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A HUMAN ORPHAN CALCITONIN RECEPTOR-LIKE STRUCTURE[1]

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Summary: A novel calcitonin receptor-like protein of 461 amino acids with seven putative transmembrane domains has been identified through molecular cloning in a cDNA library of the human cerebellum. 91% and 56% of the amino acids are identical in a rat orphan calcitonin receptor-like sequence and the human calcitonin receptor, respectively. 5.2 kb mRNA is predominantly expressed in the lung, heart and kidney. Specific binding of ^{125}I -labeled salmon calcitonin and human calcitonin gene-related peptide-I to COS-7 cells transiently transfected with the receptor cDNA was less then 0.5%. Cellular cAMP accumulation was indistinguishable in cDNA transfected and non-transfected control COS-7 and renal tubular cells from the American opossum stimulated with human and salmon calcitonin, human calcitonin gene-related peptide-I and -II, human amylin, human adrenomedullin, lizard helodermin, salmon stanniocalcin and chicken parathyroid hormone-related protein. The receptor-like protein whose ligand remains to be discovered belongs to the family of receptors of calcitonin, parathyroid hormone, secretin, vasointestinal peptide and pituitary adenylate cyclase-activating polypeptide.

Calcitonin receptors identified through molecular cloning belong to a family of seven transmembrane domain G-protein-linked receptors together with those of parathyroid hormone/parathyroid hormone related protein and secretin (1, 2). An important functional feature is coupling to both adenylate cyclase and the phosopholipase C pathways (3, 4). Within this receptor family homologous amino acids in the putative seven transmembrane domains contrast with divergent extracellular and intracellular regions thought to determine ligand specificity and interaction with different G-proteins. A functional assignment of certain regions of the receptor proteins was achieved with subtypes of CT and pituitary adenylate cyclase-activating polypeptide receptors. Two CT receptor isoforms in the rat, differing by a 37 amino acid insert in the putative second extracellular loop, exhibit characteristic ligand specificity (5,

[1]The nucleotide sequence data have been submitted to the BBJB/EMBC/Gen Bank DNA database under accession number U 17473.

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Abbreviations: CGRP, calcitonin gene-related peptide; hCRLS, human calcitonin receptor-like structure; CT, calcitonin; OK, renal tubular cell line from the American opossum; sCT, salmon CT.

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6). Five splice variants of the pituitary adenylate cyclase-activating polypeptide receptors with insertions at the C-terminal end of the third intracellular loop couple to adenylyl cyclase and phospholipase C with different efficiency (7).

Overlapping biological actions which include inhibition of bone resorption and vasodilatation of the structurally related CT and CGRP, both products of the CT gene, and amylin and the recently identified adrenomedullin have been attributed to crossreaction of the different peptides with corresponding receptors (8, 9). As a result, amylin and CGRP interact at high concentrations compared to CT with CT receptors linked to cyclic AMP accumulation (8). Receptors for CGRP, amylin and adrenomedullin have so far not been identified through molecular cloning. Since the peptides crossreact with CT receptors, sequence homology appears likely.

Recently, a CT receptor-like receptor has been cloned from cDNA libraries of the rat cerebellum (10, 11). The receptor mRNA is enriched in the lung, but the receptor ligand remains elusive. Here, we have identified a homologous structure through molecular cloning of cDNA from the human cerebellum.

MATERIALS AND METHODS

Cloning and sequencing of cDNA: A DNA fragment with 58% sequence homology to human CT receptor encoding cDNA (2) was amplified from 10^7 pfu of a human cerebellum λgt11 cDNA library (Clontech, Palo Alto, CA, USA) by polymerase chain reaction (PCR) with two pairs of nested primers. The two 5'-primers, 5'-atgacggctcagtatgaatg-3' and 5'-cctaactataattggacatgg-3', were designed to be homologous to the cDNA sequence encoding amino acids 41 to 48 and 143 to 150 of a rat calcitonin receptor-like receptor (GenBank accession number L27487) (11), and the two 3'-primers, 5'-accgtataggatcgctgcgg-3' and 5'-gatgaatgaactgggacacc-3', were complementary to sequences of the same cDNA, encoding amino acids 416 to 423 and 213 to 220. Two rounds of PCR amplification were carried out for 25 cycles (95°C - 48°C - 72°C, 1 min each) followed by incubation at 72°C for 10 min. The PCR product was purified by electrophoresis on a 2% low melting agarose gel and subcloned into the SmaI site of the pUC18 vector for sequencing. Subsequently, the fragment was reexised, labeled with [α^{32}-P]dATP with the PRIME-IT labeling system (Stratagene, La Jolla, CA, USA), and 3 x 10^5 cpm/ml were used for hybridization screening of 10^6 independent plaques of the human cerebellum cDNA library on duplicate GenescreenPlus nylon membranes (DuPont, Boston, MA, USA). Hybridization was carried out according to Church and Gilbert (12) and the membranes were washed at room temperature in 2 x SSC for 20 min and at 65°C in 0.1 x SSC and 0.1 % SDS for 30 min, and subsequently autoradiographed at -70°C for 24 h. Five independent double positive plaques were purified and inserts were subcloned into the EcoRI site of pIC19H, a pUC derivative, and mapped by PCR and DNA sequence analysis. The AutoRead kit and fluorescein-dATP and an A.L.F. DNA Sequencer (Pharmacia, Uppsala, Sweden) were used for DNA sequencing of both strands. All primers were synthesized on a Gene Assembler Plus (Pharmacia, Uppsala, Sweden).

Northern blot analysis: Northern blots of RNA from different human tissues were obtained from Clontech (Palo Alto, CA, USA). Total RNA isolated from cells transiently transfected with the hCRLS expression construct (see below) were subjected to electrophoresis on a 1% agarose formaldehyde gel and transferred to a nylon membrane (Zeta-Probe GT, BioRad, Hercules, CA, USA). An SspI fragment consisting of 539 bp encoding the C-terminal 150 amino acids of the hCRLS (Fig. 2) and 89 bp of 3'-noncocoding region was labeled with [^{32}P]dATP with the PRIME-IT labeling system (Stratagen, La Jolla, CA, USA), and hybridization was carried out according to the Clontech protocol. The membranes were autoradiographed at -70°C.

Expression of cloned cDNA: The full length hCRLS encoding cDNA was reconstructed from two subclones overlapping in the coding region between amino acid residues 131 and 322 (Fig. 2). The cDNA fragment encoding the full length hCRLS was then subcloned into the

eukaryotic expression vector pN346 carrying a chimeric cytomegalo virus/rouse sarcoma virus promoter and a SV40 promoter/mouse DHFR fusion gene, generously provided by R. Gentz, Hoffmann-La Roche, Basel, Switzerland. The structure of the full length hCRLS cDNA including the flanking sequences in pN346 were reconfirmed by DNA sequencing of both strands as described.

Expression of the hCRLS protein was investigated by transient transfection of COS-7 (ATCC CRL 1651) and OK (ATCC CRL 1840) cells with the expression construct and subsequent measurements of [^{125}I]sCT and [^{125}I]human CGRP-I binding and of cellular cyclic AMP accumulation as previously described (13). Prior to the transfections the cells were grown to subconfluency for 24 h in 24 well plates and washed twice with 500 µl/well of serum-free transfection medium (Dulbecco's modified Eagle's minimum essential medium). COS-7 and OK cells were transfected in individual wells by incubation for 4 h in 300 µl serum-free transfection medium containing 200 ng DNA and 0.6 µl Transfectam (2.5 mg/ml, Promega, Madison, WI, USA). After the transfections the cells were washed with tissue culture medium and cultured for 48 h, and assayed for ligand binding and cyclicAMP accumulation. The same protocols were used to control for transfection efficiency and reproducibility, and for receptor protein expression by transient transfection of COS-7 cells with OK parathyroid hormone/parathyroid hormone related-protein receptor encoding cDNA and OK cells with porcine CT receptor encoding cDNA generously provided by G.V. Segre and S.R. Goldring, respectively, Massachusetts General Hospital, Boston, MA, USA.

Human adrenomedullin was obtained from the Peptide Institute (Osaka, Japan), helodermin, a vasoactive peptide indentified in the venom of the Heloderma suspectum lizard (14), and chicken parathyroid hormone-related protein(1-36) from Peninsula Laboratories (Belmont, CA, USA), human CT from Ciba (Basel, Switzerland) and sCT from Sandoz (Basel, Switzerland). Human CGRP-I and -II and human amylin were purchased from Bachem (Bubendorf, Switzerland). Salmon stanniocalcin was generously provided by G.F. Wagner, University of Western Ontario, London, Canada.

RESULTS AND DISCUSSION

Hybridization screening of a human cerebellum λgt 11 cDNA library with a DNA probe that was PCR amplified from the same library, exhibiting 58% homology to the human CT receptor encoding cDNA, revealed five independent overlapping clones spanning approximately 4 kb of human cDNA. Two clones overlapped within an open reading frame encoding a protein of 461 amino acids. The amino acid sequence corresponds to a novel receptor within the parathyroid hormone/parathyroid hormone-related protein/CT/secretin receptor family (Fig. 1). The closest relative is a homologous structure cloned from a cDNA library of the rat cerebellum followed by a human CT receptor cloned from an ovarian carcinoma cell line (2, 10, 11). Alignment of the amino acid sequence with that of the rat calcitonin receptor-like receptor homologue of the hCRLS and the human CT receptor revealed 91% and 56% sequence identity, respectively (Fig. 2). This includes three out of four potential N-linked glycosylation sites identified in the human CT receptor and eight cysteines present in the putative extracellular domain and the first and second extracellular loops of human, porcine and rat CT receptors (1, 2, 5).

Important structural differences between the hCRLS and the homologous rat calcitonin receptor-like sequence and the human CT receptor of an ovarian carcinoma cell line are recognized in the putative first intracellular loop. Within this domain, the structures of the hCRLS and the rat calcitonin receptor-like receptor are more closely related to a human CT receptor isoform expressed in a human medullary thyroid carcinoma cell line (15) and to the porcine and to two rat isoforms which all lack a 16 amino acid insert unique for the human CT receptor identified in the ovarian carcinoma cell line (Fig. 2) (1, 5). The hCRLS and the rat

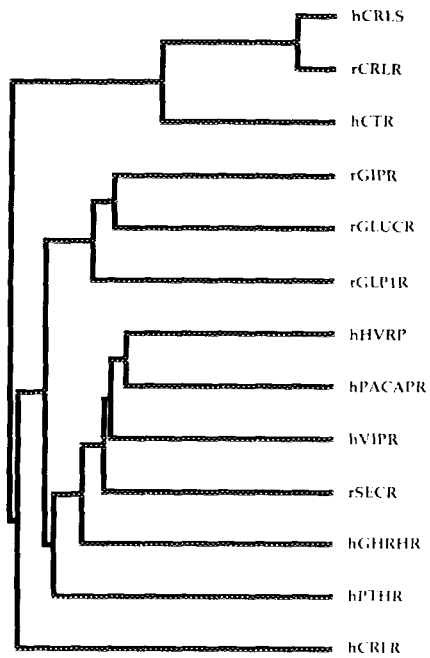

Fig. 1. Structural relationship of the hCRLS with the parathyroid hormone/parathyroid hormone-related protein/CT/secretin receptor family that includes the rat calcitonin receptor-like receptor (rCRLR), the human CT receptor (hCTR), the rat gastric inhibitory peptide receptor (rGIPR), the rat glucagon receptor (rGLUCR), the rat glucagon-like peptide 1 receptor (rGLP1R), the human helodermin-preferring vasoactive intestinal peptide receptor (hHVRP), the human pituitary adenylate cyclase-activating polypeptide receptor (hPACAPR), the human vasoactive intestinal peptide receptor (hVIPR), the rat secretin receptor (rSECR), the human growth hormone-releasing hormone receptor (hGHRHR), the human parathyroid hormone receptor (hPTHR) and the human corticotropin-releasing factor receptor (hCRFR). Amino acid sequences were compiled in the multiple sequence alignment program PILEUP of the Genetics Computer Group Software (Madison, WI).

calcitonin receptor-like receptor also lack a 37 amino acid insert unique for the putative first extracellular loop of a brain type rat CT receptor isoform (5). Additional amino acid sequence differences between the hCRLS and the human CT receptor are in the putative signal peptides and N-terminal regions and in the C-terminal intracellular domains. There, the hCRLS does not contain the unusual alanine-rich hydrophobic sequences of the human and porcine CT receptors.

The translation start site has been assigned to the second of two adjacent translation initiation codons within the DNA sequence 5'-TAATGATGGAG-3' that contains structural elements characteristic for a translation initiation site. Much like the human CT receptor encoding cDNA (2), the hCRLS cDNA also contains an additional in-frame ATG 33 nucleotides upstream of the presumed translation start site. However, translation is unlikely to start at this upstream site because the corresponding DNA sequence 5'TTTATGTTA exhibits no structural similarities to a consensus eukaryotic translation initiation sequence. Furthermore, translation initiation at this upstream AUG would result in a protein sequence extended by 11 amino acid residues with unusual structural features for a functioning signal peptide, e.g. two

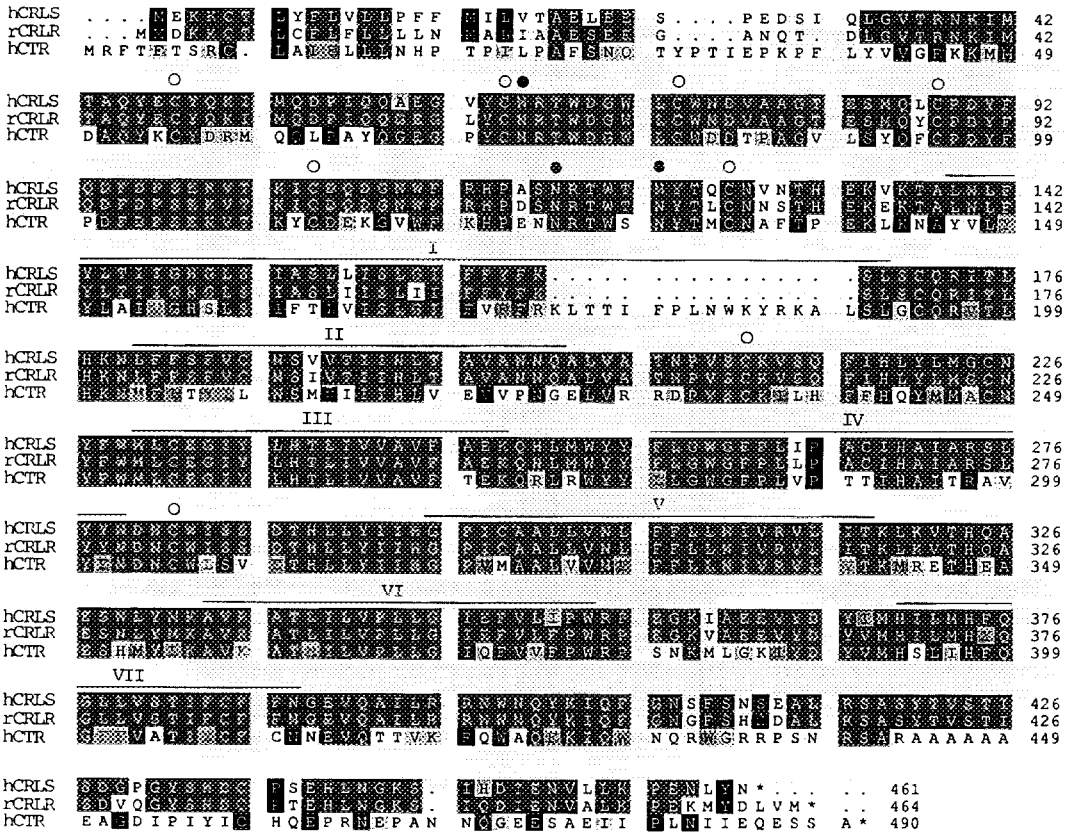

Fig. 2. Alignment of amino acid sequences of the hCRLS and the rat CT receptor-like receptor (rCRLR) and a human CT receptor (hCTR) derived from an ovarian carcinoma cell line (2, 10, 11). Conserved and similar amino acid residues are black and shaded, respectively. Solid lines indicate putative transmembrane domains I to VII. Potential N-linked glycosylation sites are indicated by closed and conserved cysteine residues by open circles. The analysis was carried out with the program of the Genetics Computer Group (Madison, WI).

hydrophobic regions interrupted by the hydrophilic sequence EKK. An upstream ATG is also present in the rat calcitonin receptor-like receptor cDNA (10, 11), but the latter reveals in contrast to the human structure an open reading frame for a dodecapeptide with a stop codon 31 nucleotides upstream of the first of also two adjacent ATG codons. The 3'-noncoding sequence identified in the hCRLS cDNA clones does not reveal a polyadenylation site.

Tissue-specific expression of hCRLS encoding mRNA was investigated by Northern blot analysis of RNA from several human tissues (Fig. 3). A major mRNA species of 5.2 kb was predominantly expressed in heart, lung and kidney. Minor 7.3 and 3.4 kb species were also observed in these tissues. Low expression of the 5.2 kb mRNA is detected in RNA from total human brain and placenta.

For identification of ligand(s) recognized by the novel receptor-like protein, COS-7 and OK cells were transiently transfected with the cDNA inserted into an eukaryotic expression vector. Specific binding of [^{125}I]-labeled sCT and -human CGRP-I to COS-7 cells transfected

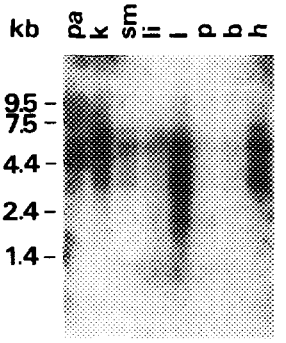

Fig. 3. Northern blot analysis of polyA$^+$ RNA of human heart (h), brain (b), placenta (p), lung (l), liver (li), skeletal muscle (sm), kidney (k) and pancreas (pa).

with the hCRLS expression construct and to nontransfected control cells was below 0.5%. Alterations of cyclic AMP accumulation in response to human and salmon CT, CGRP-I and -II, amylin, adrenomedullin, helodermin and chicken parathyroid hormone-related protein(1-36) at concentrations of up to 0.5×10^{-5} M were indistinguishable in transfected and nontransfected COS-7 and OK cells. In control transfections in COS-7 cells with the same expression vector containing parathyroid hormone/parathyroid hormone-related protein receptor cDNA efficient expression of the receptor binding protein was obtained similar to that of wildtype OK cells (13) (not shown). Correspondingly, [^{125}I]sCT binding to OK cells transfected with the porcine CT receptor cDNA was comparable to that reported in the same cell line (16). Active transcription of hCRLS encoding mRNA in transfected cell lines was confirmed by Northern blot analysis (not shown). Nonetheless, expression of the receptor protein in an inactive form or its selective interaction with signaling pathways other than adenylyl cyclase not investigated in systematic manner cannot be ruled out.

Most likely the novel orphan hCRLS is linked to a so far not discovered ligand homologous to CT, CGRP, adrenomedullin and amylin. Predominant expression of hCRLS encoding mRNA in human lung, heart and kidney is different from that of the receptors of CT, CGRP, adrenomedullin and amylin (for refs. see 8, 9).

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