fig. 1). The human pituitary receptor contains seven putative transmembrane domains and lacks a signal sequence. Overall the human and rat nucleotide sequences are 90% identical and show 96% identity at the amino acid level. Several consensus sequences for N-linked glycosylation, protein kinase A phosphorylation, and palmitoylation (18) are conserved between the human and rat receptors. There are also 18 amino acid differences (including one deletion) between these proteins and, strikingly, the human pituitary receptor contains an additional 29 amino acids in its putative third cytoplasmic loop.

Expression and Pharmacological Evaluation of hPitD₂ cDNA. To evaluate the pharmacological characteristics of the human pituitary receptor, its cDNA was subcloned into pZem3 and expressed in mouse Ltk⁻ cells (L-hPitD₂Zem). Membranes prepared from these cells showed specific binding of [3 H]domperidone, a D₂-selective antagonist (12, 13), with a $B_{\max}$ of 4.05 ± 0.3 pmol per mg of protein ($n = 2$) and a K_d of 0.74 ± 0.11 nM ($n = 2$). This K_d value is in excellent agreement with the published value of 0.74 nM in mouse brain membranes (13). A Scatchard plot of the data was linear. There was no detectable [3 H]domperidone binding in membranes prepared from cells transfected with pZem3 alone (data not shown). [3 H]Domperidone binding to L-hPitD₂Zem membranes was inhibited by a number of D₂-specific drugs (Fig. 2). Their rank order of potency was spiperone > (+)-butaclamol > haloperidol > sulpiride. The selective serotonin antagonist mianserin and the D₁-selective antagonist SCH 23390 inhibited domperidone binding only at very high concentrations, as did the inactive isomer (-)-butaclamol. These values are essentially identical to those obtained with membranes from Ltk⁻ cells transfected with the cloned rat D₂ dopamine receptor cDNA (L-RGB2Zem-1) and from rat striatum (Table 1).

Table 1. Comparison of IC₅₀ values (nM) for L-hPitD₂Zem, L-RGB2Zem1, and rat striatum

Drug	Human D ₂	Rat D ₂	Rat striatum
Spiperone	0.125	0.35	0.56
(+)-Butaclamol	0.94	1.2	1.6
Haloperidol	2.4	5.1	5.8
Sulpiride	206	160	205
Mianserin*	2,685	4,300	4,600
SCH 23390†	2,145	2,500	3,300
(-)-Butaclamol	>10,000	>10,000	>10,000
[3 H]Domperidone (K_d)	0.74	0.40	0.40

*Serotonin antagonist.

†D₁-selective dopamine antagonist.

FIG. 3. Southern blot of human genomic DNA. The genomic 1.6-kb *Bam*HI fragment containing exon 7 was prepared from λ H2G1 and used as probe (specific activity, 2×10^8 cpm/ μ g). DNA was digested with *Bam*HI (lane 1), *Bgl*II (lane 2), *Bam*HI/*Bgl*II (lane 3), or *Hind*III (lane 4).

Human D₂ Dopamine Receptor Gene. By using portions of the rat cDNA as probe, a clone was isolated from a human genomic library. This genomic clone, λ H2G1, contained a 1.6-kb *Bam*HI fragment that encoded the last 64 amino acids of the human D₂ receptor and 1.2 kb of 3' noncoding sequence. The 1.6-kb fragment was used to probe a Southern blot of human genomic DNA digested with three restriction enzymes. Each enzyme generated a single fragment that hybridized to the probe (Fig. 3) indicating that there is probably only one human D₂ dopamine receptor gene.

To isolate a genomic clone that encoded the N terminus of the human receptor protein, a 118-base-pair (bp) restriction fragment from the cloned rat D₂ receptor cDNA (corresponding to amino acid residues 1–39) was used to screen a second genomic library. λ H2G2 was isolated and found to overlap with λ H2G1 by 400 nucleotides (Fig. 4a). Together, these phage span 34 kb of the human D₂ dopamine receptor gene locus, *DRD2* (19), and contain the sequence found in the hPitD₂ cDNA plus sequences that extend 15 kb downstream of the polyadenylation signal and 3.7 kb upstream of the translation initiation site. To characterize the intron/exon structure of the gene, a genomic sequencing approach employing oligonucleotide probes and chemical cleavage was used (Fig. 4b). Since the divergence of nucleotide sequences between human and rat members of this receptor family is $\approx 10\%$ (unpublished observations), we were able to initiate the genomic sequencing by relying on hybridization probes and restriction sites that are present in the cloned rat D₂ receptor cDNA. Our results demonstrate that the coding portion of the human D₂ receptor gene is divided into seven exons (Fig. 4b). Interestingly, we found that exon 5 is 87 bp long and encodes the entire 29-amino acid sequence present in the cloned human pituitary receptor (Figs. 1 and 4c). Analysis of the six introns revealed that each contains acceptor and donor sequences that conform to the GT/AG rule (20), as summarized in Fig. 5. The approximate sizes of the introns were based on the results of Southern blotting experiments (data not shown). When compared, the genomic and cDNA sequences were found to differ by only two silent transitions, one at 939 (T to C in the gene) and the other at 957 (C to T in the gene).

FIG. 4. Schematic representation of the human D_2 dopamine receptor gene and pituitary cDNA. (a) Restriction map of the two overlapping genomic phage, λ hD2G1 and λ hD2G2; A, *Apa*LI; B, *Bam*HI; Bg, *Bgl* II; H, *Hind*III. (b) Diagram of the human gene locus *DRD2*. Exons, indicated by the boxes, are numbered. The solid boxes indicate regions of coding sequence, and open boxes, nontranslated sequence. The genomic sequencing strategy is expanded above the gene. These regions are not drawn to scale. DNA sequences (+, sense sequence; -, antisense sequence) were read from the bottom to the top of each ladder in the directions indicated by the arrows (pointing to the right, 5' to 3', and to the left, 3' to 5'). The 87-bp exon is number 5. Intron sizes were determined by Southern blotting and restriction mapping and are accurate to within 10%, with one exception: intron 1 is accurate to within 20%. (c) The structure of the human pituitary cDNA. Exon/intron splice junctions are indicated by arrowheads. Regions encoding transmembrane domains I–VII are enclosed by hatched boxes. The 87-bp sequence is vertically striped. The sites for translation initiation (ATG) and termination (TAG) are indicated, as is the polyadenylation signal sequence (AATAAAA).

DISCUSSION

Several alternative hypotheses might account for the extra sequence present in our human pituitary D_2 receptor cDNA clone. One possibility is that the human gene contains the extra 87 bases and that the rat gene does not. Another is that both human and rat have two distinct genes that code for two different D_2 dopamine receptors. Finally, alternative splicing of a single transcript could result in one mRNA with and the other without the 87 bases. In support of the latter hypothesis we have shown that there is probably only one human D_2 dopamine receptor gene, *DRD2*, and that the 87-bp sequence is contained on a distinct exon of that gene. Furthermore, we have cloned a rat brain cDNA that contains the 87-bp sequence (unpublished results). This sequence is highly sim-

ilar to that of the human cDNA and establishes that D_2 receptors containing the 29 residues are not unique to the human pituitary.

The human D_2 receptor expressed in L-hpitD₂Zem cells has essentially the same drug binding profile as do rat striatum and L-RGB2Zem-1 membranes. Therefore, since the 29 amino acids do not affect binding they may be involved in other levels of receptor function. For example, the third cytoplasmic loop of the β_2 -adrenergic receptor has been shown to be required for appropriate G-protein coupling (21). Therefore, one possibility is that this sequence may influence whether D_2 receptor stimulation inhibits adenylate cyclase, activates potassium channel conductance, or inhibits calcium mobilization (22). Another possibility is that the 29 amino acids differentiate postsynaptic D_2 receptors from presynaptic autoreceptors (23). Since a computer search (VAX/IntelliGenetics; September 1989) failed to identify another sequence of significant homology, we consider this sequence to be unique to the D_2 receptor.

Based on the comparison of genomic and cDNA sequences, the human D_2 dopamine receptor gene is divided into at least seven exons. It is possible that one or more additional exons remain to be identified at the 5' end of the gene as was shown to be the case with muscarinic receptor genes (24).

The interruption of coding sequence by introns distinguishes the human D_2 receptor gene from most other members of the G-protein-coupled receptor gene family with the exception of the opsin genes (25). One significant observation is that the placement of two introns (numbers 3 and 5) in this human gene corresponds almost precisely to intron positions conserved in bovine and *Drosophila* opsin genes (26, 27). The simplest interpretation of this finding is that their common ancestor, a gene roughly one billion years old (28), contained those introns. Our characterization of the gene structure also provides evidence that the exons encode recognizable elements of

285	286
exon1...GGAGgtagtg...	intron1...tcccagGTGG...exon2
395	396
exon2...ACAGgtgagcc...	intron2...cttgacagGTAC...exon3
532	533
exon3...GCAGgtacatt...	intron3...ccccagACCA...exon4
723	724
exon4...AAAGgtctcaa...	intron4...tcacagGGCA...exon5
810	811
exon5...AGTGtaagtg...	intron5...ggtgcagGAGG...exon6
1138	1139
exon6...CTCGgtgagtc...	intron6...ccccagGCGT...exon7

FIG. 5. Exon/intron junctions in the human D_2 dopamine receptor gene. Numbering begins with A of the putative initiation codon (see Fig. 1).

protein structure (29). That introns are found following transmembrane segments II, III, and IV (see Figs. 1 and 4) argues that the repeated structural motifs characteristic of these receptors may have evolved by internal duplication. Furthermore, the presence of several introns within the third cytoplasmic loop provides an explanation of the substantial variation in length observed across the family (30).

The possibility of alternatively spliced D₂ dopamine receptor mRNAs giving rise to structurally distinct forms of receptor is exciting. The expression of one form of the D₂ receptor mRNA or another represents a level of control that may have implications with respect to human disease.

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