PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The version of the following full text has not yet been defined or was untraceable and may differ from the publisher's version.

For additional information about this publication click this link.  
http://hdl.handle.net/2066/72271

Please be advised that this information was generated on 2019-09-20 and may be subject to change.
RESIDUES OF CORTICOTROPIN RELEASING FACTOR-BINDING PROTEIN (CRF-BP) THAT SELECTIVELY ABROGATE BINDING TO CRF BUT NOT TO UROCORTIN 1
Mark O. Huising1,2, Joan M. Vaughan1, Shaili H. Shah1, Katherine L. Grillot1, Cynthia J. Donaldson1, Jean Rivier1, Gert Flik2, Wylie W. Vale1
1 The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute for Biological Studies, La Jolla, CA 92037
2 Department of Animal Physiology, Radboud University Nijmegen, Nijmegen, The Netherlands

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 approximately 100-fold, while only marginally affecting the affinity for Ucn 1. The selective reduction in affinity for CRF depends on glutamic acid 25 (E25) in the CRF peptide, as substitution of E25 reduces the affinity for CRF-BP by approximately two orders of magnitude, but only in the presence of both R56 and D62 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) 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 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).
(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 ten cysteine residues that form five consecutive disulfide bridges (22) as well as a single asparagine (N)-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 several-fold higher than that for either CRFR, CRF-BP is generally considered an antagonist of CRFRs by virtue 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. Since 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, R46 and R59, 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; R46 interacting with the C-terminus and R59 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, while 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, Carlsbad, CA) with a FLAG-tag (DYKDDDDK) 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 up in a larger volume. Vector DNA was isolated using a maxiprep kit (Qiagen, Valencia, CA) 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 polyethyleneimine (PEI; Sigma, St. Louis, MO) as the precipitating agent. Briefly, 14.4 μg DNA was premixed with 36 μg PEI in 1 ml of serum free media (DME) containing penicillin/streptomycin and glutamine (Invitrogen, Carlsbad, CA). 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, Carlsbad, CA). 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, St. Louis, MO) 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, St. Louis, MO) and rabbit anti-human CRF-BP antiserum (#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). While alanine mutations of aspartic acids at position 98 and 114 interfered with expression or secretion of CRF-BP (supplemental Table 1), CRF-BP<sub>D98N</sub> and CRF-BP<sub>D114N</sub> 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 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 LIRMA- CRF-BP mutants were quantified by ligand immuno-radiometric assay (LIRMA) as previously described (24). Briefly, serial dilutions of each mutant were incubated overnight in 10 mM Hepes buffer pH 7.4 using dialysis tubes with 4 kDa 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.

In vitro reporter assay- HEK293T cells were seeded in a 10 cm dish at 1.5 x 10⁶ cells per dish the day prior to transfection. The following day, cells were transiently transfected with 600 ng human CRFR1 in pcDNA3.1, 5 μg pXP2 reporter construct (luciferase driven by a cAMP responsive element) and 1 μg β-galactosidase driven by a CMV promoter (29). DNA was precipitated for 10 min by incubation with 9.9 μg PEI 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, HDB (28). Cells were lysed in 100 μl luciferase buffer (10 mM MgSO4, 25 mM glycylglycine, 4 mM EGTA) supplemented with 1% Triton X-100 (EMD Biosciences, La Jolla, CA) and 1 mM dithiothreitol (DTT). Luciferase activity was determined in 50 μl cell lysate using a Lumimark plus microplate reader (Biorad, Hercules, CA) following addition of 100 μl luciferin substrate buffer (luciferase buffer supplemented with 0.3 mM luciferin, 1 mM ATP, 1mM DTT). Luciferase activity was normalized for the β-galactosidase activity measured in 20 μl cell lysate by addition of 100 μl β-galactosidase substrate buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgCl2, 50 mM β-mercaptoethanol, 1.5 mg/ml ortho-nitrophenyl-b-D-galactopyranoside; Sigma, St. Louis, MO).

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 x 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-PJ (28) media containing 2% fetal bovine serum (FBS). On day 4 in culture, cells were washed three times with β-PJ media containing 0.1% BSA and incubated for 1 h at 37°C. The media was replaced by treatment compounds diluted in β-PJ media containing 0.1% BSA. 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-[125I]iodotyrosyl) ACTH(1-39), purchased from Amersham Biosciences (Piscataway, NJ; IM216) was used as tracer with ca. 20,000 cpm added per tube. Rat ACTH(1-39), synthesized in our laboratory, was used as standard at doses ranging from 2 - 1000 pg/tube. The EC50 for rat ACTH(1-39) was 65 - 70 pg/tube; the assay displays minimal cross-reactivity with αMSH 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 (suppl. Fig. 1). This suggests that the key determinants for ligand binding are located towards 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 (suppl. Fig. 2). We determined the ability of all mutants to bind 125I-labeled r/hCRF and 125I-labeled rUcn 1 and compared each mutant to the binding capacity of wildtype (WT) CRF-BP (Fig. 1A). Mutation of several amino acids, notably W116 and Y211, completely abolished the ability of CRF-BP to bind CRF and Ucn 1, although mutant proteins were readily detectable by western immunoblotting (suppl. Fig. 3). As alanine substitutions of W116 and Y211 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 e.g. L61A, E121A, F123A and Q188A that have lost partial affinity for both r/hCRF and rUcn 1, although mutant proteins were readily detectable by western immunoblotting (suppl. Fig. 3). As alanine substitutions of W116 and Y211 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 e.g. L61A, E121A, F123A and Q188A that have lost partial affinity for both r/hCRF and rUcn 1, although mutant proteins were readily detectable by western immunoblotting (suppl. Fig. 3). As alanine substitutions of W116 and Y211 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 e.g. L61A, E121A, F123A and Q188A that have lost partial affinity for both r/hCRF and rUcn 1, although mutant proteins were readily detectable by western immunoblotting (suppl. Fig. 3). As alanine substitutions of W116 and Y211 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 e.g. L61A, E121A, F123A and Q188A that have lost partial affinity for both r/hCRF and rUcn 1, although mutant proteins were readily detectable by western immunoblotting (suppl. Fig. 3). As alanine substitutions of W116 and Y211 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 e.g. L61A, E121A, F123A and Q188A that have lost partial affinity for both r/hCRF and rUcn 1, although mutant proteins were readily detectable by western immunoblotting (suppl. Fig. 3).
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. 1B; 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 R56 and D62 and, to a lesser extent, Y54, L58, L64 and F70. Residues that selectively or more potently interfered with high affinity binding to rUcn 1 when mutated include L61, M63, F84, E88, E91, Q188 and T189 (Fig. 1B). Generally, mutants that selectively altered r/hCRF affinity were concentrated towards 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.

CRF-BP does not require R46 and R59 to bind CRF or Ucn 1. Based on photo-crosslinking experiments, a pair of N-terminal arginines of CRF-BP, R46 and R59, were suggested to contact r/hCRF (26). As the involvement of R46 and R59 in ligand binding had not been experimentally confirmed, we verified their contribution to the affinity for r/hCRF and rUcn 1. As shown in figure 2, WT recombinant C-terminally FLAG-tagged CRF-BP bound CRF and Ucn 1 with high affinity (K_i’s for CRF and Ucn 1 are 217 and 77.2 pM, respectively), consistent with previously published values for non-tagged recombinant human CRF-BP (2,16,24). However, substitution of R46 with alanine did not affect K_i values for r/hCRF or rUcn 1 whereas CRF-BP_R56A displayed a modestly (~2-fold) reduced K_i value for r/hCRF (Fig. 2). Simultaneous alanine substitution of R46 and R59 with alanine did not affect K_i values for r/hCRF or rUcn 1 whereas CRF-BP_R56A displayed a modestly (~2-fold) reduced K_i value for r/hCRF (Fig. 2).

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 to WT CRF-BP, demonstrating that the N-linked glycosylation in transfected HEK293T cells is abrogated by the N204A mutation (Fig. 2E). 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. 2C,D).

Alanine substitution of R56 and D62 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 R56 or D62 with alanine reduced the affinity for r/hCRF by more than two orders of magnitude (Fig. 3A). Both mutations significantly affected CRF binding while affinity for rUcn 1 was only two-fold reduced (Fig. 3B). The selectivity of the R56A mutation was further illustrated by substituting the adjacent arginine at position 55 (R55) with alanine, which had no effect on CRF binding (Fig. 3A). Substitution of R56 for a K only minimally improved the affinity for r/hCRF compared to CRF-BP_R56A, while the introduction of an acidic amino acid side chain at this position failed to substantially alter the affinity for r/hCRF (Fig. 3C). Substitution of D62 for an E resulted in a 10-fold loss of the affinity for r/hCRF, compared to the 100-fold loss in affinity for CRF-BP (Fig. 3D). CRF-BP mutants with a basic amino acid side chain in place of D62 did not express in detectable levels (data not shown).

As alanine substitutions of R56 and D62 resulted in remarkably similar and approximately 100-fold reductions in the affinity for r/hCRF while 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. 3A), 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. 3B). If R56 and D62 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-BPR\textsubscript{R56A/D62A}) restores the affinity for r/hCRF merely four-fold compared to CRF-BP\textsubscript{R56A/D62A}, suggesting that the relationship between both amino acids may be more complex than a straightforward ionic interaction (Fig. 3D).

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 R56 and D62 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\textsubscript{R56A} and CRF-BP\textsubscript{D62A} displayed 100-fold reduced affinity for r/hCRF while leaving the affinity for rUcn 1 largely intact (Fig. 4A,B). CRF-BP has high affinity (K\textsubscript{i} of 163 pM) for carp urotensin-I (cU1), the bony fish ortholog of mammalian Ucn 1. This affinity was reduced by approximately 10-fold in both CRF-BP\textsubscript{R56A} and CRF-BP\textsubscript{D62A} (Fig. 4C). Affinity for mUcn 2, in contrast, was increased from 10.7 nM to 2.19 nM for CRF-BP\textsubscript{R56A}, while affinity of CRF-BP\textsubscript{D62A} for mUcn 2 was unaffected (Fig. 4D). We inspected an amino acid sequence alignment of CRF family peptides (Fig. 4E) to identify differences between members that might explain the observed ligand-selective changes in affinity for CRF-BP\textsubscript{R56A} and CRF-BP\textsubscript{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 cU1 have an acidic residue (E) at position 25 and bind with lower affinity upon removal of either R56 or D62 in CRF-BP, while the affinity for rUcn 1, which has a neutral glutamine (Q) at position 25, is minimally affected by the R56A and D62A mutations. Conversely, mUcn 2 has a basic (K) residue at the equivalent amino acid position and responds to alanine substitution of R56 in CRF-BP with an increase in affinity.

To validate the involvement of E25 in r/hCRF in high affinity binding to CRF-BP, we replaced E25 with alanine in r/hCRF (r/hCRF\textsubscript{E25A}). We compared the effects of this mutation to alanine substitution of E20 (r/hCRF\textsubscript{E20A}), which is conserved in CRF, Ucn 1 and Ucn 2, as well as R23 (r/hCRF\textsubscript{R23A}). The amino acids at positions 25 and 34 in CRF have previously been shown to affect affinity for CRF-BP based on experiments with oCRF (24). The affinity of r/hCRF\textsubscript{E25A} for CRF-BP was reduced by approximately two orders of magnitude, while the K\textsubscript{i} of r/hCRF\textsubscript{E20A} was only marginally (less than 2-fold) reduced compared to r/hCRF (Fig. 5A). Alanine substitution of R23 in CRF reduced the affinity of r/hCRF for CRF-BP by approximately seven-fold. When we determined the affinity of these r/hCRF analogs for R56A and D62A mutants of CRF-BP, we found that r/hCRF\textsubscript{E25A} no longer differed from r/hCRF in its affinity for CRF-BP\textsubscript{R56A} (Fig. 5B) and had only two-fold reduced affinity for CRF-BP\textsubscript{D62A} compared to r/hCRF (Fig. 5C). These results indicate that alanine replacement of E25 in r/hCRF had no further effect on the approximately 100-fold reduction in affinity for CRF that results from the R56A or D62A mutations. In contrast, r/hCRF\textsubscript{R23A} did display a further reduction compared to r/hCRF in affinity for CRF-BP, in addition to the 100-fold reduced affinity for CRF-BP\textsubscript{R56A} and CRF-BP\textsubscript{D62A} (Fig. 5). This demonstrates that the contribution of E25 in r/hCRF to the interaction with CRF-BP depends on the presence of both R56 and D62, while R23 of r/hCRF affects affinity independently of these CRF-BP residues.

CRF-BP\textsubscript{R56A} and CRF-BP\textsubscript{D62A} inhibit Ucn 1-induced activation of CRFR1 but have selectively lost the ability to inhibit CRF. We compared the ability of CRF-BP\textsubscript{R56A} and CRF-BP\textsubscript{D62A} to inhibit r/hCRF and rUcn 1-induced activation of CRFR1. Wildtype CRF-BP inhibited
the activation of CRFR1 induced by 50 pM of r/hCRF or rUcn 1 with an IC50 of 2.65 and 8.01 nM, respectively, as measured in a cAMP-luciferase reporter assay (Fig. 6A,B). In agreement with the profound and selective loss of binding affinity for r/hCRF, the CRF-BP R56A and CRF-BPD62A mutants displayed a severely and selectively reduced potency to inhibit the r/hCRF-induced activation of CRFR1 (Fig. 6A). In contrast, inhibition of rUcn 1-induced CRFR1 activation was unaffected by R56A or D62A mutations (Fig. 6A). 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. Wildtype 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-BPD62A do not inhibit r/hCRF-induced ACTH release (Fig. 6C), while maintaining a potency similar to WT CRF-BP in inhibiting the ACTH release induced by rUcn 1 (Fig. 6D).

In a reverse approach, we tested the ability of WT CRF-BP to block the activation of CRFR1 by r/hCRF and r/hCRFE25A. Wildtype CRF-BP robustly inhibited r/hCRF, as the EC50 for r/hCRF was shifted by approximately 30-fold in the presence of CRF-BP (Fig. 7A). In contrast, CRF-BP was incapable of inhibiting the activation of CRFR1 by r/hCRFE25A in line with the profoundly reduced affinity of r/hCRFE25A for CRF-BP. The potency of r/hCRFE25A 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/hCRFE25A, we found that r/hCRFE25A was slightly more potent in inducing ACTH release compared to r/hCRF, but that this induction was no longer antagonized by CRF-BP (Fig. 7B).

**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, R46 and R59, were previously suggested to be part of the binding site on CRF-BP for CRF based on photocrosslinking 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 R46 and R59 of CRF-BP in a linear arrangement led to the postulation 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 R46 and R59 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 R46 and/or R59 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 two 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 of 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 E25 in CRF results in a comparable decrease in affinity that requires the presence of both R56 and D62 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 R56 and D62 in CRF-BP and E25 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 two 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 R56 and D62 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 four-amino acid ARAE motif at positions 22-25 of the ligand (24,36). Here we confirm the role of R23 and E25 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 E25 and R23 are the only polar residues amidst an otherwise hydrophobic face of the α-helical CRF peptide (37,38). The amino acid side chains of R23 and E25 occupy the same face of the CRF peptide but point in opposite directions (Fig. 8). Of note, the substitution of A22 in r/hCRF with glutamic acid reduces the affinity for CRF-BP by approximately 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 inter-molecular interactions that require the presence of R56 and D62 in CRF-BP and E25 in r/hCRF. It is conceivable that within the ARAE motif of r/hCRF, R23 and E25 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 the human circulation found that oCRF was consistently longer acting and was cleared at an approximately three-fold lower rate compared to 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 Ucn1 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’s disease (AD). It is expressed in brain regions that are prone to degeneration in AD and lower CRF levels in the CSF of patients correlate with greater cognitive impairment (48-51). As CRF-BP is highly expressed in areas affected by AD, 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 AD (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 R56 and D62 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.

REFERENCES


**FOOTNOTES**

We thank Peter Gray, Louise Bilezikjian, Nick Justice, Talitha van der Meulen and Kathy Falkenhagen for constructive comments and suggestions to the manuscript. Thao Dang is gratefully acknowledged for excellent technical assistance. Ron Kaiser is acknowledged for his assistance with peptide synthesis. We thank Christy Grace for supplying a figure of the NMR structure of astressin and Harvey Motulsky for his kind advice on consolidating large data sets. This work was supported in part by the Adler foundation and the Clayton Medical Research Foundation.

**FIGURE LEGENDS**

Fig. 1. Summary of the effect of alanine substitution of selected residues of human CRF-BP on maximum r/hCRF or rUcn 1 binding (A). 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 approximately 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 tracer. 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 potency were determined separately by competitive binding assays (B). Only mutants that affect affinity for either peptide by two-fold or more are shown. Note that CRF-BP<sub>W116A</sub> and CRF-BP<sub>T211A</sub> could not be determined as they do not bind detectable amounts of r/hCRF or rUcn 1 tracer. See supplemental table 1 for a comprehensive list of the relative potency for all mutants.

Fig. 2. R46, R59 and N204 in CRF-BP are dispensable for high affinity binding to r/hCRF or rUcn 1. Percent displacement (% B/B<sub>0</sub>) of <sup>125</sup>I-[D-Tyr<sup>r</sup>r/hCRF] by r/hCRF or rUcn 1, comparing wildtype (WT) CRF-BP with CRF-BP<sub>R46A</sub>, CRF-BP<sub>R59A</sub> and CRF-BP<sub>R46A/R59A</sub>. WT CRF-BP (open symbols, dashed lines) binds rUcn 1 with slightly higher affinity than r/hCRF. Alanine substitution of R46 does not affect affinity for either peptide, while the affinity for r/hCRF is less than two-fold reduced in CRF-BP<sub>R59A</sub> (A,B). Simultaneous mutation of R46 and R59 has no effect on the affinity for r/hCRF and slightly improves binding to rUcn 1 (B). Note that R46 and R59 are referred to as R23 and R36 in (26). The affinity of CRF-BP<sub>N204A</sub> (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<sub>N204A</sub> 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<sub>i</sub> values and 95% confidence intervals are derived from two or more separate experiments.

Fig. 3. Amino acid substitutions at CRF-BP position 56 and 62 differentially affect affinities for r/hCRF and rUcn 1. Alanine substitution of R56 or D62 results in profound and similar reductions in the affinity for r/hCRF (A). Simultaneous substitutions of R56 and D62 does not further reduce the affinity for r/hCRF. The affinity for r/hCRF is unaffected by alanine substitution of R55. In contrast to the profound changes in affinity for r/hCRF, CRF-BP<sub>R56A</sub> and CRF-BP<sub>D62A</sub> have only two-fold reduced affinity for rUcn 1 (B). Simultaneous substitution of R56 and D62 for alanines does not affect rUcn 1 affinity. Substitution of R56 for a K only minimally restores affinity for r/hCRF, while the introduction of acidic amino acids at this position did not further reduce the affinity for r/hCRF compared to CRF-BP<sub>R56A</sub> (C). Substitution of D62 for an E restores the affinity for r/hCRF by approximately 10-fold when compared to CRF-BP<sub>D62A</sub> (D). Switching the residues at positions 56 and 62 (CRF-BP<sub>R56D/D62R</sub>) fails to restore the affinity for r/hCRF to levels comparable to the affinity of WT CRF-BP. In all experiments <sup>125</sup>I-[D-Tyr<sup>r</sup>rUcn 1] was used as tracer with the exception of the competitive binding assays with CRF-BP<sub>R55A</sub>, where <sup>125</sup>I-[D-Tyr<sup>r</sup>r/hCRF] was used. K<sub>i</sub> values and 95% confidence intervals are derived from two or more separate experiments.

Fig. 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<sub>R56A</sub> and CRF-BP<sub>D62A</sub> have 100-fold and 10-fold reductions in affinity for r/hCRF (A) and carp urotensin 1 (C), respectively. Affinity for rUcn 1 is reduced by less than two-fold (B). In contrast, CRF-BP<sub>R56A</sub> has increased affinity for mUcn 2, while alanine substitution of D62 has no effect on mUcn 2 affinity (D). Note that the reduced affinity of r/hCRF and eUI correlates with a glutamic acid at position 25, while mUcn 2 has a basic lysine at the equivalent position and displays increased affinity for CRF-BP<sub>R56A</sub>. 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 <sup>125</sup>I-[D-Tyr<sup>r</sup>rUcn 1] was used as tracer. K<sub>i</sub> values and 95% confidence intervals are derived from two or more separate experiments.

Fig. 5. Substitution of E25 in r/hCRF affects its affinity for CRF-BP only in the presence of both R56 and D62 in CRF-BP. The affinity of r/hCRF is reduced by 80-fold following alanine substitution of E25, while alanine substitution of E20 has no effect on binding to CRF-BP (A). Alanine substitution of
R23 reduces the affinity of r/hCRF by approximately one 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 E25 in r/hCRF requires R56 and D62 to interact with CRF-BP. In contrast, replacing R23 by alanine continues to reduce affinity of r/hCRF for CRF-BP_{R56A} and CRF-BP_{D62A}, demonstrating that R23 interacts with CRF-BP independently of R56 and D62 in the binding protein. The position of the alanine substitutions within r/hCRF is illustrated in panel D. In all experiments ^{125}I-[D-Tyr^9]rUcn 1 was used as tracer. K_i values and 95% confidence intervals are derived from two or more separate experiments.

Fig. 6. CRF-BP_{R56A} and CRF-BP_{D62A} have selectively lost the ability to inhibit r/hCRF-induced activation of CRFR1. Wildtype 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). Wildtype 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).

Fig. 7. Alanine substitution of E25 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).

Fig. 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 two-fold or more are orange, R56 and D62 are red. The residues that are indicated by a bold circle indicate positions where alanine substitution reduces rUcn 1 affinity by at least two-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 E25 of CRF with R56 and D62 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 (2RMI), which is identical to r/hCRF in the central region of the peptide that is depicted (E17 to Q29) with the exception of a methionine to norleucine substitution at position 21.

Suppl. Fig. 1. Removal of the cysteines forming the fourth (C237/C264) or fifth (C277/C318) disulfide bridge of CRF-BP has no effect on the affinities for r/hCRF or rUcn 1. Percent displacement (% B/B_0) of ^{125}I-[D-Tyr^9]rCRF by r/hCRF (A) or rUcn 1 (B) demonstrates that the affinity of CRF-BP_{C237A/C264A} and CRF-BP_{C277A/C318A} for r/hCRF or rUcn 1 is indistinguishable from that of WT CRF-BP (open symbols, dashed line). K_i values and 95% confidence intervals are derived from two or more separate experiments.

Suppl. Fig. 2. Multiple amino acid alignment of CRF-BP sequences from selected vertebrate and invertebrate species. Amino acids targeted as part of our alanine scan are shaded. Asterisks indicate
amino acid identity between all sequences in the alignment, while colons and dots indicate decreasing degrees of amino acid similarity. Accession numbers are as follows: human (Homo sapiens), P24387; mouse (Mus musculus), Q60571; chicken (Gallus gallus), XM_424801; Xenopus (Xenopus laevis), Q91653; carp (Cyprinus carpio), AJ490880; honey bee (Apis mellifera), AJ780964.

Suppl. Fig. 3. Western immunoblot of wild type (WT) CRF-BP and mutants that display partial (L61A, E121A, F123A, Q188A) or complete (W116A, Y211A) loss of affinity for both r/hCRF and rUcn 1. All mutant proteins express in similar levels compared to WT CRF-BP and have indistinguishable molecular weights as determined by SDS-page and detected by western immunoblot.
% of maximal WT binding

Huising et al., Figure 1
Huising et al., Figure 4

### Table

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>R56A</th>
<th>D62A</th>
</tr>
</thead>
<tbody>
<tr>
<td>mUcn 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kᵢ (nM)</td>
<td>0.157</td>
<td>23.8</td>
<td>20.5</td>
</tr>
<tr>
<td>95% conf. interv.</td>
<td>(0.144 to 0.171)</td>
<td>(21.3 to 26.5)</td>
<td>(17.1 to 24.6)</td>
</tr>
</tbody>
</table>

### Table

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>R56A</th>
<th>D62A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ucn 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kᵢ (nM)</td>
<td>0.138</td>
<td>0.236</td>
<td>0.271</td>
</tr>
<tr>
<td>95% conf. interv.</td>
<td>(0.125 to 0.151)</td>
<td>(0.212 to 0.263)</td>
<td>(0.230 to 0.309)</td>
</tr>
</tbody>
</table>

### Table

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>R56A</th>
<th>D62A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ucn 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kᵢ (nM)</td>
<td>0.163</td>
<td>2.04</td>
<td>1.48</td>
</tr>
<tr>
<td>95% conf. interv.</td>
<td>(0.149 to 0.178)</td>
<td>(1.86 to 2.24)</td>
<td>(1.29 to 1.70)</td>
</tr>
</tbody>
</table>

### Table

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>R56A</th>
<th>D62A</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kᵢ (nM)</td>
<td>0.157</td>
<td>23.8</td>
<td>20.5</td>
</tr>
<tr>
<td>95% conf. interv.</td>
<td>(0.144 to 0.171)</td>
<td>(21.3 to 26.5)</td>
<td>(17.1 to 24.6)</td>
</tr>
</tbody>
</table>

### Table

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>R56A</th>
<th>D62A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ul</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kᵢ (nM)</td>
<td>0.138</td>
<td>0.236</td>
<td>0.271</td>
</tr>
<tr>
<td>95% conf. interv.</td>
<td>(0.125 to 0.151)</td>
<td>(0.212 to 0.263)</td>
<td>(0.230 to 0.309)</td>
</tr>
</tbody>
</table>

### Table

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>R56A</th>
<th>D62A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ul</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kᵢ (nM)</td>
<td>0.163</td>
<td>2.04</td>
<td>1.48</td>
</tr>
<tr>
<td>95% conf. interv.</td>
<td>(0.149 to 0.178)</td>
<td>(1.86 to 2.24)</td>
<td>(1.29 to 1.70)</td>
</tr>
</tbody>
</table>

### E

- rat/human CRF
- rat Ucn 1
- carp Ul
- mouse Ucn 2

**Sequence:**

- rat/human CRF: SEEPISLIDL TFHLLREVEL MARAEQLAQF AHSNRKLMEI I
- rat Ucn 1: -DDPLSIDL TFHLLRTVE LARTQSQRE R AEQNRIFDS V
- carp Ul: NDDPPISIDL TFHLLRMIE MARNENQREQ AGLNRKYLD E V
- mouse Ucn 2: ---VILSDLV PIGLRLRL QARYKAARNQ AATNQILAH V
Huisings et al., Figure 5

**A** WT CRF-BP

<table>
<thead>
<tr>
<th></th>
<th>rhCRF</th>
<th>rhCRF E20A</th>
<th>rhCRF R23A</th>
<th>rhCRF E25A</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$ (nM)</td>
<td>0.252</td>
<td>0.342</td>
<td>1.74</td>
<td>20.0</td>
</tr>
<tr>
<td>95% conf. interv.</td>
<td>(0.232 to 0.274)</td>
<td>(0.318 to 0.369)</td>
<td>(1.59 to 1.91)</td>
<td>(17.5 to 22.9)</td>
</tr>
</tbody>
</table>

**B** R55A

<table>
<thead>
<tr>
<th></th>
<th>rhCRF</th>
<th>rhCRF E20A</th>
<th>rhCRF R23A</th>
<th>rhCRF E25A</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$ (nM)</td>
<td>26.5</td>
<td>46.2</td>
<td>433</td>
<td>35.4</td>
</tr>
<tr>
<td>95% conf. interv.</td>
<td>(22.0 to 31.8)</td>
<td>(38.3 to 55.7)</td>
<td>(323 to 582)</td>
<td>(27.3 to 48.0)</td>
</tr>
</tbody>
</table>

**C** D62A

<table>
<thead>
<tr>
<th></th>
<th>rhCRF</th>
<th>rhCRF E20A</th>
<th>rhCRF R23A</th>
<th>rhCRF E25A</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$ (nM)</td>
<td>22.8</td>
<td>50.1</td>
<td>259</td>
<td>45.6</td>
</tr>
<tr>
<td>95% conf. interv.</td>
<td>(17.7 to 29.4)</td>
<td>(41.5 to 60.4)</td>
<td>(180 to 372)</td>
<td>(34.9 to 59.6)</td>
</tr>
</tbody>
</table>

**D**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>11</th>
<th>21</th>
<th>31</th>
<th>41</th>
</tr>
</thead>
<tbody>
<tr>
<td>r/hCRF</td>
<td>SEEPPISLDL TFHLLREVLE MARAEQLAQQ AHSNKRKMEI</td>
<td>SEEPPISLDL TFHLLREVLE MARAEQLAQQ AHSNKRKMEI</td>
<td>SEEPPISLDL TFHLLREVLE MAAAEQLAQQ AHSNKRKMEI</td>
<td>SEEPPISLDL TFHLLREVLE MARAAQLAQQ AHSNKRKMEI</td>
<td>SEEPPISLDL TFHLLREVLE MARAAQLAQQ AHSNKRKMEI</td>
</tr>
<tr>
<td>r/hCRF E20A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r/hCRF R23A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r/hCRF E25A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Huising et al., Figure 6

**A**  
*Graph of r/hCRF*  
- **WT**: IC50 = 2.65 (2.00 to 3.52) nM  
- **R56A**: IC50 = 460 (307 to 689) nM  
- **D62A**: IC50 >100 (n.a.) nM

**B**  
*Graph of rUcn 1*  
- **WT**: IC50 = 8.01 (5.33 to 12.0) nM  
- **R56A**: IC50 = 12.2 (9.71 to 15.4) nM  
- **D62A**: IC50 = 15.9 (12.3 to 20.6) nM

**C**  
*Graph of r/hCRF*  
- **CRF**: EC50 = 0.624 (0.366 to 1.07) nM  
- **CRF + 100 nM WT**: EC50 = 4.49 (2.68 to 7.51) nM  
- **CRF + 100 nM R56A**: EC50 = 0.680 (0.428 to 1.08) nM  
- **CRF + 100 nM D62A**: EC50 = 0.129 (0.0863 to 0.250) nM

**D**  
*Graph of rUcn 1*  
- **Ucn 1**: EC50 = 0.0833 (0.0525 to 0.132) nM  
- **Ucn 1 + 100 nM WT**: EC50 = 1.31 (0.770 to 2.22) nM  
- **Ucn 1 + 100 nM R56A**: EC50 = 1.61 (1.04 to 2.48) nM  
- **Ucn 1 + 100 nM D62A**: EC50 = 0.923 (0.539 to 1.58) nM
Huising et al., Figure 7

**A** r/hCRF vs r/hCRF E25A

- Log CRF
- EC50 (nM): 0.182 (0.103 to 0.320)
- 95% conf. interv.

- CRF
- CRF + 50 nM CRF-BP
- CRF E25A
- CRF E25A + 50 nM CRF-BP

**B** r/hCRF vs r/hCRF E25A

- Log CRF
- EC50 (nM): 0.624 (0.366 to 1.07)
- 95% conf. interv.

- CRF
- CRF + 100 nM CRF-BP
- CRF E25A
- CRF E25A + 100 nM CRF-BP
Huisings et al., Figure 8