

# The Three-Dimensional Structure of the N-Terminal Domain of Corticotropin-Releasing Factor Receptors

## Sushi Domains and the B1 Family of G Protein–Coupled Receptors

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**ABSTRACT:** The corticotropin-releasing factor (CRF) receptors, CRF-R1 and CRF-R2, belong to the B1 subfamily of G protein–coupled Receptors (GPCRs), including receptors for secretin, growth hormone-releasing hormone (GHRH), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), calcitonin, parathyroid hormone (PTH), glucagon, and glucagon-like peptide-1 (GLP-1). The peptide ligand family comprises CRF, Ucn 1, 2, and 3. CRF plays the major role in integrating the response to stress. Additionally, the ligands exhibit many effects on muscle, pancreas, heart, and the GI, reproductive, and immune systems. CRF-R1 has higher affinity for CRF than does CRF-R2 while both receptors bind Ucn 1 equally. CRF-R2 shows specificity for Ucn 2 and 3. A major binding domain of the CRFRs is the N terminus/first extracellular domain (ECD1). Soluble proteins corresponding to the ECD1s of each receptor bind CRF ligands with nanomolar affinities. Our three-dimensional (3D) nuclear magnetic resonance (NMR) structure of a soluble protein corresponding to the ECD1 of CRF-R2 $\beta$  (*I*) identified its structural fold as a Sushi domain/short consensus repeat (SCR), stabilized by three disulfide bridges, two tryptophan residues, and an internal salt bridge (Asp65–Arg101). Disruption of the bridge by D65A mutation abrogates ligand recognition and results in loss of the well-defined disulfide pattern

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**and Sushi domain structure. NMR analysis of the ECD1 in complex with astressin identified key amino acids involved in ligand recognition. Mutation of some of these residues in the full-length receptor reduces its affinity for CRF ligands. A structure-based sequence comparison shows conservation of key amino acids in all the B1 subfamily receptors, suggesting a corresponding conservation of a Sushi domain structural fold of their ECD1s.**

**KEYWORDS: CRF receptor; structure; ECD1; Sushi domains; B1 GPCR**

## INTRODUCTION

The isolation from the hypothalamus of corticotropin-releasing factor (CRF), was based on its function as an ACTH secretagogue.<sup>1</sup> CRF is the primary activator of the hypothalamic-pituitary-adrenal axis (HPA) and serves to integrate not only the endocrine, but also the autonomic and behavioral responses to stress. Subsequently, the CRF ligand family has been increased by the cloning of the three related peptides urocortins (UCNs) 1, 2, and 3, the latter two also known as stresscopin-related peptide and stresscopin, respectively.<sup>2-5</sup> The roles of the CRF ligand family continue to expand and currently include actions on the GI tract, pancreas, and muscle as well as on the reproductive, cardiovascular, and immune systems.

The actions of the ligands are initiated by binding and activation of CRF receptors. In rodents there is a single type I receptor, CRF-R1,<sup>6-8</sup> and two forms of the type II receptor, CRF-R2 $\alpha$  and CRF-R2 $\beta$ , arising from alternative splicing.<sup>9-12</sup> In humans, two splice variants for CRF-R1 have been cloned<sup>6</sup> and transcripts for other splice variants have been reported<sup>13</sup>; three splice variants for CRF-R2 have been cloned.<sup>14,15</sup> Receptors orthologous to those in mammals have been identified in amphibia, fish, and birds.

The ligands and receptors display unique mutual specificities: CRF binds with higher affinity to CRF-R1 than to CRF-R2; Ucn 1 binds with equally high affinity to both receptor types; Ucn 2 and Ucn 3 are highly specific for CRF-R2.

The CRF system also includes two soluble binding proteins, CRF-BP<sup>16</sup> and sCRF-R2 $\alpha$ ,<sup>17</sup> the first being encoded by a distinct gene, while the second results from alternative splicing of the CRF-R2 $\alpha$  gene. The CRF-BP binds CRF and Ucn 1 with high affinity. Interestingly, the ligand specificity of sCRF-R1 $\alpha$  is more like that of CRF-R1 than like that of CRF-R2 in that CRF and Ucn 1 are bound with higher affinity than is Ucn 2 and Ucn 3 is bound with very low affinity.

The expression of CRF-R1 is widespread in the central nervous system as well as many peripheral tissues such as skin, gonads, GI tract, adrenal, and immune system.<sup>18</sup> The type I receptor is the major anterior pituitary receptor mediating the CRF-stimulated release of ACTH and thus the activation of the

HPA axis. Transgenic mice in which CRF-R1 is disabled exhibit a blunted stress response and have been termed “mellow mice.”<sup>19</sup>

In the rodent, the expression of CRF-R2 $\alpha$  is largely confined to the central nervous system; CRF-R2 $\beta$  is expressed in peripheral tissues such as epididymis, GI tract, heart, vasculature, and skeletal muscle.<sup>18</sup> The type II receptor expressed in the heart mediates the effects of the urocortins on cardioprotection<sup>20</sup> and a putative type II receptor in the pancreas is presumed to mediate the effects of urocortin on pancreatic hormone release.<sup>21</sup>

The CRF receptors belong to the B1 subfamily of G protein-coupled receptors (GPCRs) known as the secretin family which includes receptors for parathyroid hormone (PTH), calcitonin, calcitonin gene-related peptide, adrenomedullin, glucagon, glucagon-like peptide-1 (GLP-1), growth hormone-releasing hormone (GHRH), pituitary adenylate cyclase-activating polypeptide (PACAP), glucose-dependent insulinotropic peptide, and vasoactive intestinal peptide (VIP). The CRF receptors couple to G<sub>s</sub> with subsequent activation of adenylate cyclase producing an increase in intracellular cyclic adenosine 3',5'-phosphate (cAMP) and protein kinase A activity. The receptors also couple to G<sub>q</sub>, and activation of protein kinase C, resulting in phosphatidylinositol hydrolysis and an increase in intracellular calcium and diacyl glycerol. In some tissues there is also ligand-stimulated activation of mitogen-activated protein (MAP) kinase.

### ***Distinct Regions of Receptors and Ligands Govern Binding and Activation***

#### ***CRF Ligands***

The CRF peptide ligands consist of 38–40 amino acids. The observation that deletion of the first 12 amino acids converts an agonist into an antagonist led to the development of high-affinity CRF antagonists such as astressin<sup>22</sup> and antisauvagine-30.<sup>23</sup> The development of the antagonists suggested that the C-terminal region of the ligand is not involved in signal transduction but rather, binds to the receptor and blocks binding of an agonist and subsequent signal transduction. Recent data on even more extensively N-truncated astressin-like antagonists<sup>24</sup> leads to the conclusion that the C-terminal 12 residues are sufficient for high-affinity binding.<sup>25</sup>

In order to investigate the region of the peptide involved in receptor signaling, a tethered-peptide receptor was created in which the N-terminal or first extracellular domain (ECD1) of CRF-R1 was replaced by the first 16 amino acids of CRF.<sup>26</sup> When expressed in mammalian cells, this chimeric receptor generated a continuous signal as measured by the accumulation of intracellular cAMP. The semipiternal signaling of this tethered-ligand receptor suggests that the N-terminal region of the ligand comprising, for example, the first 16 amino acids of CRF, is sufficient for activating the adenylate cyclase

signaling pathway. Interestingly, a similar tethered-peptide PTH receptor is also constitutively active.<sup>27,28</sup>

### *CRF Receptors*

The two types of CRF receptors are highly homologous and display ~70% sequence conservation in many regions, for example, the third intracellular loop. The majority of sequence differences occur in the extracellular domains, notably in the ECD1. Many studies using chimeric or mutant receptors have shown that the extracellular domains contribute to the binding and ligand selectivity<sup>29–31</sup> and further, that the ECD1 constitutes a major peptide-binding domain.<sup>30,32–36</sup>

The juxtamembrane receptor domain, that is, the receptor excluding the ECD1, also binds peptide ligands, albeit with significantly lower affinity.<sup>35</sup> The data from the constitutively active tethered-ligand receptor show that the juxtamembrane domain is involved in transducing the signaling response.

### *Soluble Proteins Corresponding to ECD1 of CRF Receptors*

Further support for the key role of the ECD1 as a major binding domain derives from data showing that soluble proteins corresponding to the ECD1s of either CRF-R1 or CRF-R2 bind CRF ligands with nanomolar affinities.<sup>37–39</sup> Transfection of COS cells with cDNA encoding amino acids 1–119 of CRF-R1 resulted in secretion of the corresponding protein into the medium. Following enrichment of the protein by immunoaffinity chromatography, N-terminal sequencing revealed that the first amino acid of the secreted protein is Ser24. This result confirmed the proposal that the first 23 amino acids serve as a signal peptide. The soluble protein corresponding to the ECD1 of CRF-R1 binds astressin and Ucn 1 with nanomolar affinity ( $K_d \sim 10$  nM).<sup>37</sup>

In order to obtain milligram quantities of the soluble protein, a bacterial expression system was chosen. The cDNA encoding amino acids 24–119 of hCRF-R1, ECD1-CRF-R1, (expressed as a thioredoxin fusion protein, containing an S-tag sequence for purification purposes and a thrombin cleavage site) was used to transform the Origami strain of *E. coli*. Following thrombin cleavage, the expressed protein was enriched by affinity chromatography and purified by high-performance liquid chromatography (HPLC). Biochemical characterization, using tryptic digestion and mass spectrometry, showed that the six cysteines form disulfide bonds with the following pattern: 1–3, 2–5, and 4–6.<sup>37</sup> This same disulfide pattern is observed in the soluble proteins corresponding to the ECD1s of the receptors for PTH<sup>40</sup> and GLP-1,<sup>41</sup> both of which are B1 receptor family members.

In similar fashion, a soluble protein, ECD1-CRF-R2 $\beta$ , comprising amino acids 27–133 of mCRF-R2 $\beta$  was expressed and purified from *E. coli*. Biochemical characterization showed that the disulfide pattern is the same as that

determined for ECD1-CRF-R1.<sup>39</sup> The ECD1-CRF-R2 $\beta$  binds astressin, Ucn 1, and Ucn 2 with nanomolar affinities ( $K_d \sim 10$  nM),<sup>39</sup> CRF with lower affinity ( $K_d \sim 100$  nM), and Ucn 3 with even lower affinity ( $K_d > 200$  nM). Because preliminary experiments showed that the yield of ECD1-CRF-R2 $\beta$  was greater than that of ECD1-CRF-R1, the former protein was chosen for structural studies.

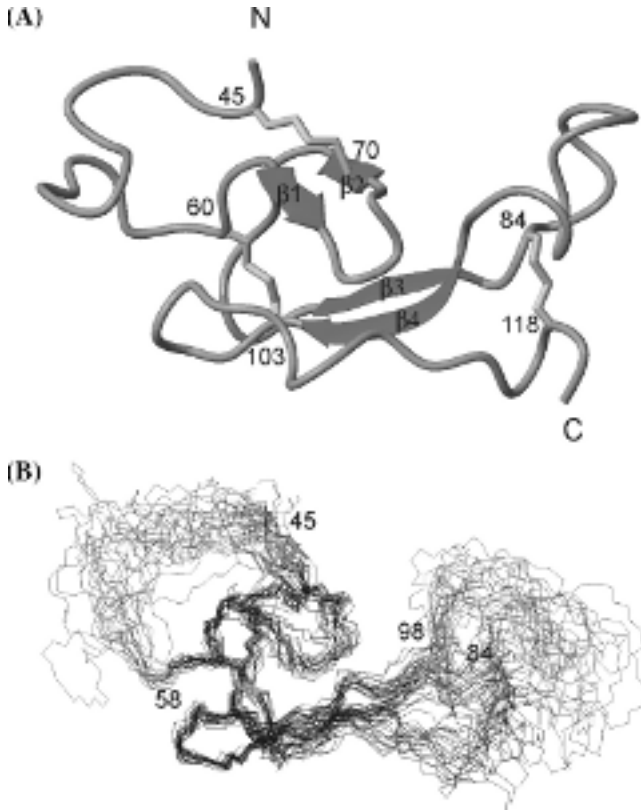
Based on the activity of the tethered-ligand receptor chimera and taking into account data on the ECD1 as a major binding domain, a model was proposed for CRF receptor activation.<sup>26</sup> This model, which has now been extended to apply to the activation of all B1 receptors,<sup>42</sup> envisions receptor activation to involve two steps: First, the ECD1 of the receptor captures the ligand through high-affinity binding of its C-terminal region. Second, the bound ligand, now positioned in an appropriate proximity, presents its N-terminal region to the juxtamembrane domain of the receptor, initiating signal transduction.

### *Three-Dimensional Structure of the ECD1 of CRF-R2 $\beta$*

The importance of the ECD1 for CRF receptor-ligand recognition serves as the impetus for obtaining the three-dimensional (3D) structure of that domain. In order to achieve this, nuclear magnetic resonance (NMR) spectroscopy was chosen because of the frequent difficulties in obtaining crystals of large proteins. For NMR spectroscopy, milligram amounts of protein, isotopically labeled with <sup>15</sup>N and <sup>13</sup>C, are required. For the structural studies, a protein corresponding to amino acids 39-133 of mCRF-R2 $\beta$  was expressed in, and purified from *E. coli*, as described above, using minimal media supplemented with (<sup>15</sup>NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and <sup>13</sup>C-D-glucose.<sup>43</sup>

#### *Structural Fold of ECD1-CRF-R2 $\beta$*

Backbone assignments were obtained from triple resonance experiments while nuclear Overhauser enhancement spectroscopy (NOESY) experiments yielded the distance restraints.<sup>43</sup> The structure determination revealed the presence of two antiparallel pairs of  $\beta$ -sheets involving residues 61–66, 69–72, 79–84, and 98–102. The polypeptide fold is stabilized by three disulfide bonds between cysteine residues 45–70, 60–103, and 84–118 and by a core composed of an internal salt bridge between Asp65 and Arg101 sandwiched between two typtophan residues (71 and 109). The N and C terminii flanking the core are disordered, whereas the central region is highly ordered in all the conformers. These structural properties are shown in FIGURE 1A, in which the lowest energy conformer is presented as a ribbon diagram. The representation in FIGURE 1B is a superposition the 20 conformers that are consistent with the NMR data. The structure is a globular one with well-defined secondary structure and a



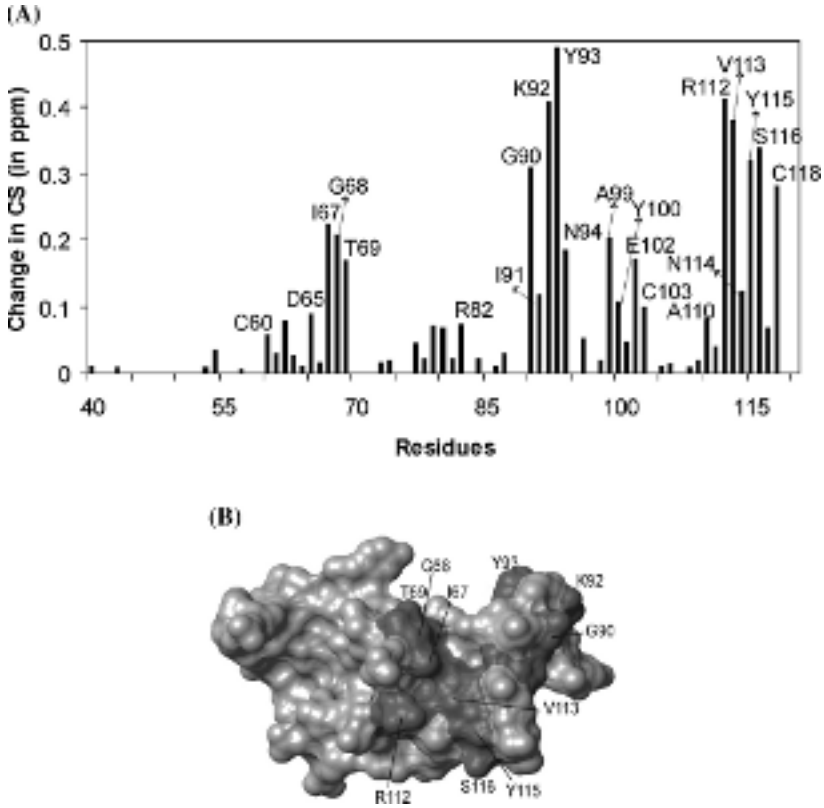
**FIGURE 1.** 3D structure of the ECD1-CRF-R2 $\beta$ . (A) Ribbon diagram of the lowest energy conformer. (B) Superposition of 20 conformers. Only amino acids 44-119 are shown.

large compact hydrophobic core. The disulfide pattern is the same as that determined biochemically for the soluble proteins corresponding to ECD1s of both CRF-R1 and CRF-R2 $\beta$ .

The core is surrounded by a layer of residues, Thr69, Val80, and Arg82, that show strict conservation and by other residues, Thr63, Ser74, and Ile67, which show conservative substitutions among the CRF receptors (FIG. 2 A). The other conserved residues are Pro72 and Pro83, which probably serve to end the  $\beta$ -strands and Gly77, Asn106, and Gly107, which are in the hinge regions and may be important for their relative orientation. Another set of conserved residues, Gly90, Tyr93, Asn94, and Thr96, are in the disordered loop between the  $\beta$ 3 and  $\beta$ 4 strands. The disordered loop comprising residues 39-58 is highly variable in sequence (FIG. 2 A) among the CRF receptor family members.

Analysis of the NMR data by the Dali program identified the structural fold of the highly ordered region as a short consensus repeat (SCR), or Sushi





**FIGURE 3.** (A) The changes in the normalized chemical shifts following binding of astressin to the ECD1-CRF-R2 $\beta$ . (B) A surface representation of the ECD1-CRF-R2 $\beta$  showing the proposed residues involved in binding astressin.

the edge of the palm of the second  $\beta$ -sheet as shown in a surface representation in FIGURE 3 B. This surface of residues is proposed to constitute the peptide-binding interface. The chemical shift changes in the disordered C-terminal loop region, including residues 85–98 represent binding-induced folding.

#### *Other CRF Receptors*

Sequence comparison of the ECD1s of CRF-R1 and CRF-R2 $\beta$  from different species (FIG. 2 A) shows the conservation of many residues that play key roles in the structure. Many of those residues are the same ones that are proposed to interact in the binding of astressin (FIG. 3 B), so that the surface defined by those residues overlaps to a large extent with the binding surface. The coincidence of these two surfaces suggests that the ligand-binding surface is conserved across all the CRF receptors.

**TABLE 1. Effect of mutation of residues in the ECD1 in the full-length receptor on the binding of astressin**

Receptor	K <sub>d</sub> (Astressin) nM	$\Delta\delta$ (ppm)
CRF-R2 $\beta$	1.1 (0.7–2.0)	—
[K92Q]CRF-R2 $\beta$	2.6 (1.9–3.6)	0.4
[R112E]CRF-R2 $\beta$	8.0 (4.3–17.4)	0.4
[I67E]CRF-R2 $\beta$	130 (85–191)	0.2
[Y115R]CRF-R2 $\beta$	>200	0.3
[D65A]CRF-R2 $\beta$	>200	0.1

K<sub>d</sub>= dissociation constant;  $\Delta\delta$  = change in NMR chemical shift of the indicated residue subsequent to binding of astressin to the ECD1-CRF-R2 $\beta$ .

### *Full-Length Receptor*

The residues I67, K92, R112, Y115 show the largest NMR chemical-shift changes following binding of astressin (FIG. 2 A). These residues are proposed to lie on the surface of the ECD1. What relevance is this binding surface for ligand recognition by the full-length receptor? In order to address this question, those residues were mutated in the full-length CRF-R2 $\beta$  and the effect of the mutations on the affinity for astressin was determined. For example, the mutation R112E in the full-length receptor produced in a sevenfold decrease in the affinity for astressin, while the mutations I67E and Y115R result in >100-fold decrease in affinity for astressin. These data are summarized in TABLE 1. The decrease in affinities, as a result of the mutations, suggests that the binding surface in the full-length receptor is similar, if not identical to that determined for the isolated ECD1.

### *Role of Salt Bridge*

Interestingly, even though Asp65 is not a surface residue in the ECD1 structure, the mutation D65A has a large effect on the binding of astressin to the full-length receptor (TABLE 1). In order to understand the reason for the importance of this salt bridge, NMR and biochemical methods were used to study the structure and ligand recognition of ECD1-CRF-R2 $\beta$  in which the salt bridge was disrupted. The protein, [D65A]ECD1-CRF-R2 $\beta$  (<sup>15</sup>N-labeled), in which the mutation is presumed to disrupt the salt bridge, was expressed and purified from *E. coli*. The NMR spectrum of the mutant protein showed a collapse of the wild-type spectrum into the random coil chemical-shift region indicating a large conformational change from the well-defined Sushi domain structure toward that of a random coil state (Perrin *et al.*, to be published). One possible explanation is that the structure of the mutant is destabilized into a metastable state comprising many different conformations that exchange with one another over the time scale of NMR measurements. Biochemical characterization of the disulfide arrangement of [D65A]ECD1-CRF-R2 $\beta$  using mass spectrometric

methods, showed no unique disulfide arrangement. Finally, [D65A]ECD1-CRF-R2 $\beta$  displayed no high-affinity binding to astressin. Thus, the internal salt-bridge in the ECD1 of the CRF receptor appears to play mainly a structural role and its presence is necessary for the high-affinity ligand recognition of both the isolated ECD1 and of the full-length receptor.

### *Model for CRF Receptor Activation*

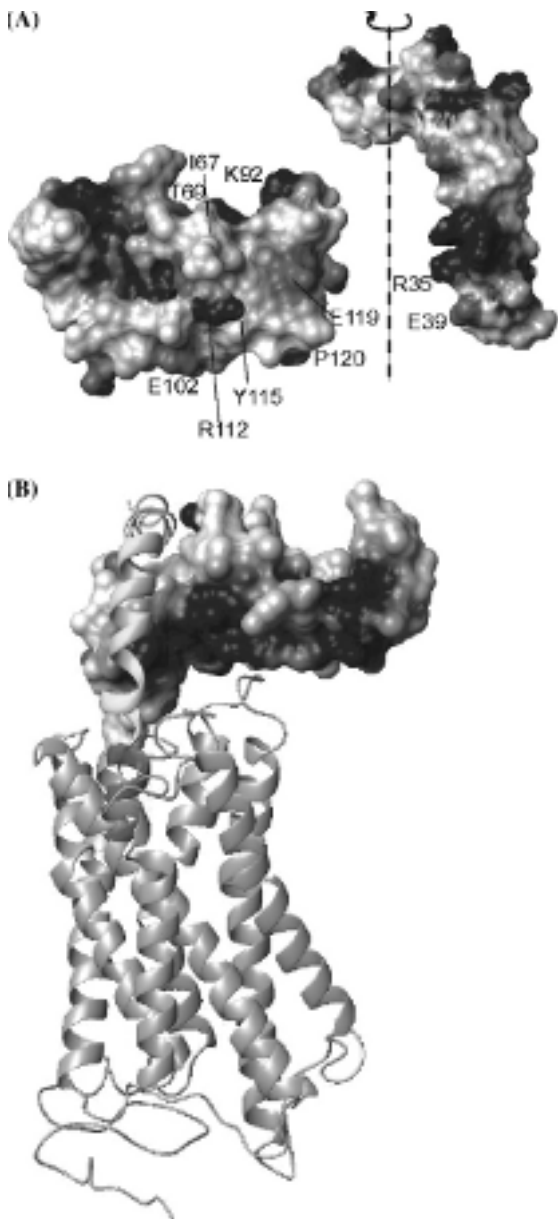
The 3D structure provides insight into the proposed mechanism of receptor activation. It is suggested from the structures of the ECD1 and of astressin 2B (Riek *et al.*, to be published) that the peptide interacts with the binding surface as shown in FIGURE 4 A. The structure of the ECD1 shows a cluster of positive charges (Arg47, Arg82, and Arg97) on the “back” of the protein’s surface. Examination of the sequences of the extracellular domains 2–4 shows that they contain a cluster of negative charges. These considerations provide a rationale for the arrangement, shown in FIGURE 4 B, in which the positively charged back surface of the ECD1 is oriented towards the negatively charged extracellular domains 2–4. A consideration of the relative orientation of the ECD1 and the juxtamembrane domains of the receptor together with the 3D structures of the ECD1 and of astressin provides a molecular basis for the proposed two-step model of the receptor activation proposed. As indicated in FIGURE 4 B, the exposed binding surface of the ECD1 of the CRF receptor binds the C-terminal region of the peptide. The peptide, thus captured, is in position in the correct proximity to present its N terminus to the remainder of the receptor, in order to initiate signaling.

### *The B1 Receptor Subfamily*

The sequences of the ECD1s of some B1 receptors are shown in FIGURE 2 B, in which the conserved residues are highlighted. These conserved residues are presumed to be responsible for maintaining the structural integrity of the ECD1. For example, the two tryptophan and six cysteine groups are strictly conserved as are the two prolines proposed to be important for ending the  $\beta$ -sheets and two glycines. In addition, there are many other residues that show only conservative changes. All the B1 subfamily receptors have either aspartic/glutamic acid and arginine/lysine residues in analogous positions for creating the structurally important internal salt bridge. Disruption of the salt bridge by mutation should produce deleterious effects on binding and signaling. Indeed, such effects have been reported for some of the B1 receptors.

### *The GHRH Receptor*

It has been known for more than 20 years that a dwarf mouse, known as the *little* mouse, displays a phenotype characterized by a hypoplastic pituitary



**FIGURE 4.** Proposed model of CRF receptor activation. **(A)** Surface representation of the ECD1-CRF-R2 $\beta$  and astressin (unpublished) showing the relative orientation of the peptide and major binding surface. **(B)** Schematic of the ECD1 oriented in the full-length receptor using rhodopsin as a model for the transmembrane domains. The model for the peptide includes an extension to include the N-terminal segment important for receptor activation.

and defective response to GHRH.<sup>46</sup> The cloning of its pituitary receptor revealed a single mutation, [D60G], in its ECD1.<sup>46</sup> This mutant GHRH receptor displays reduced ligand-induced signal transduction. The sequence comparison (FIG. 2B) shows that all the structurally important residues of the CRF receptor are conserved in the ECD1 of the GHRH receptor. Thus, the mutation [D60G], being analogous to the mutation [D65A] in CRF-R2 $\beta$ , would disrupt the internal salt bridge and result in a loss of the structural integrity of the ECD1 and of the GHRH binding surface. The consequences would then be compromised ligand recognition and impaired signal transduction.

### *Other B1 Receptors*

Mutating D69 in the calcitonin receptor (corresponding to D65 in CRF-R2 $\beta$ ) disrupted not only signaling but also the interaction of the receptor with RAMP1.<sup>47</sup> In the secretin receptor, the mutations [D49R] or [R83D] (corresponding to D65 or R101 in CRF-R2 $\beta$ ) impaired binding and signaling.<sup>48</sup> In the VIP receptor, D68 (corresponding to D65 in CRF-R2 $\beta$ ) is essential for binding VIP, and also for maintaining the constitutive activity of the [H178R] mutation.<sup>49</sup>

Thus, the conservation of the key structural residues suggests that the structural fold of the ECD1 of all the B1 receptors will be the same as that of the CRF receptor, namely a SCR/Sushi domain.

### *Extracellular Domain Interactions*

Structures of extracellular domains of cell surface proteins are modular in nature and serve to facilitate protein-protein interactions. Two common structural modules are the SCR/Sushi and the epidermal growth factor (EGF) domains. Both motifs are found in many proteins, notably those in the complement system such as CD55 and its receptor CD97,<sup>50</sup> the latter being a member of the EGF-7TM GPCR family. The ECD1 of CD97 has multiple EGF modules.<sup>51,52</sup> The X ray structure of the extracellular domain of CD55 identified four SCR/Sushi domains.<sup>53</sup> The binding of CD55 to CD97 results directly from an interaction between their Sushi and EGF domains, respectively.<sup>51</sup>

Subsequent to our identification of a Sushi domain as the structural feature of the ECD1 of the CRF receptor, two Sushi domains were identified in the ECD1 of the GABA<sub>B</sub>1a receptor, another GPCR.<sup>54</sup> Interestingly, it was found that a soluble protein corresponding to the ECD1 of the GABA<sub>B</sub>1a receptor binds to the extracellular matrix protein fibulin-2,<sup>54</sup> which belongs to a family of proteins that are characterized by tandem arrays of EGF domains.<sup>55</sup>

Thus, the identification of a Sushi domain in the structure of the ECD1 of the CRF receptor raises the possibility of interactions of CRF receptors with other receptors and proteins that contain EGF structural modules. More generally, similar interactions of EGF module-containing proteins with the other receptors in the B1 subfamily may also exist.

## CONCLUSIONS

The determination of the 3D NMR solution structure of the ECD1 of the CRF receptor has identified the structural fold adopted by this major binding domain as a SCR/Sushi domain. The structural fold is one that is found in many proteins and is responsible for their interactions with other proteins. The 3D structure identifies the peptide-binding surface on the ECD1 that appears to be relevant to ligand recognition of the full-length receptor. The structure also provides a molecular rationale for the conservation of key residues in the ECD1s of all the CRF receptors, as well as of all related B1 subfamily receptors.

The role of the internal salt bridge formed by a pair of anionic and cationic residues in ECD1 of the CRF receptor appears to be predominantly a structural one. The conservation of the corresponding residues in the ECD1s of the related receptors suggests that a corresponding salt bridge is a crucial structural component in their ECD1s also. Indeed, the compromised pituitary response to GHRH in the “*little*” mouse may now possibly be understood on the basis of the loss of the Sushi domain structure as a result of the disruption of the crucial salt bridge by mutation [D60A] in the ECD1 of its GHRH receptor.

Importantly, the 3D structure has provided important insight into the molecular model for the two-step process of peptide activation of the CRF receptor and its related B1 family members. Finally, the structure determination has opened up the possibility of novel interactions of CRF receptors, as well as of the other related receptors in the B1 subfamily, with proteins that contain extracellular EGF structural modules. The future promises to hold many surprises regarding the roles and interactions of CRF receptors and their B1 family relatives.

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