

## BEYOND CARRIER PROTEINS

# Corticotropin-releasing hormone-binding protein: biochemistry and function from fishes to mammals

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### Abstract

Corticotropin-releasing hormone (CRH) plays multiple roles in vertebrate species. In mammals, it is the major hypothalamic releasing factor for pituitary adrenocorticotropin secretion, and is a neurotransmitter or neuromodulator at other sites in the central nervous system. In non-mammalian vertebrates, CRH not only acts as a neurotransmitter and hypophysiotropin, it also acts as a potent thyrotropin-releasing factor, allowing CRH to regulate both the adrenal and thyroid axes, especially in development. The recent discovery of a family of CRH-like peptides suggests that multiple CRH-like ligands may play important roles in these functions. The biological effects of CRH and the other CRH-like ligands are mediated and modulated not only by CRH receptors, but also via a highly conserved CRH-binding protein

(CRH-BP). The CRH-BP has been identified not only in mammals, but also in non-mammalian vertebrates including fishes, amphibians, and birds, suggesting that it is a phylogenetically ancient protein with extensive structural and functional conservation. In this review, we discuss the biochemical properties of the characterized CRH-BPs and the functional roles of the CRH-BP. While much of the *in vitro* and *in vivo* data to date support an 'inhibitory' role for the CRH-BP in which it binds CRH and other CRH-like ligands and prevents the activation of CRH receptors, the possibility that the CRH-BP may also exhibit diverse extra- and intracellular roles in a cell-specific fashion and at specific times in development is also discussed.

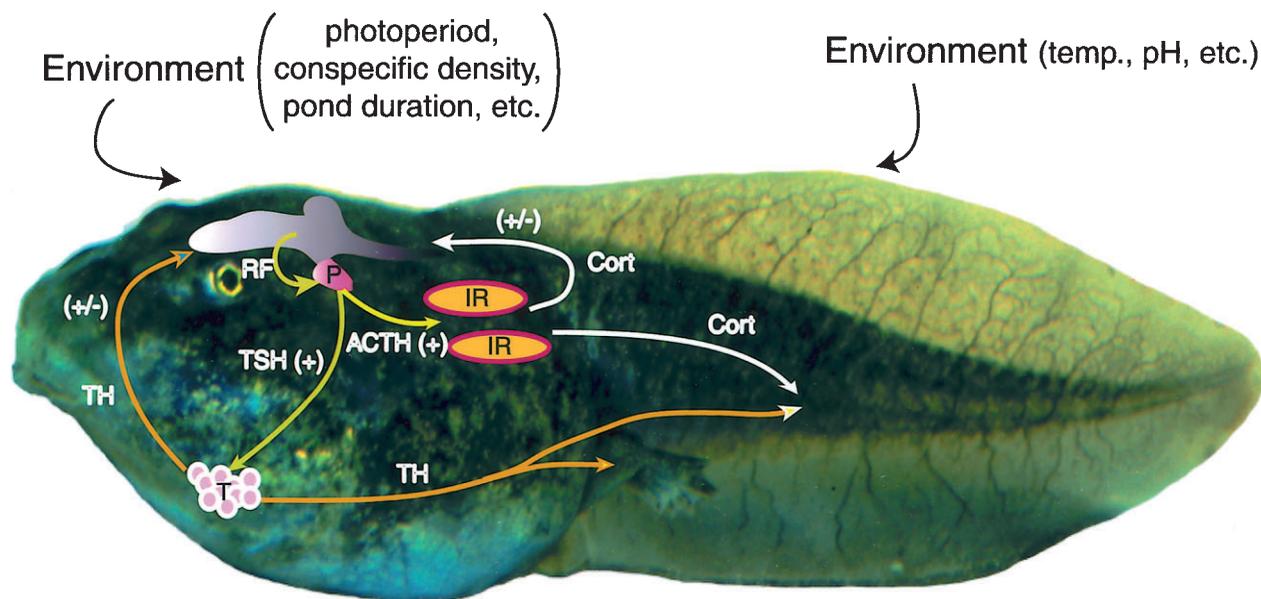
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### Introduction

Corticotropin-releasing hormone (CRH) is a key regulator of the mammalian stress response. This 41-amino acid peptide was originally isolated and characterized from ovine hypothalamus in 1981 by Vale and colleagues and shown to be the major hypothalamic peptide controlling pituitary adrenocorticotropin (ACTH) secretion (Vale *et al.* 1981). Neural input to the hypothalamus in response to stress causes an increase in the synthesis of CRH in the parvocellular division of the paraventricular nucleus and release of CRH from the median eminence. CRH acts on corticotropes in the anterior pituitary to increase the production and secretion of ACTH which stimulates glucocorticoid (cortisol or corticosterone) release from the adrenal cortex. Glucocorticoids then mediate many of the metabolic changes associated with the stress response. They also act on the hypothalamus and pituitary to decrease synthesis and release of CRH and ACTH, helping to return the stress system to homeostasis.

CRH is also expressed at many other sites in the mammalian brain including the limbic system, cortex, and brainstem nuclei associated with autonomic function. At these sites CRH is thought to act as a neurotransmitter or a neuromodulator, mediating increased anxiety-like behavior, decreased food intake, enhanced learning, increased arousal, altered blood pressure, diminished sexual behavior, and altered locomotor activity (Koob & Heinrichs 1999, Smagin *et al.* 2001). Together, these results suggest that CRH not only controls the mammalian endocrine stress response through the hypothalamo-pituitary-adrenal (HPA) axis, but also integrates the autonomic and behavioral responses to stress via its actions in the CNS. Moreover, aberrant regulation of CRH expression and activity contributes to a number of psychiatric disorders including depression, anxiety disorders, and anorexia (reviewed in Owens & Nemeroff 1991, Arborelius *et al.* 1999).

In non-mammalian vertebrates, as in mammals, CRH plays multiple roles as a hypophysiotropin and as a neurotransmitter/neuromodulator. CRH has been shown



**Figure 1** Endocrine systems controlling tadpole metamorphosis. P, pituitary gland; RF, releasing factor (CRH regulates both TSH and ACTH secretion in tadpoles); IR, interrenal gland; ACTH, adrenocorticotropic hormone; TSH, thyroid stimulating hormone; T, thyroid gland; TH, thyroid hormone; Cort, corticoids. Plus signs indicate a stimulatory effect and minus signs a negative feedback. In the case of TH and Cort effects on the brain, (+/-) indicates that these hormones promote differentiation of neurosecretory centers (and other brain regions) in addition to their negative feedback effects on neurohormone and pituitary hormone secretion. (Reprinted with permission from Denver *et al.* 2002.)

to be a potent stimulator of pituitary ACTH secretion (Tonon *et al.* 1986) in non-mammalian species, and by contrast with mammals, CRH is also a potent thyrotropin (TSH)-releasing factor (TRF; reviewed by Denver 1999) (Fig. 1). This thyroid-stimulatory activity of CRH has been demonstrated in fishes, amphibians, reptiles and birds, and a direct action of CRH on pituitary TSH secretion has been verified in at least one representative species from each vertebrate class (Denver 1999). While the tripeptide thyrotropin-releasing hormone (TRH) is active on TSH secretion in adults of most vertebrate species, TRH lacks TSH-releasing activity in amphibian larvae; however, CRH is a potent TRF in these animals (Denver & Licht 1989). Injections of CRH-like peptides, but not TRH, can accelerate metamorphosis in diverse amphibian species (Gancedo *et al.* 1992, Denver 1993, 1997a,b, Miranda *et al.* 2000, Boorse & Denver 2002).

These findings have led to the hypothesis that a primitive role of CRH was as a regulator of both the adrenal and the thyroid axes, and its role in this regard may be most important to developing animals (Denver 1999). Both central (CRH as a CRF and TRF) and peripheral interactions among the thyroid and the adrenal axes likely arose in the earliest vertebrates. For example, thyroid hormone and corticoids can synergize during development, and this is best demonstrated in the amphibian tadpole, where corticoids synergize with thyroid hormone to accelerate metamorphosis (reviewed

by Kikuyama *et al.* 1993). This functional synergy likely plays an important adaptive role. For example, many animals can modulate the timing of developmental events or the phenotype that they develop based on the prevailing environmental conditions (i.e. phenotypic plasticity). The stress axis, acting as an interface between an organism's environment and its physiology, is perfectly positioned to mediate environmental effects on development. Evidence for such a role for the stress axis in developmental plasticity comes from species as diverse as fishes (e.g. flounder metamorphosis, DeJesus *et al.* 1993), amphibians (plasticity in timing of metamorphosis in desert species, Denver 1997a,b) and mammals (timing of birth, Nathanielsz 1998, Challis & Smith 2001). However, while the mammalian CRH clearly plays a role in phenotypic plasticity, it appears to have lost its ability to stimulate pituitary TSH release during evolution while having retained its ability to stimulate ACTH release.

### Other CRH-like peptides

Between 1980 and 1982, CRH-like peptides were also discovered in the caudal neurosecretory organ (urophysis) of the teleost fish, the common sucker (*Catostomus commersoni*) (Lederis *et al.* 1982) and in the skin of the frog (*Phyllomedusa sauvagii*) (Montecucchi & Henshen 1981). These peptides, known as urotensin-I and sauvagine

## Corticotropin-Releasing Hormone Series

SEPPISLDL	TFHLLREVLE	MARAEQLAQQ	AHSNRKLMEI	I	r/h/m CRH
SQEPISLDL	TFHLLREVLE	MTKADQLAQQ	AHSNRKLLDI	A	ovine CRH
AEPISLDL	TFHLLREVLE	MARAEQIAQQ	AHSNRKLM DI	I	<i>Xenopus</i> CRH
SEPPISLDL	TFHLLREVLE	MARAEQLAQQ	AHSNRKMM EI	F	sucker CRH I

## Urotensin I/Urocortin Series

NDDPPISIDL	TFHLLRNMI E	MARIENEREQ	AGLNRKYLDE	V	sucker UI
SEPPMSIDL	TFHMLRNMI H	RAKMEGEREQ	ALINRNLMDE	V	sole UI
*DDPPLSIDL	TFHLLRTLLE	LARTQSQRER	AEQNRIIFDS	V	r/m/o UCN
*DNPSLSIDL	TFHLLRTLLE	LARTQSQRER	AEQNRIIFDS	V	human UCN
***PPLSIDL	TFXLLRNMMQ	RAEMEKLREQ	EKINREILEQ	V	pufferfish Uro
*QPPISIDL	SLELLRKMIE	IEKQEKEKQQ	AAANNRLLLDT	I	frog sauvagine
***VILSLDV	PIGLLRILLE	QARYKAARNQ	AATNAQILAH	V	mouse UCN II
***FALSVDV	PTSILSVLID	LAKNQDMRSK	AXRNAELMAR	I	pufferfish AL175143
***LTLSDV	PTNIMNVLEF	VAKAKNLRAK	AAENARLLAH	I	pufferfish AJ251323
***FTLSLDV	PTNIMNILEN	IDKAKNLRAK	AAANAQLMAQ	I	mouse UCN III
***FTLSLDV	PTNIMNILEN	IAKAKNLRAQ	AAANAHLMAQ	I	human UCN III

**Figure 2** Alignment of amino acid sequences of members of the CRH peptide family. Abbreviations: h, human; m, mouse; o, ovine; r, rat; Uro, pufferfish urotensin I-like peptide. The accession numbers are listed for the two pufferfish urocortin-like sequences.

respectively, exhibit ACTH releasing activity on the mammalian pituitary with equal efficacy to human or rat CRH. These peptides also induce vascular resistance changes (Lenz *et al.* 1985) and urotensin-I participates in osmoregulation in fish (Bern *et al.* 1985).

As shown in Fig. 2, fish urotensin-I (UI) and amphibian sauvagine show 54% and 48% amino acid identity to human (h) CRH (human, rat, and mouse CRH are identical in amino acid sequence). These peptides were originally believed to be the fish and amphibian homologs of mammalian CRH based on their structural and functional similarities. However, in 1988 and 1992, cDNA and genes were identified in fish and frog that encoded a peptide more closely related to CRH (sucker CRH and *Xenopus laevis* (frog) CRH) (Okawara *et al.* 1988, Stenzel-Poore *et al.* 1992). These results demonstrated that non-mammalian vertebrates possess at least two different CRH-like peptides, suggesting that mammals might also express another CRH-like peptide. A new mammalian CRH-like peptide, urocortin (UCN), was identified in 1995 by Vaughan and colleagues (Vaughan *et al.* 1995). This 40-amino acid amidated peptide shows 63% amino acid identity to suckerfish UI, 45% identity to hCRH, and 35% identity to sauvagine. Like the other CRH-like peptides, UCN administration can reduce mean arterial blood pressure and stimulate ACTH release, but its *in vivo*

role is presently unknown (Vaughan *et al.* 1995). In 2001, two additional CRH-like peptides were identified by database homology analyses (Hsu & Hsueh 2001, Lewis *et al.* 2001, Reyes *et al.* 2001). These mouse peptides are known as urocortin II (or stresscopin-related peptide) and urocortin III (or stresscopin) and they show approximately 30% homology to CRH and 20–40% homology to urocortin I (Fig. 2). The urocortin II and III peptides and similar sequences from pufferfish (*Takifugu rubripes* and *Tetraodon nigroviridis*) appear to represent a separate, but closely related, lineage of the CRH family. The evolution of the CRH family of neuropeptides has recently been described by several groups (Lovejoy & Balment 1999, Hsu & Hsueh 2001, Lewis *et al.* 2001). However, the recent findings of the novel urocortin-like peptides described above suggest that there is still much more to be learned before we have a complete phylogeny of this important peptide family.

### Receptors for CRH and other CRH-like peptides

The biological effects of peptides are, of course, mediated via their specific receptors on the post-synaptic or target cell. A number of CRH receptors have been identified, and all of the identified CRH-like peptides bind to at least

one of the CRH receptors. These CRH receptors are members of the seven transmembrane domain G-protein-coupled receptor family. They couple to Gs in most cell types, activating adenyl cyclase and the cAMP second messenger pathway. However, coupling of the CRH receptors to other G proteins has been reported in some cell types (reviewed in Dautzenberg & Hauger 2002). The first CRH receptor (CRH-R1) was cloned from rat, human, and mouse in 1993 (Chang *et al.* 1993, Chen *et al.* 1993, Perrin *et al.* 1993, Vita *et al.* 1993). It is expressed in anterior pituitary corticotropes, the intermediate lobe of the pituitary, and numerous sites in the CNS (Potter *et al.* 1994). The second mammalian CRH receptor to be cloned, CRH-R2, encodes a membrane receptor that is 70% identical to CRH-R1 in amino acid sequence (Kishimoto *et al.* 1995, Lovenberg *et al.* 1995, Perrin *et al.* 1995, Stenzel *et al.* 1995). This receptor has been isolated in two alternatively spliced forms in the rodent (Lovenberg *et al.* 1995) and exhibits a distinct mRNA expression profile from the CRH-R1 (Chalmers *et al.* 1996). CRH-R2 $\alpha$  is expressed primarily in brain, while CRH-R2 $\beta$  is found largely in the periphery in rats. CRH-R2 is expressed in three alternatively spliced forms in humans, with all three forms expressed in the CNS and only CRH-R2 $\alpha$  detected in peripheral sites (Kostich *et al.* 1998).

In addition to the distinct anatomical profiles of CRH-R1 and CRH-R2, the mammalian receptors also differ in their pharmacological properties. CRH-R1 has high affinity for CRH and UCN, binding both peptides with similar affinity ( $K_i=0.95$  and  $0.16$  nM respectively; Vaughan *et al.* 1995). In contrast, CRH-R2 (rodent CRH-R2 $\alpha$  and CRH-R2 $\beta$  display almost identical pharmacologies) has a significantly (10- to 40-fold) higher affinity for UCN than for CRH ( $K_i$  (uro)= $0.41$  nM;  $K_i$  (CRH)= $17$  nM) (Vaughan *et al.* 1995). The newest mammalian CRH-like peptides, urocortin II and urocortin III, bind only to CRH-R2 with affinities of  $0.5$  to  $22$  nM (Lewis *et al.* 2001).

In *X. laevis*, two CRH receptors (xCRH-R1 and xCRH-R2) have been identified and characterized (Dautzenberg *et al.* 1997). These receptors show approximately 80% amino acid identity to the mammalian CRH-R1 and CRH-R2 $\alpha$ . As in mammals, CRH binds with lower affinity to xCRH-R2 than urotensin-I, urocortin or sauvagine. In contrast, xCRH-R1 binds CRH and UCN more tightly than sauvagine (Dautzenberg *et al.* 1997). In the diploid catfish, three CRH receptors have been identified (Aria *et al.* 2001). One CRH receptor (cfCRH-R2) is very similar to mammalian CRH-R2. A second CRH receptor (cfCRH-R1) is 93% identical to mouse CRH-R1 and highly expressed in brain. However, unlike the mammalian CRH-R1, the cfCRH-R1 exhibits very low levels of expression in the pituitary. The third CRH receptor (cfCRH-R3) is more similar to CRH-R1 than CRH-R2, and is expressed in the

**Table 1** Binding properties of CRH and related peptides. Results are expressed as  $K_i$  (nM)

	Rat CRH-R1	Mouse CRH-R2	Human CRH-BP
<b>Peptide</b>			
h/r/m CRH	0.95	17	0.21
rat urocortin	0.16	0.41	0.10
hCRH (6-33)	>1000	ND	3.5
ovine CRH	2.3	ND	1100

h, human; r, rat; m, mouse; ND, not determined.

Values are taken from Sutton *et al.* (1995) and Vaughan *et al.* (1995).

pituitary gland and brain (Aria *et al.* 2001). Homologs of the third catfish CRH receptor have not been identified in mammals.

### CRH-binding protein

The biological effects of CRH and other CRH-related ligands are mediated and controlled not only by CRH receptors, but also by the CRH-binding protein (CRH-BP). This 37 kDa secreted glycoprotein was originally isolated from human plasma (Orth & Mount 1987, Behan *et al.* 1989). It is distinct from the CRH receptors and binds hCRH and UCN with an equal or greater affinity than the CRH receptors (Table 1) (Sutton *et al.* 1995, Vaughan *et al.* 1995). The structural requirements for binding of CRH to CRH-BP can be distinguished from those for the CRH receptors, allowing the design of peptide ligands such as hCRH(6-33) which binds quite tightly to CRH-BP, but very poorly to the CRH receptors (Sutton *et al.* 1995). Similarly, hCRH-R1 binds ovine (o) CRH with very high affinity, while the hCRH-BP binds oCRH very weakly.

In 1991, the CRH-BP cDNA was cloned from human liver, revealing an open reading frame encoding a protein of 322 amino acids including a 25-amino acid signal sequence (Potter *et al.* 1991). Over the last decade, the CRH-BP has been shown to exist in numerous other species and CRH-BP cDNAs have been isolated and characterized from rat, mouse, sheep, and the frog *X. laevis* (Potter *et al.* 1991, Cortright *et al.* 1995, Behan *et al.* 1996a, Brown *et al.* 1996, Valverde *et al.* 2001). The purified and/or recombinant forms of the human, rodent, and frog CRH-BP bind hCRH and UCN with very high affinity, but bind ovine CRH with very low affinity or not at all. In contrast, the ovine CRH-BP has evolved so that it is capable of binding oCRH ( $K_i=10$  nM), but still binds hCRH with a higher affinity ( $K_i=0.2$  nM; Behan *et al.* 1996a).

The amino acid sequence alignment of CRH-BP from these species is shown in Fig. 3. The size of the binding protein is highly conserved with 321 to 324 amino acids in all species characterized. Further analysis of the primary

		1				50	*		*		100
Human	CRH-BP	<u>MSPNFK</u> LQCH <u>FLILFL</u> TALR <u>GESRY</u> LELR AADYDPF.LLF SANLKRDVAG EQPYRRALRC LDMLSLQGFQF TFTADRQLH CAAFFISEPE EPITIHVDQV									
Rat	CRH-BP	-----T--L-----	-----VQ--	--V----	-----NL-E	-----P-----	-----Q-----	-----G----	-----F-L-		
Mouse	CRH-BP	-----L-----	-----VQ--	--V--L.	-----L-E	-----P-----	-----P-----	-----G----	-----L-		
Ovine	CRH-BP	-A-TL-	-----VC-L-	-----V-H--	-PH-AG G-SP--ELE-	-PL-----	V-----	-----R--	-T--A--	-----L-	
Frog	CRH-BP	-T-ASRPDWC	L--L--AV--	-----IQM--	--EDAL-L...L	NSDF--ELSE	G-I--S--	I---IE--	--Q-----	--L-L-G--	---I-E-NF-
		101*			*	150			*		200
Human	CRH-BP	SIDCQGGDFL	KVFDGWILKG	EKFPSSQDHP	LPSAERYIDF	CESGLSRRSI	RSSQNVAMIF	FRVHEPGNF	TLTIKTDPNL	FPCNVISQTP	NGKFTLVVPH
Rat	CRH-BP	-----	-----	-----	--TR--T--	-----T--V	T-----V-	-----I-----	-----I-----	S-R-A---	Y
Mouse	CRH-BP	-----	-----	-----	--TMK--T--	-----T-----	-----V-	-----I-----	-----I-----	S-R-----	Y
Ovine	CRH-BP	-----LR--I-	Q-----	-----	-----TT--V--	-D-----	-----	-----I-V--E--	-----I-----	--R--M--	
Frog	CRH-BP	N---I---I-	-----I--	-----L--	-STM--T-I	-D-DVGSIT	-----	---QQ--H--	---RKI---	-----SM	--R--MIT--
		201+*			*	250		*	*		300
Human	CRH-BP	QHRNCSFSII	YPVVIKISDL	TLGHVNLQL	KKSSA.GCEGI	GDFVELLEGT	GLDPYKMTPL	ADLCYPFHGP	AQMKVGCNDT	VVRMVSSGKH	VNRVTFEYRQ
Rat	CRH-BP	--Q-----	--T-----	A--LH----	--PA--G-T	-----G--	-----T--ML-	V-----	-----IS--A	-----M-----	
Mouse	CRH-BP	--Q-----	--A-----	--LH----	--PA--G-T	-----G--	-----M--	-----L--	-----IS--A	-----I-----	
Ovine	CRH-BP	-----	--A-----	-----L--	-----G--	-----G--	-----LL-	-----LR--	-----H--	-L-----	I-----
Frog	CRH-BP	-----	-----F--	-----F-E--	--PPK--GDA	-----G-A	-----F--	-----HS--S	-----I-----	-----NF	I-----N-
		301		*							
Human	CRH-BP	LEPYELENPN	GNSIGEFCLS	GL							
Rat	CRH-BP	---L---TST	R---P-Y---	S-							
Mouse	CRH-BP	---F---TST	---P-Y---	S-							
Ovine	CRH-BP	-----	-----Q-----	T-							
Frog	CRH-BP	-D.RQ--KKQ	---VE-A-FP	SD							

**Figure 3** Comparison of the primary structure of the vertebrate CRH-BP. The human, rat, mouse, ovine and frog binding protein sequences are shown. Hyphens indicate identical amino acids as human CRH-BP sequence and periods indicate deletions/insertions. The conserved N-linked glycosylation site is marked with a plus sign and the conserved cysteines are marked with asterisks. Underlined amino acids indicate the signal sequence.

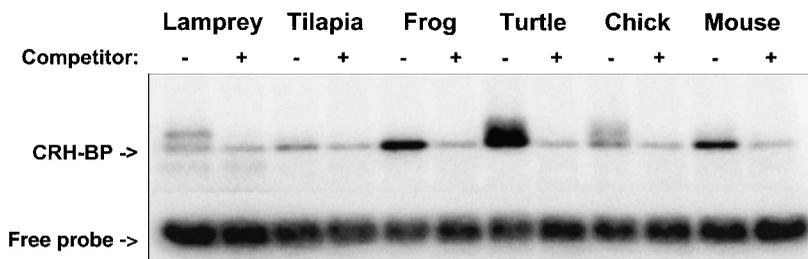
sequence from all species reveals no evidence for long hydrophobic stretches that could represent transmembrane regions, consistent with the function of CRH-BP as a secreted glycoprotein. In all five vertebrate species of CRH-BP, the full length cDNA predicts 10 cysteines in the mature CRH-BP (the cysteine in the signal peptide is removed during processing). The ten cysteine residues are sequentially bonded to form five consecutive disulfide loops that are essential for binding activity in the hCRH-BP (Fischer *et al.* 1994). The conservation of these cysteines across species suggests that the maintenance of this highly folded structure is required for activity. The N-linked glycosylation site at amino acid 204 in hCRH-BP is also conserved across all species. Finally, it should be noted that the greatest sequence diversity is found at the N-terminal (within the signal peptide sequence) and C-terminal regions of the protein. Extensive sequence homology is exhibited between amino acids 40 and 300 with 73% amino acid identity from frog to human across this sequence.

Recent studies of ours using crosslinking with [<sup>125</sup>I]-CRH have tested for CRH-BP activity in representatives from each vertebrate class. Using [<sup>125</sup>I]-*X. laevis* CRH (xCRH) as tracer, we detected CRH-BP activity in the sea lamprey, tilapia, turtle and chick brain extracts (Fig. 4; frog and mouse brain extracts were included as positive controls). The CRH-BP:CRH complex present at 41–42 kDa was significantly reduced in all cases by the addition of 200 nM unlabeled xCRH, showing the specificity of the ligand-binding protein interaction. These

results demonstrate the broad expression of CRH-BP across many different vertebrate species, suggesting that it is a phylogenetically ancient protein that exhibits extensive structural and functional conservation.

Finally, the CRH-BP is expressed in a highly tissue-specific pattern (reviewed in Kemp *et al.* 1998). Human CRH-BP is found in plasma, amniotic fluid, synovial fluid, placenta, pituitary, and brain. In contrast, CRH-BP is not found in rodent or ovine plasma, and rat, mouse, and ovine CRH-BP mRNA and protein have been detected only in brain and pituitary. *In situ* hybridization histochemical and immunocytochemical analyses have demonstrated that the CRH-BP is expressed in many specific regions of the rat brain including the cerebral cortex, hippocampus, amygdaloid complex, bed nucleus of the stria terminalis, olfactory bulb, and sensory relays associated with the auditory, olfactory, vestibular, and trigeminal systems (Potter *et al.* 1992). While CRH-BP is localized in several regions of the brain where CRH and UCN are not detected, rat CRH and CRH-BP immunoreactivity colocalize in a number of brain regions, and CRH-BP is also expressed in a number of CRH target sites, including a subset of anterior pituitary corticotropes (Potter *et al.* 1992). Colocalization studies have also demonstrated a number of sites in rat brain expressing both CRH-BP and CRH receptor.

In *X. laevis* tadpoles, the CRH-BP mRNA has been detected in brain, pituitary, liver and intestine (Valverde *et al.* 2001). The frog CRH-BP was first identified as a thyroid hormone-induced gene in *X. laevis* tadpole tail



**Figure 4** Detection of CRH-BP activity in brain extracts of representatives of each vertebrate class. The presence of CRH-BP binding activity was analyzed by chemical crosslinking using [ $^{125}$ I]-*Xenopus laevis* CRH (xCRH) as tracer as described by Valverde *et al.* (2001). Fifty micrograms brain extract protein from each species (sea lamprey, *Petramyzon marinus*; Tilapia *mossambicus*; frog, *Xenopus laevis*; turtle, *Trachemys scripta*; chick, *Gallus domesticus*; mouse, *Mus musculus*) were used in the assay and crosslinked proteins were resolved by 10% SDS-PAGE followed by autoradiography. Before crosslinking, extracts were incubated in the presence of [ $^{125}$ I]-xCRH with or without cold competitor (200 nM xCRH). Note that the intensity of the bands may or may not reflect differences in relative tissue CRH-BP content among species, since the affinities of the CRH-BPs for [ $^{125}$ I]-xCRH likely differ among species. Furthermore, the degree of competition with cold xCRH among species may also be influenced by differences in the nature of the ligand–binder interaction. To address these issues, future investigations should strive to analyze binding affinities and specificities using homologous ligands and specific immunological methods to quantify differences/changes in CRH-BP protein expression.

(Brown *et al.* 1996). The gene is strongly upregulated in the tadpole tail during metamorphic climax when this tissue is actively resorbing; however, the gene does not exhibit developmental or thyroid hormone-dependent changes in expression in the brain (Valverde *et al.* 2001). Taken together, these findings suggest that vertebrate CRH-BPs are localized at sites where they could act to modulate the actions of CRH and other CRH-like peptides in the brain, pituitary, and other peripheral sites of action.

### Roles for CRH-BP

Binding proteins have previously been identified for a variety of steroid hormones and for several polypeptide hormones including insulin-like growth factor-I (IGF-I) and growth hormone. The IGF-binding proteins (IGFBPs) are probably the most widely studied, and they not only regulate IGF action and bioavailability, but also mediate IGF-independent actions. IGFBP-1, IGFBP-3 and IGFBP-4 have been shown to inhibit IGF action in a dose-dependent manner, presumably by inhibiting IGF binding to receptors. In different cell systems, IGFBP-1 and IGFBP-3 demonstrate a stimulatory effect in the presence of IGFs. IGFBP-3 also appears to have an additional IGF-independent mechanism of action via an IGFBP-3 receptor. These binding proteins show both intracellular and extracellular roles, with activity regulated by proteolysis and phosphorylation (reviewed in Ferry *et al.* 1999). Finally, the expression of the IGFBPs is highly

regulated, allowing these binding proteins to play important regulatory roles in IGF physiology, often in a cell-type specific manner.

Consistent with these studies, multiple roles have been suggested for the CRH-BP. CRH-BP may bind CRH or other CRH-like ligands with high affinity and sequester ligand away from the receptor, thereby decreasing the actions of CRH at both the hormonal and synaptic levels (inhibitory activity). In this role, it may also function as a clearance factor to terminate the activity of CRH or UCN. Alternatively, the CRH-BP may increase the half-life of CRH or other CRH-like ligands by protecting them from degradation and delivering the ligand to receptors in the target tissue (enhancing activity). Finally, CRH-BP may signal or mediate effects on its own (with or without ligand), possibly via specific cell surface BP receptors. Currently, no receptors for CRH-BP have been identified.

Numerous *in vitro* and *in vivo* studies support an inhibitory role for the CRH-BP. In humans, CRH-BP binds placental CRH, preventing inappropriate stimulation of the stress axis by placental CRH. The binding of human CRH-BP to plasma CRH is thought to target the CRH:CRH-BP complex for clearance or degradation (Woods *et al.* 1994). A similar inhibitory role may be occurring in the pituitary. Cultured cell experiments show that recombinant CRH-BP inhibits hCRH-induced ACTH secretion from rat pituitary cultures or AtT-20 cells (Potter *et al.* 1991, Cortright *et al.* 1995) and ultrastructural studies localize CRH-BP to lysosomes and endosomes in rat pituitary corticotropes (Peto *et al.* 1999).

These results support an inhibitory role for the mammalian CRH-BP in anterior pituitary corticotropes and suggest a role for the BP in clearance of the CRH:CRH-BP complex.

Several animal models of CRH-BP overexpression or deficiency have been created to help determine the *in vivo* role of the CRH-BP (reviewed in Seasholtz *et al.* 2001). In an attempt to examine the role of pituitary CRH-BP, we created a line of transgenic mice that overexpresses CRH-BP specifically in the pituitary (Burrows *et al.* 1998). These animals demonstrate normal ACTH and glucocorticoid levels under basal and stressed conditions, but exhibit elevated hypothalamic CRH and vasopressin expression to compensate for the increased pituitary CRH-BP and maintain normal levels of 'free' CRH. This result supports the role of pituitary CRH-BP as an inhibitory molecule, binding CRH and preventing activation of the receptor. A second model of CRH-BP overexpression was created by Vale and colleagues (Lovejoy *et al.* 1998). This line of transgenic mice expresses the CRH-BP under the control of the metallothionein promoter, expressing the transgene not only in brain and pituitary, but also in the liver, kidney, heart, lung, adrenals, and spleen. Despite elevated plasma levels of CRH-BP, these mice show normal basal ACTH and corticosterone levels, but an impaired ACTH response to lipopolysaccharide (LPS)-induced inflammation. In addition, these transgenic mice show significant increases in weight gain, but with sexually dimorphic weight gain profiles. Finally, we have created a CRH-BP-deficient mouse using gene targeting in embryonic stem cells. This mouse model exhibits increased anxiety-like behavior and decreased weight gain in males (Karolyi *et al.* 1999). As CRH and UCN have both been suggested to be anxiogenic and anorectic, the phenotypes exhibited in the CRH-BP overexpressor and the CRH-BP-deficient mouse are consistent with CRH-BP acting to inhibit the activity of CRH-like ligands. In the presence of excess BP, the mice display increased weight gain and an impaired stress response, consistent with decreased CRH levels. In the absence of the binding protein, we see several behaviors consistent with elevated 'free' levels of CRH and other CRH-like ligands.

In amphibians, Brown *et al.* (1996) suggested that the upregulation of CRH-BP in the tail might serve a negative feedback role by binding CRH and thus downregulating the thyroid and adrenal axes at metamorphic climax. Such a role for this protein would require that it be secreted into the bloodstream, but we currently do not know if the CRH-BP is present in tadpole blood at metamorphic climax. It is also possible that the CRH-BP plays a local role within the tail. Recent findings (G C Boorse and R J Denver, unpublished observations) have demonstrated the existence of CRH mRNA in the tadpole tail. If CRH plays a role in the tadpole tail, either supporting cell

proliferation or prohibiting cell death, then one can hypothesize that the upregulation of CRH-BP at metamorphic climax is essential to the tail regression program. Another potential role for CRH expression in the tail could be to inhibit phagocytic cells, and neutralization of CRH by CRH-BP at metamorphic climax might then allow the appearance of such cells (thought to arise from transformation of existing mesenchyme cells; reviewed by Dodd & Dodd 1976) and/or their migration throughout the tail. Macrophages are not present in the growing tail, but appear when the tail begins to resorb (Dodd & Dodd 1976). Similar changes in macrophage activity occur in the gut (Shi 2000), and CRH-BP is expressed in this tissue in tadpoles (Valverde *et al.* 2001), but it is currently unknown whether CRH-BP is upregulated here during metamorphosis. It is noteworthy that CRH has been shown to play an important role in lymphoid tissues and at sites of inflammation in mammals (Radulovic & Spiess 2001).

Numerous other roles may exist for the vertebrate CRH-BP. Phosphorylation or proteolysis of the mature CRH-BP as suggested by Kemp *et al.* (1998) may alter its properties and functional roles. Behan *et al.* (1996b) have suggested that much of the CRH-BP is membrane-associated in rat and human brain extracts, raising the possibility of a CRH-BP receptor and receptor-associated function. Arguing against a CRH-BP receptor-associated function, however, is a recent ultrastructural study (Peto *et al.* 1999) localizing CRH-BP with lysosomes and endosomes or distributed diffusely in cell bodies and axon terminals, but not associated with plasma membrane. Other recent studies (Chan *et al.* 2000) have shown that *i.c.v.* administration of the CRH-BP specific ligand (hCRH6-33) specifically activates fos expression in CRH-BP-expressing neurons. This finding suggests an active role for the binding protein in signaling by CRH-related peptides. Thus, like the IGF-binding proteins, the CRH-BP may have diverse extra- and intracellular roles that may function in a cell-specific fashion at specific times in development. Additional studies to address the different intra- and extracellular sites of CRH-BP localization in different cell types are clearly required. Further studies are also necessary to probe the potential role of the CRH-BP in signaling following binding of CRH or CRH-like ligands. Mouse models with temporally controlled and brain region-specific over- or under-expression of the CRH-BP may provide new information on the region-specific roles of the CRH-BP. Finally, comparative analyses in diverse species will elucidate the evolutionary history of this protein as well as help define its function in extant species. The ability of CRH-BP to modulate the endocrine, synaptic, and peripheral activity of CRH and UCN throughout vertebrates suggests that it may play important roles in many processes from amphibian metamorphosis to human stress, anxiety, and obesity.

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