Distinct Structural and Functional Roles of Conserved Residues in the First Extracellular Domain of Receptors for Corticotropin-releasing Factor and Related G-protein-coupled Receptors*

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The G-protein-coupled receptor B1 family includes corticotropin-releasing factor (CRF), growth hormone-releasing hormone, incretin, and pituitary adenylate cyclase-activating polypeptide receptors. The three-dimensional NMR structure of the first extracellular domain (ECD1) of CRF receptor 2β (CRF-R2β), free and complexed with astressin, comprises a Sushi domain. This domain is stabilized in part by a salt bridge between Asp65 and Arg101. Analogous residues are conserved in other members of the B1 family. To address the importance of the salt bridge residues within this receptor family, we studied the effects of mutating the residues in full-length CRF-R2β and isolated ECD1. Mutation D65A or D65R/R101D resulted in loss of the canonical disulfide arrangement, whereas R101A retained the Cys4–Cys6 disulfide bond. The mutations resulted in misfolding within the ECD1 as determined by NMR and 1-anilino-8-naphthalenesulfonate binding but did not prevent cell surface expression. The D65A mutation in CRF-R2β greatly reduced binding and activation, but the R101A substitution had only a small effect. Similar effects were seen on astressin binding to the ECD1. The different interactions of Asp65 and Arg101, deduced from the three-dimensional structure of the complex, are consistent with the differential effects seen in the mutants. The reduction in binding of Asp65 mutants is a consequence of a distinct Asp65–Trp71 interaction, which stabilizes the ligand-binding loop. Hence, loss of the salt bridge leads to disruption of the overall fold but does not abolish function. Because homologous mutations in other B1 receptors produce similar effects, these conserved residues may play similar roles in the entire receptor family.

The B1 family of G-protein-coupled receptors (GPCRs)4 comprises 15 genes in human, including receptors for corticotropin-releasing factor (CRF), growth hormone-releasing hormone (GHRH), vasoactive intestinal peptide (VIP), parathyroid hormone, pituitary adenylate cyclase-activating polypeptide, incretins (GIP and GLP), secretin, calcitonin, and glucagon and are characterized by relatively large first extracellular domains (ECD1s). In particular, the CRF system includes two distinct receptors, CRF-R1 and CRF-R2, as well as the peptide ligands CRF, urocortins (Ucns) 1, 2, and 3 in mammals, and the related peptides, (fish) urotensin and (frog) sauavine. In addition, high affinity antagonist peptides have been synthesized, most notably astressin, which binds with equally high affinity to both receptors (1). Many studies have shown that the ECD1s of the CRF receptors constitute major ligand-binding sites. Chimeric receptors expressing the ECD1s bind CRF family ligands with high affinity, as do soluble proteins corresponding to the ECD1s (2–9). Studies of other B1 receptors report similar data (10–16).

A significant advance in understanding the structural determinants of ligand recognition for this receptor subfamily follows upon the determination of the three-dimensional NMR structure of the ECD1 of the type 2β CRF receptor, both free (17) and in complex with a peptide antagonist, astressin (18). In both forms, the ECD1 folds into a short consensus repeat (19) or Sushi domain, which contains two antiparallel β-sheets, three disulfide bonds, and a salt bridge between aspartic acid 65

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4 The abbreviations used are: GPCR, G-protein-coupled receptor; ECD1, first extracellular domain; CRF, corticotropin-releasing factor; GHRH, growth hormone-releasing hormone; VIP, vasoactive intestinal peptide; ANS, 1-anilino-8-naphthalenesulfonate; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; BSA, bovine serum albumin; HDB, Hepes dissociation buffer; HPLC, high pressure liquid chromatography; MALDI, matrix-assisted laser desorption ionization; HMGC, heteronuclear multiple quantum correlation spectroscopy; MES, 4-morpholineethane-sulfonic acid.
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and arginine 101, sandwiched between tryptophans 71 and 109 (Fig. 1). Because the anionic and cationic amino acid residues as well as the tryptophans are absolutely conserved within the B1 subfamily, it was proposed that all B1 ECD1s include Sushi domain folds (17, 18). The two subsequent reports of Sushi domain folds for the ECD1s of a pituitary adenylate cyclase-activating polypeptide receptor (20) (derived from a solution NMR data) and of an incretin receptor (21) (derived from a crystal structure data) provide strong support for our proposal of the Sushi module in the ECD1 of all B1 receptors.

The conserved amino acid residues relevant to the studies presented here are the anionic and cationic residues, Asp and Arg, forming a salt bridge in the ECD1 of the CRF receptor. In other B1 receptors, mutation of these amino acids results in loss or reduction of signaling and ligand binding (22–28). To gain further understanding of the role of the amino acids in the salt bridge, we have studied the structure, binding, and signaling capabilities of a series of mutant CRF-R2βs in which the salt bridge is either disrupted or “reversed.” Additionally, we have characterized the corresponding mutants in the isolated ECD1. These studies shed light on the structural and functional roles played by the two highly conserved amino acid residues involved in the salt bridge of the CRF receptor system and in the B1 subfamily of GPCRs.

MATERIALS AND METHODS

Mutagenesis—The amino acid mutations were introduced into the ECD1 or into (myc)CRF-R2β by overlap extension PCR using ECD1 or (myc)CRF-R2β as templates, respectively. The Myc epitope was inserted following residue 29. The PCR products were subcloned into either pET-32a(+) (Novagen) or pcDNA3, respectively, and the resulting cDNAs were used for transformation or transfection experiments described below. The complete sequences were confirmed by automated sequencing.

Protein Expression—The D65A-ECD1 was expressed in Escherichia coli in minimal media containing 4 g/liter glucose and 1 g/liter [15N]ammonium sulfate, and protein purification was carried out as described (8, 9). Twenty liters of expression media yielded one sample of 15N-labeled D65A-ECD1 with a concentration of ~0.02 mM. The other mutant ECD1s were expressed similarly but were not isotopically labeled.

Receptor Expression in COS M6 Cells—cDNAs encoding full-length wild-type or mutant (myc)CRF-R2β were transiently transfected into COS M6 cells by DEAE-dextran method as described (2). For quantitation of receptor levels, 15 μg of cDNA were transfected into COS M6 cells in 6-cm dishes. The next day, the cells were trypsinized and replated into 12-well COSTAR plates. The following day, the cells were washed with Heps dissociation buffer (HDB), incubated for 1 h in 3% BSA/HDB, and then incubated with a monoclonal antibody against the Myc epitope anti-c-Myc (clone 9E10, IgG1) (1:100) diluted in 0.1% BSA/HDB for 60 min at room temperature. The cells were then washed with 0.1% BSA/HDB and incubated with horseradish peroxidase-coupled anti-mouse antibody in 0.1% BSA/HDB for 60 min at room temperature following the procedure for 1-Step Turbo TMB-enzyme-linked immunosorbent assay (Pierce).

Radioreceptor Assays—Crude membrane preparations from COS M6 cells were used to determine the binding of various CRF ligands using radiiodinated astressin as described (2). Binding to ECD1 or to mutants thereof was performed as described (8).

cAMP Accumulation—Intracellular cAMP accumulation was determined in COS M6 cells transiently expressing the receptors as described previously (4).

Immunohistochemistry—Following transfection of COS M6 cells with cDNAs encoding the receptors, the cells were plated on poly-l-lysine-coated coverslips in 24-well culture plates (~2 × 10⁵ cells/coverslip). After 24 h, the cells were incubated with mouse anti-c-Myc (clone 9E10, IgG1) (KPBS, 1% goat serum, 1% BSA for 24 h at 4 °C). Cells were washed three times for 10 min in KPBS and then incubated with Cy3-conjugated anti-mouse IgG (1:500, Jackson ImmunoResearch) in KPBS, 1% goat serum, 1% BSA for 60 min at room temperature. A Nikon E600 light microscope (Nikon, Tokyo, Japan) equipped with fluorescent optics was employed to visualize anti-c-Myc immunoreactive cells. All images were captured using a digital camera (Photometrics, Huntington Beach, CA) and Image-Pro Plus imaging software (Media Cybernetics, San Diego). The images were cropped and adjusted to balance brightness and contrast in Adobe Photoshop (version 5.5, Adobe Systems, San Jose, CA) before import into Canvas (version 6.0) for assembly into plates.

Mass Spectrometry—To determine the disulfide arrangement, purified proteins were digested with trypsin in 50 mM MES, pH 6.2. The low pH minimized disulfide exchange reactions, which are likely to occur at higher pH values (8, 9). The resulting digests, both crude and separated by HPLC, were analyzed by MALDI-mass spectrometry on an AB Voyager DESTR instrument employing α-cyano-hydroxycinnamic acid as the matrix. Disulfide connectivities were assigned based on the observation of masses corresponding to cystine-containing peptides.

Binding of 1-Anilino-8-naphthalenesulfonate (ANS) —For analysis of ANS binding, 150 μl of 20 μM ECD1, R101A-ECD1, or D65A-ECD1 in Tris-HCl, pH 6.5, were mixed with 4 μl of 3.2 mM ANS prepared in the same buffer. The final concentration of ANS was 80 μM. Fluorescence was measured immediately after addition of ANS. The measurements were made using a spectrofluorimeter (Photon Technology International, Lawrenceville, NJ) with excitation at 370 nm and emission in the range of 400–600 nm. A rectangular 10-mm quartz microcuvette was used.

NMR Spectroscopy—Two-dimensional NMR experiments were carried out either with 13N-labeled ECD1 (concentration 0.05 mm) or 15N-labeled D65A-ECD1 (concentration 0.02 mm). One-dimensional 1H NMR experiments of the various mutants (i.e. D65A, R101A, D65R/R101D) were measured with a typical concentration of 0.02 mM. The measurements were recorded at 25 °C on a Bruker 700-MHz spectrometer equipped with four radiofrequency channels and a triple-resonance cryo-probe with shielded z-gradient coil. Buffer conditions used were 10 mM Bis-Tris (HCl), 95% H₂O, 5% D₂O, pH 5. The following parameter settings were used for the [15N,1H] HMQC experiments (29): data size 150(1) × 1024(2) complex points; t₁ max (15N) = 56 ms, t₂ max (1H) = 75.6 ms, respectively. The data sets...
were zero-filled to 1024 × 2048 complex points. Prior to Fourier transformation, the data were multiplied with a 75° shifted sine bell window in both dimensions.

RESULTS

Structural Characterization of the Salt Bridge Asp\textsuperscript{65}–Arg\textsuperscript{101}—Fig. 1 shows a schematic representation of the NMR core structure of ECD1 from CRF-R2\beta as determined by Grace et al. (18), highlighting the salt bridge formed by Asp\textsuperscript{65} and Arg\textsuperscript{101} and the hydrogen bond between Asp\textsuperscript{65} and Trp\textsuperscript{71}. To address the structural and functional role of this internal salt bridge, we have introduced a series of mutations in the isolated ECD1 that should either disrupt the salt bridge, D65A or R101A, or “reverse” it, D65R/R101D.

The extent of the proposed structural distortion induced by the mutations was characterized using mass spectrometry, multiprobe NMR spectroscopy, and ANS binding. Previously, we found that in the wild-type ECD1s (9, 17) there is a unique disulfide pattern, namely disulfide bridges between the first and third, the second and fifth, and the fourth and sixth cysteine residues (Cys\textsubscript{1}–Cys\textsubscript{3}, Cys\textsubscript{2}–Cys\textsubscript{5}, and Cys\textsubscript{4}–Cys\textsubscript{6}). Mass spectrometric analysis of D65A-ECD1 was performed following trypsin digestion of the protein. The mass spectrum shows evidence for fragments containing Cys\textsuperscript{4} connected to Cys\textsuperscript{5} as well as to Cys\textsuperscript{6} (Table 1). An additional peptide fragment containing Cys\textsuperscript{5} in its reduced state is also identified. Two more peptide fragments containing reduced cysteines Cys\textsuperscript{5} and Cys\textsuperscript{6}, either separately or joined to each other, are observed. None of these peptide fragments contained cysteines Cys\textsuperscript{2}, Cys\textsuperscript{3}, and Cys\textsuperscript{4} because these are found in a large tryptic fragment that might not easily ionize under the conditions used.

The disulfide arrangement of the R101A mutant was determined in the same way as described for the D65A mutant. It was found that Cys\textsuperscript{2} and Cys\textsuperscript{5} were connected, as were Cys\textsuperscript{3} and Cys\textsuperscript{6}. The Cys\textsuperscript{4}–Cys\textsuperscript{6} linkage is identical to the one observed in the wild-type ECD1 (9, 17), whereas the Cys\textsuperscript{2}–Cys\textsuperscript{3} linkage is not. Cysteine 1 (Cys\textsuperscript{1}) could only be observed in its reduced state and not in connection with any other cysteine (Table 1). No peptide fragment containing Cys\textsuperscript{5} was observed in any of the fractions analyzed (Table 1).

TABLE 1

| Sequence | Cysteines connected | m/z (observed) | [MH\textsuperscript{+}] calculated |
|----------|---------------------|----------------|------------------------------------|
| Mutant D65A | 113–125 | 6 (reduced) (Cys\textsuperscript{10}–Cys\textsuperscript{18}) | 1532.6 (r) 1532.70 (m) |
| | 98–112 | 5 (reduced) (Cys\textsuperscript{30}–Cys\textsuperscript{39}) | 1769.6 (r) 1769.80 (m) |
| | 29–47 | 1 (reduced) (Cys\textsuperscript{30}) | 2423.8 (r) 2244.02 (m) |
| | 102–112 and 113–125 | 5 and 6 (Cys\textsuperscript{30}–Cys\textsuperscript{39}) | 2795.0 (r) 2795.26 (m) |
| | 98–112 and 113–125 | 5 and 6 (Cys\textsuperscript{30}–Cys\textsuperscript{39}) | 3299.2 (r) 3299.51 (m) |
| | 9–47 and 102–112 | 1 and 5 (Cys\textsuperscript{30}–Cys\textsuperscript{39}) | 3506.2 (r) 3506.58 (m) |
| | 29–47 and 113–125 | 1 and 6 (Cys\textsuperscript{30}–Cys\textsuperscript{39}) | 3773.3 (r) 3773.73 (m) |
| Mutant D65R/R101D (Fraction A) | 66–92 | 3 and 4 (Cys\textsuperscript{30}–Cys\textsuperscript{39}) | 2959.3 (r) 2958.44 (m) |
| | 48–65 and 113–125 | 2 and 6 (Cys\textsuperscript{30}–Cys\textsuperscript{39}) | 3537.6 (r) 3538.65 (m) |
| | 29–47 and 113–125 | 1 and 5 (Cys\textsuperscript{30}–Cys\textsuperscript{39}) | 3974.1 (r) 3972.3 (a) |
| Mutant D65R/R101D (Fraction B) | 66–92 | 3 and 4 (Cys\textsuperscript{30}–Cys\textsuperscript{39}) | 2962.1 (r) 2961.4 (a) |
| | 98–112 and 113–125 | 5 and 6 (Cys\textsuperscript{30}–Cys\textsuperscript{39}) | 3257.6 (r) 3258.44 (m) |
| | 48–65 and 113–125 | 2 and 5 (Cys\textsuperscript{30}–Cys\textsuperscript{39}) | 3735.1 (r) 3737.1 (a) |

For D65R/R101D-ECD1, two species were separable by reversed phase HPLC. These forms were analyzed individually by MALDI–mass spectrometry after trypsin digestion. The earlier eluting peak contained fragments indicating a unique disulfide arrangement: Cys\textsuperscript{1}–Cys\textsuperscript{3}, Cys\textsuperscript{2}–Cys\textsuperscript{5}, and Cys\textsuperscript{4}–Cys\textsuperscript{6}. The later eluting peak contained the fragment in which Cys\textsuperscript{3} and Cys\textsuperscript{4} were connected and additionally a fragment containing Cys\textsuperscript{2}–Cys\textsuperscript{5} and another containing Cys\textsuperscript{5}–Cys\textsuperscript{6} (Table 1). Thus, these data indicate a scrambled disulfide arrangement.

These findings are in contrast to our previous analyses (9, 17) of protein constructs corresponding to the ECD1s, which displayed unique patterns for disulfide connectivity with the absence of cysteines in their reduced state. Taken together, these data show that the cysteines are engaged in a variety of disulfide bonding patterns indicating the absence of a unique fold or three-dimensional structure. Only the R101A-ECD1 had one disulfide (Cys\textsuperscript{4}–Cys\textsuperscript{6}) that was found in the wild-type ECD1 (8, 9).

NMR analyses confirmed that the structures of the extracellular domain are disrupted in the mutant receptors. The \[^{15}\text{N},^{1}\text{H}]-\text{HMOCQ} spectrum, which is a fingerprint of the protein structure, was used to study the possible mutagenesis-induced structural perturbation. This spectrum for ECD1 (Fig. 2) shows a well dispersed distribution of cross-peaks of amide hydrogens and indoles representing a well folded domain. The middle region of the spectrum includes a set of sharp peaks in the “random coil” chemical shift region for the amide protons. These peaks have been assigned to the unstructured N- and C-terminal segments of the ECD1 comprising residues 16–42 and 122–133, respectively.

The \[^{15}\text{N},^{1}\text{H}]-\text{HMOCQ} spectrum of D65A-ECD1 is also shown in Fig. 2 (black) superimposed on the wild-type spec-
A dramatic difference between the spectra is evident. In contrast to the well dispersed wild-type spectrum, the peaks from D65A-ECD1 collapsed into the random coil chemical shift region indicating a large conformational change from the Sushi motif toward a random coil state. A more careful analysis indicates that only cross-peaks from the flexible N- and C-terminal segments in the wild-type ECD1 spectrum have their counterpart in the D65A-ECD1 spectrum. The remaining cross-peaks of D65A-ECD1 are broadened severely and are observed in the random coil region of the spectrum. Because of the tentative assignment of the flexible N- and C-terminal segments, these remaining peaks must be assigned to residues 45–118, which form the Sushi motif in wild-type ECD1. Of the 70 expected cross-peaks for the Sushi motif (74 residues minus 4 prolines), only ~35 are present suggesting that half of the resonances of the Sushi motif are not observed because of severe line broadening. Hence, the amino acid replacement D65A induces large chemical shift changes toward random coil values and are observed in the random coil region of the spectrum. Because of the tentative assignment of the flexible N- and C-terminal segments, these remaining peaks must be assigned to residues 45–118, which form the Sushi motif in wild-type ECD1. Of the 70 expected cross-peaks for the Sushi motif (74 residues minus 4 prolines), only ~35 are present suggesting that half of the resonances of the Sushi motif are not observed because of severe line broadening. Hence, the amino acid replacement D65A induces large chemical shift changes toward random coil values and line broadening for the amide protons of the Sushi domain. A possible explanation for this observed phenomenon is that the Sushi domain of D65A-ECD1 is largely destabilized into a metastable state comprising a variety of conformations, which exchange on the millisecond time scale. This molten globule-like state lacks the core structure of CRF-ECD1 as evidenced further by the random coil chemical shifts of the indole cross-peaks (Fig. 2). Although the destabilization of the core by the amino acid replacement D65A is predicted, the extent of destabilization is intriguing.

Similar observations were made for the ECD1 variants, R101A and D65R/R101D. The one-dimensional $^1$H NMR spectra of all the ECD1 variants show a collapsed spectrum, i.e. the indole cross-peaks have random coil chemical shifts, and the upfield-shifted methyl resonances for Ile$^{67}$ and Thr$^{69}$ of wild-type ECD1, indicative of a well folded protein construct, are not observed (Fig. 3). In addition, the side chain resonances of Trp$^{71}$ and Trp$^{109}$ in the R101A-ECD1 spectrum show significant line broadening compared with that of D65A-ECD1 and D65R/R101D-ECD1 spectra. This line broadening as well as the absence of amide protons in the structured region of the ECD1 suggests that the unfolded protein in R101A-ECD1 is also undergoing a conformational exchange. All these findings suggest that ECD1 variants D65A and R101A as well as the double mutant D65R/R101D do not have the same folding as the wild type.

Another measure of the structural changes resulting from the introduction of the mutations is the binding to ANS (31). ANS is a naphthalene dye that undergoes a dramatic increase in fluorescence intensity when it binds to hydrophobic regions of proteins. ANS is routinely used in protein folding studies to detect the solvent-exposed hydrophobic surfaces of partially folded intermediates. ANS binding results in an increase of ANS fluorescence intensity together with a blue shift of its emission maximum ($\lambda_{\text{max}}$). To provide further evidence for the existence of molten globular state of D65A-ECD1 and R101A-ECD1, ANS binding was studied. The spectra in Fig. 4 show that ECD1, R101A-ECD1, and D65A-ECD1 all bind ANS with an increase in ANS emission intensity and a blue shift of its emission maximum. The low or high wavelength emissions represent ANS bound to the molten globule state of the proteins and free or less bound ANS, respectively. However, the fluorescence intensity was strikingly more pronounced when ANS is bound to the R101A and D65A mutants compared with the wild-type ECD1. Moreover, the intensity of ANS fluorescence bound to D65A-ECD1 is 1.6 times greater than that of ANS bound to
R101A-ECD1. These data strongly suggest that both mutants are incorrectly folded and most likely in molten globule states. Furthermore, the data suggest that the structural change resulting from the mutation of the aspartic acid is more severe than that of the arginine mutation.

**Functional Characterization of the Salt Bridge Variants**—To address the functional roles of Asp65 and Arg101, the binding of the mutants was studied both in full-length receptor and in the isolated ECD1, accompanied by signaling studies in the full-length receptor. In the full-length receptor, the amino acid replacement D65A greatly impairs binding to astressin. As shown in Fig. 5A, there is no significant specific binding of astressin. In contrast, the replacement R101A has a relatively minor effect (Fig. 5B). Surprisingly, the double mutation D65R/R101D, which should reverse the direction of the salt bridge, does not restore the high affinity binding of astressin (data not shown). The inhibitory binding constants for astressin are 1.1(0.7–2.0) nM for (myc)CRF-R2β and 3.3(1.5–7.1) nM for R101A(myc)CRF-R2β, whereas the binding constants are indeterminate for D65A(myc)CRF-R2β and D65R/R101D(myc)CRF-R2β. Similarly, the binding studies of the isolated ECD1 show that the mutation D65A impairs the binding of astressin to the ECD1, whereas the mutation R101A reduces the binding affinity ~5-fold (Fig. 5, C and D).

The effect of the amino acid replacements on receptor signaling was determined by measuring the accumulation of intracellular cAMP in response to the family ligands sauvagine, and the urocrsins 1–3. As shown in Fig. 6B, the mutation D65A results in a shift to the right of the response curves compared with that of the wild-type receptor (Fig. 6A); the EC₅₀ values are 10–30 times greater for the mutant than for the wild-type receptor and are not a function of the level of receptor expression. The effects of the other mutations mirror their effects on...
the binding. Interestingly, the signaling capability of sauvagine, which does not bind to the isolated ECD1 (8), is also affected by the mutation D65A.

The fact that the mutant receptors are activated by the ligands indicates that the receptors are expressed at the cell surface. Further confirmation of the cell surface expression is the robust anti-Myc antibody staining observed for cells expressing the mutant full-length receptors (Fig. 7). The Myc epitope tag was inserted into the ECD1 of the full-length receptors (see “Materials and Methods”). The level of receptor expression for the wild-type receptor is similar to that of the D65A mutant and approximately twice that of the R101A mutant (Fig. 8).

DISCUSSION

Structure-Activity Relationships—Our analysis of the three-dimensional structure of the ECD1 of CRF-R2β identified the structure as a Sushi domain and showed that the structure is stabilized in part by a salt bridge between Asp^{65} and Arg^{101} (17, 18). Based on the closeness of the corresponding residues, a salt bridge (Asp^{59}–Arg^{95}) was also proposed in the NMR structure of the ECD1 of the pituitary adenylate cyclase-activating polypeptide receptor (20). The formation of such a salt bridge is also based on the fact that a free positively charged residue, such as Arg^{101}, in the hydrophobic core of the protein is not energetically favorable. In the crystal structure of the incretin receptor ECD1, these conserved residues, Asp^{66} and Arg^{101} are separated by 6 Å, and a water-mediated salt bridge was considered possible (21).

We have shown here that disruption of this salt bridge in the ECD1 of the CRF receptor has the following consequences: (i) partial or complete disruption of the unique disulfide pattern in the isolated ECD1; (ii) collapse of the well defined three-dimensional structure of the ECD1 into a molten globule-like conformation as analyzed by NMR and ANS binding; (iii) reduction in the affinity of astressin for both the full-length receptor as well as the isolated ECD1; and (iv) impaired ligand-stimulated activation of the full-length receptor.

Several interesting observations are worth mentioning. The double mutation, D65R/R101D, intended to reverse the salt
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The strictly conserved residues are indicated by their position in the ECD1 of CRF-R2β. The cysteines are numbered sequentially.

R101A mutation is more moderate compared with that of the D65A mutant compared with that of the R101A mutation. The difference in the degree of structural distortion caused by the two mutations is also reflected in the greater ANS binding of the D65A mutant compared with that of the R101A mutant. The ANS binding of both D65A and R101A mutants indicates that both are in molten globule states, but the lesser ANS binding of the R101A mutant suggests that it is closer to the wild-type structure and may be partially structured. Thus, in the NMR sample of R101A-ECD1, the molten globule and the partially structured ECD1 are present, undergoing a slow conformational exchange. In such a case, the peaks from the structured ECD1 in the spectrum are not observed because of severe line broadening, and the peaks from the unstructured molten globule tend to dominate the spectrum, resulting in a spectrum corresponding to random coil chemical shifts. The line broadening observed for the side chain indole peaks of Trp71 and Trp109 having random coil chemical shifts (Fig. 3C) confirm the slow conformational exchange. Although it might appear surprising that a misfolded protein is able to bind ligand, the enzymatic activity of a molten globule-like protein has been reported (31).

The effects of the salt bridge disruption are much greater in the isolated ECD1 than in the full-length receptor. Furthermore, sauvagine has moderately impaired signaling capability for the D65A/myc/CRF-R2β (Fig. 6), but it displays extremely low affinity binding to the isolated ECD1 of CRF receptors (8, 9) and to the ECD1 fused to various transmembrane domains (2, 7). The reason for these observations may be that the other domains of the receptor, which are known to contribute to the overall ligand recognition, may help stabilize the ECD1 and allow for receptor activation, although of reduced potency.

The Role of the Network Asp65–Arg101–Trp71–Trp109 in the Context of the B1 GPCR Family—Comparison of the sequences of the ECD1s from all 15 human B1 members shows a strict, selective conservation of structurally important amino acids, including the six cysteines, the salt bridge residues, two tryptophans, a glycine, and a proline (Fig. 9). Exploration of a wider array of 31 diverse invertebrate B1 members, including multiple insect, nematode, and sea urchin members, shows that these residues are invariant between these divergent phyla apart from a nematode clade lacking Trp71 (not shown). Accordingly, we proposed that all B1 ECD1s assume the same fold (17): the three-dimensional structures of the other two ECD1s (20, 21) strongly support the idea that the structure of the ECD1 of CRF receptors serves as a model for all the B1 receptor ECD1s.

The results obtained here are relevant to other B1 receptors because the equivalent residues in several other receptors have been shown to be important. For example, in the calcitonin receptor, the mutation of the equivalent Asp69 disrupts not only signaling but also the interaction of the receptor with the coreceptor RAMP1 (22). In the secretin receptor, the mutations D49R or R83D impair binding and signaling (23). In the VIP receptor, Asp68 is essential for binding VIP (26) and for maintaining the constitutive activity of the H178R mutation (24). In the glucagon receptor, mutation of Asp68 abrogates ligand binding (27). Finally, the mutation underlying the little mouse was shown to be a D60G substitution in GHRH receptor. This mutation seriously disrupts GHRH binding and signaling, resulting in a hypoplastic pituitary, defective GHRH-stimulated signal transduction, and a dwarf phenotype (25). We suggest that this is an example of a “molecular structural disease” arising from a mutation that disrupts the three-dimensional structure of the major ligand recognition site. It is also possible that

bridge, does not restore either the folding or the binding. This observation may be understood by examining the complex network of interactions between Asp65, Trp71, and Arg101. Asp65 not only forms the salt bridge to Arg101 but is also involved in a hydrogen bond to Trp71 (Fig. 1). The same interaction between the side chains of the corresponding residues Asp66 and Trp71 was reported in the crystal structure of the incretin receptor ECD1 (21). Reversing the salt bridge in the CRF receptor by the replacements D65R/R101D therefore does not restore the hydrogen bond between Asp65 and Trp71 and hence does not restore the folding or ligand recognition.

Although both D65A and R101A mutations disrupt the ECD1 fold, the R101A variant in both the ECD1 and the full-length receptor binds the ligand astressin with nearly the wild-type affinity. (The same sample of R101A-ECD1 was used for both the NMR analysis and the binding studies.) This discrepancy between structure and activity requires an explanation. Based on the three-dimensional structure, it is suggested that the replacement D65A disrupts the salt bridge to Arg101, important for the fold, as well as for the hydrogen bond to Trp71. The latter interaction is important in the local arrangement of β-sheet 1 and for the formation of the correct geometry of the adjacent loop comprising residues Asp65–Trp71. This loop includes the residues Gln66 and Ile67 that are directly involved in ligand binding. Similar interactions between Asp66 and Met67 and Tyr68 and Val69 were described in the incretin ECD1 crystal structure (21). Hence, we propose that the D65A replacement results in both loss of structure and binding. In contrast, the mutation R101A disrupts the salt bridge, which is deleterious for the overall fold, but does not interfere with the local geometry around loop residues 66–68 and does not interfere with the disulfide formation between Cys4 (Cys84) and Cys6 (Cys118), as evidenced by the mass spectrometry data (Table 1). Presence of this disulfide bridge is crucial in maintaining the structure of loop 2, which is intimately involved in the ligand binding. Thus, the effect on ligand binding of the R101A mutation is more moderate compared with that of the D65A mutation.

The difference in the degree of structural distortion caused by the two mutations is also reflected in the greater ANS binding of the D65A mutant compared with that of the R101A mutant. The ANS binding of both D65A and R101A mutants indicates that both are in molten globule states, but the lesser ANS binding of the R101A mutant suggests that it is closer to the wild-type structure and may be partially structured. Thus, in the NMR sample of R101A-ECD1, the molten globule and the partially structured ECD1 are present, undergoing a slow conformational exchange. In such a case, the peaks from the structured ECD1 in the spectrum are not observed because of severe line broadening, and the peaks from the unstructured molten globule tend to dominate the spectrum, resulting in a spectrum corresponding to random coil chemical shifts. The line broadening observed for the side chain indole peaks of Trp71 and Trp109 having random coil chemical shifts (Fig. 3C) confirm the slow conformational exchange. Although it might appear surprising that a misfolded protein is able to bind ligand, the enzymatic activity of a molten globule-like protein has been reported (31).
Role of Residues in ECD1 of Receptors for CRF and B1 GPCRs

the mutation in the GHRH receptor results in incorrect processing and surface expression, although this possibility is unlikely because the data on cell-surface expression (Figs. 7 and 8) show that the mutation does not prevent transport to the cell surface. Similarly, studies on the expression of the mutant VIP (26) and glucagon (27) receptors have shown that these receptors are expressed at the cell surface. Of course, refolding and cell surface expression may be different in vivo.

The major effects of the mutations in these B1 GPCRs may now be understood as a consequence of the lack of integrity of the three-dimensional structure of the ECD1, leading to a disruption of the major ligand binding domain. The results presented here also underscore the Sushi motif of the ECD1 as a multiple protein interaction module. It binds not only peptide ligands but also interacts with other proteins, as evidenced by the lack of RAMP1 interaction with a mutated Asp69 calcitonin receptor (22). The interaction between CRF-R2β and ErbB2 (32) may also depend on the ECD1s of the receptors. Furthermore, the observations that a mutation in the VIP receptor ECD1 (Asp68) impacts the constitutive signaling of the juxtamembrane domain of the receptor and that mutations in the ECD1 of the CRF receptor affect sauvagine signaling suggest that there may be an interaction between the ECD1 of the receptor and the juxtamembrane domain.

In conclusion, we have shown that the changes in the three-dimensional fold of the receptor resulting from mutations in the ECD1 may produce multiple effects on the binding and activation of the receptors; the effects can be understood only through a detailed description of the three-dimensional structure and of the residues responsible for the ligand-receptor interaction. The structure-function relationships proposed here concerning a structurally important salt bridge and, in particular, the importance of a strictly conserved aspartic acid residue in the ECD1 of receptors in the B1 GPCR family rationalize many observations and highlights, in parallel, the complex nature between structure and function for the B1 family of receptors.

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