NMR Structure of the First Extracellular Domain of Corticotropin-releasing Factor Receptor 1 (ECD1-CRF-R1) Complexed with a High Affinity Agonist*

Received for publication, March 19, 2010, and in revised form, August 6, 2010 Published, JBC Papers in Press, September 15, 2010, DOI 10.1074/jbc.M110.121897

Christy Rani R. Grace‡, Marilyn H. Perrin‡, Jozsef Gulyas§, Jean E. Rivier‡, Wylie W. Vale‡, and Roland Riek‡,

From the ‡Structural Biology Laboratory and §Clayton Foundation Laboratories for Peptide Biology, Salk Institute for Biological Studies, La Jolla, California 92037

The corticotropin-releasing factor (CRF) peptide hormone family members coordinate endocrine, behavioral, autonomic, and metabolic responses to stress and play important roles within the cardiovascular, gastrointestinal, and central nervous systems, among others. The actions of the peptides are mediated by activation of two G-protein-coupled receptors of the B1 family, CRF receptors 1 and 2 (CRF-R1 and CRF-R2β). The recently reported three-dimensional structures of the first extracellular domain (ECD1) of both CRF-R1 and CRF-R2β (Pioszak, A. A., Parker, N. R., Suino-Powell, K., and Xu, H. E. (2008) J. Biol. Chem. 283, 32900–32912; Grace, C. R., Perrin, M. H., Gulyas, J., Digraccio, 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) complexed with peptide antagonists provided a starting point in understanding the binding between CRF ligands and receptors at a molecular level. We now report the three-dimensional NMR structure of the ECD1 of human CRF-R1 complexed with a high affinity agonist, α-helical cyclic CRF. In the structure of the complex, the C-terminal residues (23–41) of α-helical cyclic CRF bind to the ECD1 of CRF-R1 in a helical conformation mainly along the hydrophobic face of the peptide in a manner similar to that of the antagonists in their corresponding ECD1 complex structures. Unique to this study is the observation that complex formation between an agonist and the ECD1-CRF-R1 promotes the helical conformation of the N terminus of the former, important for receptor activation (Gulyas, J., Rivier, C., Perrin, M., Koerber, S. C., Sutton, S., Corrigan, A., Lahrichi, S. L., Craig, A. G., Vale, W., and Rivier, J. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 10575–10579).

Corticotropin-releasing factor (5), a 41-residue peptide hormone, and other members of the CRF family (the urocortins) are important regulators of the stress response. They are involved in the control of appetite as well as in the functioning of the cardiovascular, reproductive, gastrointestinal, immune, and central nervous systems (6–8). Their actions are mediated by binding to their G-protein-coupled receptors that belong to the peptide hormone B1 family (family B1 GPCRs). The members of this family include receptors for growth hormone-releasing hormone, secretin, calcitonin, vasoactive intestinal peptide, glucagon, glucagon-like peptide-1 (GLP-1), gastric inhibitory peptide, pituitary adenylate cyclase-activating peptide, and parathyroid hormone (PTH). For the CRF family, two receptors, CRF-R1 and CRF-R2, have been cloned and characterized (9, 10). Structure-activity studies of mutant and chimeric CRF receptors identified residues that affect the binding of the peptide ligands and are located mainly in the extracellular domains of the receptors (11–15). On the one hand, structure-activity studies of the CRF family ligands showed that the first six residues of the N-terminal portion of the peptide are not necessary for receptor signaling as long as α-helicity is maintained (3, 4, 16–18). On the other hand, the C-terminal (~15) residues contribute significantly to receptor binding affinities (16, 19). Similar data, both on signaling and binding properties, have been reported for the other members of the B1 GPCR family and their corresponding ligands (20–24).

Recently, three-dimensional structures of the ECD1s of several B1 family receptors in the presence and absence of their respective ligands have been solved. The structures reported include the NMR structure of the mouse CRF-R2β free and complexed with the CRF antagonist astressin (2), the NMR structure of the human splice variant PAC1-R, complexed with the N-terminally truncated antagonist pituitary adenylate cyclase-activating peptide (6–38) (25), the crystal structure of human GIP-R complexed with its agonist incretin GIP(1–42) (26), the crystal structure of human GLP-1R complexed with the antagonist exendin-4(9–39) (27), the crystal structure of human PTH-1R fused to maltose-binding protein (MBP) complexed with the peptide fragment PTH(15–34) (28), and the crystal structure of the human CRF-R1 fused to MBP...
complexed with CRF antagonists (1) (Fig. 1). These structures show that the short consensus repeat (SCR) is the common polypeptide fold for the ECD1 of the receptors (29), and the interaction of the C-terminal part of their cognate ligands is along the hydrophobic face of their helices.

Here, we report the three-dimensional NMR structure of the human ECD1-CRF-R1 (14) complexed with the peptide agonist \( \alpha \)-helical cyclic CRF (ohcCRF). The motivation for these studies is the identification of the binding surface involved in the recognition of a high affinity agonist, the subsequent comparison with that previously reported for the antagonists and the determination of reciprocal conformational changes in both the receptor and the agonist.

**MATERIALS AND METHODS**

**Protein Expression and Purification**—The purification and labeling of the ECD1-CRF-R1 expressed in *Escherichia coli* were carried out as described previously (15, 29).

**Synthesis of ohcCRF, with C-13 and N-15 Isotopically Labeled Amino Acids**—The synthesis was performed on a methyl-benzhydrylamine resin using the Fmoc (N-(9-fluorenyl)methoxy-carbonyl) strategy. Purification and characterization used established procedures (see Refs. 30, 31). Full details are given in the Supplemental Information section.

**Radioreceptor Assays**—Radioreceptor assays for full-length receptors expressed in mammalian cells were carried out as described previously (14) and for soluble receptors as described previously (32).

**NMR Experiments and Analysis**—All NMR spectra were recorded at 35 °C using a Bruker 700-MHz spectrometer equipped with four radiofrequency channels and a triple resonance cryo-probe with shielded \( z \)-gradient coil. The NMR samples contained either 0.3 mM \(^{13}\text{C},^{15}\text{N}\)-labeled ECD1-CRF-R1 and an equivalent concentration of unlabeled ohcCRF or 0.3 mM \(^{13}\text{C},^{15}\text{N}\)-labeled ohcCRF (labeled uniformly by \(^{15}\text{N}\) and \(^{13}\text{C}\) except for residues Thr\(^{11}\), Glu\(^{30}\), and Lys\(^{33}\)) and an equimolar concentration of unlabeled ECD1-CRF-R1 in 10 mM BisTris(HCl), 95% \( \text{H}_2\text{O} \), 5% \( \text{D}_2\text{O} \), pH 6.5. The sample for the two-dimensional \(^1\text{H},^1\text{H}\) NOESY was prepared with an equimolar concentration of 0.4 mM of unlabeled ECD1-CRF-R1 and ohcCRF, respectively, in 10 mM BisTris(HCl), pH 6.5, in 100% \( \text{D}_2\text{O} \).

Sequential assignment was performed with the standard protocol for \(^{13}\text{C},^{15}\text{N}\)-labeled samples (33). \(^{1}\text{H},^{13}\text{C}\), and \(^{15}\text{N}\) backbone resonances were assigned using triple resonance HNCA (34), HNCA(CB) (35), and three-dimensional \(^{13}\text{N}\)-resolved \(^1\text{H},^{13}\text{C}\) NOESY experiments (36). The side-chain \(^{1}\text{H}\) resonances were assigned using HCCH correlation spectroscopy (37) and \(^{13}\text{C}\)-resolved \(^1\text{H},^{13}\text{C}\) NOESY (38) experiments. Aromatic side-chain assignments were obtained with two-dimensional \(^{13}\text{C},^{1}\text{H}\) HSQC, two-dimensional \(^{1}\text{H},^{13}\text{C}\) aromatic-resolved \(^1\text{H},^{13}\text{C}\) NOESY (38) experiments. Distance constraints for the structure calculation were derived from three-dimensional \(^{13}\text{C},^{15}\text{N}\)-resolved \(^1\text{H},^{13}\text{C}\) NOESY and two-dimensional \(^1\text{H},^{13}\text{C}\) standard NOESY (39) spectra recorded with mixing times of either 100 or 120 ms. For all of the spectra quadrature detection in the indirect dimensions was achieved using States-TIPPI (40). The water signal was suppressed using spin-lock pulses (41) or the WATERGATE sequence (42). All of the spectra were processed with the program PROSA (43) and were analyzed with the program CARA (44).

**Determination of the Structure of ECD1-CRF-R1 Complexed with ohcCRF**—Meaningful distance restraints (~1795) and angle restraints (371) were collected for the calculation of
Structure of CRF-R1 N-terminal Domain Complexed with Agonist

### Table 1

| Parameters | Complex |
|------------|---------|
| Constraints | No. of upper distance limits 1795 |
| Intramolecular | ECD1 1330 |
| ohcCRF | 324 |
| Intermolecular | 141 |
| No. of dihedral angle constraints | 382 |
| No. of hydrogen bonds | 20 |
| Residual target function | 1.7 ± 0.4 Å² |
| Distance violations >0.2 Å | Minimum violation 3.6 ± 0.2 Å |
| Maximum violation | 9.4 ± 0.8 Å |
| Angle violation | Minimum | 3.5 ± 0.2° |
| Maximum | 7.7 ± 0.3° |
| Energies | Total | −2090 ± 109 kcal/mol |
| van der Waals | −310 ± 41 kcal/mol |
| Electrostatic | −1781 ± 99 kcal/mol |
| Atomic pairwise r.m.s.d. | Backbone atoms | 1.0 ± 0.2 Å |
| Heavy atoms | 1.5 ± 0.2 Å |
| Structural analysis | Residues in allowed region | 93.9% |
| Residues in disallowed region | 6.1% |

* Backbone and heavy atom root mean square deviation (r.m.s.d.) is obtained by superposing residues 42–105 of ECD1-CRF-R1 and residues 27–38 of ohcCRF in the complex.

### Table 2

| Ligand/receptor | CHO-CRF-R1 | ECD1-CRF-R1 | CHO-CRF-R2β | ECD1-CRF-R2β |
|----------------|------------|-------------|-------------|-------------|
| r/CRF | 11 | 53.6 | 28 | 97 |
| (8.4–15) | (25.7–112) | (16–62) | (22–430) |
| r/Ucn1 | 1.3 | 14.1 | 1.3 | 6.4 |
| (0.7–2.6) | (11.6–17.2) | (1.0–1.6) | (4.7–8.7) |
| ohcCRF | 1.1 | 30 | 0.8 | 10 |
| (0.8–1.4) | (23–39) | (0.6–1.4) | (4.4–25) |
| Astressin | 2 | 18 | 0.9 | 10.7 |
| (1.8–2.3) | (10–34) | (0.4–1.9) | (5.4–21.1) |

Values were derived from competitive displacement of bound [125I-Tyr]astressin. Primary structures and residue numbering are as follows: r/CRF, SEEPF- ISLSDLI407TLHV2RELEMAFRE2QALQOQ3AHSR4KIME5NH2; r/Ucn1, DDPP5ISLSDLI407TLHV2RELEMAFRE2QALQOQ3AHSR4KIME5NH2; cyclo(30–33)-r/CRF, CROSSR3QALQOQ3AHSR4KIME5NH2; and, cyclo(30–33)-r/Ucn1, CROSSR3QALQOQ3AHSR4KIME5NH2. The sequence of ohcCRF is cyclo(30–33)-Ac-PP4ISLSDLI407TLHV2RELEMAFRE2QALQOQ3AHSR4KIME5NH2. The lactam bridge connecting the side chains of residues Glu30 and Lys33 of ohcCRF stabilizes the α-helical domain first established for the antagonist astrassin (3).

### Results

**Selection of a Soluble High Affinity Agonist**—A major challenge in the three-dimensional structure determination of ECD1-CRF-R1 complexed with an agonist was to obtain a high affinity agonist that was soluble above pH 5 at concentrations required for the NMR experiments. Comparison of several CRF analogs showed that the novel CRF agonist, ohcCRF, satisfied the requirements, i.e. it bound with low nanomolar affinity to CRF-R1 and was soluble at physiological pH. This peptide is in the N-terminal residues 1–24 and the C-terminal residues 110–127 of the construct. In addition, several residues that are part of loop 2 involved in ligand binding (see below) (i.e. Arg66, Cys68, Phe71, Phe72, Gly74, Val75, Tyr77, Asn78, and Ala95) were absent in the 15N,H TROSY spectrum (Fig. 2A). Changes in pH or temperature failed to improve the quality of the spectrum. The absence of these peaks in the spectrum may be due to slow conformational exchange dynamics involving the backbone, which results in line broadening so severe that peaks cannot be detected. A similar observation was documented for the free ECD1-CRF-R2β, but it was limited to only a few residues located in loop 2 (i.e. Tyr87 (corresponding to Phe71 in CRF-R1), Phe88 (Phe72), Asn99 (Tyr73), Gly90 (Gly74), Ile91 (Val75), Lys92 (Arg76), Arg97 (Asn81)) (2). For the ECD1-CRF-R2β, this slow conformational exchange was estimated to be on the time scale of 10−2 s, and a similar rate was also estimated for the ECD1-CRF-R1. However, because the number of cross-peaks absent in the 15N,H TROSY spectrum is much larger for ECD1-CRF-R1 than for ECD1-CRF-R2β, the conformational exchange dynamics of ECD1-CRF-R1 must be of greater amplitude and/or must involve a larger segment of the ECD1-CRF-R1 complex compared with ECD1-CRF-R2β.

### Mapping the Agonist-binding Site on ECD1-CRF-R1 by Agonist-induced Chemical Shift Changes

Insight into the agonist-binding site on ECD1-CRF-R1 can be obtained from an analysis of the chemical shifts in the NMR spectra upon addition of ohcCRF (34). Fig. 2A shows the 15N,H TROSY spectrum of the ECD1-CRF-R1 in the absence and presence of equimolar ohcCRF. Following agonist binding, large chemical shift changes of resonances or appearance of cross-peaks was observed at four different regions of the ECD1 as follows: (i) Leu50 and Ile51, Val55; (ii) Arg56, Cys68, Phe71–Asn78; (iii) Asn82, Gly83, Arg85, Ala95–Ser100; and (iv) Gln103–Glu108 (Fig. 2B). Most of these residues are of hydrophobic nature and con-
served between the two CRF receptors (Ile$^{51}$, Arg$^{66}$, Cys$^{68}$, Phe$^{72}$, Gly$^{74}$, Val$^{75}$, Tyr$^{77}$, Asn$^{78}$, Asn$^{98}$, Tyr$^{99}$) (Fig. 2C). This structural study, together with similar chemical shift perturbation data observed for the ECD1-CRF-R2β-astressin complex (2), suggests that both the agonist and antagonist interact with the same residues in the ECD1s.
Three-dimensional Structure of the ECD1-CRF-R1 Complexed with hcCRF—Almost complete sequential assignment of the various resonances of the ECD1-CRF-R1-hcCRF complex was obtained using standard procedures, and the three-dimensional structure was determined (see under "Materials and Methods"). The good quality of the three-dimensional structure is represented by the small root mean square deviation of 0.88 Å for residues 42–105 of the ECD1 and for residues 27–38 of hcCRF (Fig. 3, A and B), as well as by the small value of residual constraint violations in the 20 refined conformers, and by the small deviations from ideal geometry (Table 1). In addition, the input data represent a self-consistent set, and the restraints are well satisfied in the calculated conformers, and similar energy values were obtained for all of the 20 conformers.

As documented already in the structures of the ECD1 of CRF-R2 (2, 29) and of the ECD1s of the other members of the B1 receptor family (25–28), the overall fold of the ECD1 of CRF-R1 is the short consensus repeat (SCR) motif with the three disulfide bonds between cysteine residues, Cys30–Cys54, Cys44–Cys77, and Cys68–Cys102. The SCR includes a short N-terminal α-helix (Asp27–Glu31) and two anti-parallel β-sheet regions around residues Ser4–Val48 (β1 strand), Cys34–Trp55 (β2 strand), Leu63–Arg66 (β3 strand), and Gly83–Glu86 (β4 strand). The chemical shift values for the Cα, Cβ, and Hα of the SCR motif, the aliphatic side chain of the conserved Arg85 is sandwiched between the highly conserved Trp55 and Trp93 residues and is in close proximity to the side chain of the conserved Asp49 and thus may possibly be involved in a salt bridge interaction (Fig. 3B). Furthermore, the indole nitrogen of Trp55 forms a hydrogen bond with the carboxyl group of Asp49 stabilizing the β1–β2 hairpin (Fig. 3B). These structural features are very similar to those observed for the ECD1 of CRF-R2β and are supported by the up-field shifted side chain resonances of Arg85 (βCH2, 0.42 and −0.55; γCH2, 0.65 and 1.36; δCH2, 0.97 and 0.18 ppm) that require close proximity to an aromatic side chain because aromatic ring currents cause such high field shifts. Additional up-field shifted resonances are observed for the methyl protons of Ile51 (0.12 and −0.28 ppm), Thr53 (0.08 ppm), the α-protons of Gly52 (4.42 and 2.92 ppm), and methyl protons of Val97 (0.41 and −0.29 ppm), and they are all attributed to the close proximity of the residues to the aromatic ring of Tyr99.

Conformation of the Agonist hcCRF Both Free and Complexed with the ECD1-CRF-R1—For the free 15N,13C-labeled hcCRF (amino acid numberings per Fig. 4G) at pH 6.5 and 298 K, all of the expected 30 peaks were observed in the 15N,1H HMQC spectrum (Thr11, Glu30, and Lys53 were not labeled, see under "Materials and Methods") (Fig. 4A, red contours). In addition to the major conformation, a minor conformation (~1/3 of the major conformation) is also present for most of the N-terminal residues up to residue Leu10. This minor conformation is attributed to cis/trans isomerizations of the N-terminal proline residues. The chemical shift values for the Cα, Cβ, and Hα protons (Fig. 4, C and D, red bars) of the free peptide indicate that the peptide segment from residues Phe12 to Leu37 is partially in a helical conformation.

Fig. 4A also shows the 15N,1H HMQC spectrum of 13C,15N-labeled hcCRF complexed with unlabeled ECD1-CRF-R1 (black contours). When compared with the 15N,1H HMQC spectrum of free hcCRF, two prominent features in the complex spectrum are observed as follows: (i) the larger dispersion of the cross-peaks attributed to a higher ordered structure of hcCRF in the complex, and (ii) broad cross-peaks with large line widths due to the larger size of the complex and/or due to slow conformational exchange. Most prominent chemical shift changes as well as line broadening are observed for the C-terminal residues of hcCRF with the maximum shift for Ala41 (Fig. 4E). The chemical shifts for the Cα, Cβ, and Hα protons (Fig. 4, C and D, black bars) suggest that hcCRF prefers a helical conformation when bound to the ECD1-CRF-R1 and that the helicity is more pronounced upon complex formation not only at the C terminus but also toward the N terminus of the peptide (Fig. 4, A and B). This is further supported by the α-helical NOEs (i.e. αβ(i,i+3), αN(i,i+1), and αN(i,i+4) observed for residues Phe12 to Ala41 (Fig. 4G). In addition, amide proton chemical shifts between Ala31

FIGURE 3. Three-dimensional NMR structure of ECD1-CRF-R1 complexed with hcCRF. A, superposition of 20 energy minimized conformers representing the three-dimensional NMR structure of the complex of ECD1-CRF-R1 at pH 6.5. The bundle is obtained by superimposing the backbone Cα atoms of residues 40–105 of ECD1-CRF-R1 and residues 27–41 of hcCRF. The N-terminal residues 4–22 of hcCRF do not interact with the ECD1-CRF-R1 and residues 10–41 of hcCRF prefer a helical conformation with a kink at residues 27–29. Residues 25–108 of the ECD1-CRF-R1 are shown, and the disulfide bonds are colored in yellow. B, ribbon diagram of the lowest energy conformer representing the three-dimensional NMR structure. The N-terminal α-helix of ECD1-CRF-R1 is shown in gray; β-sheets are shown in cyan and the hydrophobic core residues W55 and W93 are shown in yellow. The salt bridge involving R85 (in blue) and D49 (in red) is shown as dashed line in gray. The backbone of hcCRF is shown in gold. C, surface-charge representation of the ECD1-CRF-R1. Red represents negative; blue represents positive, and white represents neutral surface. The accumulation of negative charges on the obverse surface and of positive charges on the reverse surface suggest that the ECD1 may be oriented to the cell membrane along its positively charged surface.
Structure of CRF-R1 N-terminal Domain Complexed with Agonist

and Ala^{31} (Fig. 4F) show a wave-like pattern attributed to the amphipathic nature of the helix in the complex, observed also for astressin bound to the ECD1 of CRF-R2B (2). In addition, a kink of the long helix is observed between residues Glu^{27} to Glu^{29} of hcCRF (Fig. 3). The positioning of this kink is similar to the kinks observed for CRF family ligands in the solvent DMSO (48) but is absent in all the other ligands of family B1 when they are bound to their respective ECD1s in the crystal structures. Although the role of this kink may be understood only in the context of the full-length receptor, it enlarges the conformational space of the N-terminal peptide segment, which is possibly important for receptor-specific signaling (Fig. 3A) (49, 50).

Molecular Interactions between hcCRF and the ECD1—The helical segment of hcCRF bound to the ECD1 is along the hydrophobic face of the protein, covering an area of 2647 Å² of the ECD1 (Fig. 5). The C-terminal hcCRF residues Leu^{37}, Leu^{38}, and Ala^{41} are involved in hydrophilic contacts with Phe^{72}, Tyr^{73}, Tyr^{77}, Ile^{51}, Cys^{58}, Pro^{69}, Val^{97}, Cys^{102}, and Tyr^{99} of the ECD1. In particular, the side chain of Leu^{38} is located in a deep hydrophilic pocket surrounded by Ile^{51}, Cys^{58}, Pro^{69}, Phe^{72}, Tyr^{77}, Tyr^{79}, and Cys^{102} of the ECD1 (note that up-field shifted resonances for the β and methyl protons of Leu^{38} are indicative of its interactions with aromatic side chains of the ECD1). The amide group at the C-terminus of hcCRF is involved in an inter-molecular hydrogen bond with the backbone carboxyl of Val^{97}. In return, the backbone amide proton of Val^{97} is involved in a hydrogen bond with the backbone carboxyl of Ala^{41} in hcCRF (Fig. 5). These hydrogen bonds explain the necessity of C-terminal amidation for high affinity recognition of CRF ligands (48, 51). Although there are several NOEs observed between the side chain of Asn^{34} to Tyr^{73} and Val^{75}, in the three-dimensional structure, these two side chains are not close enough to form an intermolecular hydrogen bond. The side chain of Ala^{31} of hcCRF also interacts with Val^{75} and Tyr^{77} through hydrophobic interactions. The side chain of Arg^{35} is in close proximity to the side chain of Glu^{104}, thereby facilitating a salt bridge interaction. Because the side chain resonances of Arg^{35} could not be observed in the 13C-resolved 1H, 1H NOESY spectrum except for the δ protons, its interaction must be interpreted with care.

representation of the observed NOE interactions between hcCRF and ECD1-CRF-R1. Hydrophobic residues are colored yellow; positively charged residues are colored blue, and negatively charged residues are colored red. C–G, plots of the chemical shift difference between hcCRF and the corresponding "random coil" values for 13C (C), and for 1H (D) for the complex and free hcCRF (D), respectively. Chemical shift changes suggest that the ligand binding induces more helicity, not only in the C-terminal segment but also in the N-terminal segment. E, the normalized chemical shift changes (Δδ(1H) + 2 Δδ(13C))/2 observed for hcCRF in the complex versus the amino acid sequence (34). There is an overall 0.1 ppm chemical shift change due to the difference in the temperatures at which the free (298 K) and complex (308 K) spectra were recorded. F, plots of the observed chemical shifts of the 13C atoms of hcCRF free (in red) and bound (in black) to ECD1-CRF-R1. G, intramolecular NOEs observed in hcCRF bound to ECD1-CRF-R1 along the amino acid sequence. Thin, medium, and thick lines represent weak (4–5 Å), medium (3–4 Å), and strong (<3 Å) NOEs observed between the residues connected by the line. The bar connecting Glu^{104} and Lys^{153} represents the lactam bridge connecting the side chains of these residues. Residues Thr^{11}, Glu^{36}, and Lys^{153} are not isotopically labeled, and hence their values are not shown.
Comparison of the Structures of \( \text{ECD1-CRF-R1} \) Complexed with the Agonist \( \text{hcCRF} \) and \( \text{ECD1-CRF-R2/HC} \) Complexed with the Antagonist Astressin

Recently, our group reported the structure of the \( \text{ECD1-CRF-R2/HC} \) complexed with the peptide astressin (2). Comparison of the structures of the complexes of \( \text{ECD1-CRF-R1} \) and \( \text{ECD1-CRF-R2/HC} \) enables the identification of the common interaction sites. Both of the structures have the SCR motif characteristic of the ECD1s of family B1 members (Fig. 6A). There is a short N-terminal helix observed for \( \text{ECD1-CRF-R1} \), which was absent in the structure of \( \text{ECD1-CRF-R2/HC} \) because in the latter the N-terminal segment was truncated in the protein construct. Although conformation of loop 1 is not defined in either ECD1, loops 2 and 3 are structured and interact with the corresponding ligand in a slightly different manner (Fig. 6B). The backbone of loop 2 of \( \text{ECD1-CRF-R2/HC} \) folds closer to the ligand than the corresponding loop in \( \text{ECD1-CRF-R1} \), although the opposite holds for loop 3.

There are also structural differences of the ligand. Although both the agonist and the antagonist bind to almost the same region of the ECD1, the orientations of the ligands are slightly different with respect to loop 2 (Fig. 6, B–D). Furthermore, the C-terminal residues of astressin prefer a \( 3_{10} \) helix, whereas in \( \text{hcCRF} \), they are in an \( \alpha \)-helical conformation. In both ligands, the backbone carbonyl of the last residue (Ala/Ile\(^{41}\)) is involved in a hydrogen bond with the amide proton of Val\(^{97/113}\). The side chains of the ligand residues, Glu\(^{39}\), Leu/Lys\(^{36}\), Ala/His\(^{32}\), and Gln\(^{29}\) are completely solvent-exposed in both of the structures. Also, the side chain of Glu\(^{40}\) of \( \text{hcCRF} \) is completely solvent-exposed, whereas Ile\(^{40}\) of astressin interacts with Gln\(^{66}\) of \( \text{CRF-R2/HC} \). The side chain of Leu/Ile\(^{38}\) is completely buried in a hydrophobic core in both structures (Fig. 6). Although the side chains of Phe\(^{72/88}\) of ECD1 are conserved in their positions and these residues interact with residues Leu\(^{37}\) and Asn\(^{34}\) of the ligand in both of the structures, the positions of Tyr\(^{77/93}\) and Asn\(^{89}\) and Tyr\(^{77/93}\) are distinct. The orientation of Tyr\(^{77}\) of \( \text{CRF-R1} \) is toward the ligand, whereas in \( \text{CRF-R2/HC} \), Tyr\(^{93}\) points away from the ligand. Consistent with this is the observation that the mutation
Y77A in CRF-R1 abrogated high affinity binding of both astressin and sauvgaine and significantly increased the EC₅₀ for sauvgaine- and urocortin1-stimulated intracellular cAMP accumulation (data not shown). The side chain of Asn³⁴ of astressin is in close proximity to the ECD1 to form a hydrogen bond with the backbone carbonyl of Phe⁸⁸ in ECD1-CRF-R2β. Such a hydrogen bond is missing in the structure of the ECD1-CRF-R1 complex with ahcCRF. The residues Val⁷⁹/Ile⁸⁵ are in almost the same position, and they interact with Ala³¹ of the ligand. The side chain of Arg³⁵ does not directly interact with Glu¹⁹⁴ of the ECD1-CRF-R1, although it is close enough to form a solvent-exposed inter-molecular salt bridge. In contrast, in ECD1-CRF-R2β, Arg³⁵ is involved in a buried salt bridge with Glu⁸⁶, which, in ECD1-CRF-R1, is replaced by Ala⁷⁰, and its side chain is solvent-exposed.

Comparison of the NMR Structure of ECD1-CRF-R1-ahcCRF Complex and X-ray Crystallographic Structures of ECD1-CRF-R1 Peptide Complex—The NMR structure of ECD1-CRF-R1 complexed with the high affinity agonist ahcCRF may be compared with the crystal structures of ECD1-CRF-R1 complexed with the low affinity fragments CRF(22–41) and CRF(27–41), which are presumed to be antagonists (1). Interestingly, the ECD1 of CRF-R1 was crystallized in a ligand-dependent manner in three different forms that differ mainly in the conformations of loop 2, to which the ligand binds (Fig. 7). Pioszak et al. (1) suggest that crystal form II is possibly more relevant, physiologically, than the others because only in crystal form II is loop 2 unhindered from crystal packing constraints. Indeed, our NMR structure of ECD-CRF-R1-ahcCRF (Fig. 7, E and F) is more similar to the crystal form II than to forms I and III (Fig. 7, A–D) (1). The differences between the NMR and x-ray structures are discussed in detail in the supplemental material. The comparison between all of the four structures suggests the presence of a structural plasticity of the SCR motif of the ECD1-CRF-R1 (see below).

Structural Plasticity of Both the ECD1 and the Ligand—The absence of cross-peaks for amide moieties of residues in loop 2 in the ¹⁵N,¹H TROSY spectra of both of the ECD1s of CRF-R1 and CRF-R2β suggests the presence of slow conformational exchange dynamics in the ECD1s of CRF receptors. Although the cross-peaks appear in the ¹⁵N,¹H TROSY spectra in the complex with the peptide for both receptor studies, the significant line broadening observed for these peaks suggests that the conformational exchange is only partially suppressed. The amide moieties of the ligand ahcCRF also show broad, weak cross-peaks in the ¹⁵N,¹H HMBC spectra upon complex formation (Fig. 4). The presence of slow conformational exchange dynamics in the millisecond time range for segments of both the ECD1 and the ligand is in agreement with the presence of conformational heterogeneity observed in both the NMR and x-ray structures. Furthermore, the nanomolar binding affinity of the antagonist astressin for a CRF-R2β mutant whose corresponding ECD1 shows molten globule-like conformational states (52) supports the dynamic character of the ECD1. Although we can only speculate about the biological role
of these conformational exchange dynamics, their presence could account for the multiple recognition, binding, and signal-
ing observed for the various hormone ligands.

Refinement of the Two-step Binding Mechanism of CRF Peptides to Its Receptors—The two-step model for ligand binding and sig-
naling of type B1 GPCRs (29, 53, 54) proposes that the C-terminal
segment of the ligand binds to the ECD1, which then may position
the N-terminal portion of the peptide hormone in close proximity
to the serpentine regions of the receptor to initiate signaling. The
ECD1 is therefore the major peptide-binding domain, and con-
versely, the C-terminal segment of the ligand is important for high
binding affinity and selectivity to the receptors. All of the three-
dimensional structures of the ECD1-receptor-ligand complexes
are consistent with this model, because the C-terminal segment of
the peptide ligand interacts with the ECD1 (1, 2, 26–28). Our
NMR studies of the complex between ECD1-CRF-R1 and an ago-
nist further indicate that the recognition of the ligand by the ECD1
not only binds the hormone and positions its N-terminal residues
for signal activation but also induces helix formation toward the N
terminus of the ligand to generate a conformationally active state.
In a recent review (55), this additional function in receptor activa-
tion of the ECD1 in family B1 GPCRs was proposed to be based on
the following: (i) a structural comparison between various hor-
monal ligands, free and complexed with ECD1s and (ii) the impor-
tance of helix-capping residues in the N-terminal region of the
numerous corresponding ligands (56–58). The induction of the
helix in the ligand upon complex formation with the ECD1 not only
binds the hormone and positions its N-terminal residues for signal activation but also induces helix formation toward the N
terminus of the ligand to generate a conformationally active state.

CONCLUSION

The information gained from our structural studies comple-
ments earlier knowledge of the molecular interactions between
ligands and GPCRs of the B1 family. Specifically, the three-dimen-
sional NMR structure presented here of ECD1-CRF-R1 complexed with an agonist suggests a refined two-step model for receptor activation.

Agonist Versus Antagonist—Previous studies on chemically
modified and truncated CRF ligands showed that the first seven
residues at the N termini of CRF are not necessary for GPCR
signaling (3, 4) and that residue 8 was critical, whereas the
C-terminal (~15) residues are important for binding (47, 53,
54). Hence, CRF analogs truncated by 8 residues or more at the
N terminus are antagonists. This finding can easily be under-
stood with the help of the two-step model for ligand binding
and signaling of type B1 GPCR discussed above (16, 19, 29). If
the N-terminal segment of the ligand is missing, the C-terminal
fragment still binds to the receptor but is not able to produce
activation. Such a ligand is then evidently an antagonist because
it occupies the major binding site thereby blocking peptide ago-
nist binding. The three-dimensional structure of the ECD1-
CRF-R1 complexed with the agonist ghCRF presented here is
the first direct experimental proof that supports this hypothesis
(Figs. 7 and 8) and shows clearly the similarity of the C-terminal
binding of the peptide agonists and antagonists.
