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Cloning and Characterization of Human Urocortin

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ABSTRACT Urocortin, a new member of the CRF peptide family which also includes urotensin I and sauvagine, was recently cloned from the rat midbrain. The synthetic replicate of urocortin was found to bind with high affinity to type 1 and type 2 CRF receptors and, based upon its anatomic localization within the brain, was proposed to be a natural ligand for the type 2 CRF receptors. Using a genomic library, we have cloned the human counterpart of rat urocortin and localized it to human chromosome 2. Human and rat urocortin share 95% identity within the mature peptide region. Synthetic human urocortin binds with high affinity to CRF receptor types 1, 2α, and 2β, stimulates cAMP accumulation from cells stably transfected with these receptors, and acts *in vitro* to release ACTH from dispersed rat anterior pituitary cells. In addition, the CRF-binding protein binds human urocortin with high affinity and can prevent urocortin-stimulated ACTH secretion *in vitro*. The inhibitory effect of the CRF-binding protein on human urocortin can be blocked by biologically inactive CRF fragments, such as CRF(9-33).

Corticotropin releasing factor (CRF), a 41 amino acid peptide first isolated from ovine hypothalamus (1), is the principal neuroregulator of the mammalian pituitary-adrenal axis. CRF has high homology to the non-mammalian peptides urotensin I (Uro), a 41 amino acid peptide originally isolated from the urophyses of the teleost fish, *Catostomus commersoni* (2), and sauvagine (Svg), a 40 amino acid peptide isolated from the skin of the tree frog *Phyllomedusa sauvagei* (3). These peptides were considered to be non-mammalian homologs of CRF based on structural and functional similarities. All three peptides can stimulate ACTH secretion *in vitro* and *in vivo* in the rat (4) and selectively dilate the mesenteric artery causing a decrease in systemic blood pressure in the dog (5). However, fish Uro and amphibian Svg were noted to have greater hypotensive effect in mammals than CRF (6).

Characterization of genes encoding peptides with >90% homology to CRF in the teleost fish (7) and in the frog (8) indicated that at least two members of the CRF family coexist in each of these vertebrate classes, raising the possibility that Uro- or Svg-like peptides might exist in mammals. Accordingly, discrete localization of Uro-like immunoreactivity was found in a midbrain region of the rat known as the Edinger-Westphal nucleus. Using a carp Uro cDNA probe, a unique cDNA was cloned from a library constructed from a rat midbrain dissection encompassing the Edinger-Westphal nucleus. This cDNA encoded a precursor and a putative peptide, which we named Urocortin (Ucn) (9), related more to Uro than to CRF.

Rat Ucn, similar to all members of the CRF ligand family, binds to the seven transmembrane domain CRF receptors, which are functionally coupled to adenylate cyclase. Two distinct CRF receptor genes have been identified. The first, CRF-R1, was cloned from human and mouse pituitary (10,11) and rat brain (12,13). Two splice variants of a second CRF receptor, which differ in their N-terminal domains, were isolated. CRF-R2α was cloned from the rat brain (14) and CRF-R2β was isolated from the mouse heart (15-17) and identified in the rat brain and lung (14). Urocortin, CRF, Uro and Svg bind to and stimulate adenylate cyclase activity of the CRF receptor subtypes to different degrees (9,14-17); the type 2 CRF receptors are selective for Ucn, Uro, and Svg relative to CRF. These peptides also bind with varying affinities to the binding protein for CRF (CRF-BP) (18,19), which has been shown to inactivate CRF (18).

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A cDNA probe encoding the peptide region of rat Ucn was used to screen a human genomic library by hybridization. We report the nucleotide and amino acid sequence of human Ucn. We also evaluate the binding and some biological activities of this synthetic peptide on cells expressing CRF receptors 1, 2α, and 2β, as well as on the CRF-BP.

Materials and Methods

Library Screening: Approximately 0.6 x 10^6 phage plaques of a human placental genomic library in the EMBL3 SP6/T7 vector (Clontech, Palo Alto, CA) were screened by hybridization using a probe encoding the peptide region of rat Ucn. The 160 bp probe was synthesized by PCR using the oligonucleotides (sense: 5'-TGCAGGCGAGCGGCAACGACGAGACGA-3' and antisense: 5'-ATACGGGGCCGATCACTTGCCCACCGAG-3'), [α^{32}P-dCTP], and rat Ucn cDNA (9) as template. Hybridization was carried out at 42°C in standard buffers with 20% formamide. Final washes were at 42°C in 2XSSC/0.1% SDS. The phage DNA from an individual positive plaque was purified and subcloned into pBluescript (Stratagene, La Jolla, CA). Dideoxy sequencing was done using the Sequenase kit (US Biochemical, Cleveland, OH).

Chromosomal Localization: The human chromosomal localization of Ucn was obtained by probing a Southern blot containing 24 isolated human/rodent somatic cell hybrids (Coriell Cell Repositories, Camden, NJ). The blot was hybridized with the rat Ucn probe described in the library screening above. The final washes for the blots were at 60°C in 0.2XSSC/0.1%SDS.

Binding Assays: CHO cell lines were transfected by electroporation with the pRc/RSV or pRc/CMV expression vector (Invitrogen, San Diego, CA) containing the cDNA of either human CRF-R1 (10), rat CRF-R2α (courtesy of Tim Lovenberg,

Neurocrine Biosciences, La Jolla , CA) (14), or mouse CRF-R2β (15). Stable transfectants were selected based on resistance to G418 (1 mg/ml) and clones expressing high CRF receptor levels were identified (Sutton and Vale, unpublished). These stable cell lines were used to determine the affinities of synthetic test peptides (1). Competitive displacement of [^{125}I-Nle^{21}, Tyr^{32}]oCRF (for CRF-R1) or of [^{125}I-Tyr^{0}]rUcn (for CRF-R2α and CRF-R2β) by test peptides was used to compare the affinities of the peptides for the various CRF receptors (9,10). The affinity of recombinant human CRF-BP for test peptides was measured based on the displacement of [^{125}I-DTyr^{0}]r/hCRF as previously described (19). The inhibitory dissociation constants (Ki) and 95% confidence limits were determined by the LIGAND program (20).

In vitro bioassays: Stably transfected CHO cells expressing human CRF-R1, rat CRF-R2α , or mouse CRF-R2β were treated with test peptides as described (9,10). Cells were lysed and the intracellular cAMP levels were measured with an RIA kit (Biomedical Technologies, Stoughton, MA). Potencies were determined by the Allfit program (21) with results expressed as the average ±sem for three or more independent assays. Rat anterior pituitary cells were established in culture (22) and treated in triplicate with test peptides with or without recombinant CRF-BP for three hours as described (19). The media were collected and secreted ACTH was measured with an RIA kit (Diagnostic Products Corp., Los Angeles, CA).

Results and Discussion

A positive clone determined to encode Ucn was obtained from a low stringency hybridization screen of a human genomic library with a probe that encoded the mature peptide region of rat Ucn. The clone had an insert size of approximately 15Kb which was subcloned into pBluescript. Using primers specific to rat Ucn, internal sequence that corresponded to human Ucn was obtained and the complete coding region was sequenced. Similar to the organization of other genes for rat and xenopus CRFs (8,23), the coding region for human Ucn is found on one exon. The nucleotide sequence for human Ucn encodes a 123 amino acid protein comprised of a 79 amino acid precursor and a putative 40 amino acid mature peptide. The mature peptide is preceded by the proteolytic processing site Arg-Arg and followed by amino acids Gly-Lys, presumed to be involved in C-terminal amidation and cleavage of peptides from a precursor. The precise processing of this precursor will not be formally established until the native peptide itself is characterized in human tissues.

The putative mature 40 amino acid human Ucn peptide has 88% identity to rat Ucn at the nucleotide level and 95% identity at the amino acid level. For the full-length protein, human and rat Ucn show 75% identity at the nucleotide level and 73% identity at the amino acid level. Human Ucn is one amino acid longer than rat Ucn in the precursor region. The human Ucn gene is localized to human chromosome 2.

There are two amino acid differences between the human and rat Ucn peptides: at position 2 (Asn for Asp) and at position 4 (Ser for Pro) (Fig. 1). The change at position 4 is interesting because the Pro-Pro motif is highly conserved in the CRF peptide family, seen in all CRFs, Uros, and Svg. This change is further intriguing because a Pro-Ser, rather than the Pro-Pro consensus, occurs in some of the insect diuretic hormones (24,25), which have also been considered to be part of the CRF superfamily. Among the diuretic hormones, *Manduca sexta* has the highest homology to human Ucn with 30% identity at the amino acid level.

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1   M R Q A G R A A L L A A L L L L V Q L C  hUcn
1   M R Q R G R A T L L V A L L L L V Q L R  rUcn

21  P G S S Q R S P E A A G - - - V Q D P S  hUcn
21  P E S S Q W S P A A A A A N V V Q D P N  rUcn

38  L R W S P G A R N Q G G G A R A L L L L  hUcn
41  L R W N P G V R N Q G G G V R A L L L L  rUcn

58  L A D A S R A A G P G R L G L G T A G E  hUcn
61  L A E R F - - - - P R R A G S E P A G E  rUcn

78  R P R R D N P S L S I D L T F H L L R T  hUcn
77  R Q R R D D P P L S I D L T F H L L R T  rUcn

98  L L E L A R T Q S Q R E R A E Q N R I I  hUcn
97  L L E L A R T Q S Q R E R A E Q N R I I  rUcn

118 F D S V G K ■                            hUcn
117 F D S V G K ■                            rUcn
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Fig. 1. Amino acid comparison of human and rat urocortin. The alignment was done by the Lasergene alignment system and the Clustal method with an identity residue weight table. Identical residues are shaded; the square indicates a putative amidation site; and the arrows indicate the putative mature peptide. The nucleotide sequence for human Ucn has been deposited into Genbank under accession number U43177.

Human Ucn is a potent ligand for all three functional CRF receptors identified to date (CRF-R1, CRF-R2α, and CRF-R2β). The affinity of human Ucn for CRF-R1 is comparable to that of rat Ucn (Table 1). While CRF-R1 does not appear to markedly discriminate among CRF-related peptides, CRF-R2α and CRF-R2β show definite selectivities. Human and rat (9) Ucn exhibit much greater affinity than rat/human (r/h) CRF for CRF-R2β. Similarly, both human and rat Ucn have approximately 10-fold higher affinity than r/hCRF for CRF-R2α (Table 1). Rat Ucn has a slightly higher affinity than Uro and Svg for CRF-R2β (9) and CRF-R2α (Table 1); the affinity of human Ucn is in the same range as the two non-mammalian peptides. The type 2 CRF receptors thus favor Ucn, Uro, and Svg over r/hCRF.

Human Ucn is similarly potent in inducing intracellular cAMP accumulation in CHO cells stably transfected with CRF-R1, CRF-R2α, or CRF-R2β (Table 2). Human Ucn acts in the same range as rat Ucn and r/hCRF to induce cAMP levels in cells transfected with CRF-R1. By contrast, cells expressing CRF-R2β accumulate cAMP in response to human Ucn with approximately 20-fold greater sensitivity than in response to r/hCRF. This effect has also been reported for rat Ucn (9). Human and rat Ucn are at least 10-fold more potent than r/hCRF in stimulating cAMP accumulation in CRF-R2α transfected cells (Table 2).

Rat Ucn was found to be a highly potent secretagog for ACTH *in vitro*, and induced a prolonged

elevation of plasma ACTH in the rat *in vivo* (9). Human Ucn is equipotent to rat Ucn and more potent than r/hCRF, Uro, or Svg in its ability to stimulate ACTH secretion from rat anterior pituitary cells *in vitro* [Fig. 2 and (9)].

Table 1. Binding to stably transfected CHO cells expressing CRF receptors or to recombinant human CRF binding protein.

Peptide	human CRF-R1 Ki	rat CRF-R2α Ki	mouse CRF-R2β Ki	human CRF-BP Ki
hUcn	0.41 nM (0.23-0.74)	1.8 nM (1.2-2.8)	1.5 nM (0.89-2.4)	0.22 nM (0.19-0.27)
rUcn	0.16 nM (0.08-0.32)	0.58 nM (0.42-0.82)	0.41 nM (0.26-0.66)	0.12 nM (0.11-0.15)
r/hCRF	0.95 nM (0.47-2.0)	13 nM (7.2-22)	17 nM (10-29)	0.21 nM (0.17-0.26)
sfUro	0.43 nM (0.23-0.81)	3.4 nM (2.6-4.4)	3.0 nM (1.8-4.8)	0.028 nM (0.021-0.036)
Svg	1.2 nM (0.54-2.5)	1.4 nM (1.1-1.8)	2.0 nM (1.1-3.6)	65 nM (46-92)

Binding properties of human urocortin compared to rat urocortin, rat/human CRF, suckerfish urotensin I, and frog sauvagine on the three known CRF receptor types: 1, 2α, and 2β; and on the CRF-BP. Dissociation constants (Ki) were calculated from three or more experiments for each test peptide. The values are shown with 95% confidence limits.

Table 2. Accumulation of intracellular cAMP in stably transfected CHO cells expressing CRF receptors.

Peptide	human CRF-R1 EC_{50}	rat CRF-R2α EC_{50}	mouse CRF-R2β EC_{50}
hUcn	0.22 nM (±0.08 nM)	0.32 nM (±0.10 nM)	0.13 nM (±0.02 nM)
rUcn	0.12 nM (±0.02 nM)	0.39 nM (±0.10 nM)	0.13 nM (±0.03 nM)
r/hCRF	0.26 nM (±0.05 nM)	5.3 nM (±2.4 nM)	3.0 nM (±1.0 nM)

Stimulation of intracellular cAMP by human urocortin compared to rat urocortin and rat/human CRF in CHO cells stably transfected with CRF receptors. Shown are EC_{50} values ±sem, calculated from three or more experiments.

CRF-BP (18), a 37K circulating protein in human plasma, is also distributed throughout the central nervous system (26) of all vertebrates studied thus far, including human beings (27). CRF-BP binds human Ucn with high affinity and in the same range as that seen for rat Ucn and r/hCRF (Table 1). As has been noted for CRF (19), coincubation with CRF-BP prevents the release of ACTH by human Ucn (Fig. 3). The actions and distribution of CRF-BP have led us to suggest that CRF-BP may serve to temporally and anatomically limit the action of Ucn. Furthermore, biologically inactive CRF fragments such as CRF(9-33), which we reported earlier to interfere with the ability of CRF-BP to bind to CRF (19), can also block the inactivation of Ucn by CRF-BP and restore the ability of Ucn to release ACTH (Fig. 3). Recently, it was reported that CRF-BP-blockers can arouse rats and improve their performance on cognitive tests, by elevating levels of free CRF (27). Appropriate blockers of the CRF-BP might be used therapeutically to elevate levels of free Ucn, as was proposed for CRF (19,27).

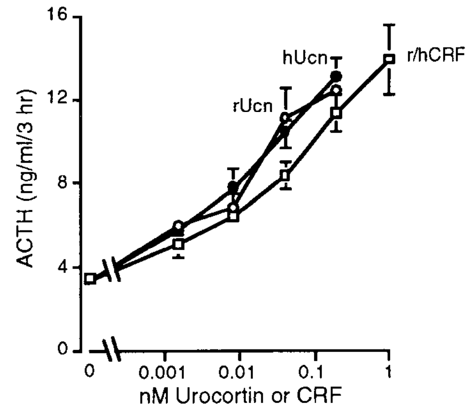

Fig. 2. Secretion of ACTH from cultured rat anterior pituitary cells. Monolayer cells were treated for 3h with human urocortin (●), rat urocortin (○), or rat/human CRF (□) and secreted ACTH was measured.

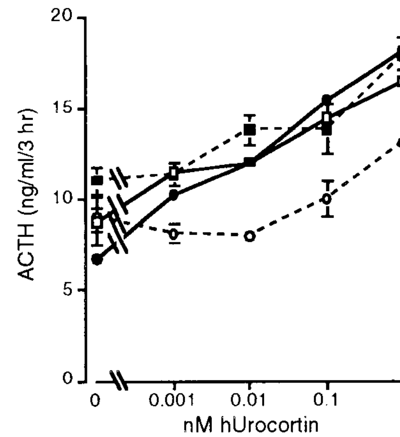

Fig. 3. Effects of CRF-BP, in the presence or absence of CRF(9-33), on urocortin-induced ACTH secretion. Cultured rat anterior pituitary cells were treated for 3h with human urocortin (●) in the presence of 100 nM CRF-BP (○), 5μM CRF(9-33) (□), or 100 nM CRF-BP plus 5μM CRF(9-33) (■).

Clearly, more physiological, biochemical and anatomical studies are needed before the functions and pharmacologic potential of this new mammalian peptide can be defined. The high affinity of Ucn for all known CRF receptors and its likely role as the native ligand for the brain type 2 CRF receptors in the rat compel further exploration of this ligand/receptor signaling system.

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