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Cloning and Characterization of the Human Corticotropin-Releasing Factor-2 Receptor Complementary Deoxyribonucleic Acid

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ABSTRACT

Two CRF receptor subtypes (CRF_1 and CRF_2 receptors) with distinct brain localizations and pharmacological profiles have recently been cloned and characterized. For the CRF_2 receptor subtype, at least 2 splice forms with different 5′-coding sequences ($CRF_{2\alpha}$ and $CRF_{2\beta}$) have been identified in rat. In this article, we report the genomic structure and the corresponding complementary DNA (cDNA) sequence of the human CRF_2 receptor. The gene coding for human CRF_2 receptor consists of at least 12 exons and spans approximately 30 kilobases. The cDNA sequence in the protein-coding region is 94% identical to that of the reported rat $CRF_{2\alpha}$ receptor. At present, there is no evidence for the existence of a $CRF_{2\beta}$ receptor homolog in humans. The encoded receptor is 411 amino acids in length and is 70% identical to the human CRF_1 receptor, with least sequence homology in the N-terminal extracellular domain (47% identical). Cells transfected with the full-length human CRF_2 receptor cDNA responded to rat/human CRF and sauvagine by increasing the intracellular cAMP level, with EC_{50} values of approximately 20 and 1 nM, respectively. The CRF- and sauvagine-induced accumulation of intracellular cAMP could be competitively inhibited by the CRF receptor antagonist D-Phe-CRF. This pharmacological profile was comparable to that of the rat $CRF_{2\alpha}$ receptor. The relative abundance of the CRF_2 receptor messenger RNA appears to be lower in humans than in rats for the tissues studied thus far. (*Endocrinology* **137:** 72–77, 1996)

CRF, A 41-AMINO acid hypothalamic peptide (1), is the main regulator of release of ACTH and other POMC-derived peptides from the anterior pituitary. In addition to its endocrine role at the pituitary, CRF has been shown to function as a neurotransmitter in brain to integrate the electrophysiological, autonomic, and behavioral responses to stress (2–4). Localization of CRF and its binding sites in immune cells and inflamed tissues also supports autocrine/paracrine roles for CRF in the immune system (5–7).

CRF mediates its effects by binding to high affinity membrane receptors that are coupled to G_s and transduces its signal through stimulation of cAMP production (8–13). The complementary DNAs (cDNAs) for one CRF receptor subtype (CRF_1 receptor) with a high degree of interspecies homology were recently cloned from human pituitary (14) and brain (15), mouse pituitary (15), and rat brain (16, 17). A second CRF receptor (CRF_2 receptor) with distinct brain localization, tissue distribution, and pharmacological profile was recently cloned from a rat hypothalamus cDNA library (18) and a mouse heart cDNA library (19, 20). In the rat, two splice forms of CRF_2 receptor ($CRF_{2\alpha}$ and $CRF_{2\beta}$) with distinct tissue distributions were identified (18). The $CRF_{2\alpha}$ receptor is expressed predominantly in brain, whereas the $CRF_{2\beta}$ receptor shows the highest level of expression in heart and skeletal muscle and to a lesser extent is expressed in brain, lung, and intestine (21). In the mouse, only the homolog of the rat $CRF_{2\beta}$ form has been identified (19, 20). To determine the physiological role of CRF_2 receptor in humans, it was necessary to obtain the cDNA encoding the human homolog. In this article, we report the genomic structure and putative cDNA sequence of the human CRF_2 receptor. We demonstrate the functionality of the putative full-length cDNA by transfecting the cDNA into VIP2.0Zc cells (22), a cell line containing a cAMP-responsive β-galactosidase (β-gal) reporter gene, and show that the transfected cells respond to CRF with increasing intracellular cAMP and, thus, β-gal activity. The human CRF_2 receptor is shown to have a structure and pharmacological profile similar to those of the rat $CRF_{2\alpha}$ receptor. The cDNA and genomic sequences encoding a human homolog of rat $CRF_{2\beta}$ remain to be identified.

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Materials and Methods

Materials

The human genomic library and cDNA libraries from frontal cerebral cortex, cerebellum, hippocampus, and heart were obtained from Stratagene (La Jolla, CA); the human fetal brain, hypothalamus, amygdala, skeletal muscle, and lung cDNA libraries were purchased from Clontech (Palo Alto, CA); the human kidney cDNA library was obtained from Dr. Daniel J. Noonan (University of Kentucky, Lexington, KY); diethylaminoethyl (DEAE) dextran and chloroquine were obtained from Sigma Chemical Co. (St. Louis, MO); chlorophenol red β-D-galactopyranoside was purchased from Boehringer Mannheim (Indianapolis, IN); Sequenase was purchased from U.S. Biochemical Corp. (Cleveland, OH); rat/human CRF (r/hCRF) and [D-Phe^{12},Nle^{21,38},Ala^{32}]r/hCRF (D-Phe-CRF) were synthesized in-house; the LVIP2.0Zc cell line was a generous gift from Drs. Michael J. Brownstein and Monika König, Laboratory of Cell Biology, NIMH; human CRF_1 receptor cDNA was kindly provided by

FIG. 1. Genomic structure of human CRF_2 receptor. The human CRF_2 receptor gene spans approximately 30 kb, with the protein-coding sequence interrupted by 11 introns (I1 to I11). The exons (E1 to E12) are indicated by *vertical bars*. The sizes of each exon and intron are shown in *parentheses*. 5'UT, 5'-Untranslated region; 3'UT, 3'-untranslated region.

Dr. Wylie W. Vale, The Clayton Foundation Laboratories, The Salk Institute (La Jolla, CA).

Library screening

Using the entire rat CRF_2 receptor cDNA as probe, between 1–2 million phage plaques from each of the following human cDNA libraries were screened: frontal cerebral cortex, hippocampus, cerebellum, hypothalamus, amygdala, fetal brain, heart, lung, skeletal muscle, and kidney. Approximately one half-million phage plaques from the human genomic library were screened. Positive clones were plaque purified, subcloned, and sequenced by the dideoxy chain termination method using Sequenase.

RT-PCR

Polyadenylated RNA was isolated from human frontal cerebral cortex, reverse transcribed, dG-tailed, and used as template for first round PCR, as previously described (18). The primer with nucleotide sequence 5'-GTGGCCCAGGTAGTTGACGACA-3' (corresponding to the complementary strand of nucleotides 351–372 in Fig. 2) in combination with a $d(C)_{18}$ oligonucleotide [5'-ACTGTAAGCTT$(C)_{18}$-3'] were used in the first round PCR. The products of this reaction served as template for a second round of amplification reaction using the same $d(C)_{18}$ oligonucleotide and an internal primer with a sequence of 5'-GGATCCTGGGCTCACACTGTGAGTAGT-3' (corresponding to the complementary strand of nucleotides 281–301 attached to a *Bam*HI restriction endonuclease recognition sequence). The products of this reaction was probed by Southern blot analysis using an oligonucleotide corresponding to the first 20 nucleotides of coding sequence based on genomic sequence. Regions that showed positive hybridization signals were isolated, digested with *Hin*dIII and *Bam*HI, and subcloned into pBluescript (Stratagene) for sequence analysis.

Cell transfection

As screening of cDNA libraries failed to yield a cDNA clone containing the most 5'-coding sequence, the full-length cDNA was constructed by filling the missing 5'-sequence with synthetic oligonucleotides based on the sequence information obtained from the genomic clone and from RT-PCR using human frontal cerebral cortex messenger RNA (mRNA). The full-length cDNA was then subcloned into an expression vector under the control of Rous sarcoma virus promoter and transfected into VIP2.OZc cells by either the standard DEAE dextran method (transient transfection) or the calcium phosphate coprecipitation method (stable transfection). For transient transfection, VIP2.OZc cells were plated at 3×10^6 cells/10-cm dish on the day before transfection. The cells were exposed to serum-free DMEM containing 100 μg/ml DEAE dextran, 100 μM chloroquine, and 10 μg DNA for 3–4 h; washed with serum-free DMEM; and replenished with fresh complete medium. The transfected cells were plated onto a 96-well plate at 5×10^4 cells/well 24 h after transfection and assayed for CRF responsiveness 48 h after transfection. For stable transfection, the cells were cotransfected with human CRF_2 receptor cDNA and pSV2Neo, and the resulting geneticin-resistant cells were selected and cloned, as previously described (23).

Functional assay of the cloned receptor

For indirect measurement of intracellular cAMP through reporter β-gal gene, the transfected VIP2.0Zc cells in 96-well plates were exposed to r/hCRF for 7–8 h, and the cAMP-induced β-gal activity was assayed, as previously described (22). In short, the cells were lysed by a hypotonic solution, and the assay buffer and substrate (5 mg/ml chlorophenol red β-D-galactopyranoside) solution were added. The color reaction was developed at 37 C for 30 min before the optical density at 595 nm was measured with a Bio-Rad microplate reader (Bio-Rad Laboratories, Richmond, CA). In cells stably transfected with either human CRF_1 or CRF_2 receptor cDNA, intracellular cAMP levels were measured directly by RIA, as previously described (18). All experiments were performed in triplicate.

Results

Cloning of human CRF_2 receptor gene and cDNA

In the rat, CRF_2 receptor message has been detected in discrete brain regions (hypothalamus, cerebral cortex, septum, and amygdala) by *in situ* hybridization and in some peripheral tissues, such as lung, heart, skeletal muscle, and kidney, by PCR (18) and RNA protection analysis (21). Initial screening of the human frontal cerebral cortex cDNA library yielded two overlapping cDNA clones. Compared to the cDNA sequences of the rat CRF_2 receptor, one of the two clones appeared to contain two introns, and overall, these two clones yielded 1023 nucleotides of 3'-end coding sequence and approximately 1 kilobase (kb) of 3'-untranslated region. To search for the missing 5'-coding sequences, four additional cDNA libraries from different brain regions, one fetal brain cDNA library, and four peripheral tissue cDNA libraries were screened. Two independent cDNA clones were obtained from the kidney cDNA library. However, both clones contained less than 200 bp of coding sequences and

mainly intron sequences. The fetal brain library yielded one cDNA clone, which again contained mainly intron sequences with 306 bp of coding sequence. None of the kidney or fetal brain cDNAs gave more coding sequence information than the frontal cerebral cortex cDNAs.

The limited number of cDNA clones and coding information obtained from these cDNA libraries prompted us to screen a genomic library to determine whether the gene coding for the human CRF_2 receptor fully encodes the information for a functional receptor. Three positive clones were analyzed by restriction mapping and Southern blot analysis, and the regions containing coding information were sequenced. As shown in Fig. 1, the gene coding for human CRF_2 receptor spans approximately 30 kb, with 12 exons (assuming there are no introns in the 5′-untranslated region) and 11 introns including 1 of approximately 14.5 kb in size. When appropriately spliced, it should yield a message with 1233 bp of open reading frame, with no difference from the available corresponding cDNA sequence. All exon/intron junctions conform with the consensus sequences, with exons flanked by AG and GT at 5′- and 3′-ends, respectively. To confirm the intron/exon junctions for the 5′-coding region predicted from genomic clones, RT-PCR was performed using human frontal cerebral cortex mRNA as template. By subcloning and sequencing the PCR product, we were able to identify several clones containing the fully processed 5′-cDNA of the human $CRF_{2\alpha}$ receptor.

The full-length cDNA coding sequence and the putative protein sequence are shown in Fig. 2. The first 213-nucleotide sequence was derived from the genomic clone and RT-PCR. As expected, the putative human CRF_2 receptor contains 8 hydrophobic regions, including the N-terminal signal peptide and 7 putative transmembrane domains. The receptor protein showed 94% sequence identity to its rat homolog, with all 5 potential glycosylation sites and cysteine residues conserved. The protein sequences of human CRF_1 and CRF_2 receptor were 70% identical, with greater sequence homology toward the C-terminus and only 47% identity in the N-terminal extracellular domain.

Functional assay of the cloned receptor

The full-length human CRF_2 receptor cDNA was subcloned into an expression vector and transfected into VIP2.0Zc cells, a cell line containing a cAMP-responsive β-gal gene. The transfected cells responded to r/hCRF and sauvagine by increasing β-gal activity in a dose-dependent manner, as shown in Fig. 3A. The EC_{50} values for r/hCRF and sauvagine were 20 and 1 nM, respectively. For comparison, we also transfected the human CRF_1 receptor cDNA into VIP2.0Zc cells. As shown in Fig. 3B, both r/hCRF and sauvagine had higher affinity for the CRF_1 receptor than for the CRF_2 receptor, as measured by cAMP responsiveness. The EC_{50} values of r/hCRF and sauvagine for the CRF_1 receptor were both approximately 0.1 nM. To confirm the above results obtained by indirect assay of cAMP accumulation, we also measured the intracellular cAMP levels of a VIP2.0Zc cell line stably expressing human CRF_2 receptor in response to r/hCRF and sauvagine directly by RIA (Fig. 3C). As a control, Fig. 3D shows the stimulation of intracellular cAMP

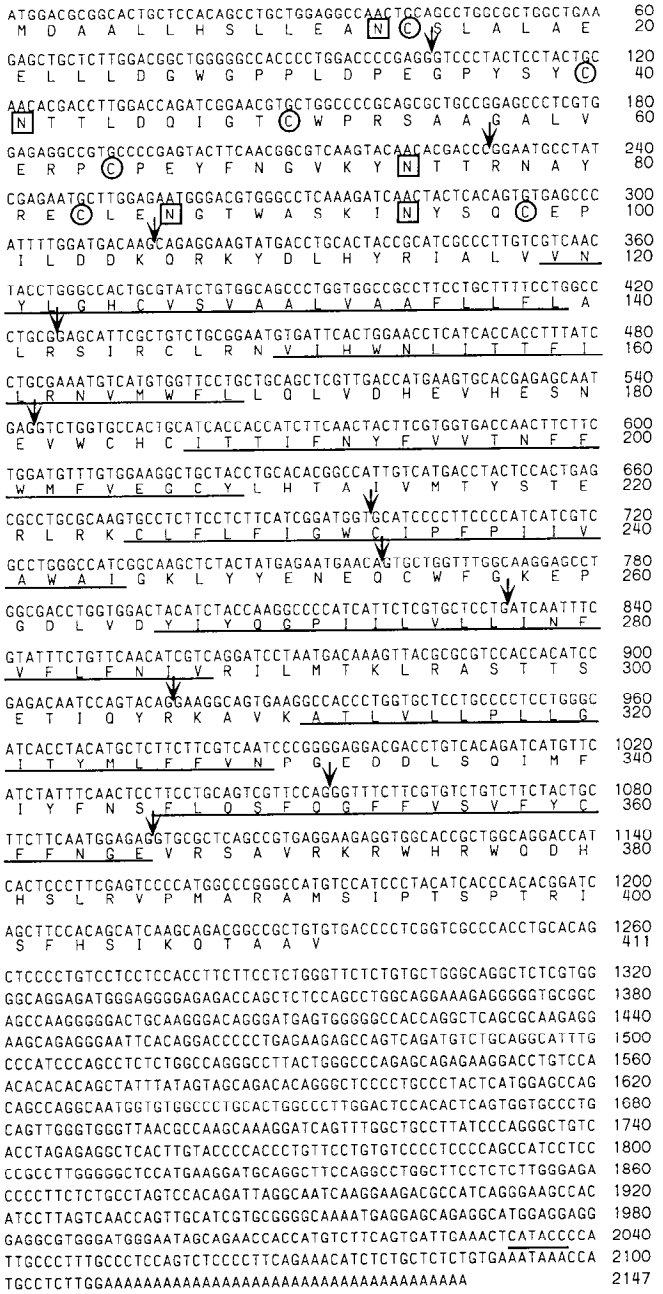

FIG. 2. Nucleotide and amino acid sequence of the human CRF_2 receptor. The potential *N*-linked glycosylation sites are *boxed*, and the cysteine residues in the extracellular domain are *circled*. *Underlined sequences* are the putative transmembrane domains. *Arrows* indicate the positions of introns. The polyadenylation signal is *overlined*. The sequence has been deposited in the GenBank data base (accession no. U34587).

production measured by RIA in an L cell line stably expressing the human CRF_1 receptor. As Fig. 3 shows, for both human CRF_1 and CRF_2 receptors, direct measurement of cAMP levels gave rank order profiles of r/hCRF and sauvagine identical to those produced by the indirect β-gal assay. CRF-induced β-gal activity could be competitively inhibited by the putative CRF receptor antagonist D-Phe-CRF, with approximately equal affinity for CRF_1 and CRF_2 receptor (Fig. 4).

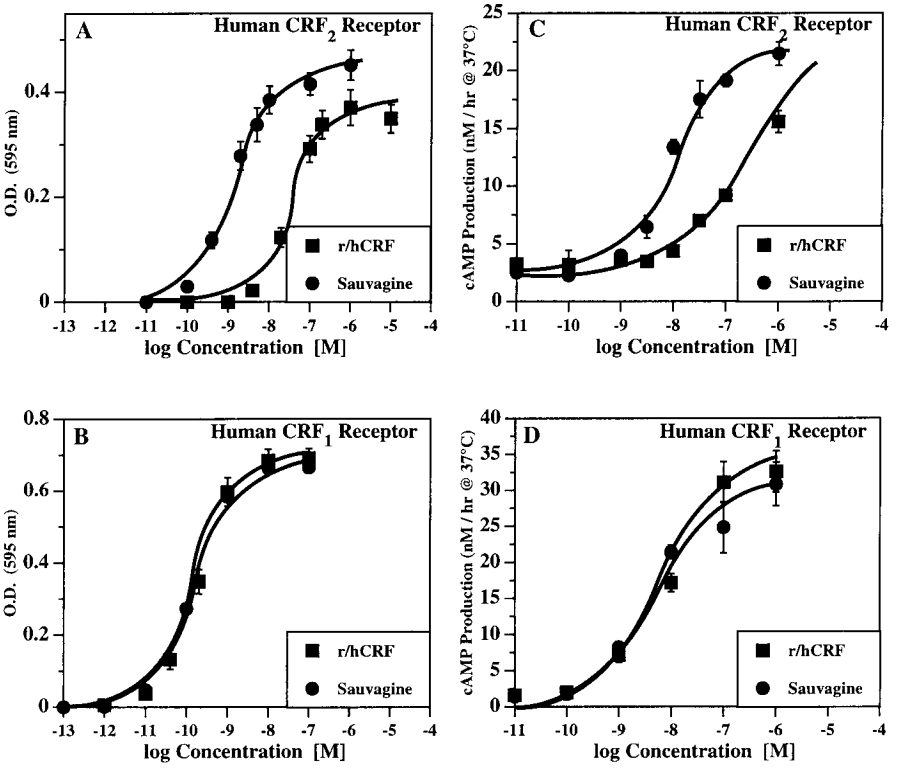

FIG. 3. Stimulation of intracellular cAMP by sauvagine (*circles*) and r/hCRF (*squares*) in cells transfected with human CRF_2 (A and C) and CRF_1 (B and D) receptor cDNA. A and B, The transiently transfected VIP2.0Zc cells were incubated with various concentrations of sauvagine or r/hCRF for 7 h, and the cAMP-induced β-gal activity was measured and expressed as optical density at 595 nm. C and D, The intracellular cAMP levels of VIP2.0Zc cells stably expressing the human CRF_2 receptor (C) and L cells stably expressing the human CRF_1 receptor (D) were directly measured by RIA after incubation with sauvagine and r/hCRF at 37 C for 1 h. Data are the mean ± SD from a representative experiment.

Discussion

The cloning and characterization of a second CRF receptor subtype (CRF_2 receptor) in the rat and mouse have recently been described (18–20). The rat CRF_2 receptor mRNA is expressed in specific regions of the brain, such as hypothalamus, lateral septum, and amygdala (18), as well as in peripheral tissues, such as heart and skeletal muscle (21). The present study attempted to use this information to identify the human homolog of CRF_2 receptor to validate its physiological role in humans. However, screening of cDNA libraries from human tissues that corresponded to the rat tissues expressing the CRF_2 receptor mRNA failed to yield any full-length human CRF_2 receptor cDNAs. The cDNAs that gave the most coding information were obtained from a cDNA library constructed from human frontal cerebral cortex, a region that showed only scattered positive hybridization signals in the rat (18, 24), suggesting that CRF_2 receptor might have a different tissue distribution pattern in humans.

Analysis of the human CRF_2 receptor gene reveals a genomic structure similar to that of mouse CRF_1 receptor (25), which has 12 introns, the last 10 of which interrupt the coding region at identical positions as human CRF_2 receptor gene introns 2–11. The cDNA sequences for CRF_1 and CRF_2 receptor diverge significantly at the 5′-end. This is also true for the genomic structure, such that mouse CRF_1 receptor gene has one extra intron (intron 1) in the most 5′-coding region. Intron 2 of mouse CRF_1 receptor and intron 1 of human CRF_2 receptor also occur at different positions of the coding region. A splice variant with a 154-nucleotide deletion in the rat CRF_1 receptor has been identified (16). This 154-nucleotide segment turns out to correspond to the entire

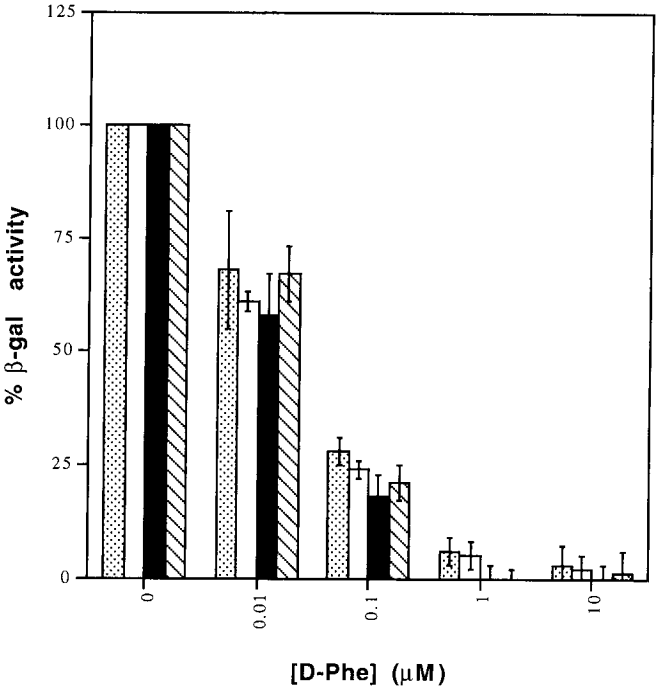

FIG. 4. Inhibition of CRF- and sauvagine-induced β-gal activity by CRF antagonist D-Phe-CRF. Cells transfected with CRF_1 receptor cDNA were incubated with 1 nM r/hCRF (*dotted bars*) or 1 nM sauvagine (*open bars*), whereas CRF_2 receptor cDNA-transfected cells were incubated with 200 nM r/hCRF (*closed bars*) or 5 nM sauvagine (*hatched bars*) in the absence and presence of various concentrations of D-Phe-CRF. The CRF and sauvagine concentrations used produced approximately 90% of the maximal CRF/sauvagine-induced activity. The β-gal activity was expressed as a percentage of that in the absence of D-Phe. Data are the mean ± SD from a representative experiment.

exon 6 of human CRF_2 receptor. Thus, the splice variant is probably derived from alternative usage of a splicing donor/acceptor pair. On the other hand, Chen *et al.* (14) reported a splice variant for human CRF_1 receptor with an 87-nucleotide insertion in the first intracellular loop. This insertion occurs exactly at the corresponding position of the exon 4 and 5 junction of the human CRF_2 receptor. However, the 87-nucleotide segment showed no sequence homology to any part of intron 4 (the intron between exons 4 and 5) of the CRF_2 receptor gene. Thus, there is no structural counterpart of this CRF_1 receptor splice variant for the CRF_2 receptor.

In the rat, two splice variants for CRF_2 receptor ($CRF_{2\alpha}$ and $CRF_{2\beta}$) with different 5′-sequences have been identified (18). The two forms appear to diverge at the corresponding position of human CRF_2 receptor where the first intron occurs. The putative human CRF_2 receptor reported here is the homolog of rat $CRF_{2\alpha}$. As a probe specific for the rat $CRF_{2\beta}$ was used to screen the human genomic library, no positive clone containing the coding sequence for a possible human $CRF_{2\beta}$ form was found.

The putative full-length human CRF_2 receptor cDNA was cloned into an expression vector and transfected into cells. The transfected cells expressing the CRF_2 receptor transduced the CRF binding signal into increasing the level of intracellular cAMP. The cell line used for the transfection (VIP2.0Zc) is a mouse L cell line containing an exogenous β-gal gene under control of the cAMP-responsive VIP promoter/enhancer (22), such that the intracellular cAMP level can be indirectly monitored by measuring the β-gal activity. We have previously characterized VIP2.0Zc cell lines stably expressing the human and rat CRF_1 receptor and demonstrated the pharmacological specificity of their response to CRF agonists and antagonists (23). Here, by transiently transfecting human CRF_1 and CRF_2 receptor cDNAs into VIP2.0Zc cells and measuring the intracellular cAMP levels, either indirectly through the β-gal reporter gene or directly by RIA, we show that, similar to rat CRF_1 *vs.* $CRF_{2\alpha}$ receptors, the two human receptor subtypes clearly have distinct pharmacological characteristics, with the CRF_2 receptor having much lower affinity for CRF and sauvagine than the CRF_1 receptor. This difference in the affinity for CRF and sauvagine between the two receptor subtypes is reflected in the fact that the extracellular domain, the region presumably at least partially responsible for the binding of ligands, is the least conserved region between the two molecules. In contrast to the different affinities of CRF and sauvagine for human CRF_1 and CRF_2 receptors, the putative CRF receptor antagonist D-Phe-CRF has approximately equal affinity for the two human CRF receptor subtypes. Thus, the pharmacological profile of the human CRF_2 receptor corresponds to that of rat $CRF_{2\alpha}$, which appears to be different from that of rat (our unpublished results) or mouse (19, 20) $CRF_{2\beta}$. It is interesting to note that although the affinities for CRF and sauvagine are lower with CRF_2 receptor than CRF_1 receptor, the affinities of these receptors for D-Phe-CRF are identical. Therefore, studies using this antagonist to assess the relevant effects of exogenously added or endogenously produced CRF may not distinguish the two receptor subtypes.

In summary, we have characterized the genomic structure and coding sequence for the human CRF_2 receptor. When transfected into cells, the putative full-length cDNA is capable of directing the expression of a functional CRF receptor with distinct pharmacology from the human CRF_1 receptor. The negative screening results from a number of cDNA libraries of human brain regions suggest that CRF_2 receptor might have a different regulation and/or distribution pattern in humans and rats. Detailed characterization of the CRF_2 receptor message distribution in monkeys and humans is currently underway.

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