Volume 335, number 1, 1–5 FEBS 13277 November 1993

Primary structure and functional expression of mouse pituitary and human brain corticotrophin releasing factor receptors

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Received 2 October 1993

Corticotrophin-releasing factor (CRF) is the principal hypothalamic factor governing the pituitary–adrenal axis, but the wide extra-pituitary distribution of CRF and its receptors suggest a major role for this neuropeptide in the integration of the overall physiological and behavioral responses of an organism to stress. We have cloned a CRF receptor complementary DNA (cDNA) by expression in COS-7 cells of a cDNA library from the AtT20 mouse pituitary tumour cell line. The cloned mouse cDNA was then used as a probe to isolate a human CRF receptor cDNA from a human brain cDNA library. The mouse and human cDNAs both encode 415 amino acid proteins that are 97% identical, containing seven putative transmembrane domains characteristic of G protein-coupled receptors. The CRF receptor shows homology with the receptors for growth hormone-releasing factor, vasoactive intestinal peptide, secretin, parathyroid hormone, and calcitonin. COS-7 cells transfected with the mouse CRF receptor cDNA bind radiolabelled ovine CRF with high affinity and respond specifically to CRF by accumulation of intracellular cAMP. A 2.7 kb mRNA coding for the CRF receptor could be detected in AtT20 cells and human cortex tissue. PCR analysis also detected the receptor transcript in human pituitary, brainstem, and testis.

Cloning; cDNA; Neuropeptide; Stress; Binding

1. INTRODUCTION

Corticotrophin-releasing factor (CRF) is a 41 amino acid peptide originally isolated from ovine hypothalamus extracts [1]. CRF-producing neurons, which have their origin in the parvocellular region of the paraventricular nucleus of the hypothalamus, are the major physiological regulators of the basal and stress-induced secretion of ACTH, β-endorphin and other pro-opiomelanocortin-related peptides from the anterior pituitary [2]. However, the presence of high-affinity CRF receptors is not restricted to the pituitary, since CRF receptors have also been detected in numerous areas of the central nervous system and some peripheral tissues [2,3]. This wide distribution of CRF receptors suggests an important role for CRF in the mediation of the behavioral and autonomic responses to stress, in addition to its endocrine role at the pituitary–adrenal axis.

Because the molecular characterization of the CRF receptor will lead to a more detailed understanding of the physiology and pharmacology of CRF action, we undertook the cloning of this receptor, and in this report we describe the isolation, functional expression, and tissue distribution of mouse and human CRF receptor cDNAs. The mouse cDNA was isolated from the ATt20 pituitary cell line, and the human from brain tissue. CRF bound specifically to COS-7 cells transfected with the mouse CRF receptor cDNA and stimulated the intracellular accumulation of cAMP. PCR analysis of human tissues revealed receptor transcripts not only in the brain and pituitary gland, but also in the testis.

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2. MATERIALS AND METHODS

2.1. *cDNA library constructions, isolation of cDNAs and sequence analysis*

Total RNA was extracted from AtT20 cells or human brain tissue as described [4]. Poly(A) RNA was isolated from total RNA with oligo$(dT)_{25}$ magnetic beads (Dynal). The AtT20 size-fractionated cDNA library was constructed using the primer-adapter procedure [5] and the vector, pSE1 [6]. The expression cloning strategy used to isolate the mouse CRF receptor cDNA was as previously reported [7]. To isolate the human CRF receptor cDNA, a human brain cDNA library was constructed using the same primer-adapter method and the vector, pTZ18R (Pharmacia); then 4×10^5 clones were screened with the cloned mouse cDNA. Several positive clones were isolated and sequenced [8]. The mouse and human cDNA sequences have been submitted to the EMBL Data Library (accession numbers X72305 and X72304, respectively).

2.2. *Cell transfection and functional analysis*

For the functional characterization of the recombinant receptor, COS cells were transfected in Petri dishes (15 cm diameter) as described [7], and 24 h later trypsinized and plated at 5×10^5 cells per well in 6-well plates. After growth for 48 h at 37°C the cells were used either for binding experiments with [^{125}I]Tyr^{0}oCRF (New England Nuclear) as described [7], or for cAMP accumulation. For cAMP accumulation the cells were incubated for 1 h at 37°C with the appropriate concentrations of peptides or forskolin, then cAMP was ex-

tracted [9] and quantified in triplicate using a [^{125}I]cAMP assay system (Amersham). Triplicate determinations showed a variation of less than 10%. All results shown are representative of at least two independent experiments.

2.3. *Tissue distribution*

RNA samples were copied with reverse transcriptase and submitted to PCR using the sense primer corresponding to sequence +365 to +381 and the antisense primer corresponding to +1,345 to +1,362 (numbering is based on the human cDNA sequence submitted to the EMBL data library, and corresponds to the protein sequence Ser^{47}-to-Gly^{52} and Ile^{374}-to-His^{379}). PCR-amplified products were hybridized with a probe complementary to sequence +649 to +666 of the cDNA (protein sequence Leu^{142}-to-Ile^{147}). Size markers are indicated at the left of the figure.

3. RESULTS AND DISCUSSION

To isolate the CRF receptor we used an expression cloning strategy previously reported [7]. We constructed a cDNA library, containing 6×10^5 recombinant clones, from AtT20 mouse pituitary tumour cells known to express CRF receptors [10]. The library was divided into 100 pools and plasmid DNA from each pool was introduced into COS-7 cells [7]. The pools were screened for binding with a labelled ovine (o)CRF, [^{125}I]Tyr^{0}o-CRF. Three of the 100 pools tested showed a significant binding activity. A positive pool was subdivided and transfected until a unique cDNA conferring CRF-binding activity was obtained. The isolated mouse CRF receptor cDNA is 2,443 bp long with an open reading frame that encodes a 415 amino acid protein with seven putative hydrophobic transmembrane regions characteristic of G protein-coupled receptors (Fig. 1).

COS-7 cells transfected with the isolated cDNA encoding the CRF receptor showed specific binding for [^{125}I]Tyr^{0}oCRF. Scatchard analysis of the saturation curve showed a single component site with a K_d value of 1.6 ± 0.3 nM and a maximal binding capacity of 5×10^5 receptors/cell (Fig. 2A). The affinity displayed by the recombinant receptor is in good agreement with the K_d value of 1.8 nM for the AtT20 CRF receptor [10], and 0.8 nM for the rat anterior pituitary receptor [11]. In competition studies, oCRF and human (h)CRF were equally effective in inhibiting the labelled oCRF binding to the cloned receptor, with an inhibitory affinity constant (K_i) of 4.5 ± 0.5 nM. The receptor antagonist α-helical oCRF 9–41 (oCRF9–41) [12], was less potent

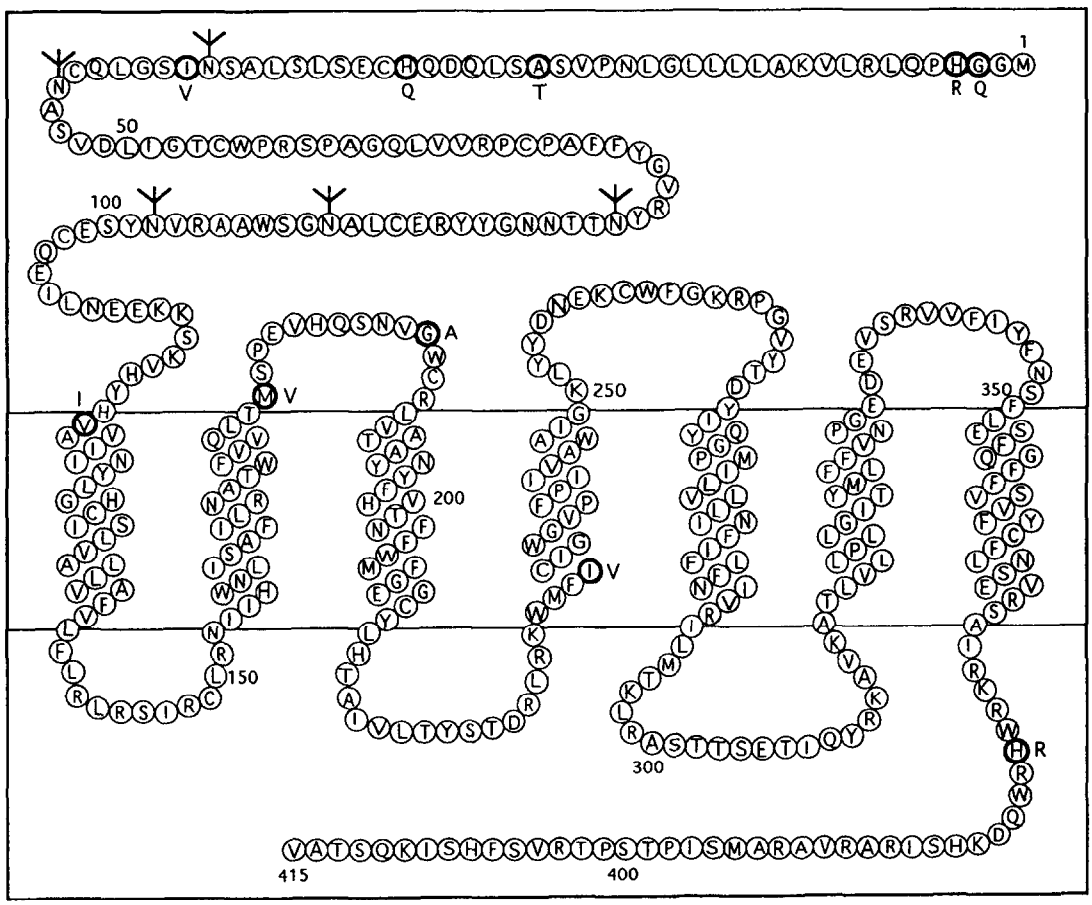

Fig. 1. Schematic representation of the deduced human and mouse CRF receptor peptide sequences. The letters within the circles represent the sequence of the human protein, the letters outside the circles represent the amino acids that are different in the mouse sequence. The potential glycosylation sites are indicated (ψ). The mouse and human cDNA sequences have been submitted to the EMBL Data Library (accession numbers X72305 and X72304, respectively).

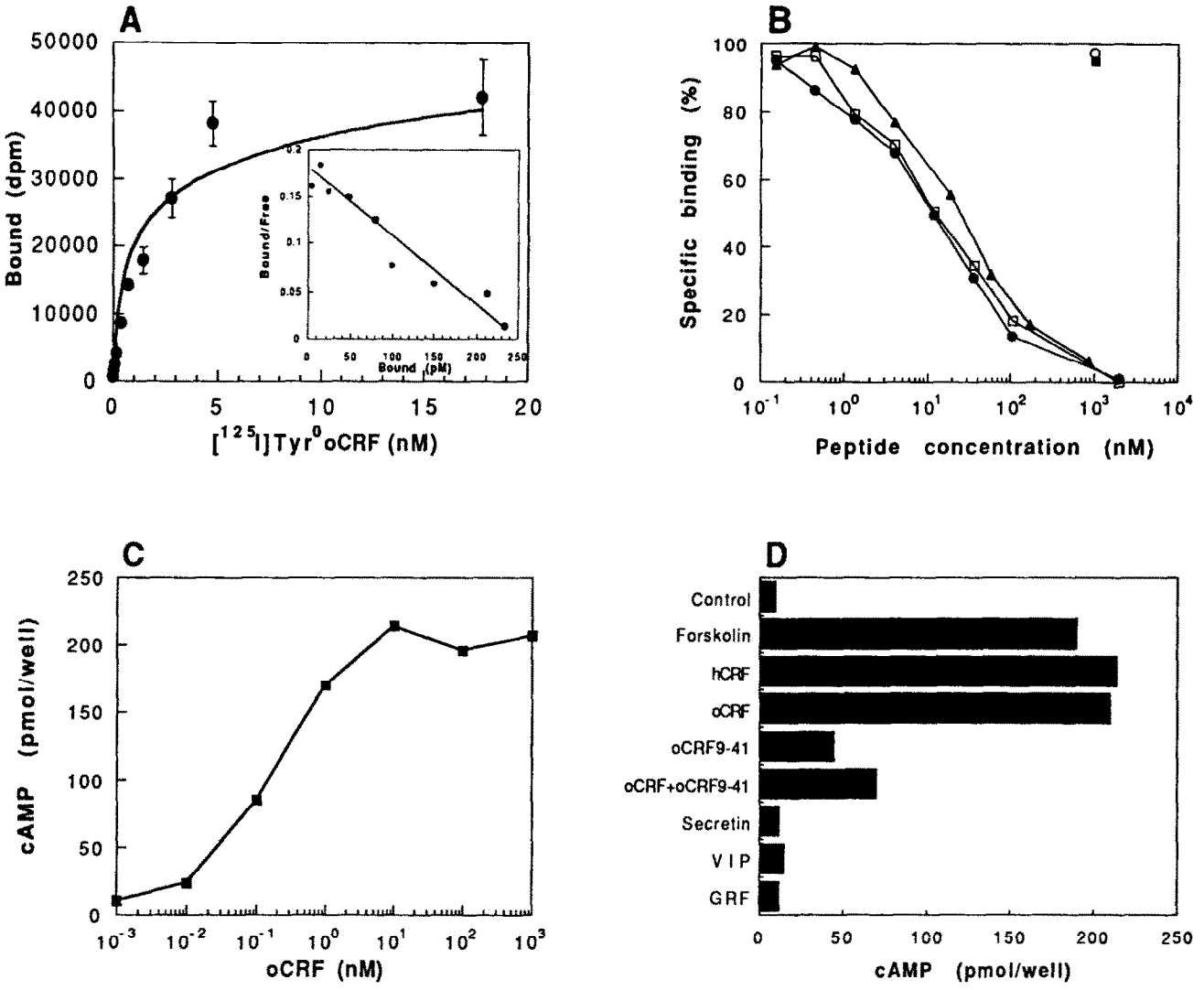

Fig. 2. Functional characterization of the recombinant receptor. COS-7 cells were transfected with the mouse CRF receptor cDNA and assayed for (A) binding of radiolabelled oCRF (inset) Scatchard analysis of the saturation curve; (B) radioreceptor assay of radiolabelled oCRF in the presence of increasing concentrations of competing oCRF (●), hCRF (□), oCRF9–41 (▲), GRF (■) and neurotensin (○); (C) intracellular cAMP accumulation in the presence of increasing concentrations of oCRF; and (D) intracellular cAMP accumulation in the presence of forskolin (12 μM), oCRF (1 μM), hCRF (1 μM), oCRF9–41 (10 μM), oCRF (10 nM) plus oCRF9–41 (10 μM), GRF (10 μM), VIP (10 μM), secretin (10 μM).

(K_i 12 nM), while the unrelated peptides, growth hormone-releasing factor (GRF) and neurotensin, did not inhibit binding (Fig. 2B). The pharmacology of the cloned receptor is therefore similar to that previously described for the CRF receptor [13]. Since it has been shown that the interaction of agonists with the CRF receptor modulates intracellular levels of cAMP [14], we measured the accumulation of this second messenger in the transfected COS-7 cells after oCRF binding. The results showed a dose-dependent increase in intracellular cAMP following oCRF binding (Fig. 2C). hCRF also raised the intracellular cAMP to the level induced by oCRF. The antagonist, oCRF9–41, alone had only a minor stimulatory effect but antagonised cAMP accumulation induced by oCRF. Secretin, vasoactive intestinal peptide (VIP), and GRF did not raise the intracellular cAMP levels. The cAMP accumulation induced by forskolin, a known stimulator of adenylate cyclase, was similar to that of hCRF and oCRF (Fig. 2D). In mock-transfected COS-7 cells ovine or human CRF did not increase intracellular cAMP, while forskolin, as expected, did (not shown). Since these results showed that we had cloned a functional mouse CRF receptor, we used the cloned mouse cDNA to screen a human brain cDNA library. The screening resulted in the identification of a clone containing a 2,536 bp cDNA with an open reading frame that encodes a 415 amino acid protein strikingly similar to the mouse protein. The deduced sequences differ only in 10 amino acids, and both have several potential N-glycosylation sites in the N-terminal extracellular segment (Fig. 1). These results are in agreement with previous data that suggested that the

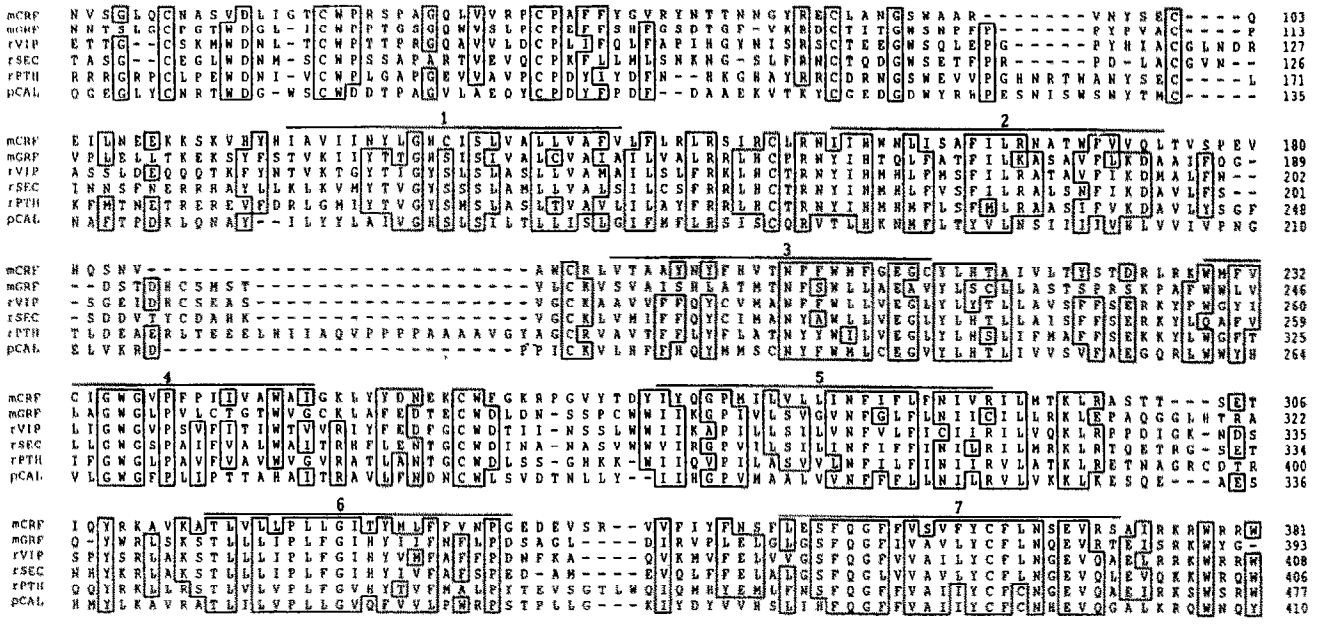

Fig. 3. Alignment of the mouse CRF receptor sequence with the mouse (m) GRF, rat (r) VIP, secretin (sec) and PTH and porcine (p) calcitonin (cal) receptor sequences. The N- and C-terminal regions, which show only slight homology, are not shown. Putative transmembrane domains are indicated.

CRF receptor is a 70 kDa protein [15], which, when deglycosylated, migrates on gel electrophoresis as a 40–45 kDa protein [16]. The glycosylation has been previously suggested to be responsible for the size differences observed between the brain and pituitary CRF receptor [16]. A database search showed that the CRF receptor belongs to a subfamily of G protein-coupled receptors for small peptides like GRF [17], VIP [18], secretin [19], parathyroid hormone (PTH) [20], and calcitonin [21] (Fig. 3). Among the conserved features in this group are 5 cysteines in the N-terminal extracellular segment, and 2 in the first and second extracellular loops. The protein does not share any significant homology with the plasma CRF-binding protein previously described [22].

By Northern blot analysis of RNA from mouse AtT20 cells and human cortex we identified a 2.7 kb transcript for the CRF receptor (not shown). PCR analysis (Fig. 4) showed that the transcript is also present, at a lower level than in the cortex, in human pituitary, brainstem, and testis, results that are consistent with the known distribution of CRF receptors [2,3]. No transcript was detected in placenta, peripheral blood lymphocytes, kidney, or liver. It should be noted that among these tissues, peripheral blood lymphocytes, kidney, and liver had previously been described as CRF receptor positive [23]. These discrepancies may be due to either species differences, since the binding sites were described in rat tissues, or to very low levels of transcripts, or to differences in the transcript sequences. In fact, even if our results suggest that the same mRNA may encode the CRF receptor in the brain, pituitary, and testis, they do not exclude the existence of other CRF receptors. The sequences described here should be helpful in searching for alternative CRF receptors.

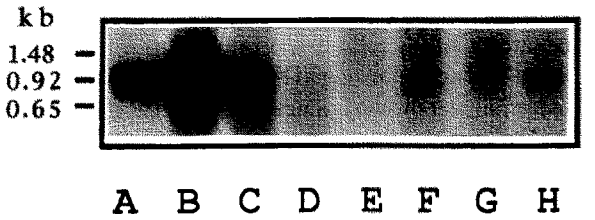

Fig. 4. Expression patterns of the CRF receptor mRNA. Human RNA was prepared from brainstem (lane A), cortex (lane B), pituitary (lane C), peripheral blood lymphocytes (lane D), placenta (lane E), kidney (lane F), liver (line G) and testis (lane H). RNA samples were copied with reverse transcriptase and submitted to PCR. PCR-amplified products were hybridized with a probe complementary to sequence +649 to +666 of the cDNA (protein sequence Leu^{142}-to-Ile^{147}). Size markers are indicated at the left of the figure.

The cloning of the CRF receptor should also help to address the question of the regulation of CRF receptor gene expression in the normal stress response, as well as in pathological conditions, such as Alzheimer's disease and major depression, in which abnormal amounts of CRF receptors have been reported [24,25]. Furthermore, the availability of the cDNA will facilitate the definition of structural features critical to the CRF receptor interaction and may help the rational design of new drugs of potential clinical utility in anxiety and other stress-related behaviors including depression.

Acknowledgements: We thank D. Shire and A. Minty for review of the manuscript, D. Gully for discussions, and A. Coquerel, M.B. Delisle and J. Hanoune for tissue sources.

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