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Molecular and Cellular Endocrinology 173 (2001) 29–40

MCE
Molecular and Cellular Endocrinology

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Biochemical characterization and expression analysis of the *Xenopus laevis* corticotropin-releasing hormone binding protein

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Received 14 June 2000; accepted 12 October 2000

Abstract

Corticotropin-releasing hormone (CRH) plays a key role in the regulation of responses to stress. The presence of a high affinity binding protein for CRH (CRH-BP) has been reported in mammals. We have characterized the biochemical properties and expression of CRH-BP in the South African clawed frog, *Xenopus laevis*. Apparent inhibition constants ($K_{i[app]}$) for different ligands were determined by competitive binding assay. *Xenopus* CRH-BP (xCRH-BP) exhibited a high affinity for xCRH ($K_{i[app]} = 1.08$ nM) and sauvagine (1.36 nM). Similar to rodent and human CRH-BPs, the frog protein binds urotensin I and urocortin with high affinity, and ovine CRH with low affinity. RT-PCR analysis showed that xCRH-BP is expressed in brain, pituitary, liver, tail, and intestine. Brain xCRH-BP mRNA is expressed at a relatively constant level throughout metamorphosis and increases slightly in the metamorphic frog. By contrast, the gene is strongly upregulated in the tail at metamorphic climax. Thus, regulation of xCRH-BP gene expression is tissue specific. Because xCRH-BP binds CRH-like peptides with high affinity the protein may regulated, the bioavailability of CRH in amphibia as it does in mammals.

Keywords: CRH; CRH-BP; Stress; *Xenopus*; Development

1. Introduction

Corticotropin-releasing hormone (CRH) is a 41 amino acid polypeptide that functions as the primary neuroendocrine integrator of the vertebrate stress response. CRH is released following emotional or physical stress and initiates a cascade of endocrine signaling events involving the anterior pituitary and the adrenal cortex (the hypothalamo-pituitary-adrenal [HPA] axis). This activation ultimately gives rise to an elevation in plasma concentrations of glucocorticoids (Vale et al., 1981). Glucocorticoids mediate physiological and behavioral adaptive responses to stress and are essential to survival (Sapolsky et al., 2000). In mammals, CRH is expressed in multiple sites throughout the brain and may play an essential role as a neurotransmitter or neuromodulator in addition to its role as a hypophysiotropin. For instance, CRH is known to influence appetite, locomotion, and behavioral responses to stress and anxiety (Altemus et al., 1994; Gargiulo and Donoso, 1996; Glowa et al., 1992; Linthorst et al., 1997; Neumann et al., 1998). Recent findings support a critical role for CRH of fetal and/or placental origin in regulating the length of the gestational period in humans and sheep (McLean et al., 1995).

An accumulating body of evidence shows that in nonmammalian vertebrates CRH controls both the HPA and the HP-thyroid axes. CRH peptides act at the level of the pituitary in birds, reptiles, amphibians and fishes to stimulate the release of both adrenocorticotropin (ACTH) and thyrotropin (TSH) (Denver, 2000) and consequently increase the production of both glucocorticoids and thyroid hormone (Denver, 1997,

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0303-7207/01/$ - see front matter
PII: S0303-7207(00)00437-8

1998). Glucocorticoids and thyroid hormone play critical roles in vertebrate development, and thus CRH has been proposed to be an evolutionarily conserved developmental signaling molecule (Denver, 2000).

CRH and related peptides represent the only family of neuropeptides for which a specific binding protein (CRH-BP) has been identified. The CRH-BP was first detected, isolated and characterized from human plasma by several groups (Behan et al., 1988; Orth and Mount, 1987; Suda et al., 1988). Since then genomic (human: Behan et al., 1993a; and rat: Cortright et al., 1997) and mRNA (sheep: Behan et al., 1996a; mouse: Cortright et al., 1995 human and rat: Potter et al., 1991) sequences of several mammalian CRH-BPs have been reported. The CRH-BP and CRH receptors are structurally unrelated and they exhibit different pharmacological profiles (Behan et al., 1996a,b, 1995). All CRH-BPs have 10 highly conserved cysteines and in the human protein these cysteines have been shown to form five intramolecular disulfide bonds whose integrity is required for binding; i.e., treatment with reducing agents abolishes CRH binding (Fischer et al., 1994; Suda et al., 1988). Mammalian CRH-BPs are N-glycosylated and their binding function is temperature-sensitive and pH-dependent (Cortright et al., 1995; Orth and Mount, 1987; Suda et al., 1988, 1989).

In humans CRH-BP is produced in the brain, anterior pituitary, liver, and placenta (Petraglia et al., 1993; Potter et al., 1991); whereas, in rodents it is produced only in the brain and pituitary. Only in humans are there appreciable levels of CRH-BP in the blood, likely owing to its production by the liver and by the placenta. It has been proposed that a major role for the CRH-BP in mammals is to sequester CRH and thus neutralize its bioactivity. In support of this hypothesis CRH-BP exhibits a higher affinity for CRH than the CRH receptor (Sutton et al., 1995) and CRH-BP inhibits CRH-mediated ACTH secretion in vitro (Linton et al., 1990; Potter et al., 1991; Suda et al., 1988). Both CRH and CRH-BP are produced in large quantities by the human placenta and are secreted into the bloodstream. Placental CRH-BP has been implicated in the protection of the human pituitary gland against elevated CRH levels during normal pregnancy and in pregnancies complicated by hypertension (Linton et al., 1993; Perkins et al., 1993; Petraglia et al., 1996). Studies of transgenic mice that overexpress CRH-BP or mice with a targeted disruption of the CRH-BP gene have provided evidence for its role as an important regulator of the HPA axis and a modulator of CRH actions in behavioral responses to stress (Burrows et al., 1998; Karolyi et al., 1999; Lovejoy et al., 1998).

Recently, a cDNA for a frog CRH-BP was isolated from a subtractive library derived from the tail of *Xenopus* tadpoles (Brown et al., 1996). Interestingly, the *Xenopus* (x)CRH-BP was identified as one of 17 genes upregulated by thyroid hormone in the tail resorption program during tadpole metamorphosis. The xCRH-BP mRNA encodes a 321 amino acid polypeptide with a highly conserved primary structure compared with mammalian CRH-BPs. However, neither the functional characteristics of the protein nor the expression patterns of the gene have been described. Towards understanding the evolution of this protein and its role in regulating CRH bioavailability we have analyzed the biochemical characteristics of *Xenopus* brain CRH-BP and compared these with the mouse CRH-BP. In addition, we examined the developmental and tissue-specific expression of the xCRH-BP gene.

2. Material and methods

2.1. Animals and tissue collection

Adult *Xenopus laevis* were maintained in large holding tanks at 22°C under a 12:12 light:dark cycle and fed beef liver twice a week. Spawning was induced by injection of gonadotropin releasing hormone agonist (Sigma). Newly hatched tadpoles were maintained in 10% Holtfreter's solution for 5 days and then transferred to a large holding tank and fed pulverized rabbit chow. Animals at different stages of development were euthanized by immersion in 0.1% benzocaine and tissues collected for protein and RNA analyses (see below). Tadpoles were staged based on the staging system of (Nieukoop and Faber, 1994).

2.2. Cloning of Xenopus laevis CRH-BP cDNA and plasmid construction

The full-length xCRH-BP cDNA was isolated by RT-PCR using 1 μg total brain RNA as template. Total RNA was extracted using Trizol (Gibco BRL). Reverse transcription of total RNA was conducted using random hexamers and Superscript II reverse transcriptase (Gibco BRL). 10% of the reverse transcription reaction was amplified by PCR using gene specific primers (Genbank accession # U41858). The forward primer 5′ATAAGAATGCGGCCGCTCTGCATCATGACTCC TGCTTCCA3′ contains a *Not I* site and corresponds to position 64 through 87 of the mRNA (which spans the translation initiation site). The reverse primer 5′GGGCTCGAGGATTCTTTGCAAATCAGTCACTG G3′ contains a *Xho I* site and corresponds to position 1027 through 1050 of the mRNA (which spans the translation stop site). PCR conditions were 94°C 1 min, 72°C 2 min and 55°C 1 min for 30 cycles. The EXPAND polymerase mix (Roche, BMB) which contains a mixture of *Taq* and *Pwo* DNA polymerases was used to minimize the chance of PCR errors. The PCR product was phenol-chloroform extracted, precipitated,

digested with *Not I* and *Xho I*, gel purified and ligated into *Not I* and *Xho I* digested expression vector (CMV-neo, a derivative of the CS2 family of expression plasmids; a gift of Drs Michael, D. Uhler and Robert C. Thompson). The construct was verified by DNA sequencing.

2.3. Generation of recombinant Xenopus and mouse CRH-BP

The mouse pituitary corticotrope cell line AtT20 was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37°C. The *Xenopus* XTC-2 epithelial cell line was cultured in Leibovitz's 15 (L 15) medium diluted 1:1.5 (to adjust for the reduced osmolarity of amphibian body fluids) and 10% fetal bovine serum at 25°C. Both cell lines were maintained in an atmosphere of 5% CO_2 and were transiently transfected using the calcium phosphate method (Gorman et al., 1983) with the CMVneo-xCRH-BP expression plasmid described above. Twenty four hours after transfection the growth medium was replaced with serum-free medium and cells were incubated for an additional 48 h. Conditioned medium was collected, protease inhibitor cocktail (Sigma) was added to a concentration of 1X and aliquots frozen at −80°C until analysis. Cells were harvested, suspended in homogenization buffer containing protease inhibitors (0.02 M phosphate buffer [PB], 0.1% Triton X-100, 0.5% protease inhibitor cocktail [Sigma]), homogenized using a polytron and extracts frozen at −80°C until analysis. Recombinant mouse CRH-BP (rmCRH-BP) was obtained from stably transfected AtT20 cells that were described previously (Cortright et al., 1995). These cells were cultured and media and cells harvested as described above.

2.4. Radioiodination of CRH

Iodination of xCRH or rodent-human CRH (r/hCRH; both provided by Jean Rivier and Wylie Vale, The Salk Institute, La Jolla, CA) was achieved by the iodogen method (Pierce). Free ^{125}I was removed by Sep-Pack C_{18} column chromatography (Waters Inc.) and the radiolabeled peptide was purified by high performance liquid chromatography essentially as described by Vale et al. (1983). Briefly, the reaction was loaded onto a 25 cm × 4.6 mm C_{18} reverse phase analytical column (Supelco) and eluted with a solvent system of 88% formic acid with triethylamine (TEAF; pH 3.0) in pump A and a mixture of 40% TEAF plus 60% acetonitrile in pump B. A gradient of 40–100% B was run over 35 min and 0.75 ml fractions were collected. Several fractions corresponding to the peak of radioactivity were collected, pooled and used in the crosslinking assay (see below).

2.5. Crosslinking assay

Tissues were suspended in homogenization buffer and homogenized using a polytron (see above) followed by sonication for 10 s and centrifugation in a microfuge for 2 min to clarify the extract. Total protein contents of crude tissue extracts were measured by protein assay (Pierce) using bovine serum albumin (BSA) as standard prepared in the homogenization buffer. The native *Xenopus* brain CRH-BP obtained in this manner is hereafter referred to as xCRH-BP to distinguish it from the recombinant CRH-BP (rxCRH-BP). A large pool of *Xenopus* adult brain extract was produced, divided into small aliquots and frozen at −80°C. This ensured accuracy and repeatability of the $K_{i[app]}$ measurements for binding of different CRH-like peptides to the xCRH-BP (see below).

Binding of [^{125}I]-xCRH to the xCRH-BP was studied by crosslinking assay as described by Cortright et al. (1995) after Suda et al. (1988), using the brain tissue extract pool described above. This pool was used to produce a saturation curve to determine the amount of protein to use in the binding reactions. Similarly, an association curve of [^{125}I]-xCRH and xCRH-BP established the kinetics of the binding reaction at 4°C. Based on these results each tube contained 45 μg of total protein (which gave approximately half-maximal binding). The reaction contained 15 000 cpm of [^{125}I]-xCRH in a final volume of 95 μl (crosslinking buffer: 100 mM sodium phosphate, pH 7.4, 0.1% BSA, 0.01% sodium azide, 50 mM sodium chloride). After the overnight incubation the reactions were allowed to reach room temperature for 10–12 min before addition of 5 μl of 20 mM disuccinimidyl suberate (DSS; Pierce) dissolved in dimethyl sulfoxide (Fisher Co.). Crosslinking was conducted at room temperature 15 min and the reaction terminated by addition of 35 μl reducing SDS sample buffer (50 mM Tris, pH 6.8, 35% glycerol, 30% β-mercaptoethanol, 2% SDS, 0.05% bromophenol blue) which was then heated to 95°C for 5 min. Ninety microliters of each reaction were fractionated on a 12.5% SDS-polyacrylamide gel (Laemmli, 1970). Gels were dried and analyzed by phosphorimager (Bio-Rad). Band densities were quantified using Scion Image software. For tests of the effect of pH on [^{125}I]-xCRH binding to xCRH-BP, crosslinking buffers at different pHs were prepared and used in the assay. The inclusion of detergent in the homogenization buffer (which would disrupt microsomal membranes) and the absence of divalent cations in the crosslinking buffer precluded the detection of CRH receptors in our assay.

2.6. Competitive binding assay

Competitive binding assay was conducted as described by Cortright et al. (1995). Previous experiments

have shown that this assay, which involves chemical crosslinking after allowing the binding reaction to reach equilibrium, can be used to obtain apparent binding inhibition constants ($K_{i[app]}$) that are similar to the equilibrium binding constants for hCRH-BP (see Orth and Mount, 1987; Suda et al., 1988; Sutton et al., 1995). Thus, we used this method to obtain apparent K_i values for xCRH-BP and several different ligands. Various radioinert peptides (xCRH, r/hCRH, αhelCRH$_{[9-41]}$, ovine CRH, sauvagine [SV; from Jean Rivier and Wylie Vale, Salk Institute, La Jolla, CA], r/hCRH$_{[6-33]}$ [from Calbiochem], urotensin I, urocortin, thyrotropin-releasing hormone, gonadotropin-releasing hormone; from Sigma Chemical Co.) were used as competitors for [^{125}I]-xCRH binding in the assay. Displacement of [^{125}I]-xCRH was analyzed by crosslinking assay as described above. Binding data were analyzed as described by Cortright et al. (1995) by fitting the inverse hyperbolic equation using Sigma Plot software (see below).

2.7. RNA extraction, Northern blotting and RT-PCR

RNA was extracted from frog tissues using Trizol (Gibco BRL) and 20 μg of total RNA was fractionated by gel electrophoresis in a formaldehyde 1% agarose gel. RNAs were transferred to a nylon membrane by capillary transfer in 20X SSC (Sambrook et al., 1989) and UV-crosslinked. Blots were hybridized overnight in Hybrisol I (Intergen) with xCRH-BP cDNA labeled with ^{32}P-dCTP by random priming (Boehringer Mannheim Corp). Hybridized blots were washed with 2X SSC, 0.5% SDS at room temperature for 10 min, then 0.1X SSC, 0.5% SDS at 65°C for 1 h before exposure to X-ray film for 1–14 days. Blots were stripped and reprobed with a ^{32}P-labeled rpL8 cDNA to normalize for RNA loading. The rpL8 codes for a ribosomal protein that is expressed constitutively and is unaltered by hormone treatments (Shi and Liang, 1994).

We examined the expression of the xCRH-BP gene in different tissues by reverse transcriptase-polymerase chain reaction (RT-PCR). First, the integrity of the RNA was verified by fractionating 5 μg of total RNA from each tissue in a formaldehyde 1% agarose gel and staining with ethidium bromide. For RT-PCR, 2.5 μg of total RNA from each tissue was digested with 3 U RNAse-free DNAse I (GibcoBRL) to remove genomic DNA. The reverse transcription reaction contained oligo(dT)$_{27}$ (0.5 μg) and 200 U SuperScript II (GibcoBRL) in a final volume of 20 μl. 10% of the RT reaction was included in the PCR; two rounds of amplification were done. The following gene-specific primers were used in the PCR to yield a 452 bp amplification product: forward primer 5′TGACTCCTGCTTCCAGACCT3′, encompassing positions 73 through 92 of the mRNA, and reverse primer 5′TGACCTTGTAATGCTCCCCAC3′, encompassing positions 504 through 524 of the mRNA. Because the gene structure of the xCRH-BP is unknown, we were unable to design primers to span intron-exon boundaries. However, PCR amplification of *X. laevis* genomic DNA using primers that we designed for cloning the full-length xCRH-BP cDNA (see above) yielded a single product of ~1 kb which is the expected size of the cDNA; i.e. this result suggests the absence of intronic sequences in the xCRH-BP gene (data not shown). Thus, only a single 452 bp product was expected from our RT-PCR experiments. This fact necessitated that we verify that genomic DNA did not contaminate our RNA preparations. Thus, in addition to the DNAse digestion described above, we controlled for residual genomic DNA contamination by conducting RT-PCR without the addition of reverse transcriptase to the negative controls.

2.8. Western blotting

Twenty micrograms of protein extracted from late prometamorphic tadpole brain (NK stage 62; Nieukoop and Faber, 1994) was fractionated by SDS-PAGE in a 12.5% gel (Laemmli, 1970) and electroblotted onto a nitrocellulose membrane. Immuno-detection of CRH-BP was accomplished using a rabbit polyclonal antiserum (1:3000 dilution) raised against a maltose binding protein (MBP)-mouse CRH-BP fusion protein (rm-CRH-BP). The mCRH-BP cDNA was subcloned into the pMAL-c vector (New England Biolabs) to produce the fusion protein. The fusion protein was expressed in *E. coli* and purified by SDS-PAGE and electroelution. Rabbits were immunized with the fusion protein to generate a polyclonal antiserum that recognizes the mCRH-BP (Hazelton Research Products, Hazelton, PA). The secondary antibody was goat anti-rabbit-HRP and detection of immune complexes was done by chemiluminescence (NEN).

2.9. Data analysis and statistics

Competitive binding experiments were repeated three to five times for each peptide. Binding data were fitted to the regression equation below using the computer program SigmaPlot.

$$y = \frac{1 - ax}{b + x}, \quad (1)$$

where y is the relative intensity of the specific band in the gel as determined by optical density, x is the peptide concentration, a is the intensity of the band in the absence of competitor, and b is the $K_{i(app)}$. The $K_{i(app)}$ values were log-transformed to achieve homogeneity of variance, analyzed by a one way analysis of variance and subjected to Tukey's post hoc multiple comparison

test using the computer program SuperANOVA (Abacus Concepts, Inc.). Northern blots were repeated three times on tissue from three different experiments.

3. Results

3.1. Sequence comparison of the Xenopus and mammalian CRH-BPs

Xenopus and mouse CRH-BPs share 65.6% sequence similarity within the coding sequence at the nucleotide level and 67.2% sequence similarity at the amino acid level (Fig. 1). The greatest divergence in sequence between the amphibian and the mammalian proteins is in the N-terminal and C-terminal regions. The mature xCRH-BP protein possesses 10 cysteines that are conserved in all vertebrates CRH-BPs that have been characterized thus far. These cysteines are known to give rise to five intramolecular disulfide bonds in the human CRH-BP (Fischer et al., 1994) which confer a rigid secondary structure on the molecule and are essential for peptide binding (Suda et al., 1989). The putative N-glycosylation site in the mature xCRH-BP protein is also conserved across taxa. The extensive structural conservation exhibited by the vertebrate CRH-BPs suggests that the function of the protein is evolutionarily conserved.

3.2. Detection of the CRH-BP in extracts of Xenopus brain

Because of the extensive structural conservation between the frog and mouse CRH-BPs we predicted that the anti-rmCRH-BP polyclonal serum would crossreact with the frog CRH-BP, thus allowing us to detect the protein by Western blot. Western blotting detected the monomeric form of the native frog brain and recombinant mouse CRH-BPs at the expected molecular mass of 37 kDa (Fig. 2(A)). The antiserum also detected a higher molecular mass protein of ~70 kDa in both the frog brain extract and the recombinant mouse CRH-BP. This high molecular mass protein likely represents a dimer of CRH-BP, which has been previously reported to form in the recombinant human protein (Woods et al., 1994).

Crosslinking of [^{125}I]-xCRH to frog brain extract or recombinant mouse CRH-BP detected bands of identical molecular mass (Fig. 2(B)). This binding was partially displaced by coincubation with cold xCRH (5 nM) before crosslinking and fractionation by SDS-PAGE. After fractionating the crosslinked complexes derived from frog brain extracts, we transferred the proteins to nitrocellulose and then exposed the blot to X-ray film to detect the crosslinked complexes. We processed the same membrane for immunodetection of CRH-BP. This experiment verified that the radiolabeled

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                                                         50                                                  100
frog  MTPASRPDWCLILLFLAVLRGESRYIQMREAAEDALFL···LNSDFKREL SEGQIYRRSLRCIDMLSIEGQFTFQADRPQLHCALFLIGEPEEFIIIEYN
mouse -S-NFKLQCHF--IL-TA-------LEVQ---VYDPL··LLFSANL--D- A-E-P---A---L----LP-----T---------A-F--------T-H-D
rat   -S-NFKLQCHFT-IL-TA-------LEVQ---VYDP-··LLFSANL--N- A-E-P---A---L----LP-----T--Q------A-F--------T-HFD
human -S-NFKLQCHF--I--TA-------LEL----DYDP-··LLFSANL---- AGE-P---A---L----LQ-----T---------A-F-S------T-H-D
sheep -A-TLKLQCHF--VC-LA-------LEL---VDHDP-PHLAGGASP---- EGEPL---A---V----LQ-----T---------T-F-A------T-H-D

                                                         150                                                 200
frog  FVNIDCIGGDILKVFDGWIIKGEKFPSSLDHPLSTMERYTDICEDGDVGS ITRSSQNVAMIFFRVQQPGHGFTLTIRKIPNLFPCNVISQSMNGRFTMIT
mouse L-S---Q---F--------L--------Q----P--K----F--S-LTRR SI--------V----HE--N---I--KTD-----------TPS----LVV
rat   L-S---Q---F--------L--------Q----P-R-----F--S-LTRR SVT-------V----HE--N---I--KTD-------I---TPS---ALVV
human Q-S---Q---F--------L--------Q----PSA---I-F--S-LSRR SI-------------HE--N------KTD-----------TP--K--LVV
sheep L-S---LR----Q------L--------Q----P-T---V-F-DS-LSRR SI-------------HE--N---I-VKTE-------I---TP-----LVM

          *                                              250                                                 300
frog  PHQHRNCSFSIIYPVVIKIFDLTLGHFNELQLKKPPPKGCGDAGDFVELL GGAGLDPSKMFPLADLCHSFHGSAQMKIGCDNTVVRMVSSGNFINRVTFE
mouse -Y--Q----------A---S------LHG------AA·---GT------- --T-------M------YP-L-P-----S---A--------KH-------
rat   -Y--Q----------T---S--A---LHG------AA·---GT------- --T---T---ML-V---YP---P-----S---A--------KHM------
human -------------------S------V-G-----SSA·--EGI------- --T-------T------YP---P----V-------------KHV------
sheep ---------------A---S------L-G-----SSA·---GI------- --T-------LL-----YPLR-P----V---H--L------KH-------

frog  YNQLD·RQLEKKQGNSVEEACFPSD  321
mouse -R--EPFE--TST---IP-Y-LS-L  322
rat   -R--EPLE--TSTR--IP-Y-LS-L  322
human -R--EPYE--NPN---IG-F-LSGL  322
sheep -R--EPYE--NPN---IQ-F-LSTL  324
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Fig. 1. Amino acid alignment of the primary structures of the vertebrate CRH binding proteins. The mouse and the frog binding protein exhibit 67.2% sequence similarity. The conserved, putative N-glycosylation site (Asn^{206}) is indicated with an asterisk and the ten conserved cysteine residues are indicated by the arrowheads. Hyphens indicate identical amino acids as those of the frog sequence and periods indicate deletions/insertions.

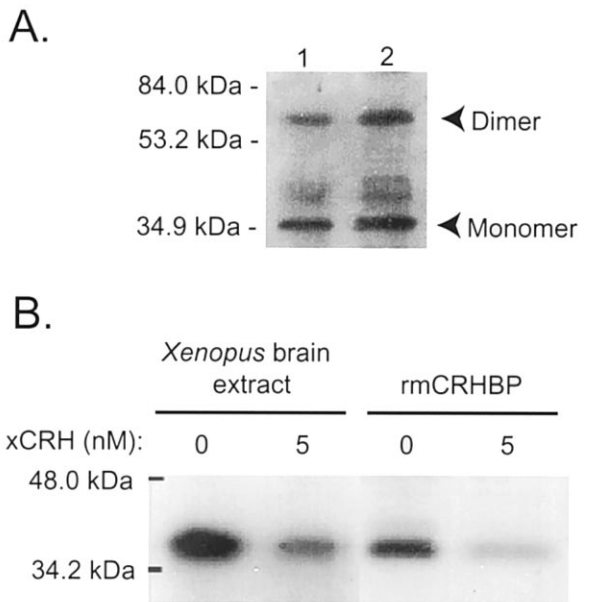


Fig. 2. Immunological and radioisotopic detection of the xCRH-BP from crude brain extracts. (A) xCRH-BP was detected by Western blot using anti-recombinant mouse CRH-BP (rmCRH-BP). Lane 1: purified rmCRH-BP (30 ng); lane 2: *Xenopus* brain extract (10 μg). The lower band is the monomeric form of the protein while the upper band corresponds to the dimer. (B) Crosslinking assay detects a band in *Xenopus* brain extract that is of similar molecular mass to the rmCRH-BP and the binding of [^{125}I]-xCRH is displaceable by radioinert xCRH. *Xenopus* brain extract or rmCRH-BP were incubated with [^{125}I]-xCRH in the absence or presence of radioinert xCRH as indicated in the figure. Bound complexes were crosslinked and resolved by SDS-PAGE (Section 2).

band in the crosslinking assay corresponded to an immunoreactive band of the same molecular mass (data not shown). Taken together, these results support the conclusion that the crosslinking assay is capable of detecting the monomeric form of *Xenopus* brain CRH-BP.

3.3. Competitive binding assay of CRH-like peptide binding to native Xenopus brain CRH-BP

We first established conditions for the competitive binding assay by determining the quantity of brain protein that would give approximately half maximal binding of a fixed amount of [^{125}I]-xCRH (15 000 cpm) in the assay. Under these conditions titration of the pooled brain extract produced maximum binding with 75 μg of total protein/tube (Fig. 3(A)); we used 45 μg of brain protein/tube for all subsequent tests. We also determined the kinetics of association of [^{125}I]-xCRH and CRH-BP at 4°C. This experiment showed that equilibrium binding of [^{125}I]-xCRH to xCRH-BP was achieved by 2 h and remained constant up to 20 h (Fig. 3(B)). Subsequently, all binding reactions were conducted at 4°C overnight to assure equilibrium. We also examined the effect of pH on the binding of [^{125}I]-xCRH to xCRH-BP (Fig. 4). This experiment revealed that binding is pH dependent, and it decreases dramatically below pH 7. Binding increased significantly above pH 7, reached a maximum at 9.5 and remained constant through pH 11.

Using a competitive binding assay we analyzed the pharmacological characteristics of the brain xCRH-BP. Extracts were coincubated with [^{125}I]-xCRH and different CRH-like peptides or two unrelated peptides (TRH and GnRH) at a single dose of 10 nM for each peptide before crosslinking. This experiment showed that xCRH, urotensin I, SV, αhelCRH$_{[9-41]}$, urocortin, r/hCRH and r/hCRH$_{[6-33]}$ all competed to varying degrees with [^{125}I]-xCRH for binding to xCRH-BP; whereas, neither oCRH, TRH, nor GnRH displaced the label at the dose used in this experiment (Fig. 5).

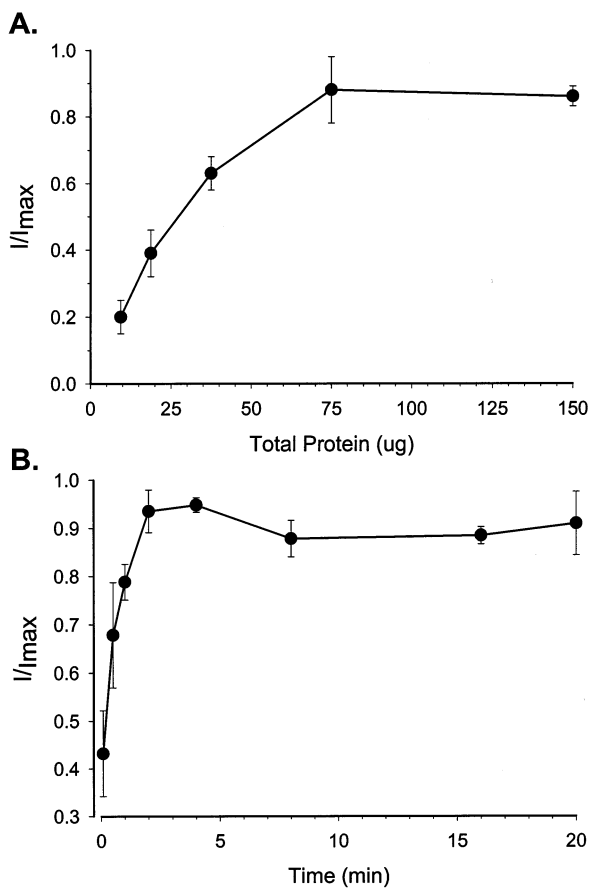


Fig. 3. The dependence of [^{125}I]-xCRH binding to xCRH-BP in *Xenopus* crude brain extracts on protein concentration and incubation time. Binding analyses were done by crosslinking assay (Section 2). (A) Effects of varying brain extract protein concentration on [^{125}I-xCRH] binding to xCRH-BP. Varying concentrations of brain protein were incubated with 15 000 cpm [^{125}I-xCRH] at 4°C overnight before chemical crosslinking. (B) Kinetics of [^{125}I]-xCRH binding to xCRH-BP at 4°C. 45 μg of *Xenopus* brain extract protein was incubated with 15 000 cpm [^{125}I]-xCRH for various times before chemical crosslinking. In both graphs, each point represents the mean and the vertical lines the SEM for 3 replicates.

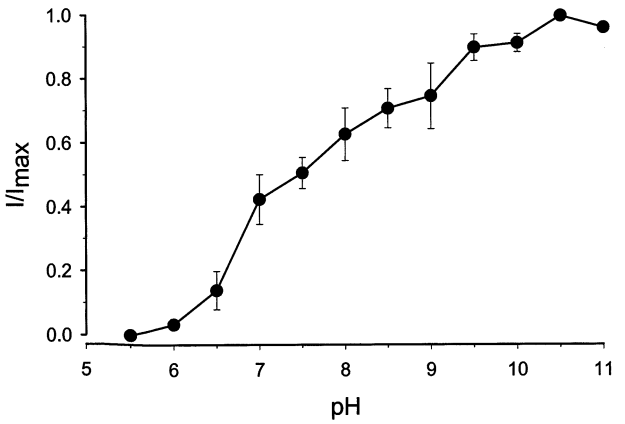


Fig. 4. Effect of pH on the binding of [^{125}I]-xCRH to xCRH-BP in *Xenopus* brain extract. Binding analyses were done by crosslinking assay (Section 2). Each data point represents the mean ± SEM of three replicates.

We next conducted a series of competitive binding assays in order to calculate the apparent inhibition constants ($K_{i[app]}$) for binding of different CRH-like peptides to the xCRH-BP (Fig. 6). We used the same pool of adult *Xenopus* crude brain extract in all of the competitive binding assays. This was done both as a quality control and to ensure a constant level of endogenous CRH-like peptides that might compete for binding to the CRH-BP. xCRH-BP bound all CRH-like peptides tested with high affinity except oCRH (Fig. 6). xCRH-BP bound urotensin I with the highest affinity (0.45 ± 0.07 nM) and r/hCRH$_{[6-33]}$ with the lowest affinity (6.32 ± 1.10 nM). We were unable to determine the $K_{i[app]}$ of xCRH-BP for oCRH since this peptide did not displace the [^{125}I]-xCRH, even at the highest dose tested (20 nM).

To determine whether compounds present in the brain extract might modify the binding of [^{125}I]-xCRH to native xCRH-BP and thus skew our affinity estimates, we compared the calculated affinities of the native xCRH-BP for xCRH with that of the recombinant *Xenopus* (rx)CRH-BP expressed in either the rodent corticotroph cell line AtT20 or the *Xenopus* epithelial cell line XTC-2. Expression of the recombinant xCRH-BP was confirmed by crosslinking assay and compared with *Xenopus* brain extract and the recombinant mouse (rm)CRH-BP (Fig. 7; data shown only for expression in AtT20 cells; neither conditioned medium nor cell extracts of untransfected AtT20 cells possessed binding activity for [^{125}I]-xCRH). In these experiments, we were unable to detect rxCRH-BP in the culture medium, possibly due to a low level of expression in the transient transfection assay. However, we detected rxCRH-BP in extracts of transfected cells, and this protein had an identical molecular mass to the native xCRH-BP and the rmCRH-BP (which is detectable in conditioned medium obtained from the stably transfected cells). In competitive binding assays we estimated the $K_{i[app]}$ for the [^{125}I]-xCRH binding to the rxCRH-BP (from AtT20 cells) to be 2.8 ± 0.8 nM (2.8 ± 0.6 nM for the XTC-2 cells) which is slightly higher than the calculated $K_{i[app]}$ for the native *Xenopus* brain CRH-BP (1.08 ± 0.11 nM).

We also estimated the binding affinity of rmCRH-BP for the homologous r/hCRH and compared it with the affinity of xCRH binding to the native and recombinant frog CRH-BPs. For these experiments, we used radioinert r/hCRH to displace [^{125}I]-r/hCRH from the rmCRH-BP. We estimated the $K_{i[app]}$ for r/hCRH binding to the rmCRH-BP to be 0.8 ± 0.2 nM, which is close to the value reported (Cortright et al., 1995; 0.37 nM).

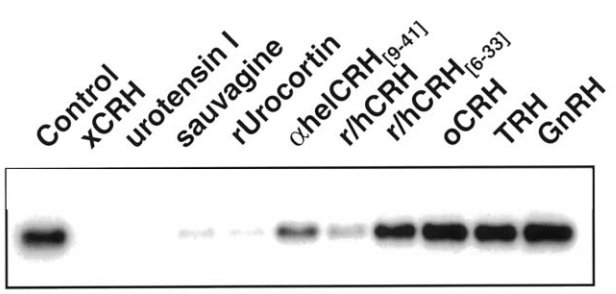
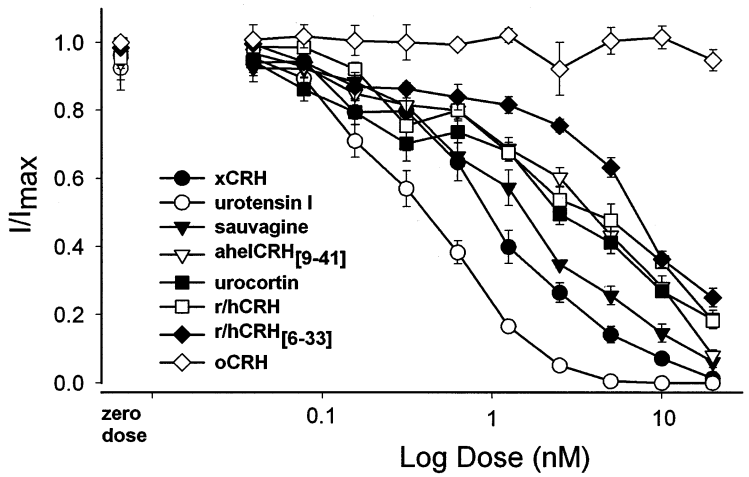


Fig. 5. Displacement of [^{125}I]-xCRH binding to xCRH-BP from *Xenopus* brain extract by different peptides. 15 000 cpm of [^{125}I]-xCRH and 10 nM of each radioinert peptide were incubated with 45 μg brain protein before crosslinking and analysis by SDS-PAGE and autoradiography (Section 2).

3.4. Analysis of developmental and tissue-specific expression of xCRH-BP mRNA

We analyzed the expression of xCRH-BP mRNA in different tadpole and frog tissues by RT-PCR and Northern blotting. RT-PCR analysis showed that the gene is expressed in brain, pituitary, intestine, liver and tail (Fig. 8). Southern blot analysis of the PCR gel was required in order to detect the signal in the pituitary. By Northern blotting we detected a single mRNA transcript of ~4 kb in the brain and two transcripts in the tail of ~1 and 4 kb (Fig. 9). Northern blot analysis showed that the expression of the xCRH-BP gene is low in the brain during premetamorphosis (stage 48), rises at the onset of prometamorphosis (stage 52) and remains constant throughout metamorphosis (stages 54–64), increasing slightly in the metamorphic frog (stage 66; Fig. 9(A)). In the tail, low expression of xCRH-BP mRNA can be detected during pre- and prometamorphosis, but rises dramatically at metamorphic climax and is maximal at stage 64 when the tail is actively resorbing (Fig. 9(B)).

A.



B.

	10	20	30	40	Mean Ki±SEM(nM)	Significance
xCRH	AEEPPISLDL	TFHLLREVLE	MARAEQIAQQ	AHSNRKLMDI I	1.08 ± 0.11	b
urotensin I	DD----I--	------NMI-	---N-NQRE-	-GL---YL-E V	0.45 ± 0.07	a
urocortin	DD--L-I--	------TL--	L--TQSQRER	-EQ--IIF-S V	1.18 ± 0.32	b
sauvagine	QG----I--	SLE---KMI-	IEKQ-KEK--	-AN--L-LDT -	1.36 ± 0.10	bc
r/hCRH	S---------	----------	------L---	--------E- -	1.84 ± 0.29	bc
αhelCRH[9-41]	.........--	-------M--	--K---E-E-	-AL--L-LEE A	3.35 ± 0.71	cd
r/hCRH[6-33]	.....-----	----------	------L---	---..... .	6.32 ± 1.10	d
oCRH	SQ--------	----------	-TK-D-L---	-------L-- A	>20	

Fig. 6. Competitive binding analysis of different CRH-like peptides binding to xCRH-BP. (A) Competitive binding curves for CRH-like peptides and [^{125}I]-xCRH binding to brain xCRH-BP. Competitive binding and crosslinking assay were done as described in Section 2. Points represent the mean ± SEM for 3–5 replicates. (B) Comparison of primary sequences of CRH-like peptides tested in the competitive binding assay and calculation of mean inhibition constants ($K_{i[app]}$). Mean $K_{i[app]}$ ± SEM for 3–5 replicates; data were analyzed by ANOVA and letters separate the means based on Tukey's posthoc test ($P < 0.05$). Hyphens indicate identical amino acid residues as those of xCRH and periods indicate deletions.

4. Discussion

Here we report the detection in the brain, for the first time, of a nonmammalian binding protein for CRH (CRH-BP), the *X. laevis* CRH-BP. We detected the xCRH-BP in the brain by Western immunoblot, crosslinking assay with [^{125}I]-xCRH, Northern blotting, and RT-PCR. Radioligand detection of the covalently bound complex revealed the presence of a protein in *Xenopus* crude brain extracts with an apparent molecular mass of ~41 kDa, which is of similar size to the recombinant mouse CRH-BP (Fig. 2). Subtracting the molecular mass contributed by xCRH (~4500 Da), the xCRH-BP exhibits an apparent molecular mass of 37 kDa, which is in agreement with its predicted size (321 a.a.; ~35 kDa). It is important to note that the *X. laevis* CRH receptors, which are G protein-linked receptors with expected molecular masses of >50 kDa based on their predicted amino acid sequences and glycosylation (Dautzenberg et al., 1997; Grigoriadis and De Souza, 1989), were not detected by this method.

The sequence similarities among the vertebrate CRH-BPs predict similar biochemical and antigenic characteristics. As with the mammalian CRH-BPs the binding of [^{125}I]-xCRH to xCRH-BP was low under acidic conditions but increased with increasing pH. Unlike the mouse CRH-BP, whose binding of [^{125}I]-r/hCRH was reduced at high pH (> pH 10), the frog binding protein continued to bind [^{125}I]-xCRH at pHs as high as 11 (Cortright et al., 1995; Suda et al., 1988). These findings suggest that the *Xenopus* CRH-BP (xCRH-BP) is more stable than the mammalian homologues at high pH.

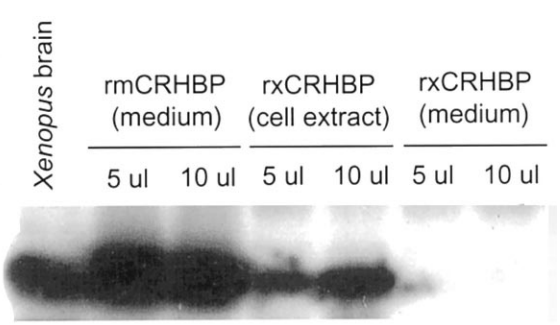


Fig. 7. Crosslinking analysis of [^{125}I]-xCRH binding to recombinant *Xenopus* and mouse CRH-BPs. Recombinant proteins were obtained by transfection of AtT20 cells (Section 2). Conditioned medium from AtT20 cells stably transfected with CMVneo-rmCRH-BP and medium and cell extracts from AtT20 cells transiently transfected with CMVneo-rxCRH-BP were analyzed. No rxCRH-BP was detected in the medium of transiently transfected cells.

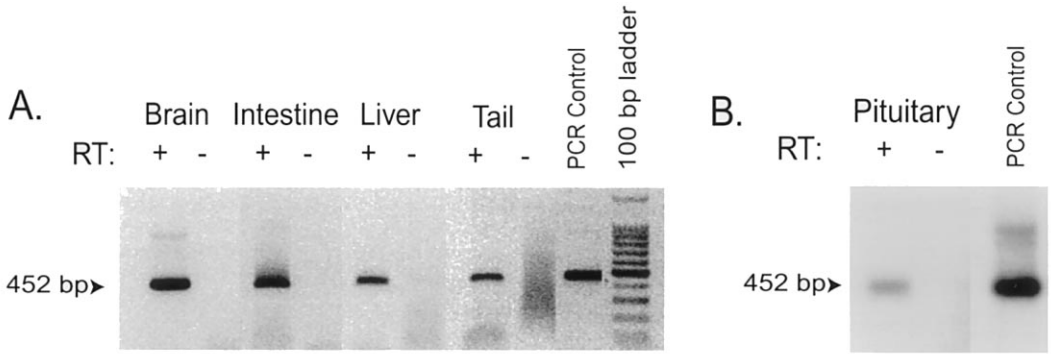


Fig. 8. RT-PCR analysis of xCRH-BP mRNA expression in different tadpole tissues. 2.5 μg of total RNA from *Xenopus* brain, pituitary, intestine, liver and tail was first treated with RNAse-free DNAse and then reverse transcribed (RT) or not (controls for genomic DNA contamination) before PCR amplification (Section 2). (A) Ethidium bromide-stained gel. (B) Southern blot (using [^{32}P]-xCRH-BP cDNA as probe) showing expression of the xCRH-BP mRNA in the pituitary. The PCR controls utilized $\sim$10 ng of the full-length xCRH-BP cDNA.

The specificity and reversibility of the binding was demonstrated by competing [^{125}I]-xCRH with radioinert xCRH which, together with the apparent molecular mass of the complex, further supports the conclusion that the *Xenopus* brain extract contains a specific CRH-BP. Furthermore, only CRH-like peptides competed for binding while unrelated peptides such as TRH or GnRH did not (Fig. 5).

Our initial tests of the binding specificity of the xCRH-BP showed that urotensin I, xCRH, SV, r/hCRH, and urocortin are strong competitors for [^{125}I]-xCRH binding to xCRH-BP (Fig. 5). The αhelCRH$_{[9-41]}$ and r/hCRH$_{[6-33]}$ exhibited lower affinities for the xCRH-BP in competition with [^{125}I]-xCRH, findings that were later confirmed by detailed analyses of the affinities for binding of the different peptides. The r/hCRH$_{[6-33]}$ binds the human CRH-BP with moderately high affinity (3.5 nM) but does not bind to the hCRH receptor (R1; Sutton et al., 1995). The affinity of the frog CRH-BP for r/hCRH$_{[6-33]}$ is comparable to the human CRH-BP but currently it is unknown whether this peptide binds to the frog CRH receptor. The lower binding affinity of r/hCRH$_{[6-33]}$ compared with the entire r/hCRH indicates that the N-terminal and/or the C-terminal residues are important for binding of the r/hCRH to the frog CRH-BP. As with most known vertebrate CRH-BPs (except the ovine CRH-BP; Behan et al., 1996a; Cortright et al., 1995; Suda et al., 1988; Sutton et al., 1995), oCRH exhibited very low affinity for the xCRH-BP, and we were unable to estimate the $K_{i[app]}$ for this peptide. The combined effect of amino acid substitutions Thr^{22}, Lys^{23} and Asp^{25} in the oCRH molecule has been shown to account for the low affinity binding to the hCRH-BP (Sutton et al., 1995) and could also explain the low binding affinity of this peptide for the frog protein.

We found that the xCRH-BP binds the frog peptide SV with tenfold or greater affinity than does the human CRH-BP (frog $K_{i[app]} = 1.36$ nM vs. human $K_{i[app]} =$ 17.5; Behan et al., 1993b; Kahl et al., 1998; Sutton et al., 1995), which may reflect the coevolution of the neuropeptide and its binding site. However, it should be recognized that there is currently no direct evidence (i.e., from peptide or nucleic acid sequencing) for the presence of an urotensin/urocortin/sauvagine-like molecule in *Xenopus* brain. Indeed, the phylogeny of this paralogous lineage of CRH-like peptides in amphibia is completely unexplored. Sauvagine was isolated from the skin of the monkey frog (*Phyllomedusa sauvageii*; Montecucchi and Henschen, 1981) but a similar molecule has not been reported in any other amphibian species nor in any vertebrate. Thus, whether the high affinity binding of xCRH-BP for SV reflects

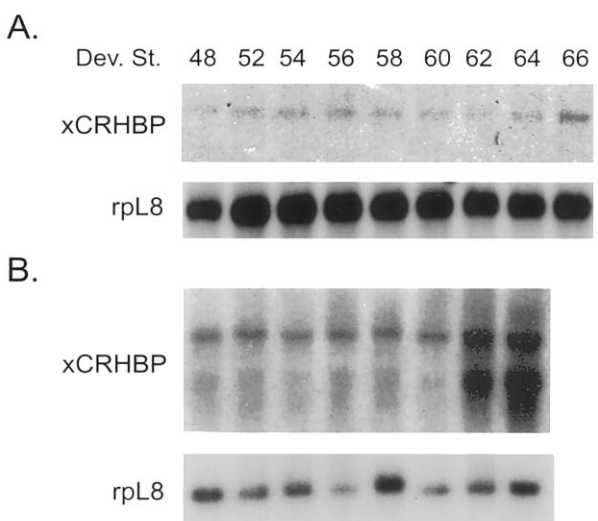


Fig. 9. Developmental expression of xCRH-BP mRNA in *Xenopus* tadpole brain (A) and tail (B). Total RNA was extracted from tadpole tissues at different stages of development (NK; Nieuwkoop-Faber, 1967) and analyzed by Northern blotting with a full-length [^{32}P]-labeled xCRH-BP cDNA (Section 2). Blots were stripped and hybridized with a probe for the *Xenopus* ribosomal L8 gene (rpL8) to control for RNA loading.

the coevolution of structure/function awaits clarification of the structures of members of this peptide lineage in *Xenopus*. Interestingly, the xCRH-BP bound fish urotensin I with highest affinity (0.45 nM) which may hint at the presence of a peptide with similar structure in *Xenopus*.

Similar to the mammalian binding proteins (Sutton et al., 1995), our data show that the xCRH-BP binds xCRH with higher affinity than do the *Xenopus* CRH receptors (1.08 nM for xCRH-BP vs. 7.8 for xCRH-R1 and 9.4 for xCRH-R2; Dautzenberg et al., 1997; Sutton et al., 1995). The prevailing view is that CRH-BP modulates CRH biological activity by binding the peptide with high affinity, thus preventing its interaction with the CRH receptors (Linton et al., 1990; Turnbull and Rivier, 1997; Zhao et al., 1997). The high affinity binding of xCRH to the xCRH-BP suggests that, as in mammals, the frog binding protein may regulate the bioavailability of xCRH (and perhaps-other CRH-like peptides belonging to the urotensin/urocortin/sauvagine family, the identities of which have not yet been determined in the frog). Whether the xCRH-BP plays a physiological role in modulating CRH bioavailability in *Xenopus* remains to be determined.

To directly compare the binding affinities of the frog and rodent CRH-BPs and to determine if our findings are comparable to those reported in the literature, we measured the $K_{i[app]}$ values for the binding of xCRH or r/hCRH to rxCRH-BP or rmCRH-BP, respectively. In our assay, the rmCRH-BP exhibited a slightly lower ($\sim$twofold) affinity for [^{125}I]-r/hCRH than previously reported (Cortright et al., 1995). This difference may be a result of differences in the specific activities of the tracers used. The $K_{i[app]}$ for [^{125}I]-xCRH binding to the rxCRH-BP was approximately twofold higher than that obtained for the native brain xCRH-BP. While these differences are small, they could point to differences in the postranslational processing of the native and recombinant proteins. Nevertheless, these data support the estimates of the high affinity binding of [^{125}I]-xCRH to the xCRH-BP obtained with the native brain protein.

RNA analyses showed that the CRH-BP gene is expressed in the brain, pituitary, intestine, liver and tail of *Xenopus*. The expression of CRH-BP mRNA in brain and pituitary has been shown in other vertebrates (Cortright et al., 1995; Peto et al., 1999; Potter et al., 1992). Immunohistochemical and in situ hybridization studies have shown that CRH, CRH-BP and CRH receptors are colocalized in several brain regions, suggesting that CRH-BP can modulate CRH bioavailability within the brain (Potter et al., 1992). Similarly, the presence of xCRH-BP mRNA in frog brain and pituitary as shown in this study suggests the possibility that the frog binding protein can modulate CRH bioavailability and thus influence both the neuromodulatory and hypophysiotropic actions of CRH-like peptides. CRH-BP gene expression in the frog intestine is a novel finding among vertebrates and suggests a potential role for CRH-BP in modulating CRH signaling within the diffuse neuroendocrine system of the amphibian gut. Northern blot analysis failed to detect CRH-BP mRNA in the intestine of the rat (Potter et al., 1991), and RT-PCR analysis failed to detect the transcript in mouse liver (Cortright et al., 1995). To our knowledge, the expression of CRH-BP in mouse, sheep, or human intestine has not been examined. Suda et al. (1988) used chemical crosslinking to detect CRH-BP in human plasma but we were unable to detect the xCRH-BP by this method in frog plasma (Valverde and Denver, unpublished). Also, we could not detect CRH-BP by crosslinking in *Xenopus* intestine, liver or in mouse intestine (Valverde and Denver, unpublished). It may be that the concentration of xCRH-BP in frog blood and the level of expression of the protein in intestine and liver is too low for us to detect by crosslinking assay, and more sensitive methods such as radioimmunoassay will be necessary to resolve this question.

Analysis of the developmental expression of xCRH-BP mRNA shows that the transcript is expressed at low levels in the brain during premetamorphosis, and increases during prometamorphosis during which time it is maintained at a constant level. The gene is expressed at a slightly higher level in the brain of juvenile frogs. By contrast, the transcript is expressed at very low levels during pre and prometamorphosis in the tail and rises dramatically at metamorphic climax, confirming the findings of Brown et al. (1996). We detected no developmental changes in transcript level in the intestine (data not shown) and we have not yet completed a developmental analysis of expression in the pituitary. The CRH-BP was identified as one of 17 genes upregulated by thyroid hormone in the tail of *Xenopus* tadpoles (Brown et al., 1996; Wang and Brown, 1991). However, we found no effect of thyroid hormone (3,5,3′-triiodothyronine; 5 or 50 nM added to the aquarium water) on CRH-BP gene expression in the tadpole brain or intestine (Valverde and Denver, unpublished). Our findings show that the xCRH-BP gene is regulated in a developmental and tissue-specific manner. Although the significance of this tissue-specific regulation is not known, it is conceivable that tail CRH-BP could modulate CRH bioavailability during metamorphic climax.

In summary, we report for the first time the biochemical characteristics and the expression of a CRH-BP in a nonmammalian vertebrate and have compared these characteristics with those of mammalian CRH-BPs. Taken together, the high affinity of the xCRH-BP for xCRH and other CRH-like peptides and the expression of the xCRH-BP in the brain and pituitary suggests that the modulation of the bioavailability of CRH

peptides by a specific binding protein arose early in vertebrate evolution. In addition to their role in regulating ACTH, CRH-like peptides have been implicated in the control of the thyroid axis through direct regulation of TSH secretion in amphibians and other non-mammalian vertebrates (see Denver, 2000). As thyroid hormone is the primary morphogen controlling amphibian metamorphosis, the CRH-BP could potentially play an important role in regulating the bioavailability of CRH and thus influence the timing and progression of metamorphosis.

Acknowledgements

Drs Jean Rivier and Wylie Vale generously provided many of the CRH-like peptides used in this study. We thank Graham Boorse for assistance with the PCR analysis of *Xenopus* genomic DNA. This research was supported in part by NSF grant IBN9974672 to RJD, NIH grant DK42730 to AFS and grant NIH 5P60 DK20572 from the NIDDK to the Michigan Diabetes Research and Training Center. RAV was supported by a Postdoctoral Fellowship for the Diversity in the Sciences from the College of Literature, Science and the Arts, The University of Michigan and a NIH training grant (T32 HD07505-02) awarded to the Center for Organogenesis at The University of Michigan.

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