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Residues of Corticotropin Releasing Factor-binding Protein (CRF-BP) That Selectively Abrogate Binding to CRF but Not to Urocortin 1*[S]

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Corticotropin releasing factor-binding protein (CRF-BP) binds CRF and urocortin 1 (Ucn 1) with high affinity, thus preventing CRF receptor (CRFR) activation. Despite recent progress on the molecular details that govern interactions between CRF family neuropeptides and their cognate receptors, little is known concerning the mechanisms that allow CRF-BP to bind CRF and Ucn 1 with picomolar affinity. We conducted a comprehensive alanine scan of 76 evolutionarily conserved residues of CRF-BP and identified several residues that differentially affected the affinity for CRF over Ucn 1. We determined that both neuropeptides derive their similarly high affinity from distinct binding surfaces on CRF-BP. Alanine substitutions of arginine 56 (R56A) and aspartic acid 62 (D62A) reduce the affinity for CRF by ~100-fold, while only marginally affecting the affinity for Ucn 1. The selective reduction in affinity for CRF depends on glutamic acid 25 in the CRF peptide, as substitution of Glu^{25} reduces the affinity for CRF-BP by approximately 2 orders of magnitude, but only in the presence of both Arg^{56} and Asp^{62} in human CRF-BP. We show that CRF-BP_{R56A} and CRF-BP_{D62A} have lost the ability to inhibit CRFR1-mediated responses to CRF that activate luciferase induction in HEK293T cells and ACTH release from cultured rat anterior pituitary cells. In contrast, both CRF-BP mutants retain the ability to inhibit Ucn 1-induced CRFR1 activation. Collectively our findings demonstrate that CRF-BP has distinct and separable binding surfaces for CRF and Ucn 1, opening new avenues for the design of ligand-specific antagonists based on CRF-BP.

Corticotropin releasing factor (CRF)[3] is a 41-amino acid neuropeptide characterized in 1981 as the principal hypothalamic factor to induce the release of adrenocorticotropic hormone (ACTH) from the pituitary gland (1). Three CRF-related peptides, urocortin (Ucn) 1, 2 and 3, have since been discovered (2–5). CRF and urocortins are pleiotropic neuropeptides that govern functions in the central nervous system as well as at peripheral sites (6–8). CRF family peptides signal via two G-protein coupled receptors, CRFR1 and CRFR2. CRF and Ucn 1 activate both receptors, whereas Ucn 2 and Ucn 3 are selective agonists for CRFR2. Considerable progress has been made in recent years to unravel the molecular interactions that dictate the binding of CRF family peptides to their cognate receptors. The extracellular domain of CRFRs primarily interacts with the C-terminal residues of CRF (9–11). The N-terminal residues of CRF are required for receptor activation and are proposed to interact with the transmembrane region of the receptor to induce conformational changes that enable G-protein activation (12).

Corticotropin releasing factor-binding protein (CRF-BP) is a 37-kDa glycoprotein that was originally found to circulate in high concentrations in late gestational maternal plasma where it likely prevents inappropriate release of ACTH from the pituitary gland by placental-derived CRF (13–15). CRF-BP was named for its ability to bind to CRF with high affinity (16), but it also binds to other members of the CRF family of neuropeptides. Human CRF-BP has high (pM) affinity for rat Ucn 1 (rUcn 1) and rat/human CRF (r/hCRF) and intermediate (nM) affinity for mouse Ucn 2. CRF-BP does not appreciably bind Ucn 3. The fact that CRF-BP displays no significant sequence similarity to any other known protein facilitated its characterization in early vertebrates and insects (17–21). Among the conserved structural features of CRF-BP are 10 cysteine residues that form five consecutive disulfide bridges (22) as well as a single asparagine (Asn)-linked glycosylation site at position 204 reported to be required for CRF binding (23). As the affinity of CRF and Ucn 1 for CRF-BP is severalfold higher than that for either CRFR, CRF-BP is generally considered an antagonist of CRFRs by vir-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and Table S1.

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[3] The abbreviations used are: CRF, corticotropin releasing factor; ACTH, adrenocorticotropic hormone; Ucn 1, urocortin 1; CRF-BP, corticotropin releasing factor-binding protein; CRFR, corticotropin releasing factor receptor; oCRF, ovine corticotropin releasing factor; rCRF, rat corticotropin releasing factor; hCRF, human corticotropin releasing factor; WT, wild-type; HEK, human embryonic kidney.

tue of its potential to sequester ligands. Despite our increasing understanding of the molecular interactions that underlie high potency activation of CRFRs, we know little about the interactions that facilitate binding between CRF-BP and its high affinity endogenous peptide ligands CRF and Ucn 1. Early work on a panel of truncated CRF-derived compounds revealed that CRF-(6–33) retained most of the binding affinity for CRF-BP and therefore contained the key residues for interaction with CRF-BP (13, 24). Scrutiny of the large difference in affinity between ovine CRF (oCRF) and r/hCRF for human CRF-BP subsequently pinpointed the four amino acid ARAE (alanine-arginine-alanine-glutamic acid) motif at positions 22–25 of r/hCRF as crucial for the high affinity interaction with CRF-BP (24, 25).

By contrast, insight into the regions and residues of CRF-BP that are responsible for ligand binding is scant and the CRF-BP structure is not known. Because CRF-BP has no known paralogous genes, we cannot derive structural information from similar folds in related proteins. On the basis of photoaffinity labeling experiments with r/hCRF-(6–33), a pair of arginines in CRF-BP, Arg^{46} and Arg^{59}, was identified as part of the ligand binding site of rat CRF-BP for CRF (26). The binding interface of CRF-BP was proposed to consist of a linear conformation of a stretch of N-terminal amino acids in CRF-BP that interacts with the α-helical CRF peptide in antiparallel fashion: Arg^{46} interacting with the C terminus and Arg^{59} with the N terminus of the peptide (26). However, this model awaits experimental verification. In addition to our fragmented understanding of the mode of interaction between CRF-BP and CRF, little is known about the mechanism that allows Ucn 1 to bind CRF-BP with high affinity.

In the present study we have adopted an alanine scanning mutagenesis approach to identify key residues on CRF-BP that mediate binding to r/hCRF and rUcn 1. Interestingly, this approach allowed us to identify several amino acid residues on CRF-BP that selectively mediate binding of CRF but not Ucn 1. The selectively disrupted affinity for r/hCRF of these CRF-BP mutants abrogates the ability to block CRF-induced activation of CRFR1, whereas the inhibition of rUcn 1 is unaffected.

EXPERIMENTAL PROCEDURES

Mutagenesis Approach—Human CRF-BP was cloned into the EcoRV and NotI sites of the pcDNA3.1 expression vector (Invitrogen) with a FLAG tag (DYKDDDK) at the N terminus. Single amino acid mutations were introduced by site-specific primers in a PCR-based mutagenesis strategy using high fidelity *Taq* DNA polymerase (Bio-X-act, Bioline USA Inc., Randolph, MA). Vector from individual clones was purified (miniprep, Qiagen, Valencia, CA) and verified by automated sequencing of both strands. Clones that carried the desired mutation only were grown in a larger volume. Vector DNA was isolated using a maxiprep kit (Qiagen) according to the manufacturer's protocol and verified once more by automated sequencing.

Protein Expression and Purification—The pcDNA3.1 expression vector containing CRF-BP was introduced into human embryonic kidney 293T (HEK293T) cells by transient transfection with polyethylenimine (Sigma) as the precipitating agent. Briefly, 14.4 μg of DNA was premixed with 36 μg of polyethylenimine in 1 ml of serum-free media (Dulbecco's modified Eagle's medium) containing penicillin/streptomycin and glutamine (Invitrogen). DNA was allowed to precipitate for 10 min before dispersal over the surface of a 40–60% confluent 15-cm Petri dish containing serum-free media. The following morning media was replaced by serum-free expression media without phenol red (Freestyle 293 expression media; Invitrogen). Expression media was harvested after 48 h and cells and cell debris were removed by centrifugation. CRF-BP was purified by overnight incubation with 50 μl of a 50% slurry of anti-FLAG-agarose beads (Sigma) and eluted from the resin using 100 mM glycine, pH 3.0. The pH was neutralized by the addition of 20% (v/v) 0.5 M Tris-HCl, 1.5 M NaCl, pH 7.4. Protein expression was confirmed by Western blot using a mouse anti-FLAG monoclonal (1:2000 Sigma) and rabbit anti-human CRF-BP antiserum (number 5144; 1:2000) (27). We initially constructed a series of truncated CRF-BP mutants to identify regions of CRF-BP involved in ligand binding, but none of these truncated CRF-BP mutants was detectable in the culture media following transient transfection (data not shown). All but eight of the CRF-BP alanine mutants were secreted following transient transfection in levels detectable by Western blot (data not shown). Whereas alanine mutations of aspartic acids at positions 98 and 114 interfered with expression or secretion of CRF-BP (supplemental Table 1), $CRF\text{-}BP_{D98N}$ and $CRF\text{-}BP_{D114N}$ were expressed and did not display gross abnormalities in the affinity for r/hCRF and rUcn 1 (data not shown). CRF-BP was dialyzed overnight in 10 mM Hepes buffer, pH 7.4, using dialysis tubes with 4 kDa of MWCO (GBioscience, St. Louis, MO) and stored at −20 °C. Controls transfected with vector DNA alone were included in all experiments and were consistently negative for the presence of FLAG-tagged protein or peptide binding activity.

CRF-BP Ligand Immunoradiometric Assay—CRF-BP mutants were quantified by ligand immunoradiometric assay as previously described (24). Briefly, serial dilutions of each mutant were incubated overnight at 4 °C in 50 mM sodium phosphate buffer, pH 7.5, containing 100 mM sodium chloride, 25 mM EDTA, 0.1% sodium azide, 0.1% crystalline bovine serum albumin (ImmunO grade, MP Biomedicals, Irvine, CA) and 0.01% Triton X-100 (EMD Biosciences, La Jolla, CA)) with 50,000 cpm of radiolabeled tracer in the presence of rabbit anti-human CRF-BP antiserum (number 5144; 1:1000) (27). For all experiments ^{125}I-[D-Tyr^0]r/hCRF was used as tracer, except for CRF-BP with mutations at positions 56 or 62 that interfere with r/hCRF binding, where ^{125}I-[D-Tyr^0]rUcn 1 was used instead. We verified that the choice of tracer, ^{125}I-[D-Tyr^0]r/hCRF *versus* ^{125}I-[D-Tyr^0]rUcn 1, has no significant effect on K_i values (data not shown). Also, r/hCRF was capable of completely displacing ^{125}I-[D-Tyr^0]rUcn 1 and rUcn 1 completely displaced ^{125}I-[D-Tyr^0]r/hCRF (data not shown). Iodination was carried out as previously described (28). Total counts bound were measured by precipitation for 2 h with sheep anti-rabbit γ-globulin (1:20), normal rabbit serum (1:100), and 4% polyethylene glycol (average M_r 8,000; Sigma). For every mutant an appropriate working dilution was determined in the linear

range of the assay, where an increase in CRF-BP was accompanied by linear increase in tracer binding. At that dilution, the affinities of CRF-BP mutants for CRF and Ucn 1 were determined by competition with increasing amounts of unlabeled peptide. Binding curves and IC_{50} values including 95% confidence intervals were obtained using Prism 4.0c for Macintosh (Graphpad Software Inc., San Diego, CA). Curves were fitted by non-linear regression assuming one-site binding. All peptides were synthesized in-house using a *tert*-butyl-oxy-carbonyl strategy on an automated peptide sequencer (CS536 peptide synthesizer; C. S. Bio Co., San Carlos, CA) and purified by reverse phase high pressure liquid chromatography and characterized by mass spectrometry.

In Vitro Reporter Assay—HEK293T cells were seeded in a 10-cm dish at 1.5×10^6 cells per dish the day prior to transfection. The following day, cells were transiently transfected with 600 ng of human CRFR1 in pcDNA3.1, 5 μg of pXP2 reporter construct (luciferase driven by a cAMP responsive element), and 1 μg of β-galactosidase driven by a cytomegalovirus promoter (29). DNA was precipitated for 10 min by incubation with 9.9 μg of polyethylenimine and added to the cells under serum-free conditions. The following day, cells were trypsinized and seeded in poly-L-lysine-coated wells of a 48-well plate at 100,000 cells/well in media containing 10% fetal bovine serum. After an overnight rest, cells were stimulated for 3 h followed by a single wash with ice-cold Hepes dissociation buffer (28). Cells were lysed in 100 μl of luciferase buffer (10 mM $MgSO_4$, 25 mM glycylclycine, 4 mM EGTA) supplemented with 1% Triton X-100 (EMD Biosciences, La Jolla, CA) and 1 mM dithiothreitol. Luciferase activity was determined in 50 μl of cell lysate using a Lumimark plus microplate reader (Bio-Rad) following addition of 100 μl of luciferin substrate buffer (luciferase buffer supplemented with 0.3 mM luciferin, 1 mM ATP, and 1 mM dithiothreitol). Luciferase activity was normalized for the β-galactosidase activity measured in 20 μl of cell lysate by addition of 100 μl of β-galactosidase substrate buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol, 1.5 mg/ml *ortho*-nitrophenyl-β-D-galactopyranoside; Sigma).

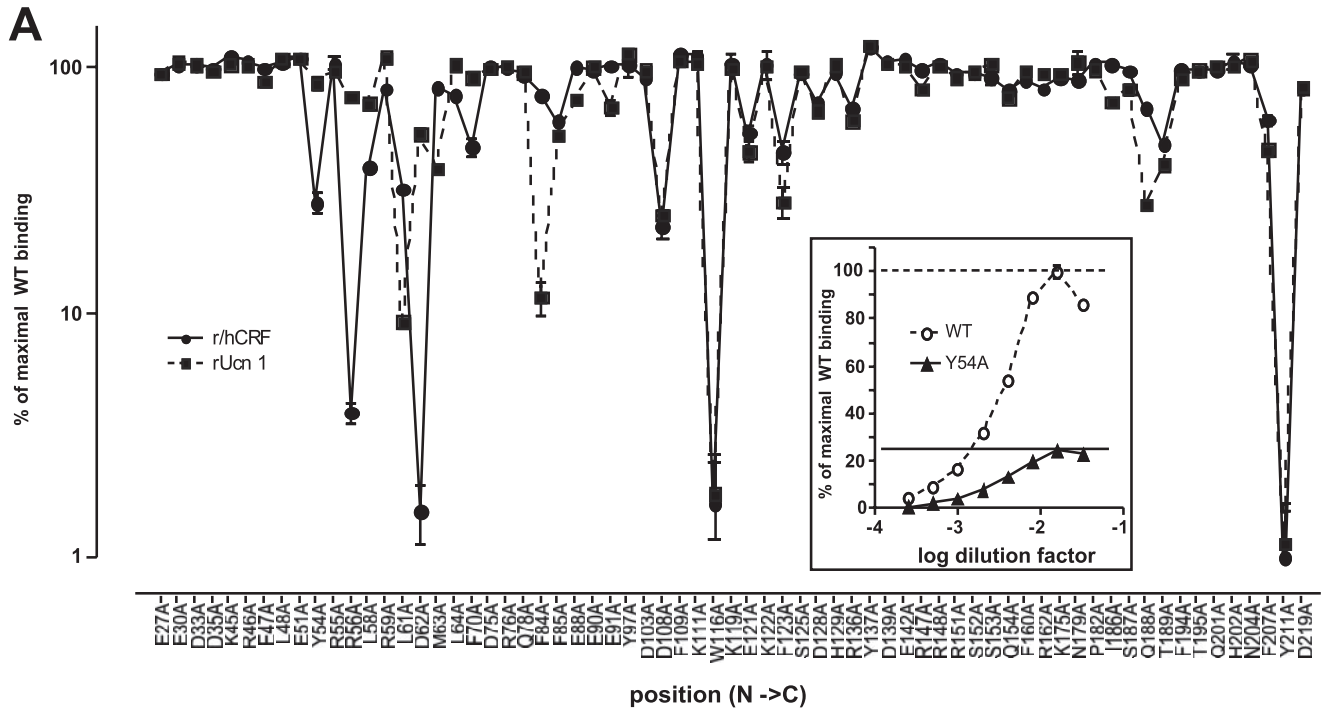
Rat Anterior Pituitary Assay—Purified CRF-BP mutants were tested on cultured primary rat anterior pituitary cells isolated from male Sprague-Dawley rats and dispersed into single cells with collagenase as previously described (28). Cells were cultured at 6.2×10^4 cells per well in poly-L-lysine-coated 96-well tissue culture plates (Costar, Cambridge, MA). Cultures were maintained in 0.1 ml/well β-Pit-julep (28) media containing 2% fetal bovine serum. On day 4 in culture, cells were washed three times with β-Pit-julep media containing 0.1% bovine serum albumin and incubated for 1 h at 37 °C. The media was replaced by treatment compounds diluted in β-Pit-julep media containing 0.1% bovine serum albumin. Media was harvested after 3 h and stored at −20 °C until analysis for ACTH content. The procedure for ACTH radioimmunoassay was similar to that previously described for melanin-concentrating hormone (30), except that all buffers contained 0.05% Triton X-100. Rabbit anti-rat ACTH serum (Peninsula Laboratories, San Carlos, CA; T-4002) was used at 1:30,000 final dilution. [3-[^{125}I]Iodotyrosyl^2]ACTH(1–39), purchased from Amersham Biosciences (IM216) was used as tracer with about 20,000 cpm added per tube. Rat ACTH(1–39), synthesized in our laboratory, was used as standard at doses ranging from 2 to 1000 pg/tube. The EC_{50} for rat ACTH(1–39) was 65–70 pg/tube; the assay displays minimal cross-reactivity with α-melanocyte-stimulating hormone and ACTH(1–24).

RESULTS

An Alanine Scan Identifies Residues That Selectively Affect Binding of CRF or Ucn 1—We specifically disrupted the disulfide bridges of CRF-BP by mutating both cysteines of each pair to alanine. Only the CRF-BP mutants lacking the fourth or fifth disulfide bridge were expressed in detectable quantities, and their affinities for r/hCRF and rUcn 1 were unaffected (supplemental Fig. 1). This suggests that the key determinants for ligand binding are located toward the N-terminal part of CRF-BP. Based on this observation we initiated a comprehensive alanine scan of the N-terminal domain of hCRF-BP (31). In keeping with the argument that evolutionarily conserved residues are more likely to be involved in protein function, we targeted a panel of 76 residues that are conserved or conservatively substituted in CRF-BP of early vertebrate and insect species (supplemental Fig. 2). We determined the ability of all mutants to bind ^{125}I-labeled r/hCRF and ^{125}I-labeled rUcn 1 and compared each mutant to the binding capacity of wild-type (WT) CRF-BP (Fig. 1*A*). Mutation of several amino acids, notably Trp^{116} and Tyr^{211}, completely abolished the ability of CRF-BP to bind CRF and Ucn 1, although mutant proteins were readily detectable by Western immunoblotting (supplemental Fig. 3). As alanine substitutions of Trp^{116} and Tyr^{211} interfered with bioactivity in general, rather than specifically affecting affinity for r/hCRF or rUcn 1, it is possible that these mutations cause CRF-BP to misfold, resulting in loss of function. Similarly, mutants, such as L61A, E121A, F123A, and Q188A, that have lost partial affinity for both r/hCRF and rUcn 1 may have done so because these mutations result in partial misfolding rather than specifically affecting the binding surface for the peptide ligands.

We subsequently determined the relative potency of all CRF-BP mutants for binding to r/hCRF and rUcn 1 using competitive binding assays. For r/hCRF and rUcn 1 we identified 13 and 14 alanine mutants, respectively, that had reduced affinity for the ligand by 2-fold or more (Fig. 1*B* and supplemental Table 1). Interestingly, we identified several amino acids that, when mutated, selectively affected the K_i for r/hCRF, but not rUcn 1, and vice versa. Residues that selectively affected the K_i for r/hCRF when substituted by alanine include Arg^{56} and Asp^{62} and, to a lesser extent, Tyr^{54}, Leu^{58}, Leu^{64}, and Phe^{70}. Residues that selectively or more potently interfered with high affinity binding to rUcn 1 when mutated include Leu^{61}, Met^{63}, Phe^{84}, Glu^{88}, Glu^{91}, Gln^{188}, and Thr^{189} (Fig. 1*B*). Generally, mutants that selectively altered r/hCRF affinity were concentrated toward the N terminus, whereas mutations that disproportionately affected the binding of rUcn 1 were distributed more evenly throughout the linear sequence of the N-terminal domain of CRF-BP.

A



B

mutant	r/hCRF fold reduction in relative affinity	r/hCRF 95% confidence intervals	rUcn 1 fold reduction in relative affinity	rUcn 1 95% confidence intervals
WT	1.00	(0.849 to 1.18)	1.00	(0.765 to 1.31)
Y54A	3.94	(3.32 to 4.68)	1.12	(0.953 to 1.31)
R56A	133	(123 to 145)	2.05	(1.87 to 2.25)
L58A	3.39	(2.84 to 4.04)	1.76	(1.41 to 2.20)
L61A	6.69	(5.58 to 8.02)	22.0	(18.2 to 26.7)
D62A	95.7	(85.1 to 107)	1.44	(1.20 to 1.72)
M63A	1.11	(0.967 to 1.27)	7.76	(6.53 to 9.21)
L64A	2.33	(2.03 to 2.67)	1.13	(0.951 to 1.34)
F70A	6.45	(5.59 to 7.45)	1.35	(1.20 to 1.52)
F84A	1.79	(1.56 to 2.05)	33.9	(28.5 to 40.3)
F85A	3.11	(2.70 to 3.58)	4.08	(3.63 to 4.59)
E88A	1.33	(1.16 to 1.54)	2.34	(2.09 to 2.64)
E91A	1.14	(1.01 to 1.29)	2.28	(2.04 to 2.54)
E121A	3.88	(3.46 to 4.34)	5.92	(5.29 to 6.63)
F123A	5.19	(4.51 to 5.98)	5.66	(5.03 to 6.36)
D128A	1.83	(1.60 to 2.10)	2.52	(2.12 to 3.00)
Q154A	1.82	(1.61 to 2.06)	2.02	(1.77 to 2.30)
Q188A	2.44	(2.21 to 2.70)	8.03	(7.16 to 8.99)
T189A	3.52	(3.05 to 4.05)	5.17	(4.60 to 5.81)
F207A	2.23	(1.97 to 2.53)	2.54	(2.23 to 2.89)

FIGURE 1. **Summary of the effect of alanine substitution of selected residues of human CRF-BP on maximum r/hCRF or rUcn 1 binding.** Serial dilutions of each mutant were incubated with a fixed amount of ^{125}I-[D-Tyr^0]r/hCRF or ^{125}I-[D-Tyr^0]rUcn 1. The amount of bound radioligand increases with increasing concentrations of CRF-BP, until it reaches a maximum and starts to decrease with increasing CRF-BP concentration, when the capacity of the CRF-BP antiserum is no longer sufficient to capture all CRF-BP (*inset*). The maximum tracer binding capacity is an approximate indicator of affinity. For example (*inset*), CRF-BP$_{Y54A}$ binds ~25% of the tracer that is bound by WT CRF-BP, indicative of reduced affinity for r/hCRF. Using this method we determined the maximum tracer binding capacity for each alanine mutant in duplicate for independently expressed and purified CRF-BP preparations using r/hCRF and rUcn 1 tracers (*A*). We expressed these maxima relative to the maximal ^{125}I-[D-Tyr^0]r/hCRF (*circles*, *solid line*) and ^{125}I-[D-Tyr^0]rUcn 1 (*boxes*, *dashed line*) binding capacity of WT CRF-BP, which was defined as 100%. Alanine substitutions that affect the affinity for r/hCRF and/or rUcn 1 are characterized by a decrease in their percent of maximal binding. Changes in relative affinity were determined separately by competitive binding assays (*B*). Only mutants that affect affinity for either peptide by 2-fold or more are shown. Note that the affinities of CRF-BP$_{W116A}$ and CRF-BP$_{Y211A}$ could not be determined as neither mutant binds detectable amounts of r/hCRF or rUcn 1 tracers. See supplemental Table 1 for a comprehensive list of the relative affinity for all mutants.

CRF-BP Does Not Require Arg^{46} and Arg^{59} to Bind CRF or Ucn 1—Based on photocross-linking experiments, a pair of N-terminal arginines of CRF-BP, Arg^{46} and Arg^{59}, were suggested to contact r/hCRF (26). As the involvement of Arg^{46} and Arg^{59} in ligand binding had not been experimentally confirmed, we verified their contribution to the affinity for r/hCRF and rUcn 1. As shown in Fig. 2, WT recombinant C-terminal FLAG-tagged CRF-BP bound CRF and Ucn 1 with high affinity (K_i values for CRF and Ucn 1 were 217 and 77.2 pM, respectively), consistent with previously published values for non-tagged recombinant human CRF-BP (2, 16, 24). However, substitution of Arg^{46} with alanine did not

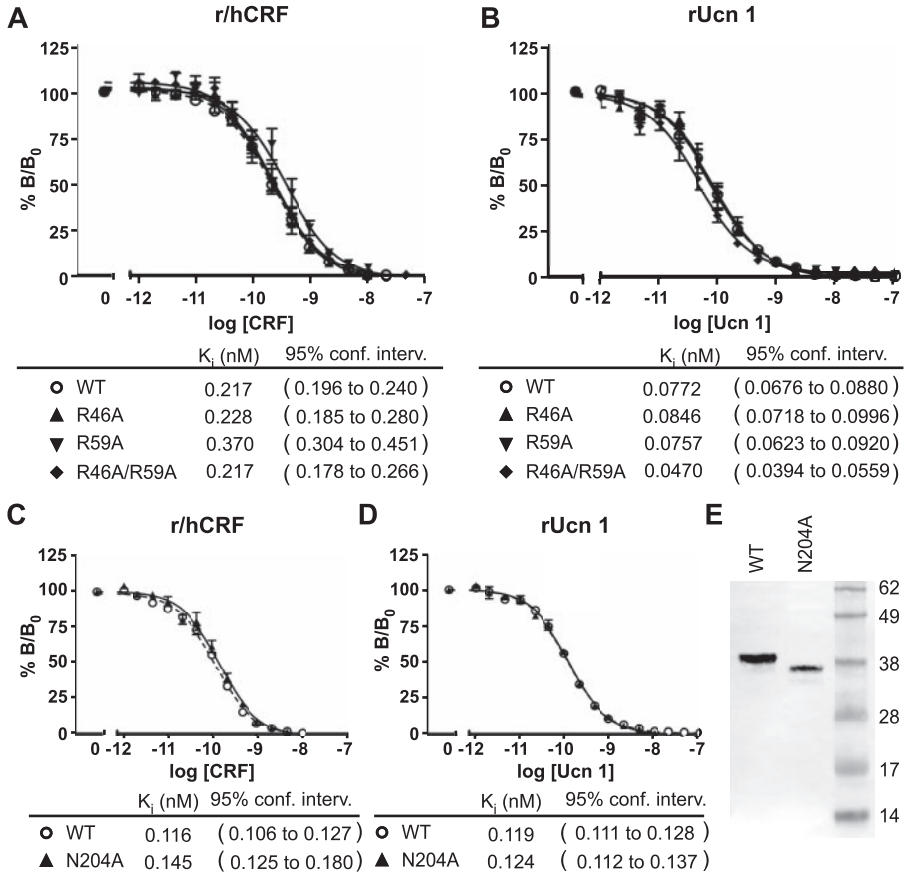


FIGURE 2. **Arg^{46}, Arg^{59}, and Asn^{204} in CRF-BP are dispensable for high affinity binding to r/hCRF or rUcn 1.** Percent displacement (% B/B_0) of ^{125}I-[D-Tyr^0]r/hCRF by r/hCRF or rUcn 1, comparing WT CRF-BP with CRF-BP$_{R46A}$, CRF-BP$_{R59A}$, and CRF-BP$_{R46A/R59A}$. WT CRF-BP (*open symbols*, *dashed lines*) binds rUcn 1 with slightly higher affinity than r/hCRF. Alanine substitution of Arg^{46} does not affect affinity for either peptide, whereas the affinity for r/hCRF is less than 2-fold reduced in CRF-BP$_{R59A}$ (*A* and *B*). Simultaneous mutation of Arg^{46} and Arg^{59} has no effect on the affinity for r/hCRF and slightly improves binding to rUcn 1 (*B*). Note that Arg^{46} and Arg^{59} are referred to as Arg^{23} and Arg^{36} in Ref. 26. The affinity of CRF-BP$_{N204A}$ (*closed symbols*, *solid line*) for r/hCRF (*C*) or rUcn 1 (*D*) is not different from that of WT CRF-BP (*open symbols*, *dashed line*). CRF-BP$_{N204A}$ has a lower apparent molecular weight than WT CRF-BP as determined by SDS-PAGE and detected by Western immunoblot (*E*), confirming that *N*-linked glycosylation in HEK293T cells is prevented by the N204A mutation. K_i values and 95% confidence intervals are derived from two or more separate experiments.

affect K_i values for r/hCRF or rUcn 1, whereas CRF-BP$_{R59A}$ displayed a modestly (<2-fold) reduced K_i value for r/hCRF (Fig. 2). Simultaneous alanine substitution of Arg^{46} and Arg^{59} resulted in a CRF-BP protein that was indistinguishable from hCRF-BP in its K_i for CRF and had slightly increased affinity for rUcn 1 (Fig. 2). Collectively, these data indicate that neither Arg^{46} nor Arg^{59} are key for binding to r/hCRF or rUcn 1.

N-Linked Glycosylation at Position 204 Is Not Required for Ligand Binding—Alanine replacement of the asparagine comprising the single *N*-linked glycosylation site of CRF-BP (N204A) results in a reduction in molecular weight compared with WT CRF-BP, demonstrating that the *N*-linked glycosylation in transfected HEK293T cells is abrogated by the N204A mutation (Fig. 2*E*). In contradiction to an early report that *N*-linked glycosylation is required for CRF binding (23), the affinities of the N204A mutant for r/hCRF and rUcn 1 were indistinguishable from those of WT CRF-BP (Fig. 2, *C* and *D*).

Alanine Substitution of Arg^{56} and Asp^{62} Selectively Abrogates CRF Binding—We focused in more detail on the profound and specific loss in affinity for CRF, but not Ucn 1, observed for CRF-BP$_{R56A}$ and CRF-BP$_{D62A}$. Replacing either Arg^{56} or Asp^{62} with alanine reduced the affinity for r/hCRF by more than 2 orders of magnitude (Fig. 3*A*). Both mutations significantly affected CRF binding, whereas affinity for rUcn 1 was only 2-fold reduced (Fig. 3*B*). The selectivity of the R56A mutation was further illustrated by substituting the adjacent arginine at position 55 (Arg^{55}) with alanine, which had no effect on CRF binding (Fig. 3*A*). Substitution of Arg^{56} for a Lys only minimally improved the affinity for r/hCRF compared with CRF-BP$_{R56A}$, whereas the introduction of an acidic amino acid side chain at this position failed to substantially alter the affinity for r/hCRF when further compared with CRF-BP$_{R56A}$ (Fig. 3*C*). Substitution of Asp^{62} for Glu resulted in a 10-fold loss of the affinity for r/hCRF, compared with the 100-fold loss in affinity for CRF-BP$_{R56A}$ (Fig. 3*D*). CRF-BP mutants with a basic amino acid side chain in place of Asp^{62} did not express in detectable levels (data not shown).

As alanine substitutions of Arg^{56} and Asp^{62} resulted in remarkably similar and ~100-fold reductions in the affinity for r/hCRF, whereas only marginally affecting the affinity for rUcn 1, we expressed CRF-BP with an R56A/D62A double mutation to test if the effects of the single mutations were additive. CRF-BP$_{R56A/D62A}$ bound r/hCRF with an affinity that was indistinguishable from that of either single mutant (Fig. 3*A*), demonstrating that the effects of the R56A and D62A mutations on r/hCRF binding were not cumulative. The affinity of R56A/D62A for rUcn 1 was unaffected (Fig. 3*B*). If Arg^{56} and Asp^{62} together form an intramolecular salt bridge, one would anticipate that switching both amino acids could restore the loss in affinity for r/hCRF caused by the removal of either amino acid. However, switching the amino acid residues at positions 56 and 62 (CRF-BP$_{R56D/D62R}$) restores the affinity for r/hCRF merely 4-fold compared with CRF-BP$_{R56A/D62A}$, suggesting that the relationship between both amino acids may be more complex than a straightforward ionic interaction (Fig. 3*D*).

The Side Chain Charge at Ligand Position 25 Determines the Direction of the Shift in Affinity for R56A and D62A—To identify candidate peptide residues or regions that could potentially act through Arg^{56} and Asp^{62} of CRF-BP, we compared the affinity of additional members of the CRF peptide family and investigated if these affinities are affected by the R56A or D62A mutations. As demonstrated earlier, CRF-BP$_{R56A}$ and CRF-

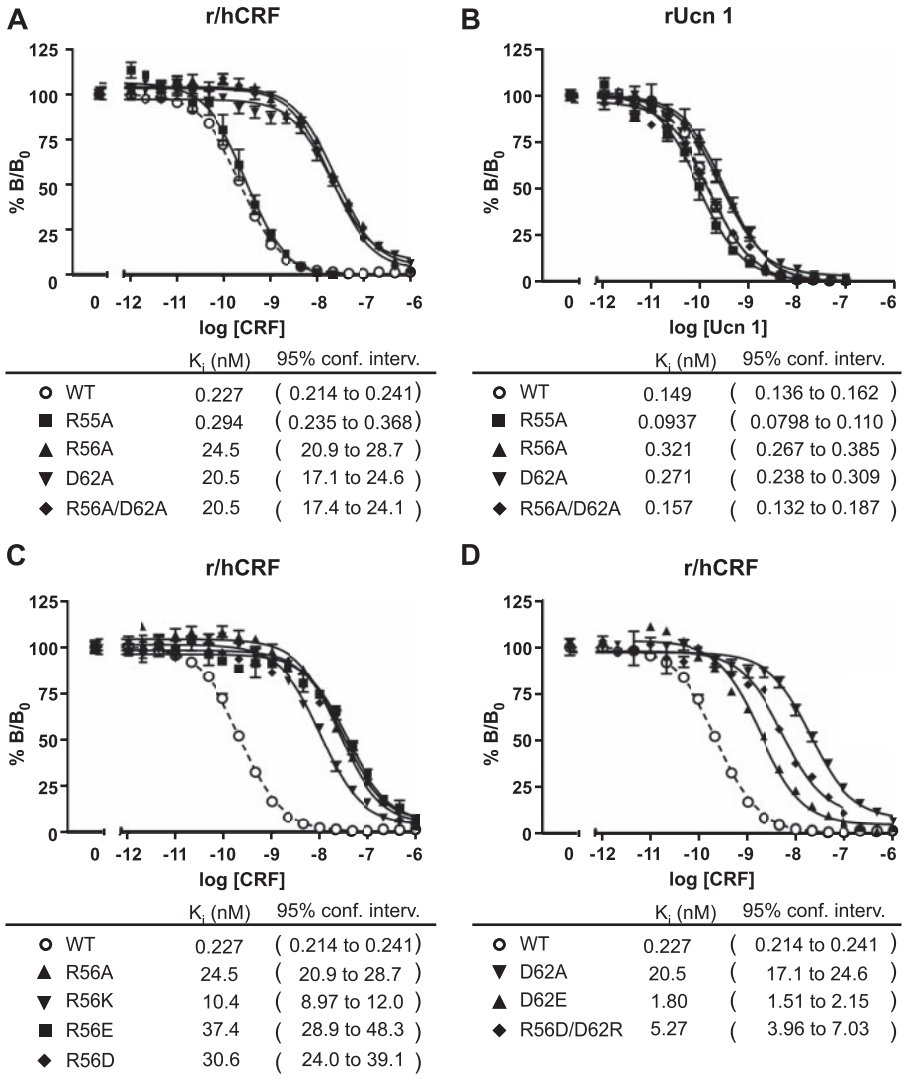
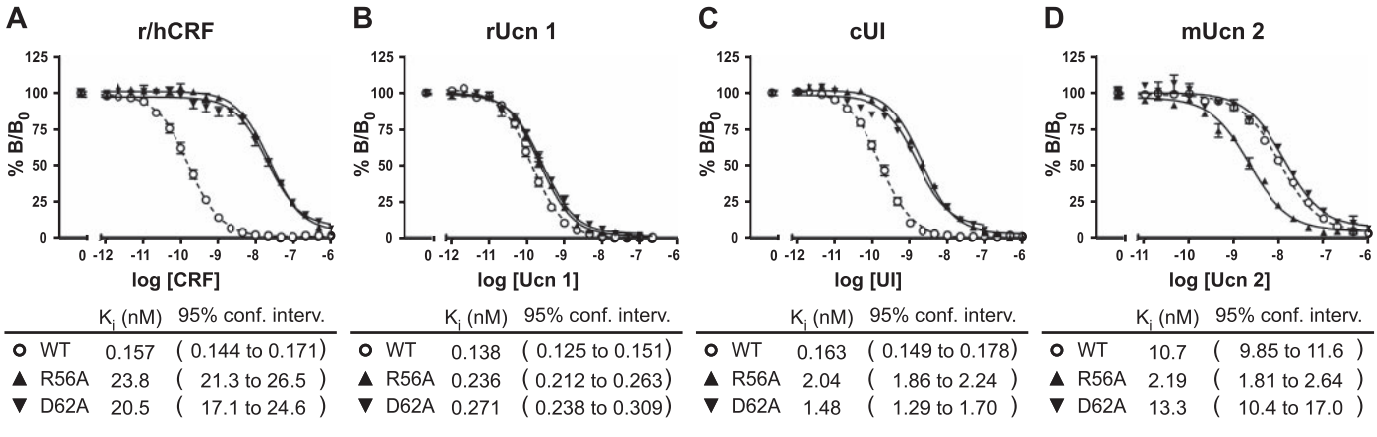


FIGURE 3. **Amino acid substitutions at CRF-BP positions 56 and 62 differentially affect affinities for r/hCRF and rUcn 1.** Alanine substitution of Arg^{56} or Asp^{62} results in profound and similar reductions in the affinity for r/hCRF (*A*). Simultaneous substitutions of Arg^{56} and Asp^{62} does not further reduce the affinity for r/hCRF. The affinity for r/hCRF is unaffected by alanine substitution of Arg^{55}. In contrast to the profound changes in affinity for r/hCRF, CRF-BP_{R56A} and CRF-BP_{D62A} have only 2-fold reduced affinity for rUcn 1 (*B*). Simultaneous substitution of Arg^{56} and Asp^{62} for alanines does not affect rUcn 1 affinity. Substitution of Arg^{56} for a Lys only minimally restores affinity for r/hCRF, whereas the introduction of acidic amino acids at this position did not further reduce the affinity for r/hCRF compared with CRF-BP_{R56A} (*C*). Substitution of Asp^{62} for Glu restores the affinity for r/hCRF by ~10-fold when compared with CRF-BP_{D62A} (*D*). Switching the residues at positions 56 and 62 (CRF-$BP_{R56D/D62R}$) fails to restore the affinity for r/hCRF to levels comparable with the affinity of WT CRF-BP. In all experiments ^{125}I-[D-Tyr^0]rUcn 1 was used as tracer with the exception of the competitive binding assays with CRF-BP_{R55A}, where ^{125}I-[D-Tyr^0]r/hCRF was used. K_i values and 95% confidence intervals are derived from two or more separate experiments.

BP_{D62A} displayed 100-fold reduced affinity for r/hCRF while leaving the affinity for rUcn 1 largely intact (Fig. 4, *A* and *B*). CRF-BP has high affinity (K_i of 163 pM) for carp urotensin-I (cUI), the bony fish ortholog of mammalian Ucn 1. This affinity was reduced by ~10-fold in both CRF-BP_{R56A} and CRF-BP_{D62A} (Fig. 4*C*). Affinity for mUcn 2, in contrast, was increased from 10.7 to 2.19 nM for CRF-BP_{R56A}, whereas affinity of CRF-BP_{D62A} for mUcn 2 was unaffected (Fig. 4*D*). We inspected an amino acid sequence alignment of CRF family peptides (Fig. 4*E*) to identify differences between members that might explain the observed ligand-selective changes in affinity for CRF-BP_{R56A} and CRF-BP_{D62A}. The charge of the amino acid side chain at ligand position 25 correlated well with the direction and magnitude of the observed changes in affinity of CRF-BP for the different CRF-related ligands. Both r/hCRF and cUI have an acidic residue (Glu) at position 25 and bind with lower affinity upon removal of either Arg^{56} or Asp^{62} in CRF-BP, whereas the affinity for rUcn 1, which has a neutral glutamine (Gln) at position 25, is minimally affected by the R56A and D62A mutations. Conversely, mUcn 2 has a basic (Lys) residue at the equivalent amino acid position and responds to alanine substitution of Arg^{56} in CRF-BP with an increase in affinity.

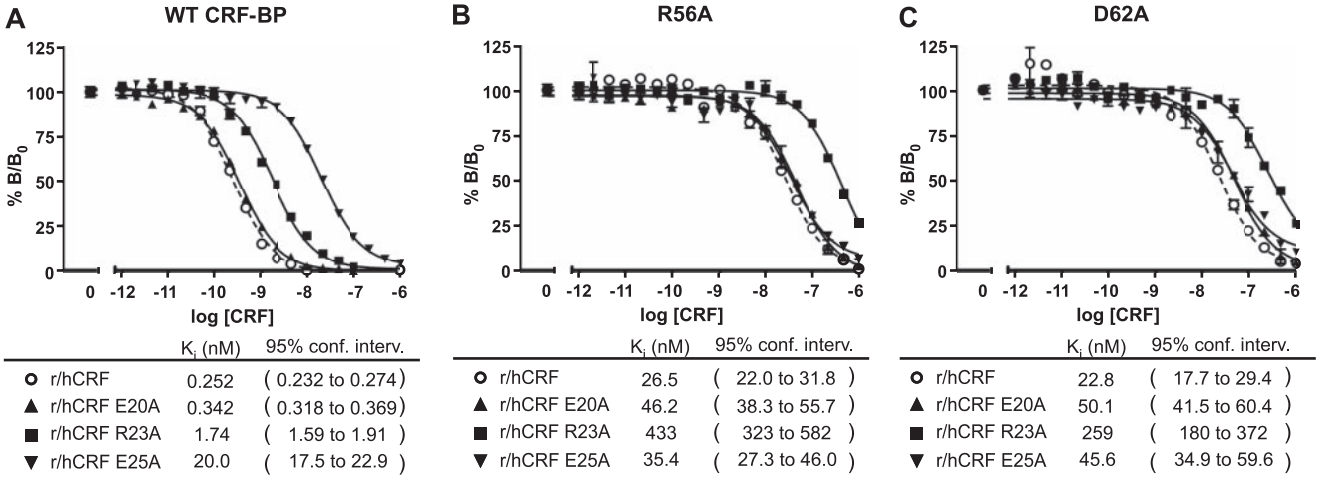
To validate the involvement of Glu^{25} in r/hCRF in high affinity binding to CRF-BP, we replaced Glu^{25} with alanine in r/hCRF (r/$hCRF_{E25A}$). We compared the effects of this mutation to alanine substitution of Glu^{20} (r/$hCRF_{E20A}$), which is conserved in CRF, Ucn 1, and Ucn 2, as well as Arg^{23} (r/$hCRF_{R23A}$). The amino acids at positions 25 and 23 in CRF have previously been shown to affect affinity for CRF-BP based on experiments with oCRF (24). The affinity of r/$hCRF_{E25A}$ for CRF-BP was reduced by approximately 2 orders of magnitude, whereas the K_i of r/$hCRF_{E20A}$ was only marginally (less than 2-fold) reduced compared with r/hCRF (Fig. 5*A*). Alanine substitution of Arg^{23} in CRF reduced the affinity of r/hCRF for CRF-BP by ~7-fold. When we determined the affinity of these r/hCRF analogs for R56A and D62A mutants of CRF-BP, we found that r/$hCRF_{E25A}$ no longer differed from r/hCRF in its affinity for CRF-BP_{R56A} (Fig. 5*B*) and had only 2-fold reduced affinity for CRF-BP_{D62A} compared with r/hCRF (Fig. 5*C*). These results indicate that alanine replacement of Glu^{25} in r/hCRF had no further effect on the ~100-fold reduction in affinity for CRF that results from the R56A or D62A mutations. In contrast, r/$hCRF_{R23A}$ did display a further reduction compared with r/hCRF in affinity for CRF-BP, in addition to the 100-fold reduced affinity for CRF-BP_{R56A} and CRF-BP_{D62A} (Fig. 5). This demonstrates that the contribution of Glu^{25} in r/hCRF to the interaction with CRF-BP depends on the presence of both Arg^{56} and Asp^{62}, whereas Arg^{23} of r/hCRF affects affinity independently of these CRF-BP residues.



E

```
                1          11         21         31         41
rat/human CRF   SEEPPISLDL TFHLLREVLE MARAEQLAQQ AHSNRKLMEI I
rat Ucn 1       -DDPPLSIDL TFHLLRTVLE LARTQSQRER AEQNRIIFDS V
carp UI         NDDPPISIDL TFHLLRNMIE MARNENQREQ AGLNRKYLDE V
mouse Ucn 2     ---VILSLDV PIGLLRILLE QARYKAARNQ AATNAQILAH V
```

FIGURE 4. **The direction and severity of the change in affinity for CRF family peptides correlates with the charge of the amino acid side chain at position 25 of the ligand.** CRF-BP_{R56A} and CRF-BP_{D62A} have 100- and 10-fold reductions in affinity for r/hCRF (*A*) and carp urotensin I (*C*), respectively. Affinity for rUcn 1 is reduced by less than 2-fold (*B*). In contrast, CRF-BP_{R56A} has increased affinity for mUcn 2, whereas alanine substitution of Asp^{62} has no effect on mUcn 2 affinity (*D*). Note that the reduced affinity of r/hCRF and cUI correlates with a glutamic acid at position 25, whereas mUcn 2 has a basic lysine at the equivalent position and displays increased affinity for CRF-BP_{R56A}. The minor effects of either CRF-BP mutant on rUcn 1 affinity correspond with the absence of an organic base or acid in the side chain of amino acid position 25 (*E*). In all experiments ^{125}I-[D-Tyr^0]rUcn 1 was used as tracer. K_i values and 95% confidence intervals are derived from two or more separate experiments.



D

```
                1          11         21         31         41
r/hCRF          SEEPPISLDL TFHLLREVLE MARAEQLAQQ AHSNRKLMEI I
r/hCRF E20A     SEEPPISLDL TFHLLREVLA MARAEQLAQQ AHSNRKLMEI I
r/hCRF R23A     SEEPPISLDL TFHLLREVLE MAAAEQLAQQ AHSNRKLMEI I
r/hCRF E25A     SEEPPISLDL TFHLLREVLE MARAAQLAQQ AHSNRKLMEI I
```

FIGURE 5. **Substitution of Glu^{25} in r/hCRF affects its affinity for CRF-BP only in the presence of both Arg^{56} and Asp^{62} in CRF-BP.** The affinity of r/hCRF is reduced by 80-fold following alanine substitution of Glu^{25}, whereas alanine substitution of Glu^{20} has no effect on binding to CRF-BP (*A*). Alanine substitution of Arg^{23} reduces the affinity of r/hCRF by approximately 1 order of magnitude. The profound loss in affinity of r/$hCRF_{E25A}$ for CRF-BP is absent on the background of CRF-BP_{R56A} (*B*) or CRF-BP_{D62A} (*C*), suggesting that Glu^{25} in r/hCRF requires Arg^{56} and Asp^{62} to interact with CRF-BP. In contrast, replacing Arg^{23} by alanine continues to reduce affinity of r/hCRF for CRF-BP_{R56A} and CRF-BP_{D62A}, demonstrating that Arg^{23} interacts with CRF-BP independently of Arg^{56} and Asp^{62} in the binding protein. The position of the alanine substitutions within r/hCRF is illustrated in *D*. In all experiments ^{125}I-[D-Tyr^0]rUcn 1 was used as tracer. K_i values and 95% confidence intervals are derived from two or more separate experiments.

CRF-BP_{R56A} and CRF-BP_{D62A} Inhibit Ucn 1-induced Activation of CRFR1 but Have Selectively Lost the Ability to Inhibit CRF—We compared the ability of CRF-BP_{R56A} and CRF-BP_{D62A} to inhibit r/hCRF and rUcn 1-induced activation of CRFR1. Wild-type CRF-BP inhibited the activation of CRFR1 induced by 50 pM r/hCRF or rUcn 1 with an IC_{50} of 2.65 and 8.01 nM, respectively, as measured in a cAMP-luciferase reporter assay (Fig. 6, *A* and *B*). In agreement with the profound

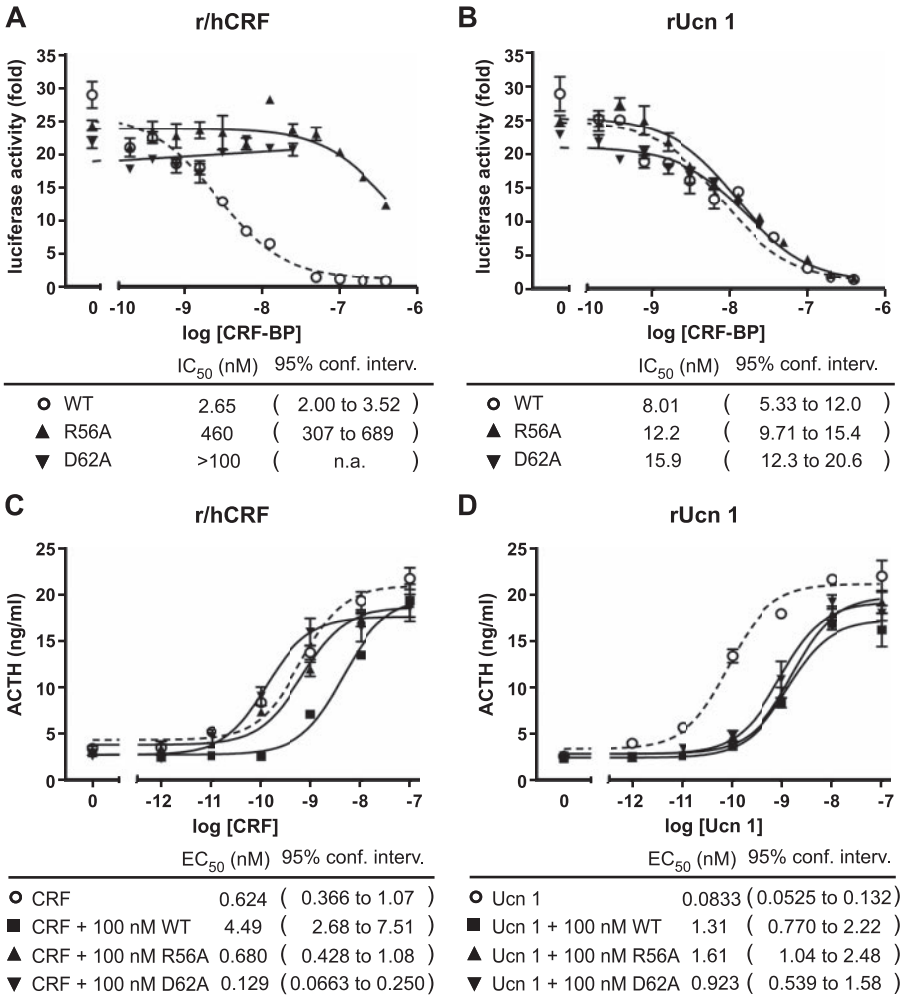


FIGURE 6. **CRF-BP$_{R56A}$ and CRF-BP$_{D62A}$ have selectively lost the ability to inhibit r/hCRF-induced activation of CRFR1.** Wild-type CRF-BP dose dependently inhibits r/hCRF-induced (50 pM) activation of CRFR1 as measured by cAMP-responsive element-driven luciferase activity (*A*). This dose-dependent inhibition is greatly impaired in CRF-BP$_{R56A}$ and CRF-BP$_{D62A}$. In contrast, both CRF-BP mutants retain the ability to inhibit rUcn 1-induced (50 pM) activation of CRFR1 with similar potency to WT CRF-BP (*B*). Wild-type CRF-BP inhibits the r/hCRF-induced release of ACTH from primary rat anterior pituitary cultures, but both CRF-BP$_{R56A}$ and CRF-BP$_{D62A}$ have lost the ability to inhibit r/hCRF-induced ACTH release (*C*). In contrast, both CRF-BP mutants retain the ability to inhibit rUcn 1-induced release of ACTH with the same potency as WT CRF-BP (*D*).

and selective loss of binding affinity for r/hCRF, the CRF-BP$_{R56A}$ and CRF-BP$_{D62A}$ mutants displayed a severely and selectively reduced potency to inhibit the r/hCRF-induced activation of CRFR1 (Fig. 6*A*). In contrast, inhibition of rUcn 1-induced CRFR1 activation was unaffected by R56A or D62A mutations (Fig. 6*B*). We then tested the ability of CRF-BP to inhibit the release of ACTH from primary rat anterior pituitary cultures induced by CRF or Ucn 1 via endogenous CRFR1. Wild-type CRF-BP at a concentration of 100 nM significantly inhibited the release of ACTH induced by increasing doses of CRF and Ucn 1. However, CRF-BP$_{R56A}$ and CRF-BP$_{D62A}$ do not inhibit r/hCRF-induced ACTH release (Fig. 6*C*) while maintaining a potency similar to WT CRF-BP in inhibiting the ACTH release induced by rUcn 1 (Fig. 6*D*).

In a reverse approach, we tested the ability of WT CRF-BP to block the activation of CRFR1 by r/hCRF and r/hCRF$_{E25A}$. Wild-type CRF-BP robustly inhibited r/hCRF, as the EC_{50} for r/hCRF was shifted by ~30-fold in the presence of CRF-BP (Fig. 7*A*). In contrast, CRF-BP was incapable of inhibiting the activation of CRFR1 by r/hCRF$_{E25A}$ in line with the profoundly reduced affinity of r/hCRF$_{E25A}$ for CRF-BP. The potency of r/hCRF$_{E25A}$ to activate CRFR1 was equal to that of r/hCRF. When we compared the induction of ACTH release from rat primary anterior pituitary cells by r/hCRF and r/hCRF$_{E25A}$, we found that r/hCRF$_{E25A}$ was slightly more potent in inducing ACTH release compared with r/hCRF, but that this induction was no longer antagonized by CRF-BP (Fig. 7*B*).

DISCUSSION

Considerable progress has been made in recent years to identify the molecular determinants that direct the interaction of CRF family ligands with their cognate receptors (9–12). Yet, little attention has been paid to the binding surface on CRF-BP responsible for the high affinity interactions between CRF-BP and its endogenous ligands CRF and Ucn 1. To address this hiatus, we initiated a mutagenesis approach that involved a comprehensive alanine scan of CRF-BP. We focused on the N-terminal 27-kDa domain of CRF-BP, as disruption of either of the two C-terminal disulfide bridges had no effect on the affinity for r/hCRF and rUcn 1. This is in agreement with earlier observations that CRF-BP undergoes spontaneous cleavage after serine 234, resulting in an inactive 10-kDa C-terminal fragment and a 27-kDa N-terminal fragment that retains the ability to bind CRF (31). Our approach revealed multiple amino acids in CRF-BP that differentially or selectively affect the binding of r/hCRF or rUcn 1 when replaced by alanine. As r/hCRF and rUcn 1 compete for binding to CRF-BP it is probable that both peptides occupy partially overlapping areas on the surface of CRF-BP. From the differences in amino acid positions of CRF-BP that affect the affinity of r/hCRF and rUcn 1, and the discovery that many of these residues differentially affect the affinity for either peptide, it follows that r/hCRF and rUcn 1 depend in part on distinct molecular interactions to bind to CRF-BP with similarly high affinity.

Two N-terminal arginine residues, Arg^{46} and Arg^{59}, were previously suggested to be part of the binding site on CRF-BP for CRF based on photocross-linking studies (26). The coincidental similarities of the distances between the N and C terminus of α-helical CRF-(6–33) and the side chains of Arg^{46} and Arg^{59} of CRF-BP in a linear arrangement led to the postulation

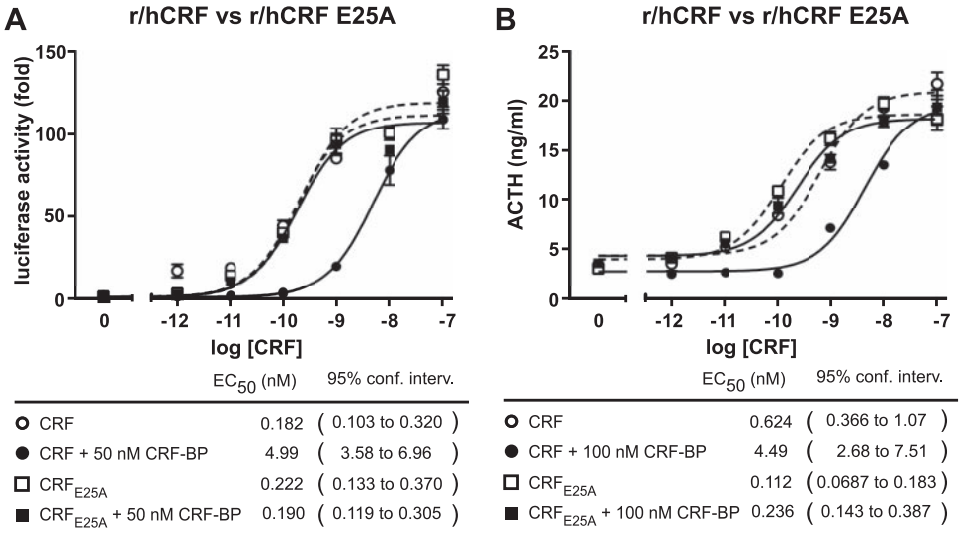


FIGURE 7. **Alanine substitution of Glu^{25} in r/hCRF creates a ligand that activates CRFR1 with equal or greater potency than r/hCRF but is no longer inhibited by CRF-BP.** CRFR1 is activated in a dose-dependent fashion and with equal potency by r/hCRF and r/hCRF$_{E25A}$ as measured by luciferase activity (*A*). However, addition of CRF-BP inhibits only the r/hCRF-induced CRFR1 activation. Similarly, r/hCRF and r/hCRF$_{E25A}$ both induce ACTH release from primary rat anterior pituitary cell cultures, but addition of CRF-BP no longer inhibits r/hCRF$_{E25A}$-induced ACTH release (*B*).

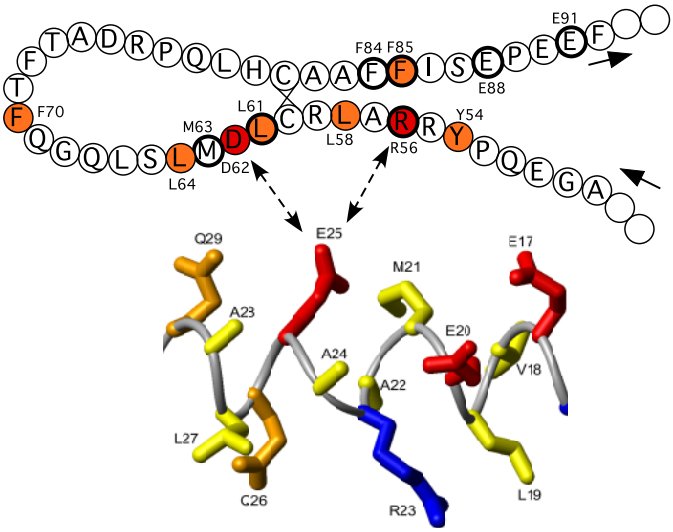


FIGURE 8. **Schematic representation of part of the N terminus of human CRF-BP, highlighting the amino acid positions where alanine substitution affects the affinity for r/hCRF or rUcn 1.** Positions where alanine substitution results in a reduction of the affinity for r/hCRF of 2-fold or more are *orange*, Arg^{56} and Asp^{62} are *red*. The residues that are indicated by a *bold circle* indicate positions where alanine substitution reduces rUcn 1 affinity by at least 2-fold. The three-dimensional structure of the central part of CRF illustrates that the amino acid side chains of glutamine 25 and arginine 23 are situated on the same face of the ligand and point in opposite directions. The *dashed lines* connecting Glu^{25} of CRF with Arg^{56} and Asp^{62} of CRF-BP indicate that high affinity binding of r/hCRF by CRF-BP depends on interactions that directly or indirectly require all three residues. The three-dimensional structure is derived from the NMR structure of astressin (PDB 2RMI), which is identical to r/hCRF in the central region of the peptide that is depicted (Glu^{17} to Gln^{29}) with the exception of a methionine to norleucine substitution at position 21.

that the interface between CRF and CRF-BP consists of two antiparallel polypeptides (26). However, no mutagenesis experiments were conducted to test the validity of this model. We have now replaced Arg^{46} and Arg^{59} with alanine, both individually and in combination, and found that mutation of these residues only minimally affects the affinity of CRF-BP for r/hCRF or rUcn 1. Although this does not rule out the possibility that Arg^{46} and/or Arg^{59} are situated in proximity to the actual binding surface for CRF in CRF-BP, it demonstrates that neither residue contributes substantially to peptide binding.

A dramatic example of the ligand-specific events involved in binding CRF, but not Ucn 1, is provided by CRF-BP$_{R56A}$ and CRF-BP$_{D62A}$. These CRF-BP mutants each display a selectively reduced affinity for r/hCRF of approximately 2 orders of magnitude, coupled with only marginally reduced affinity for rUcn 1. Furthermore, the remarkably similar effects of both mutations are not cumulative, prompting the possibility of a direct ionic interaction. However, switching the amino acids at positions 56 and 62 fails to restore the affinity for r/hCRF, suggesting that these residues may interact in a more complex fashion. Alanine substitution of Glu^{25} in CRF results in a comparable decrease in affinity that requires the presence of both Arg^{56} and Asp^{62} in CRF-BP, as the alanine replacement of either CRF-BP residue abrogates the large difference in affinity between r/hCRF and r/hCRF$_{E25A}$. Collectively, this suggests that Arg^{56} and Asp^{62} in CRF-BP and Glu^{25} in r/hCRF participate in the same molecular interaction. It is possible that these amino acids engage in a composite interaction that requires all three residues, as removal of any one of them is sufficient for the full shift in affinity of 2 orders of magnitude. One possible explanation is the formation of a salt bridge triad that has been observed in a number of proteins (32–35). In these proteins, two residues with organic acid side chains and one with a basic side chain (or vice versa) interlock in a small network of salt bridges and hydrogen bonds that greatly stabilize protein structure. Alternatively, it is possible that substitution of Arg^{56} and Asp^{62} by alanine confers a structural change in CRF-BP that indirectly interferes with high affinity binding to r/hCRF.

Previous experiments with modified oCRF peptides demonstrated a key role for the 4-amino acid ARAE motif at positions 22–25 of the ligand (24, 36). Here we confirm the role of Arg^{23} and Glu^{25} in the interaction between r/hCRF and CRF-BP, as alanine substitution of these peptide residues results in a loss of affinity of 7- and 80-fold, respectively. A closer inspection of the core residues of CRF in their α-helical conformation reveals that Glu^{25} and Arg^{23} are the only polar residues amid an otherwise hydrophobic face of the α-helical CRF peptide (37, 38). The amino acid side chains of Arg^{23} and Glu^{25} occupy the same face of the CRF peptide but point in opposite directions (Fig. 8). Of note, the substitution of Ala^{22} in r/hCRF with glutamic acid reduces the affinity for CRF-BP by ~100-fold (25). Perhaps replacing the small side chain of an alanine that has high α-helical propensity with the larger and acidic side chain of glutamic

acid interferes with the same intra- and intermolecular interactions that require the presence of Arg^{56} and Asp^{62} in CRF-BP and Glu^{25} in r/hCRF. It is conceivable that within the ARAE motif of r/hCRF, Arg^{23} and Glu^{25} directly interact with the binding surface of CRF-BP, whereas the role of the alanines at positions 22 and 24 may be to prevent steric hindrance, promote peptide α-helicity, or both.

Early studies comparing the duration of oCRF and r/hCRF action following bolus injection in human circulation found that oCRF was consistently longer acting and was cleared at an ~3-fold lower rate compared with r/hCRF (39). CRF-BP is suspected of actively clearing r/hCRF, but not oCRF for which it has only low affinity, from the circulation (40, 41). By introducing a single E25A amino acid substitution in r/hCRF, we generated a peptide that is equipotent to endogenous r/hCRF in its activation of CRFR1 but that may no longer be actively cleared from the circulation or inhibited from receptor activation by CRF-BP.

Our discovery that different regions of CRF-BP contribute to the binding of CRF and Ucn 1 opens new avenues for the specific abrogation of selected CRF family members. Traditionally, intervention of pathologies associated with dysregulated signaling by CRF family peptides has aimed at the selective activation or antagonism of CRFRs. Selective receptor antagonists are available for CRFR1 (*e.g.* antalarmin) and CRFR2 (antisauvagine-30, $Astressin_2$-B) (7, 42–44). The identification of residues that selectively affect the affinity of CRF-BP for CRF family peptides facilitates the design of ligand-specific antagonists that could be used as alternatives for, or complimentary to, selective receptor antagonists.

With the introduction of a single alanine mutation (R56A) in CRF-BP we effectively created a Ucn 1-specific antagonist. Although the generally beneficial effects of Ucn 1 on cardiovascular performance (2, 8, 45, 46) may limit the clinical potential of a CRF-BP-based Ucn 1-specific antagonist, this antagonist could be a valuable tool to discriminate between the effects of Ucn 1 and CRF on CRF receptors. In light of the recent observation that Ucn 2 reduces peripheral insulin sensitivity (47), the design of a Ucn 2-selective antagonist based on CRF-BP holds promise to protect from or alleviate metabolic insults that lead to obesity and type II diabetes.

CRF is implicated in the etiology of Alzheimer disease. It is expressed in brain regions that are prone to degeneration in Alzheimer disease and lower CRF levels in the cerebrospinal fluid of patients correlate with greater cognitive impairment (48–51). As CRF-BP is highly expressed in areas affected by Alzheimer disease, but is sparse at sites where liberation of endogenous CRF would result in unfavorable stress and anxiety-like side effects, the administration of ligands that are incapable of receptor activation but can dissociate endogenous CRF from CRF-BP has been proposed for the treatment of Alzheimer disease (52). We anticipate that intimate knowledge of the mechanisms by which CRF and CRF-BP interact provides impetus for the development of CRF-BP antagonists that may locally compete with endogenous CRF for CRF-BP. The identification of Arg^{56} and Asp^{62} as amino acids key for binding CRF, but not Ucn 1, may further the design of antagonists that selectively prevent the interaction between CRF and CRF-BP. A complete understanding of the interactions between CRF-BP and its endogenous ligands, including those residues responsible for ligand selectivity, awaits the resolution of the three-dimensional structure of CRF-BP.

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REFERENCES

1. Vale, W., Spiess, J., Rivier, C., and Rivier, J. (1981) *Science* **213,** 1394–1397
2. Vaughan, J., Donaldson, C., Bittencourt, J., Perrin, M. H., Lewis, K., Sutton, S., Chan, R., Turnbull, A. V., Lovejoy, D., Rivier, C., Rivier, J., Sawchenko, P. E., and Vale, W. (1995) *Nature* **378,** 287–292
3. Lewis, K., Li, C., Perrin, M. H., Blount, A., Kunitake, K., Donaldson, C., Vaughan, J., Reyes, T. M., Gulyas, J., Fischer, W., Bilezikjian, L., Rivier, J., Sawchenko, P. E., and Vale, W. W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 7570–7575
4. Reyes, T. M., Lewis, K., Perrin, M. H., Kunitake, K. S., Vaughan, J., Arias, C. A., Hogenesch, J. B., Gulyas, J., Rivier, J., Vale, W. W., and Sawchenko, P. E. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 2843–2848
5. Hsu, S. Y., and Hsueh, A. J. (2001) *Nat. Med.* **7,** 605–611
6. Smith, S. M., and Vale, W. W. (2006) *Dialogues Clin. Neurosci.* **8,** 383–395
7. Zorrilla, E. P., Tache, Y., and Koob, G. F. (2003) *Trends Pharmacol. Sci.* **24,** 421–427
8. Fekete, E. M., and Zorrilla, E. P. (2007) *Front. Neuroendocrinol.* **28,** 1–27
9. Grace, C. R., Perrin, M. H., Gulyas, J., Digruccio, M. R., Cantle, J. P., Rivier, J. E., Vale, W. W., and Riek, R. (2007) *Proc. Natl. Acad. Sci. U. S. A.* **104,** 4858–4863
10. Rijkers, D. T., Kruijtzer, J. A., van Oostenbrugge, M., Ronken, E., den Hartog, J. A., and Liskamp, R. M. (2004) *ChemBioChem* **5,** 340–348
11. Yamada, Y., Mizutani, K., Mizusawa, Y., Hantani, Y., Tanaka, M., Tanaka, Y., Tomimoto, M., Sugawara, M., Imai, N., Yamada, H., Okajima, N., and Haruta, J. (2004) *J. Med. Chem.* **47,** 1075–1078
12. Grace, C. R., Perrin, M. H., DiGruccio, M. R., Miller, C. L., Rivier, J. E., Vale, W. W., and Riek, R. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 12836–12841
13. Orth, D. N., and Mount, C. D. (1987) *Biochem. Biophys. Res. Commun.* **143,** 411–417
14. Suda, T., Iwashita, M., Tozawa, F., Ushiyama, T., Tomori, N., Sumitomo, T., Nakagami, Y., Demura, H., and Shizume, K. (1988) *J. Clin. Endocrinol. Metab.* **67,** 1278–1283
15. Linton, E. A., Wolfe, C. D., Behan, D. P., and Lowry, P. J. (1988) *Clin. Endocrinol.* **28,** 315–324
16. Potter, E., Behan, D. P., Fischer, W. H., Linton, E. A., Lowry, P. J., and Vale, W. W. (1991) *Nature* **349,** 423–426
17. Huising, M. O., Metz, J. R., van Schooten, C., Taverne-Thiele, A. J., Hermsen, T., Verburg-van Kemenade, B. M., and Flik, G. (2004) *J. Mol. Endocrinol.* **32,** 627–648
18. Huising, M. O., and Flik, G. (2005) *Endocrinology* **146,** 2165–2170
19. Alderman, S. L., and Bernier, N. J. (2007) *J. Comp. Neurol.* **502,** 783–793
20. Doyon, C., Trudeau, V. L., and Moon, T. W. (2005) *J. Endocrinol.* **186,** 123–130
21. Boorse, G. C., Crespi, E. J., Dautzenberg, F. M., and Denver, R. J. (2005) *Endocrinology* **146,** 4851–4860
22. Fischer, W. H., Behan, D. P., Park, M., Potter, E., Lowry, P. J., and Vale, W. (1994) *J. Biol. Chem.* **269,** 4313–4316
23. Suda, T., Sumitomo, T., Tozawa, F., Ushiyama, T., and Demura, H. (1989) *Biochem. Biophys. Res. Commun.* **165,** 703–707
24. Sutton, S. W., Behan, D. P., Lahrichi, S. L., Kaiser, R., Corrigan, A., Lowry, P., Potter, E., Perrin, M. H., Rivier, J., and Vale, W. W. (1995) *Endocrinology*

136, 1097–1102
25. Eckart, K., Jahn, O., Radulovic, J., Tezval, H., van Werven, L., and Spiess, J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 11142–11147
26. Jahn, O., Eckart, K., Brauns, O., Tezval, H., and Spiess, J. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 12055–12060
27. Potter, E., Behan, D. P., Linton, E. A., Lowry, P. J., Sawchenko, P. E., and Vale, W. W. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4192–4196
28. Vale, W., Vaughan, J., Yamamoto, G., Bruhn, T., Douglas, C., Dalton, D., Rivier, C., and Rivier, J. (1983) *Methods Enzymol.* **103,** 565–577
29. Bilezikjian, L. M., Corrigan, A. Z., Blount, A. L., Chen, Y., and Vale, W. W. (2001) *Endocrinology* **142,** 1065–1072
30. Vaughan, J. M., Fischer, W. H., Hoeger, C., Rivier, J., and Vale, W. (1989) *Endocrinology* **125,** 1660–1665
31. Woods, R. J., Kemp, C. F., David, J., Sumner, I. G., and Lowry, P. J. (1999) *J. Clin. Endocrinol. Metab.* **84,** 2788–2794
32. Kobayashi, T., Kageyama, Y., Sumitomo, N., Saeki, K., Shirai, T., and Ito, S. (2005) *World J. Microbiol. Biotechnol.* **21,** 961–967
33. Sauer, R. T., Milla, M. E., Waldburger, C. D., Brown, B. M., and Schildbach, J. F. (1996) *FASEB J.* **10,** 42–48
34. Horovitz, A., Serrano, L., Avron, B., Bycroft, M., and Fersht, A. R. (1990) *J. Mol. Biol.* **216,** 1031–1044
35. Perutz, M. F. (1990) *Annu. Rev. Physiol.* **52,** 1–25
36. Jahn, O., Eckart, K., Sydow, S., Hofmann, B. A., and Spiess, J. (2001) *Peptides* **22,** 47–56
37. Grace, C. R., Cervini, L., Gulyas, J., Rivier, J., and Riek, R. (2007) *Biopolymers* **87,** 196–205
38. Pallai, P. V., Mabilia, M., Goodman, M., Vale, W., and Rivier, J. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 6770–6774
39. Schurmeyer, T. H., Schulte, H. M., Avgerinos, P. C., Tomai, T. P., Loriaux, D. L., Gold, P. W., and Chrousos, G. P. (1987) *Horm. Metab. Res.* (suppl.) **16,** 24–30
40. Saphier, P. W., Faria, M., Grossman, A., Coy, D. H., Besser, G. M., Hodson, B., Parkes, M., Linton, E. A., and Lowry, P. J. (1992) *J. Endocrinol.* **133,** 487–495
41. Kemp, C. F., Woods, R. J., and Lowry, P. J. (1998) *Peptides* **19,** 1119–1128
42. Webster, E. L., Lewis, D. B., Torpy, D. J., Zachman, E. K., Rice, K. C., and Chrousos, G. P. (1996) *Endocrinology* **137,** 5747–5750
43. Ruhmann, A., Bonk, I., Lin, C. R., Rosenfeld, M. G., and Spiess, J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15264–15269
44. Rivier, J., Gulyas, J., Kirby, D., Low, W., Perrin, M. H., Kunitake, K., DiGruccio, M., Vaughan, J., Reubi, J. C., Waser, B., Koerber, S. C., Martinez, V., Wang, L., Tache, Y., and Vale, W. (2002) *J. Med. Chem.* **45,** 4737–4747
45. Rademaker, M. T., Charles, C. J., Espiner, E. A., Frampton, C. M., Lainchbury, J. G., and Richards, A. M. (2005) *Eur. Heart J.* **26,** 2055–2062
46. Rademaker, M. T., Charles, C. J., Espiner, E. A., Fisher, S., Frampton, C. M., Kirkpatrick, C. M., Lainchbury, J. G., Nicholls, M. G., Richards, A. M., and Vale, W. W. (2002) *J. Am. Coll. Cardiol.* **40,** 1495–1505
47. Chen, A., Brar, B., Choi, C. S., Rousso, D., Vaughan, J., Kuperman, Y., Kim, S. N., Donaldson, C., Smith, S. M., Jamieson, P., Li, C., Nagy, T. R., Shulman, G. I., Lee, K. F., and Vale, W. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 16580–16585
48. May, C., Rapoport, S. I., Tomai, T. P., Chrousos, G. P., and Gold, P. W. (1987) *Neurology* **37,** 535–538
49. De Souza, E. B., Whitehouse, P. J., Kuhar, M. J., Price, D. L., and Vale, W. W. (1986) *Nature* **319,** 593–595
50. De Souza, E. B. (1995) *Psychoneuroendocrinology* **20,** 789–819
51. Leake, A., Perry, E. K., Perry, R. H., Fairbairn, A. F., and Ferrier, I. N. (1990) *Biol. Psychiatry* **28,** 603–608
52. Behan, D. P., Heinrichs, S. C., Troncoso, J. C., Liu, X. J., Kawas, C. H., Ling, N., and De Souza, E. B. (1995) *Nature* **378,** 284–287