Urocortin is the principal ligand for the corticotrophin-releasing factor binding protein in the ovine brain with no evidence for a sauvagine-like peptide

S M Baigent and **P J Lowry**

School of Animal and Microbial Sciences, University of Reading, Reading, Berkshire RG6 6AJ, UK

(Requests for offprints should be addressed to S M Baigent; Email: s.m.baigent@reading.ac.uk)

ABSTRACT

To purify novel ligands for the corticotrophin-releasing factor binding protein (CRF-BP) from ovine brain, whole brain was homogenised in methanol and the supernatant extracted on Sep-pak C18 cartridges followed by a preliminary HPLC step. Three peaks of ovine CRF-BP ligand activity were detected in the HPLC fractions, the first two of which were also detected by a specific corticotrophin-releasing factor two-site immuno-radiometric assay, the third peak being detected by a human CRF-BP ligand assay, which will not detect ovine CRF. Human CRF-BP ligand-containing fractions were further purified by affinity chromatography on a human recombinant CRF-BP column with two additional HPLC steps. The human CRF-BP ligand was found to: (a) possess a molecular mass of 4707 Daltons, (b) have an N-terminal amino acid sequence (5 residues) identical to rat urocortin, (c) be detected by a specific urocortin radioimmunoassay, (d) have high affinity for both the human and ovine CRF-BPs and (e) be present in many regions of the ovine brain. Additionally, a 300 bp cDNA fragment sharing 83% homology with the rat urocortin gene was cloned from ovine brain, the product of which was predicted to have an identical amino acid sequence to that of rat urocortin. These pieces of information confirmed the identity of the human CRF-BP ligand as an ovine urocortin. The specially developed CRF-BP ligand assays showed that the rank orders of affinity of the CRF family members for human CRF-BP were: carp urotensin-1>>human CRF=rat/ovine urocortin>human urocortin>>frog sauvagine>>ovine CRF, and those for the ovine CRF-BP were: carp urotensin-1> human CRF=rat/ovine urocortin>human urocortin> frog sauvagine>>ovine CRF. This study describes a successful technique for the purification and detection of peptide ligands for the CRF-BP. We conclude that urocortin is the principal ligand for the CRF-BP in ovine brain and we could find no evidence for a centrally located mammalian sauvagine-like peptide.

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INTRODUCTION

Corticotrophin-releasing factor (CRF) is a 41 amino acid neuropeptide which was first isolated from ovine hypothalami in 1981 (Vale *et al*. 1981). CRF is released from the hypothalamus in response to stressful stimuli and its secretion results in activation of the hypothalamo–pituitary–adrenal axis (Linton *et al*. 1985), alterations in behavioural characteristics (Mele *et al*. 1987, Johnson *et al*. 1994) and changes in autonomic functions (Gunion & Taché 1987, Nink *et al*. 1994). In higher primates, peripheral CRF concentrations are normally low, but during pregnancy the peptide is released by the placenta into the maternal circulation, and the plasma levels rise progressively to reach a peak at term (Campbell *et al*. 1987, Sasaki *et al*. 1987). This placental CRF is believed to induce parturition (McLean *et al*. 1995), with a liver-derived circulating CRF binding protein (CRF-BP) (Linton *et al*. 1988, Behan *et al*. 1989, Potter *et al*. 1991) ensuring that any undesirable stress response to excess plasma CRF is inactivated during pregnancy (Linton *et al*. 1990). Plasma CRF-BP circulates only in higher primates, although a membrane-bound form resides in the brain of all mammals studied (Potter *et al*. 1991, Behan *et al*. 1996*a*).

Anomalies in the relationship between the CRF family members and the CRF-BP led to the belief

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that additional ligands for the CRF-BP may exist in mammals. For example, CRF and CRF-BP mRNA do not always co-localise in regions of the brain (Potter *et al*. 1992). Additionally, peripheral CRF-BP circulates in males and non-pregnant females (Linton *et al*. 1988) in the absence of ligand (Linton *et al*. 1987). The higher affinity of the CRF homologue, fish urotensin-1, for the human (h) CRF-BP compared with that of hCRF also raises doubts as to whether CRF is the principal ligand for its binding protein (Lederis *et al*. 1982, 1983, Sutton *et al*. 1995). The gene encoding a urotensin-like peptide, urocortin, has been cloned from rat and human brain regions (Vaughan *et al*. 1995, Donaldson *et al*. 1996). Urocortin possesses a similar affinity to hCRF for the hCRF-BP (Vaughan *et al*. 1995), yet its distribution and abundance has been reported to be limited both in the brain (Vaughan *et al*. 1995, Kozicz *et al*. 1998) and in the periphery of rats (Oki *et al*. 1998). Our experiments help to establish that the addition of urocortin to the CRF family accounts for the molar excesses of CRF-BP over its ligands in the mammalian brain.

The aim of this study was to isolate, purify and characterise novel high affinity mammalian ligands for the hCRF-BP from ovine brain tissue using classical biochemical purification techniques and Edman Degradation amino acid sequencing, followed by a molecular biological approach to isolate the genes coding for such peptides. Brain was chosen as the starting material because in this tissue disparate distributions of CRF-BP mRNA and CRF mRNA imply the presence of additional CRF-BP ligands (Potter *et al*. 1992). Ovine tissue was chosen because differences in affinities of the ovine (o) CRF-BP for oCRF and hCRF (Behan *et al*. 1996*a*), might also indicate the existence of a novel preferred ligand for the CRF-BP in ovine brain. Additionally, the very low affinity of oCRF for the hCRF-BP (Linton *et al*. 1988, Sutton *et al*. 1995) meant that it would not be expected to interfere with the hCRF-BP ligand assay used to measure novel CRF-BP ligands, or with the affinity purification procedure involving use of a human recombinant CRF-BP column.

MATERIALS AND METHODS

Immunoassays

At each stage of purification, fractions were assayed for both CRF immunoreactivity and hCRF-BP ligand activity. All reagents were diluted in assay buffer (0·05 M phosphate buffer containing 0·5% bovine serum albumin, wt/vol, 0·1% sodium azide, wt/vol).

The h/o CRF two-site immunoradiometric assay was a modification of that described by Linton *et al*. (1987) using an unlabelled rabbit anti-hCRF(1–20) antibody in conjunction with a radioiodinated sheep anti-hCRF(21–41) antibody; oCRF was used as standard. Complexes were precipitated with a sheep anti-rabbit antibody. This assay is specific for hCRF and oCRF. All antibodies were raised, purified and iodinated in our laboratory.

For the hCRF-BP ligand assay, hCRF standard (range 0·4–100 ng/ml) or sample was incubated with 2·5 ng recombinant hCRF-BP (50 µl) (Linton *et al*. 1993) containing 0·1% Nonidet P-40 (vol/vol) (Sigma, Poole, Dorset, UK) at room temperature for 3 h. After incubation with ^{125}I-hCRF (35 000 c.p.m./22·7 nCi per tube) overnight at 4 °C, complexes were precipitated with a sheep antibody raised against the hCRF-BP and also with a donkey anti-sheep antibody. This assay detects high affinity ligands for the hCRF-BP including fish urotensin-1, human and rat urocortin and hCRF. The amphibian homologue, sauvagine, is less readily detected and oCRF is undetectable. All components for this assay were made in our laboratory except for the donkey anti-sheep antibody, which was obtained from IGi (Gateshead, Tyne and Wear, UK) and the standard peptides were kindly donated by Jean Rivier and co-workers at the Salk Institute, CA, USA.

In a variation of the above BP ligand assay, oCRF-BP isolated from ovine brain membranes (Behan *et al*. 1996*a*) was used instead of recombinant hCRF-BP. Briefly, the oCRF-BP was isolated by homogenising 30 g ovine brain in 30 ml 10% sucrose containing 1% trasylol (Sigma) and 2 mM phenymethyl-sulphonyl-fluoride (PMSF) (Sigma). The homogenate was centrifuged at 3000 × ***g*** for 5 min at 4 °C to pellet nuclei and particulate matter. The supernatant was centrifuged for 30 min at 28 000 × ***g*** at 4 °C. The resulting membrane pellets were resuspended in 4 ml 10% sucrose and stored at − 20 °C. Just prior to use 1% Nonidet P-40 was added to the thawed membrane preparation and the sample vortexed to dissociate the oCRF-BP from the membranes. The assay was performed with both oCRF and hCRF being used as separate standards. The oCRF-BP ligand assay was better than the hCRF-BP ligand assay at detecting oCRF and sauvagine, as well as the high affinity hCRF-BP ligands. This 'universal' assay detected all characterised CRF family members.

A specific urocortin radioimmunoassay became available soon after we had reached the end of the purification procedure. Standard urocortin (Salk

Institute, USA) or sample was incubated with a sheep anti-human urocortin antibody (1:64 dilution, 0·1% Nonidet P-40, donated by Roger Smith of the John Hunter Hospital, Newcastle, Australia) at 4 °C overnight. After the addition of ^{125}I-labelled rat urocortin (15 000 c.p.m./11·3 nCi per tube, Amersham, Little Chalfont, Bucks, UK) and a further incubation at 4 °C for 48 h, donkey anti-sheep antibody was added to precipitate the assay.

Peptide purification

Two major attempts to purify peptides were made, each following a similar protocol. Whole ovine brains (75 for the first and 150 for the second attempt) were homogenised in three volumes of methanol containing 0·1% trifluoroacetic acid (TFA) (BDH, Poole, Dorset, UK), 1 mM PMSF and 1 mM N-ethylmaleimide (NEM) (Sigma). After centrifugation at 3000 × ***g***, the supernatant was rotary evaporated to one sixth of its original volume and diluted threefold with distilled water. This extract was divided into 10 g tissue weight equivalents and loaded onto Sep-pak C18 cartridges (Waters, Milford, USA). Peptides were eluted manually with increasing concentrations of acetonitrile containing 0·1% TFA. One set of fractions were dried to pellet form in a vacuum centrifuge with 1 mg mannitol, resuspended in buffer, and assayed. Remaining fractions shown to contain hCRF-BP ligand were also dried by vacuum centrifugation, then dissolved in 20% aqueous acetonitrile containing 0·1% TFA and subjected to a preliminary reverse phase HPLC using a Hi-pore C3 column of dimensions 10 × 250 mm (Bio-Rad, Hemel Hempstead, Herts, UK). Peptides were eluted using a gradient of acetonitrile containing 0·1% TFA. The gradient ran from 20–70% over a period of 30 min at a flow rate of 3 ml/min. One millilitre fractions were collected. Fractions were dried by vacuum centrifugation with mannitol and at this stage were assayed for oCRF-BP ligand activity as well as for CRF immunoreactivity and hCRF-BP ligand activity. Fractions with hCRF-BP ligand activity were resuspended in 0·05 M phosphate buffer and affinity purified on a column containing human recombinant CRF-BP cross linked to cyanogen bromide-activated Sepharose (Sigma). Six fractions were eluted, each with 300 µl 1% acetic acid (vol/vol) containing 1 mM octyl glucoside (Pierce, Rockford, IL, USA). Aliquots of each fraction were dried, resuspended in buffer and assayed as before. hCRF-BP ligand was further purified on two HPLC columns (Spherisorb, C18, 4·4 × 250 mm, Hichrom, Reading, Berks, UK and microbore C18, 2 × 250 mm, Vydac, CA, USA) using acetonitrile gradients as described previously, and flow rates of 1 ml/min and 250 µl/min respectively. Approximately 50 ng purified hCRF-BP ligand were subjected to electrospray mass spectrometry and the remainder (400 ng) was subjected to N-terminal amino acid sequencing, the first purified sample being analysed entirely at the National Institute of Medical Research, Mill Hill, London, UK (Electro-spray spectrometer, ABI sequencer) and the second sample at the Ludwig Institute, London, UK (Time-flight spectrometer, Procise sequencer).

Brain distribution of high affinity hCRF-BP ligand

The distribution of hCRF-BP ligand activity in ovine brain was assessed crudely in six regions. Regions were obtained as follows. (a) The main mass of brain was neatly removed from the skull with no protruding tissues attached except for the brain stem and a short length of spinal cord. (b) The cerebellum was removed from this mass of brain tissue. (c) The pink sphere of the thalamic intermediate mass was dissected, together with underlying tissue containing the hypothalamus and tegmentum ('thalamic/hypothalamic region'). (d) The brain stem just ventral to the tegmentum (but including the pons and medulla) and a short region of spinal cord were removed ('brain stem'). (e) The remainder of the brain mass was bisected in the transverse plane just ventral to the optic chiasma ('fore and rear cerebral sections'). (f) The pituitary was removed from a small pocket in the skull. (g) Finally, two lengths of optic nerve tissue, which also contained the retinal vein and artery, were dissected from narrow passages of cartilage in the skull after the main mass of brain had been removed; therefore, these represented only the 2 cm lengths of tissue external to the brain closest to the eye ('optic nerves'). The aim of this experiment was not to identify the exact regions of the brain in which the hCRF-BP ligand was expressed, but to establish whether the hCRF-BP ligand activity was confined to a single area of the brain or was more widely distributed. Each region was extracted to the Sep-pak column stage as previously described and assayed for h/oCRF and hCRF-BP ligand activity.

Cloning

Half of a fresh fetal ovine brain was homogenised in Tri-reagent (Sigma) and total RNA extracted following the Tri-reagent protocol, a modification of the technique used by Chomczynski & Sacchi

(1987). mRNA was isolated using Promega's PolyAT Tract mRNA Isolation System (Promega, Southampton, Hants, UK) (Wallace 1987) and Marathon adapted ds cDNA was synthesised using the technique described by the Clontech Marathon cDNA Synthesis protocol (Chenchik *et al*. 1995). KlenTaq polymerase containing Taq antibody (Clontech, Basingstoke, Hants, UK) was used in all PCR reactions. Marathon PCR is designed to allow rapid amplification of cDNA ends (3′ or 5′ RACE) using a specific primer together with an adapter primer, which hybridises to the Marathon adapters at either end of the cDNA sequence. Initially, in a non-RACE reaction, a human urocortin forward primer (5′ GACAACCCTTCTCTGTCCATTG ACC 3′) based on the N-terminal region of the mature urocortin peptide, was used in conjunction with a degenerate urocortin/urotensin reverse primer (5′ TATGATGCGGTTCWGYYCSGCS YGYTCCC 3′) to amplify a 120 base pair fragment of the urocortin gene. The subsequent primers used separately to amplify full-length ovine urocortin were a 3′ RACE forward primer (5′ CTACTACG GACCCTGCTGGAACTCGCGC 3′) and a 5′ RACE reverse primer (5′ TCTGGCTCTGCGT CCGCGCGAGTTCCACG 3′), both of these primers being used in separate touchdown RACE PCR reactions with the Marathon adapter primers. All primers were synthesized by Genosys, Cambridge, Cambs, UK. Two rounds of Touch-down PCR (Don *et al*. 1991) were carried out before the PCR products could be visualised on 1·2% agarose gels. Bands were extracted from the gel using a Gene Clean Kit (Bio-101, Vista, USA) and cDNA fragments were cloned, transformed and sequenced (School of AMS sequencing service, University of Reading). Sequences were analysed using the BLAST sequence homology search program.

Urocortin analysis

The urocortin radioimmunoassay was used to aid identification of the hCRF-BP ligand. A whole ovine brain was extracted and purified to the preliminary HPLC stage, where fractions were assayed for urocortin and hCRF-BP ligand activity using human urocortin standard and ^{125}I-labelled rat urocortin. Fractions were also assayed for h/oCRF immunoreactivity.

Human and ovine CRF-BP ligand-binding curves

To compare relative affinities of the CRF family members for both ovine and human CRF-BP, assays were performed as described above with peptide concentrations ranging from 0·2 to 100 ng/ml using ^{125}I-hCRF as tracer. The peptides used were carp urotensin, human urocortin, rat/ovine urocortin, hCRF, oCRF and sauvagine.

RESULTS

Peptide purification

After Sep-pak extraction, a broad peak of CRF immunoreactivity amounting to 40 ng/g brain tissue was detected, whereas hCRF-BP ligand activity appeared to be much lower at 4 ng/g in hCRF equivalents. Three peaks of activity in fractions 54, 56–58 and 64–67 were separated by HPLC (Fig. 1A and B). Fractions 54 and 56–58 were identified as oxidised and reduced forms of oCRF in the oCRF-BP ligand assay and the h/oCRF IRMA. Fractions 64–67 had ligand activity in the oCRF-BP and hCRF-BP ligand assays, but had no CRF immunoreactivity. These results indicated that the hCRF-BP ligand was more hydrophobic than both oxidised and reduced forms of oCRF.

At the affinity purification step (Fig. 1C), the majority of oCRF passed through the column without binding (void fraction). However, the fact that 5% of total oCRF was able to bind to the affinity column, despite its negligible affinity for the hCRF-BP, was attributed to the large excess of the binding protein available for interaction. It was also at the affinity purification step that the greatest loss of hCRF-BP ligand activity was incurred. Although greater than 90% of the ligand bound to the column, it was only possible to elute approximately 10% of the captured peptide (fractions 2 and 3). These results indicated that the ligand had a high affinity for the hCRF-BP and illustrated the degree of absorptive loss associated with the purification of a highly hydrophobic peptide. hCRF-BP ligand-containing fractions, accumulated from ten or more separate affinity purification runs, were combined for the final two HPLC steps, after which the ligand was considered to be suitably pure for mass spectrometry and amino acid sequencing.

The first major purification attempt yielded just 424 ng hCRF-BP ligand in hCRF equivalent from 75 ovine brains (Fig. 1D), a 6% recovery from the original 8 μg obtained after completion of the Sep-pak extractions. The second purification attempt, in which 150 ovine brains were extracted as starting material, resulted in 970 ng hCRF-BP ligand after the final HPLC step. It should be emphasised that only one peak of hCRF-BP ligand activity was detected on each final HPLC run and on both occasions the purified ligand eluted at

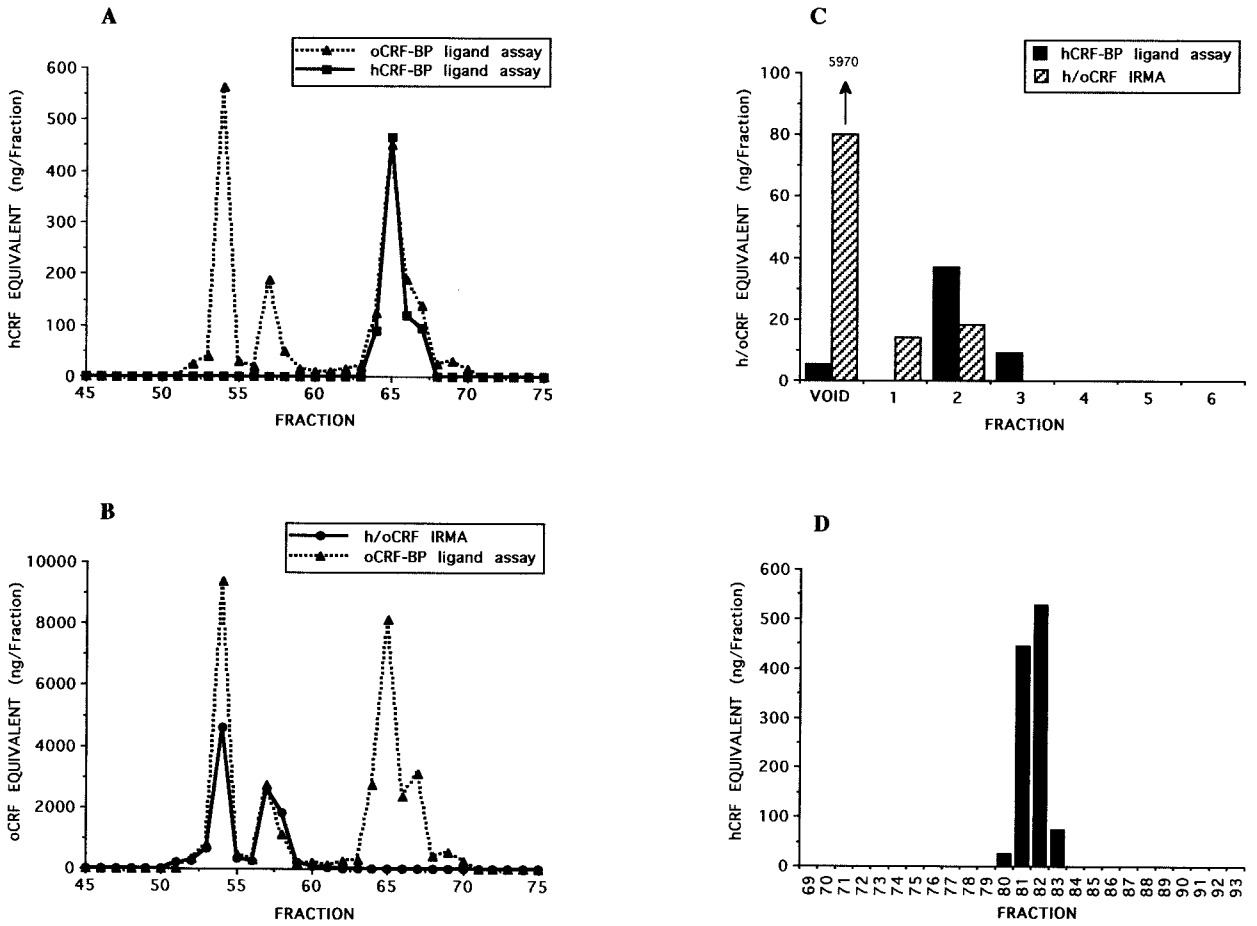

FIGURE 1. Purification of high affinity hCRF-BP ligand. Whole ovine brain was extracted in methanol containing enzyme inhibitors and 0·1% TFA, and the supernatant was extracted on Sep-pak C18 cartridges. Selected fractions were then subjected to a preliminary HPLC on a C3, Bio-Rad, Hi-pore, 10 × 250 mm column and eluted fractions assayed for (A) human and ovine CRF-BP ligand activity using hCRF as standard and (B) h/o CRF immunoreactivity and ovine BP ligand activity using oCRF as standard. HPLC fractions containing hCRF-BP ligand activity were further purified by affinity chromatography on a human recombinant binding protein column and peptides eluted with 300 µl fractions of 1% acetic acid (C). Fractions 2 and 3 were subjected to HPLC on a C18 Spherisorb, Hichrom, 4·4 × 250 mm column followed by an HPLC on a C18, Microbore, Vydac, 2·0 × 250 mm column (D).

precisely the same position on the acetonitrile gradient (60% acetonitrile). Mass spectrometry provided additional proof that the same peptide was present in each of the two purified samples as they were both found to possess a molecular mass of 4706/4707 Daltons (the same as that for rat urocortin). Given this information, identical amino acid sequences for the two samples would be expected. The short N-terminal sequences obtained on each occasion were identical to rat urocortin for the first five amino acids (DDPPL) but, as the signals became faint, the residues identified thereafter were not deemed reliable by the sequencers. Such short sequences had been expected due to the low concentrations of the hCRF-BP ligand available for sequencing. The sequences of the other CRF family members are aligned in Fig. 2.

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hCRF     SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII
oCRF     SQEPPISLDLTFHLLREVLEMTKADQLAQQAHSNRKLLDIA
cUT-1    NDDPPISIDLTFHLLRNMIEMARNENQREQAGLNRKYLDEV
rUCN       DDPPLSIDLTFHLLRTLLELARTQSQRERAEQNRIIFDSV
SVG        EGPPISIDLSLELLRKMIEIEKQEKEKQQAANNRLLLDTI
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FIGURE 2. Amino acid sequences of the CRF family of peptides. cUT-1=carp urotensin-1, rUCN=rat urocortin, SVG=frog sauvagine.

TABLE 1. The levels of h/oCRF immunoreactivity and hCRF-BP ligand activity detected in different regions of the ovine brain. A single ovine brain was crudely divided into seven distinct sections and each region was separately homogenised and extracted on Sep-pak C18 cartridges. The values listed in the table represent the total concentrations of hCRF-BP ligand activity (in hCRF equivalent) and oCRF immunoreactivity eluted from the cartridges, calculated as nanograms per gram of tissue

	Mass (g)	**oCRF** (ng/g)	**hBP ligand** (ng/g)
Brain region			
Fore cerebrum	18·7	31·6	12·3
Rear cerebrum	42·6	34·5	13·5
Optic nerves	0·8	26·2	35·0
Thalmus/hypothalamus	12·3	25·2	8·9
Pituitaries × 10	3·0	17·0	10·3
Cerebellum	14·0	22·2	7·3
Brain stem	7·0	16·6	13·7

Brain distribution

The levels of oCRF and hCRF-BP ligand per gram of brain section are summarised in Table 1. Both peptides appeared to be distributed across all the brain regions studied, although highest levels of oCRF were found in the cerebral regions (31·6 and 34·5 ng/g), whilst hCRF-BP ligand activity was slightly elevated in the brain stem (13·7 ng/g) and greatly raised in the optic nerves (35·0 ng/g). This experiment was repeated and similar results were obtained.

Subcloning

The non-degenerate human urocortin forward primer used in conjunction with a reverse direction urocortin/urotensin primer yielded a PCR product of the expected 120 bp. Three separate identical clones containing this cDNA fragment failed to match exactly with any other known gene, but were all found to share an 83% degree of identity with rat urocortin and a 90% degree of identity with the human gene. The clone was, therefore, believed to be that of an ovine form of urocortin. In an attempt to clone full-length cDNA, two primers, one for 5′ RACE and the other for 3′ RACE, were designed based on the central region of the cloned fragment sequence, and were used in a Touchdown PCR reaction together with the Marathon adapter primer. 5′ RACE PCR did not reveal the anticipated product, but 3′ RACE resulted in a clear 300 bp DNA band on an agarose gel after two 30-cycle rounds of PCR. The sequences of three identical clones derived from this band all showed a high degree of identity with rat and human urocortin.

At about the same time that our cDNA sequence was obtained, two other groups managed to clone regions of precisely the same gene, which were extended at the 5′ end to encode the complete mature peptide region and some of the precursor (Genbank Database accession numbers: AF051807, D Cepoi, S Sutton & W Vale and AF085334, M P Richardson, unpublished sequences at time of writing). The placement of the two extended cDNA sequences onto the database (and the translated product, which was identical to rat urocortin in the mature peptide region) have allowed us to positively identify our clone as that belonging to ovine urocortin. All three overlapping database sequences for the ovine urocortin gene (including our own sequence) have been combined and are aligned with the rat urocortin and human urocortin cDNA sequences in Fig. 3. The translated ovine urocortin product is also illustrated.

Urocortin analysis

The result of this experiment is illustrated in Fig. 4 and suggests that the hCRF-BP ligand activity detected in fractions 10–13 can be accounted for by similar levels of urocortin immunoreactivity. Additionally, urocortin standard peptide was shown to elute at exactly the same point on the acetonitrile gradient as the hCRF-BP ligand activity detected, confirming our belief that the high affinity hCRF-BP ligand purified from ovine brain was in fact ovine urocortin.

Human and ovine CRF-BP ligand binding curves

The CRF-BP ligand binding curves are plotted in Fig. 5. Concentrations of peptides are plotted as ng/ml, but since the molecular masses of the CRF family members are all very similar (between 4596 and 4866 Daltons), the calculated ED_{50} values can closely represent comparative affinities of the peptides for the ovine or human CRF-BPs. Although these experiments alone do not allow us to compare the affinities of a particular ligand for the hCRF-BP with its affinity for the oCRF-BP, they do enable us to rank the CRF family members separately in order of affinity for the two CRF-BPs. Behan *et al*. (1996*a*) previously demonstrated that hCRF has similar affinities for the human recombinant CRF-BP and native ovine CRF-BP and, therefore, the positions of the other peptide standard curves in relation to the hCRF standard curves will approximately reflect their relative

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rUCN   GCGGCCGCTC TCCATCTTGC ACTGGATAGA CACTCCGATA ACCTGGCAGG
hUCN   ---------- -CACCCTGCG CTGCCCCTGT GTGTCCAGGG CCGGCGGCAC
oUCN   ---------- ---------- ---------- ---------- ----------

rUCN   CGGCACCATG AGGCAGAGGG GACGCGCTAC GCTCCTGGTA GCGTTGCTGC
hUCN   CATGAGGCAG GCGGGACGCG CAGCGCTGCT GGCCGCGCTG CTGCTCCTGG
oUCN   ---------- ---------- ---------- ---------- ----------

rUCN   TTCTGGTTCA GCTGCGCCCG GAGAGCAGCC AGTGGAGCCC AGCGGCTGCG
hUCN   TACAGCTGTG CCCTGGGAGC AGCCAGAGGA GCCCCGAGGC GGCCGGGGTC
oUCN   ---------- ---------- ---------- ---------- ----------

rUCN   GCGGCGAATG TGGTCCAGGA TCCGAATCTG CGATGGAACC CCGGAGTGCG
hUCN   CAGGACCCGA GTCTGCGCTG GAGCCCCGGG GCACGGAACC AGGGTGGCGG
oUCN   ---------- ---------- ---------- ---------- ----------

                10         20         30         40         50
rUCN   GAATCAGGGC GGAGGTGTCC GCGCACTCCT CTTGCTGTTA GCGGAGCGCT
hUCN   GGCCCGCGCG CTCCTCTTGC TGCTGGCGGA GCGCTTCCCG CGCCGCGCGG
oUCN   CCACGCTCTC CTCTTGCTGC TGGCGGAGCG CTTCCCGCGC CGCCGGGCGG
oUCN    H  A  L   L  L  L  L   A  E  R   F  P  R    R  R  A

                60         70         80         90         100
rUCN   TCCCGCGCCG CGCGGGATCT GAGCCTGCAG GCGAGCGGCA ACGACGAGAC
hUCN   GGCCCGGCCG ATTGGGACTC GGGACGGCAG GCGAGCGGCC GCGGCGGGAC
oUCN   AGCAGGGCCG ATGGGGATCC ACGACGGCAG GTGAGCGGCA GCGACGAGAC
oUCN   E  Q  G  R   W  G  S   T  T  A   G  E  R  Q    R  R  D

                110        120        130        140        150
rUCN   GACCCGCCGT TGTCCATCGA CCTCACCTTC CACCTGCTGC GGACCCTGCT
hUCN   AACCCTTCTC TGTCCATTGA CCTCACCTTT CACCTGCTGC GGACCCTGCT
oUCN   GACCCTCCCC TGTCCATTGA CCTCACCTTC CACCTACTAC GGACCCTGCT
oUCN    D  P  P   L  S  I  D   L  T  F   H  L  L   R  T  L  L

                160        170        180        190        200
rUCN   AGAGCTAGCT CGGACACAGA GCCAGCGCGA GCGCGCAGAG CAGAACCGCA
hUCN   GGAGCTGGCG CGGACGCAGA GCCAGCGGGA GCGCGCCGAG CAGAACCGCA
oUCN   GGAACTCGCG CGGACGCAGA GCCAGAGGGA GCGCGCCGAG CAGAACCGCA
oUCN     E  L  A   R  T  Q   S  Q  R  E   R  A  E   Q  N  R

                210        220        230        240        250
rUCN   TCATATTCGA TTCGGTGGGC AAGTGATCGG CCCCGTATGG GGTCCAGAAA
hUCN   TCATATTCGA CTCGGTGGGC AAGTGATGGC CCGGTTTGGG GCTGCGAAAA
oUCN   TCATATTCGA CTCGGTGGGC AAGTGATCGG CGGGTCTCGG GCCCCGACAA
oUCN   I  I  F  D   S  V  G   K  •

                260        270        280        290        300
rUCN   GCCTTGACCC TTTCCCCCAC CTACCCCGGG GCTGGAGCGC GCACAACCGG
hUCN   CGTTGACCCC TTTCCCCCAC CCCAGAGTTG GGATGCGGGG CAGAGCCACC
oUCN   TCTCGACCCC CATGCTCCAC CTCAGGGTTG AGACGTGAGC GACAGGAACT

                310        320        330        340        350
rUCN   AGCTGGCTCA GTCCCGCGTT GCAGCGCCGC CCAGAGTTAC CCTGAACACT
hUCN   AGGGCACTGT CTGCGTGACT A
oUCN   GACCAAGTCC TGCAGGGCTA GAGCGGCCTA GGGA---TAC CCTGAGCACT

                360        370        380        390        400
rUCN   CTGACTGGAT A--TTTTTTA ATAAAACTGC C
hUCN   ---------- ---TTTTTTA ATAAAAGTAC TGAAGACCCG TTGGC
oUCN   CTCCGCGTTA CCGGTTTTTA ATAAAAGTGC TGAAGAGCCG TTGGCCTCTG
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FIGURE 3. Comparison of the ovine urocortin cDNA sequence with full length rat and human sequences. The complete region sequenced from our overlapping 120 bp and 300 bp fragments is underlined (bases 136–388, accession number AFO84258). The sequence contains the majority of the coding region for the mature peptide and the complete 3′ noncoding region leading up to the polyA tail. The two other sequences on the database ran from bases 1–208 (M P Richardson, Centre for Reproductive Biology, Edinburgh – accession number AF085334) and bases 93–400 (D Cepoi, S Sutton & W Vale, Salk Institute, USA – accession number AF051807). The mature peptide sequence for ovine urocortin is identical to the published rat urocortin sequence. UCN=urocortin, h=human, r=rat, o=ovine.

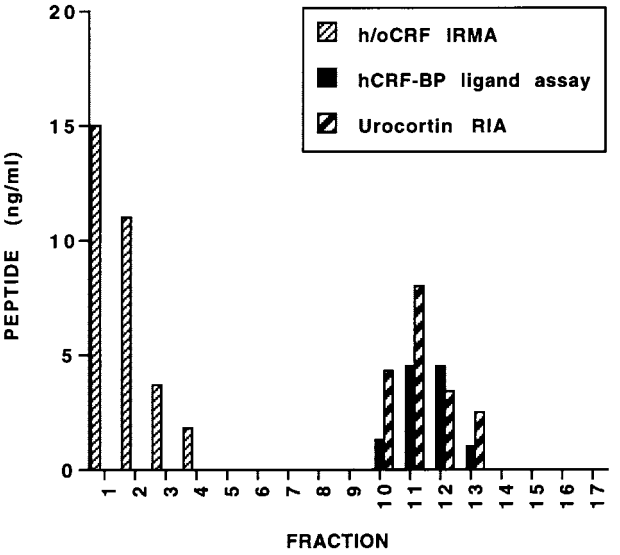

FIGURE 4. Preliminary HPLC of ovine brain extract using the urocortin radioimmunoassay (RIA) to help ascertain the identity of the hCRF-BP ligand. Two whole ovine brains were homogenised and extracted on Sep-pak cartridges as described previously prior to being subjected to a preliminary HPLC on a C3 Bio-Rad, Hi-pore 10 × 250 mm column. All fractions collected were taken to dryness, resuspended in assay buffer and assayed using the h/oCRF immunoradiometric assay (IRMA), the hCRF-BP ligand assay and the urocortin radioimmunoassay.

affinities for the two CRF-BPs. The binding proteins share similar rank orders of potency in their abilities to bind the CRF family of peptides, the order for hCRF-BP being: carp urotensin>> hCRF=rat/ovine urocortin>human urocortin>> sauvagine>>oCRF, and for the oCRF-BP: carp urotensin>hCRF=rat/ovine urocortin>human urocortin>sauvagine>>oCRF. The results demonstrate that carp urotensin, hCRF and the urocortins are all potent ligands for the human and ovine CRF-BPs. Interestingly, sauvagine has moderate affinity for the oCRF-BP, but low affinity for the hCRF-BP, whilst oCRF has a low affinity for the oCRF-BP and negligible affinity for the hCRF-BP.

DISCUSSION

We have shown that extracted ovine brain not only contains oCRF, but also a second peptide ligand for the oCRF-BP. Unlike oCRF, this peptide had high affinities for both recombinant hCRF-BP and native oCRF-BP. Purification of this ligand showed that it had a molecular mass and N-terminal amino acid sequence identical to those predicted for rat

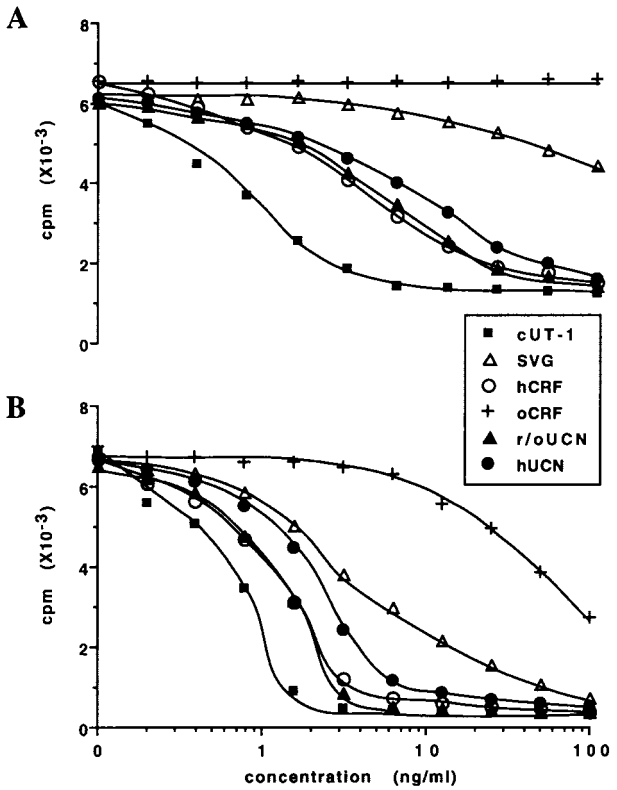

FIGURE 5. Peptide binding curves for the human (A) and ovine (B) CRF-BP ligand assays. Synthetic peptides (concentration range 0·4–100 ng/ml) were incubated with 2·5 ng of either native ovine brain oCRF-BP or recombinant hCRF-BP for 3 h at room temperature prior to the addition of 35 K c.p.m. ^{125}I-hCRF and a further overnight incubation at 4 °C. Complexes were precipitated by addition of a sheep anti-hCRF-BP antibody and a donkey anti-sheep antibody. See legends for Figs 2 and 3 for abbreviations.

urocortin, that it was detected by a urocortin radioimmunoassay, and that it was widely distributed across the ovine brain. Later, the cloning of several overlapping ovine urocortin 3′ gene fragments from ovine brain (combined length 350 bp), provided additional proof that urocortin is expressed in ovine brain and confirmed that the mature peptide amino acid sequence of ovine urocortin is identical to that of rat urocortin. Although urocortin has now been cloned from a variety of mammalian tissues, the peptide itself has never before been purified completely from crude tissue. This paper is the first to describe a successful purification procedure for urocortin, which could also be applied to other CRF-BP ligands, and demonstrates that much can be learnt about the properties of native peptides during the purification processes. For example, we show here that urocortin peptide is found at concentrations tenfold lower than that of oCRF in ovine brain and is very hydrophobic, making it difficult to purify in significant quantities. Additionally, N-terminal sequencing confirmed the site of cleavage of the mature peptide from its precursor protein, just as mass spectrometry accurately revealed the mass of this active peptide.

The CRF-BP ligand assays were used to provide information on the relative affinities of ovine urocortin and the other CRF family members for the ovine and human binding proteins. The endogenous ovine brain CRF-BP, which shares 86% amino acid homology with the hCRF-BP (Behan *et al*. 1996*a*), possesses different binding properties to its human counterpart, especially with respect to its greatly enhanced ability to bind sauvagine and oCRF. Sutton *et al*. (1995) showed that residues Ala^{22}, Arg^{23} and Glu^{25} in hCRF and carp urotensin are important for high affinity binding of these two ligands to hCRF-BP. The corresponding residues in low affinity oCRF are Thr^{22}, Lys^{23} and Asp^{25}, and the substitution of these three key amino acids with the corresponding hCRF residues results in the formation of a high affinity hCRF-BP ligand. Interestingly, urocortin contains two of these important residues (Ala^{22} and Arg^{23}) and sauvagine only one (Glu^{25}). Our human and ovine CRF-BP ligand assay binding curves show that hCRF, carp urotensin and the urocortins have ED_{50} values that are between 1·5- and 8-fold higher in the hCRF-BP ligand assay than in the oCRF-BP ligand assay. However, sauvagine and oCRF have ED_{50} values that are at least 200-fold higher in the hCRF-BP ligand assay compared with the oCRF-BP ligand assay. This suggests that sauvagine and oCRF may contain common essential amino acids in their central binding regions, which allow them to have considerably higher affinities for the oCRF-BP than for the hCRF-BP. We propose that Lys^{23}, present in both sauvagine and oCRF (Arg in all other family members), is the best candidate for such a residue, although amino acids located elsewhere in the peptide sequences would undoubtedly be expected to contribute to this effect.

It is now generally accepted that urocortin is the mammalian equivalent to fish urotensin, mainly due to the high degree of identity between the two peptides at the amino acid level, although urotensin does have a 5- to 10-fold higher affinity for the hCRF-BP. However, the existence of a mammalian peptide more closely related to frog sauvagine in peptide sequence, which may be expected to have lower affinity for the CRF-binding protein, previously remained a possibility. In our studies on

ovine brain tissue, the oCRF-BP ligand assay readily detected both high affinity rat/ovine urocortin (ED_{50}=1·2 ng/ml) and low affinity oCRF (ED_{50}=65). This assay would, therefore, be expected to detect peptides with a high degree of homology to sauvagine (ED_{50}=5·0). However, no significant residual oCRF-BP ligand activity, which would indicate the presence of a sauvagine-like peptide, was detected in the eluted HPLC fractions and we therefore conclude that oCRF and ovine urocortin are the only natural ligands for the sheep brain CRF-BP. We show here that ovine urocortin has a 50-fold higher affinity for the oCRF-BP than oCRF and, with the acceptance that urocortin may be more widely distributed across the brain than was initially suggested (as demonstrated by our brain distribution experiments discussed below), we propose that urocortin may be the predominant ligand for the oCRF-BP in ovine brain.

Previous studies have shown that, in the rat, urocortin immunoreactivity and mRNA predominates in the Edinger Westphal (EW) nucleus and the lateral superior olive, with less obvious staining in the olfactory bulb, lateral hypothalamus and lateral septal nucleus (Vaughan *et al*. 1995, Kozicz *et al*. 1998). Other groups have also detected urocortin mRNA in rat pituitary (Wong *et al*. 1996, Iino *et al*. 1997). These findings would account for the hCRF-BP ligand activity which we detected in the fore and rear cerebral regions, the thalamic region, the pituitary and, perhaps, the brainstem (which is innervated by projections arising in the EW nucleus). However, the presence of moderate concentrations of the ligand in the cerebellum and comparatively high concentrations of the peptide in the optic nerves appear to be novel findings. It is possible that our technique would be able to detect small quantities of dispersed urocortin peptide, which would not be visible using immunohistochemistry. CRF-like immunoreactivity has previously been reported to be present in the photoreceptors and inner plexiform layers of the eye (Mastorakos *et al*. 1995), but the source of CRF-BP ligand activity in the optic neural tissue/blood vessels remains uncertain. Given that oCRF was also detected in many regions of the central nervous system, the extensive distribution of brain CRF-BP may now be accounted for by the combined presence of cells containing CRF and urocortin.

It has been suggested that the role of membrane bound CRF-BP in the brain is to modify the activity of CRF and urocortin at nerve terminals where they are released, thereby preventing the peptides from activating CRF R1 and R2 receptors (Chang *et al*. 1994, Lovenburg *et al*. 1995). This process may be reversible and, in particular conditions, the binding protein may release the captured ligands, allowing them to act at receptor sites in close proximity. Behan *et al*. (1996*b*) suggest that urocortin release in the human brain may elevate endogenous levels of 'free' hCRF by displacing hCRF from the binding protein. This theory may be even more relevant with respect to the ovine brain, due to the low affinity of oCRF for the oCRF-BP. Alternative hypotheses include the possibility of CRF-BP being released from brain membranes on association with ligand, and acting as a ligand delivery system for distant CRF brain receptors. Cleavage of the CRF-BP may offer a further mechanism by which modulation of ligand activity may occur (Kemp *et al*. 1996). Despite a variety of valid suggestions concerning the role of the brain CRF-BP, experimental evidence to support these hypotheses remains limited.

In conclusion, we propose that urocortin and CRF are the only ligands for the CRF-binding protein in the ovine brain. Although CRF was measured at concentrations 10-fold higher than those of urocortin, it was found to have a 50-fold lower affinity for the native ovine binding protein and, therefore, urocortin appears to be the main ligand for the brain CRF-BP. We cannot, however, rule out the possibility of there being sauvagine-like peptides in the human periphery where the hCRF-BP circulates in males and non-pregnant women. The absence of significant amounts of either CRF or urocortin in the peripheral tissues or blood of these individuals still remains unexplained. The recent finding that urocortin is expressed in human peripheral lymphocytes (Bamberger *et al*. 1998) can only partially account for the presence of circulating CRF-BP in these individuals, but, nevertheless, we speculate that the peripheral tissues of humans remain the most likely source from which a third mammalian ligand for the CRF-BP will eventually be isolated.

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