Extracellular Cysteines of the Corticotropin-Releasing Factor Receptor Are Critical for Ligand Interaction†

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ABSTRACT: The corticotropin-releasing factor receptor (CRF-R) contains six conserved cysteines in its amino-terminal domain (C30, C44, C54, C68, C87, and C102) and one cysteine in its first and second extracellular loops (C188 and C258, respectively). Additionally, several other cysteines are located in the transmembrane domains (C128, C211, C233, and C364) and first intracellular loop (C150). Reduction of disulfide bonds with DTT decreased CRF binding to detergent-solubilized membranes, suggesting an important role for disulfide bonds in ligand recognition. Therefore, site-directed mutagenesis was used to introduce single and paired Cys (C) to Ser (S) or Ala (A) mutations. A silent nine amino acid tag from *c myc* was introduced in the amino terminus of the mouse CRF-R. With the exception of C258S and C188S/C258S mutations, all C to S or to A receptor mutants had good surface expression that was at least 52.5% of control. C30S, C54S, and C30S/C54S mutations had good CRF binding and CRF-stimulated cAMP accumulation. No CRF binding was detected for the C44S, C68S, C87S, C102S, C188S, C258S, C30S/C44S, C30S/C68S, C54S/C68S, C87S/C102S, and C188S/C258S mutants, while CRF-stimulated cAMP accumulation occurred with high EC_{50} values. In particular, receptors carrying double mutations, C44S/C102S and C68S/C87S, had an improved signaling property as compared to receptors carrying the respective single cysteine mutations. These data, together with the effects of DTT on CRF binding, indicate that disulfide bridges are important for receptor functions. Functional data from single and paired cysteine mutations suggest potential pairings between C44 and C102, C68 and C87, and C188 and C258 that are critical for ligand−receptor interactions.

Corticotropin-releasing factor (CRF) (Vale et al., 1981) stimulates the secretion of ACTH in the anterior pituitary gland through binding to specific receptors. The CRF receptor belongs to a large family of proteins which have seven hydrophobic, putative membrane-spanning domains and which interact with cellular effector systems via guanine nucleotide binding proteins (G proteins). Two distinct subtypes of CRFRs, designated CRFR1 and CRFR2, along with a number of alternatively spliced variants, have been cloned from human (Chen et al., 1993; Liaw et al., 1996), rat (Perrin et al., 1993, 1995; Lovenberg et al., 1995), mouse (Vita et al., 1993; Stenzel et al., 1995; Xiong et al., 1995), and chicken (Yu et al., 1996). The CRF receptors have been shown to activate two signaling pathways: adenylate cyclase (Labrie et al., 1982; Labrie et al., 1982a,b, 1983) and phospholipase C (Xiong et al., 1995).

The CRFRs share significant sequence homology (42−57% similarity) with the members of the newly recognized R7G2 family of receptors (Tsai-Morris et al., 1996). A key structural feature of this receptor family is the conserved arrangement of six cysteine residues in the extracellular domain. Additionally, there are two other cysteine residues in its first and second extracellular loops; these two cysteines are conserved among nearly all G protein coupled receptors. At least some of the conserved cysteines, if not all, may participate in disulfide bond linkages which may play an important role in receptor function.

Studies of other G protein-coupled receptors have demonstrated that conserved cysteines play a critical role in receptor function. Extracellular cysteines were shown to be involved in β-adrenergic receptor (βAR) activation and ligand binding (Fraser, 1989). The high-affinity state of the β_2AR requires unique interaction between conserved and nonconserved extracellular cysteines (Noda et al., 1994). Moreover, cysteine residues in the seventh transmembrane helix of the muscarinic acetylcholine receptor (m1 mAChR) may influence agonist binding and the efficiency of receptor activation (Savarese et al., 1992). Biochemical studies had shown that a disulfide bond between conserved extracellular cysteines in the thyrotropin-releasing hormone (TRH) receptor is critical for ligand binding (Perlman et al., 1995). On the other hand, intracellular conserved cysteine residues may play a role in receptor expression (Zhu et al., 1995), turnover, and localization (Kennedy & Limbird, 1993).

The role of the cysteines residues in the overall structure and function of the CRF receptor family has not yet been studied. The extracellular cysteines are likely to be involved in disulfide bridges that maintain the structure of the extracellular domain. However, cysteine pairing of the R7G2 receptor family is unknown and is a difficult problem to address. We are approaching this problem by comparing ligand binding and CRF-stimulated cAMP levels of CRF

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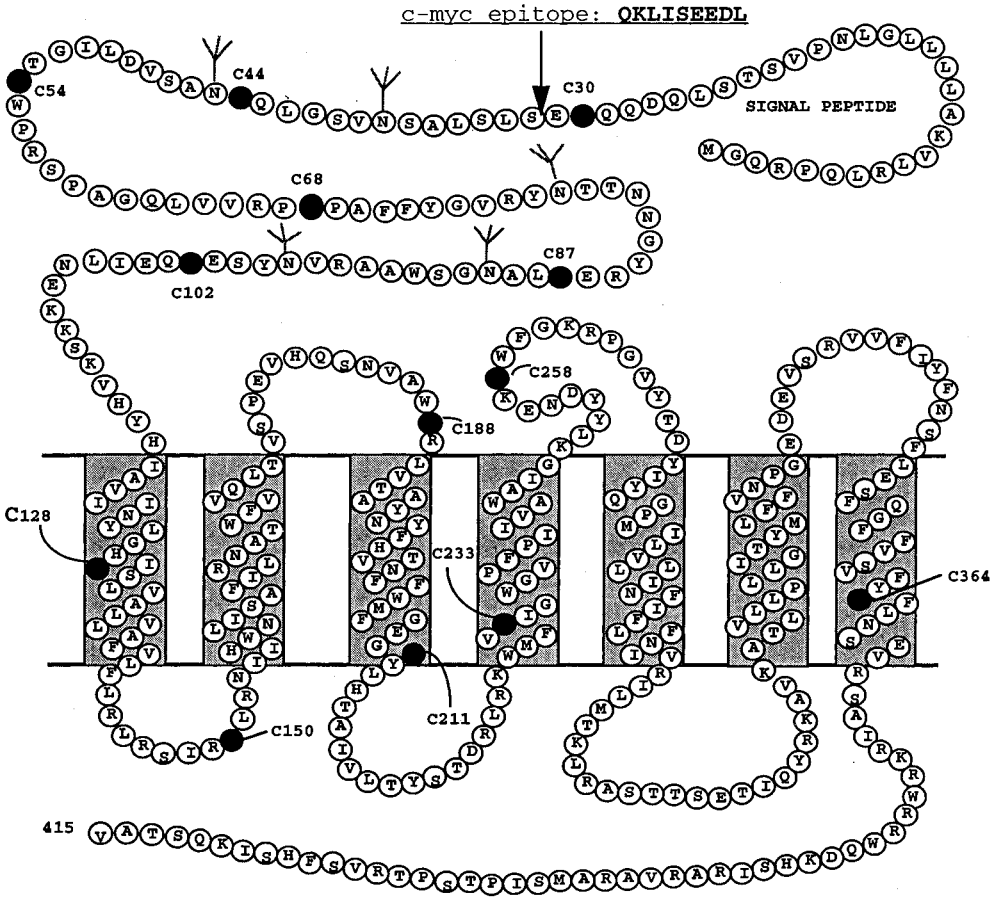

FIGURE 1: Schematic illustration of mouse CRF receptor map shows the cysteine residues (black spot) throughout the receptor molecule and the position of *c-myc* epitope insertion.

receptors with single or paired mutations of extracellular cysteines. If the EC_{50} and/or ligand binding affinity and capacity are not deteriorated, or rather rescued by a paired mutation, then the two cysteines are likly to be part of the same disulfide bridge. We have identified some cysteine residues that can be mutated without causing a complete loss of the binding and the signaling properties. Additionally, some paired cysteine mutations resulted in improved receptor functions as compared to the functional properties of receptors with single cysteine mutations. The data indicate that at least three extracellular disulfide bond(s) is (are) essential for CRF receptor interaction.

EXPERIMENTAL PROCEDURES

Preparation of Detergent-Solubilized Receptor Protein and Treatment with Dithiothreitol (DTT). Detergent-solubilized cell extracts were prepared based on a modified method (Liu et al., 1995). Transfected COS-7 cells were chilled on ice for 30 min, and disrupted in buffer A (150 mM NaCl, 20 mM HEPES, pH 7.4, containing 1 μM leupeptin, 1 μM pepstatin, 0.1 mg/mL aprotinin, 3 mM EGTA, 10 mM phenylmethanesulfonyl fluoride, 10 mM EDTA, and 5 mM *N*-ethylmaleimide) with a polytron Tissuemizer. The cell extract was centrifuged at 100*g* for 15 min at 4 °C, and the supernatant was pelleted by centrifugation at 47000*g* for 40 min. The membrane pellet was resuspended in buffer B (buffer A containing 0.5% NP-40, 40% glycerol, and protease inhibitors), by 10 strokes using a glass homogenizer. The detergent-solubilized extracts were then incubated for 2 h at room temperature in the presence or absence of the desired concentration of DTT.

^{125}I-CRF Binding to Detergent-Solubilized Membranes. The detergent-solubilized membranes (100 μg/tube) were incubated with rat/human (r/h) CRF (10^{-11}—10^{-6} M) and ^{125}I-r/hCRF (Du Pont/New England Nuclear Co., Boston, MA) (100 000 cpm/tube) overnight at 4 °C. Two hundred microliters of charcoal solution (6.25 mg/mL charcoal and 6.25 mg/mL dextran in sodium phosphate buffer, pH 7.4) was added; the samples were vortexed and incubated at 4 °C for 7 min, and centrifuged for 20 min at 12 000 rpm in a microcentrifuge. The supernatant was then transferred into tubes, and the radioactivity was measured in a gamma counter.

Construction of Epitope-Tagged Mouse (m) CRFR (ECR) and CRFR Mutants. To select a "silent" region within the CRFR extracellular amino terminus, we compared the sequences of the CRFR with those of the PTH/PTHrP receptor, a member of the CRFR family which has an extracellular exon (exon E2) that is tolerant for mutation and deletion and that is absent in other members of this receptor family (Lee et al., 1994). Therefore, we selected a site within the CRF receptor extracellular domain that corresponds to the insertion site of the "tolerant" sequences of the PTH/PTHrP receptors. Nine amino acid residues (EQKLISEEDL) from the *c myc* epitope were inserted between residues Glu31 and Ser32 within the extracellular domain of the mouse CRF receptor (Figure 1).

Site-Directed Mutagenesis. ECR was used for single-strand plasmid preparation. All the receptor mutations were then created on the ECR backbone by site-directed mutagenesis according to the method of Kunkel (1985). Mutants were confirmed by sequence analysis.

Cell Culture and Receptor Expression. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Ninety percent confluent cells were transfected with mutant and wild-type (ECR) receptor cDNAs using the DEAE-dextran method (Xiong et al., 1995). Three days after transfection, the cells were tested for receptor expression on the cell surface by double antibody assay, CRF-stimulated cAMP accumulation, and CRF radioligand binding.

Antibody Binding to Epitope-Tagged Receptors. Ascites fluid was developed in pristane-primed Balb-c mice (Charles River Laboratories, Wilmington, MA) by injecting 9E10 hybridoma cells, obtained from American Type Tissue Culture (ATTC), in the peritoneal cavity. Transfected COS-7 cells were rinsed with phosphate-buffered saline (PBS, pH 7.40) containing 5% heat-inactivated fetal bovine serum and incubated with the monoclonal antibody 9E10 in ascites fluid (1:1000). After 2 h of incubation at room temperature, the cells were rinsed 3 times to remove excess unbound antibody, ^{125}I-labeled sheep anti-mouse immunoglobulin G (Du Pont/New England Nuclear Co., Boston, MA) was then added (200 000 cpm/well), and the incubation was continued for an additional 2 h at room temperature. At the end of the second incubation, the cells were rinsed with PBS ($\times$3) and lysed with 1 N NaOH (750 μL). The lysates were collected and counted in a Micromedic gamma counter.

^{125}I-CRF Binding to Intact Cells. CRF binding assays were performed as previously described (Xiong et al., 1995). Briefly, intact COS-7 cells in 24-well plates were rinsed with binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 5% heat-inactivated horse serum containing 0.5% heat-inactivated fetal bovine serum, 0.1% bovine serum albumin, and 0.1% bacitracin) and incubated with ^{125}I-r/hCRF (100 000 cpm/well) in the presence of increasing concentrations of unlabeled r/hCRF (0, 0.01, 1.0, 3.0, 10, 30, 100, and 1000 nM) at room temperature for 2 h. At the end of the incubation period, the cells were rinsed 3 times with binding buffer and lysed with 1 N NaOH. The lysates were collected and counted for radioactivity.

CRF-Stimulated cAMP Accumulation. Intact COS-7 cells in 24-well plates, transfected with the CRF receptor constructs, were chilled on ice for 30 min, rinsed with ice-cold PBS, and challenged with r/hCRF in DMEM containing 2 mM 3-isobutyl-1-methylxanthine (IBMX), 1 mg/mL BSA, and 35 mM HEPES, pH 7.4. The cells were then incubated at 37 °C for 15 min. The supernatant was removed, and the cells were rapidly frozen by placing the plates on dry ice for 10 min. Intracellular cAMP was extracted by thawing the cells in 50 mM HCl (1.0 mL). An aliquot of the acid extract was diluted (1:100) in sodium acetate buffer (50 mM, pH 5.5), and the cAMP content was determined by RIA (Abou-Samra et al., 1987).

RESULTS

Biochemical Evidence for Disulfide Bonds within the CRF Receptor. To examine the possibility that disulfide bonds in the CRF receptor were important for function, we tested the effects of DTT, a strong disulfide bond reducing agent, on radiolabeled CRF binding to detergent-solubilized CRF receptors. Treatment of solubilized COS-7 cell membranes with DTT decreased ^{125}I-CRF binding in a dose-dependent manner. Specific binding was reduced to 82% and 25% of control in membranes treated with 5.0 mM and 50.0 mM DTT, respectively (Figure 2). However, Scatchard analysis indicated that the apparent binding affinity of CRF was not significantly affected by DTT treatment (K_d = 8.0 nM). These data thus suggest that the CRF receptor contains disulfide bonds that are important for maintaining proper receptor structure and/or forming a binding pocket that is critical for interactions with the hormone.

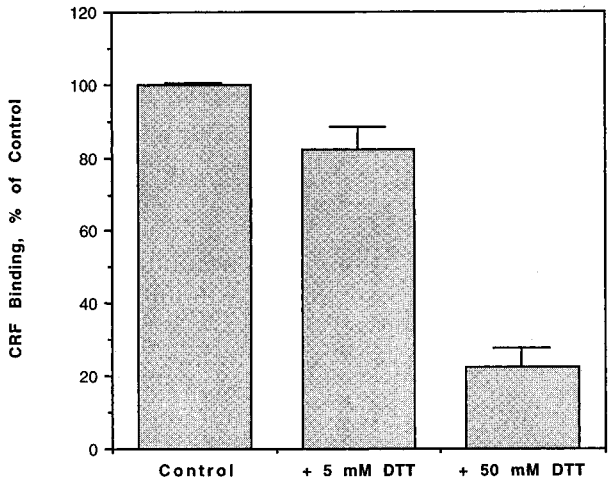

FIGURE 2: Effects of DTT on CRF binding. CRF binding to DTT-treated detergent-solubilized CRF receptor protein was measured and expressed as a percentage of control. Data are means ± SD of triplicates, in a representative of at least three independent experiments.

To assess the surface expression of the receptor mutants, we constructed a *c myc* epitope-tagged CRF receptor, henceforth named ECR, that has binding and signaling properties that were similar to that of the unmodified receptor (Figure 3). Cell surface expression of the mutant receptors and ECR was thus determined by a double-antibody binding assay performed on intact COS-7 cells. All the mutations were thus constructed on the ECR backbone, and the properties of each mutant receptor were determined and compared to those of ECR.

Properties of Cysteine to Serine Mutations within the Amino-Terminal Domain. All the mutant receptors carrying cysteine to serine mutations of the amino-terminal domains had an expression level that was at least 52% of that of ECR (Table 1). Among these mutations, only C30S and C54S mutant receptors bound the CRF radioligand. CRF binding to C30S and C54S was 40% and 90% of control, respectively (Table 1). The apparent binding affinity to C30S and C54S mutant receptors was similar to that of ECR (Table 1 and Figure 4A,B). We then compared the binding capacity and affinity and the EC_{50}s of CRF-stimulated cAMP accumulation among single and double mutant receptors. In this regard, double mutations of C30S and C54S (C30S/C54S) did not further impair CRF binding; maximal CRF binding and CRF binding affinity to the C30S/C54S were similar to the respective values of the C54S single mutant receptor (Table 1 and Figure 4A,B). Since single mutations of C44, C68, C87, and C102 completely abolished CRF binding but did not impair expression (Table 1), the adverse effects of these four mutations on receptor function could involve disruption of disulfide bonds.

To assess the signaling properties of the mutant receptors, transfected COS-7 cells were exposed to increasing concentrations of CRF, and intracellular cAMP accumulation in the presence of IBMX was measured. Maximum cAMP stimu-

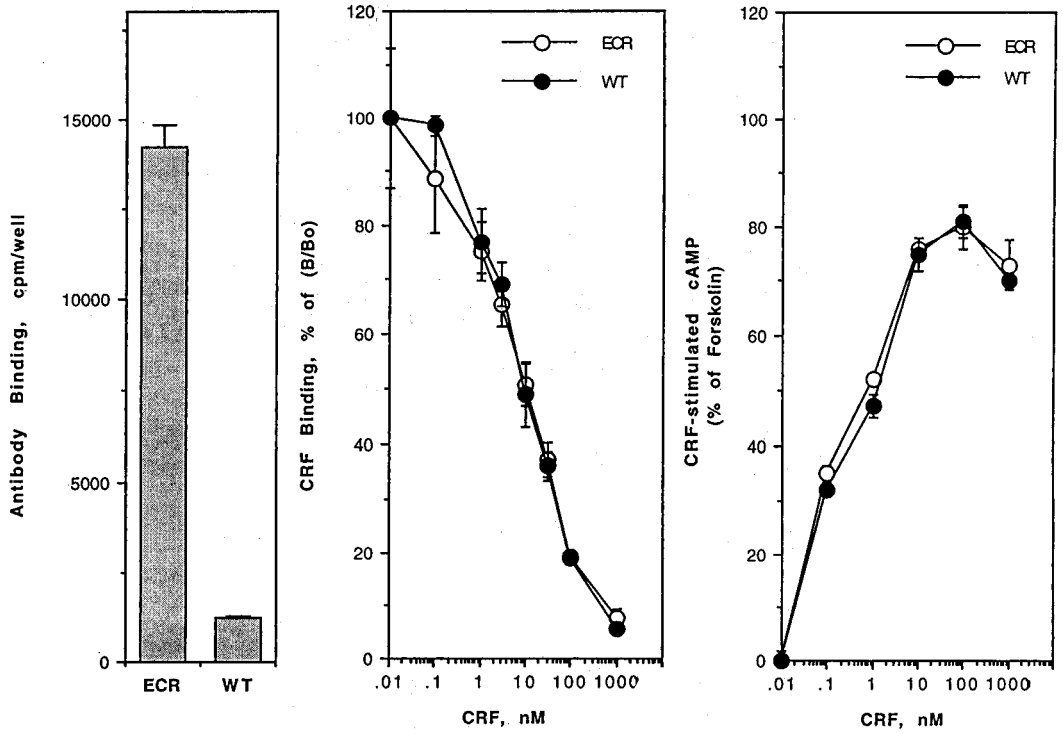

FIGURE 3: Comparison between the functional properties of the wild-type mouse CRF receptor and the epitope-tagged mouse CRF receptor (ECR) is shown. Left panel: double antibody binding to intact cells using 9E10 as a first antibody followed by ^{125}I-CRF binding. Right panel: CRF-stimulated cAMP accumulation. Data are means ± SD of triplicates, in a representative of at least three independent experiments.

Table 1: Properties of CRF Receptors with Mutations of the Amino-Terminal Cysteines

receptor	expression (% ECR)	CRF binding		CRF-stimulated cAMP accumulation		
		% ECR	K_d (nM)	basal (pmol)	EC_{50} (nM)	% ECR (wt)
ECR	100 ± 2.13	100 ± 2.30	9.6 ± 0.80	8.1 ± 0.16	3.0 ± 1.2	100 ± 3.2
C30S	67.5 ± 2.60	35.3 ± 2.48	10 ± 1.10	8.1 ± 0.21	0.1 ± 0.02	77.9 ± 4.33
C44S	67.2 ± 2.03	0.8 ± 0.35	ND[a]	8.4 ± 0.11	>1000	1.9 ± 0.23
C54S	150.4 ± 8.71	91.2 ± 1.73	10.6 ± 1.70	8.2 ± 0.02	1.0 ± 0.05	75.4 ± 1.70
C68S	62.0 ± 2.84	1.7 ± 0.99	ND	7.9 ± 0.20	1000	42.2 ± 4.20
C87S	62.4 ± 0.60	6.7 ± 1.38	ND	8.0 ± 0.12	1000	36.2 ± 0.03
C102S	52.5 ± 2.78	7.2 ± 0.39	ND	8.3 ± 0.14	>1000	3.1 ± 0.01
C30S/C44S	84.8 ± 1.47	1.2 ± 0.25	ND	8.0 ± 0.02	1000	60.4 ± 3.50
C30S/C54S	84.6 ± 2.10	90.1 ± 4.30	9.4 ± 0.70	8.5 ± 0.09	<0.1 ± 0.01	67.9 ± 0.13
C30S/C68S	68.1 ± 6.75	3.2 ± 0.83	ND	8.0 ± 0.10	>1000	64.3 ± 0.08
C44S/C68S	69.3 ± 2.82	1.2 ± 1.00	ND	8.2 ± 0.08	>1000	4.5 ± 0.02
C44S/C87S	60.0 ± 0.48	1.6 ± 0.20	ND	8.0 ± 0.06	>1000	34.6 ± 1.34
C44S/C102S	64.1 ± 0.52	0.2 ± 0.02	ND	8.3 ± 0.14	50.0 ± 6	53.1 ± 2.52
C68S/C87S	64.5 ± 0.52	1.2 ± 0.04	ND	8.2 ± 0.11	750 ± 25	67.7 ± 2.61
C68S/C102S	62.3 ± 0.50	1.6 ± 0.21	ND	8.1 ± 0.09	>1000	45.0 ± 5.20
C87S/C102S	69.3 ± 2.82	1.0 ± 0.01	ND	8.0 ± 0.10	>1000	3.9 ± 0.24

[a] ND, not detected.

lation in C30S was slightly decreased; this decrease reflects the low expression of this mutant (Table 1). In contrast, C54S mutation increased expression to 150% of control and decreased maximum cAMP stimulation to 75% of control (Table 1). Double mutations of C30S/C54S increased the sensitivity of cAMP stimulation by CRF with an EC_{50} that was less than 0.1 nM; maximal CRF-stimulated cAMP accumulation of the double mutant receptor was not different from those of C30S or C54S single mutations (Table 1).

Single mutations of C44S or C102S led to complete loss of ligand-stimulated cAMP accumulation (Table 1), while C44S/C102S combined mutations rescued the receptor response to CRF stimulation with a maximum response that was 53% of that of the ECR and an EC_{50} of about 50.0 nM (Table 1). The remaining possible pairwise combinations that may occur between C44S or C102S and the other C to S mutations (C30S/C44S, C44S/C68S, C44S/C87S, C68S/C102S, and C87S/C102S) showed an EC_{50} for CRF stimulation that was higher than 1000 nM (Table 1).

The remaining cysteines in the amino-terminal domains are C68 and C87. When both cysteines were singly mutated to serines, CRF-stimulated cAMP accumulation of the receptor mutants was 42% and 36% of the control values, respectively, with an EC_{50} of 1000 nM (Table 1). However, when these two cysteines were mutated together, the effect was not additive. Instead maximum CRF-stimulated cAMP accumulation was increased to 68% of control, and the EC_{50} of CRF was improved to 700 nM, respectively (Table 1).

Properties of Cysteine to Serine Mutations within the Extracellular Loops. Mutant receptors carrying C258S or C188S/C258S had only 35% and 20% of the ECR expression, respectively. However, when C258 mutated to alanine in these receptors, the expression was markedly improved. The C258A and C188S/C258A mutant receptors had expres-

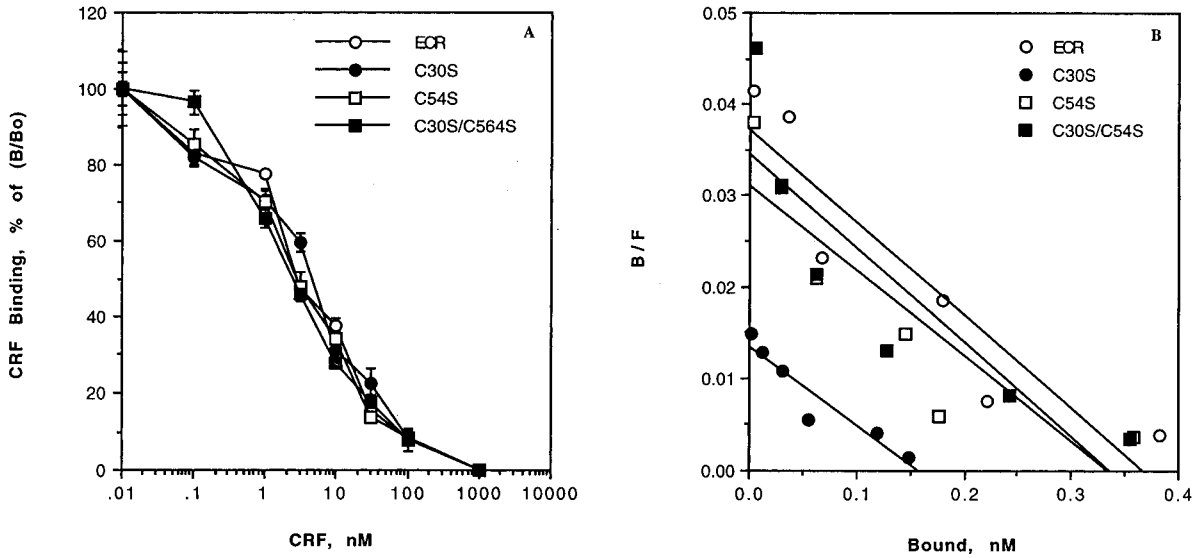

FIGURE 4: CRF binding properties of some mutant receptors. Competition binding (A) and Scatchard analysis (B) for C30S, C54S, and C30S/C54S, respectively. Data are means ± SD of triplicates, in a representative of at least three independent experiments.

Table 2: Properties of CRF Receptors with Mutations of the First and Second Extracellular Loop Cysteines

			CRF-stimulated cAMP accumulation		
receptor	expression (% ECR)	CRF binding (% ECR)	basal (pmol)	EC_{50} (nM)	% ECR (wt)
ECR	100 ± 2.10	100 ± 2.20	7.9 ± 0.02	3.0 ± 1.2	100 ± 2.20
C188S	117 ± 3.63	6.9 ± 0.92	8.2 ± 0.02	>1000	22.2 ± 0.42
C258A	72.7 ± 0.58	4.3 ± 1.30	8.3 ± 0.04	2.0 ± 0.06	35 ± 0.34
C188S/C258A	74.1 ± 0.60	3.1 ± 0.40	8 ± 0.01	2.0 ± 0.03	53.3 ± 2.51

Table 3: Properties of CRF Receptors with Mutations of the Transmembrane and Intracellular Cysteines

		CRF binding		CRF-stimulated cAMP accumulation		
receptor	expression (% ECR)	% ECR	K_d (nM)	basal (pmol)	EC_{50} (nM)	% ECR (wt)
ECR	100 ± 3.70	100 ± 2.20	9.4 ± 1.06	7.9 ± 0.01	7 ± 1.10	100 ± 5.23
C128S	99.8 ± 6.50	124.2 ± 2.96	12.3 ± 1.32	8.2 ± 0.01	10.2 ± 0.50	100.9 ± 4.37
C150S	113 ± 4.90	108.5 ± 1.31	10.1 ± 1.40	8.1 ± 0.01	10.7 ± 0.62	107.4 ± 5.10
C211S	90.2 ± 4.03	100 ± 5.22	11.5 ± 0.72	11.2 ± 0.02	6.5 ± 0.03	112.5 ± 4.24
C233S	64.5 ± 1.33	69.8 ± 6.5	10.2 ± 1.10	7.9 ± 0.01	10.6 ± 1.15	71.4 ± 6.23
C364S	131.2 ± 6.1	130.3 ± 5.09	9.8 ± 1.12	7.3 ± 0.04	10.2 ± 0.55	96.5 ± 4.30

sion levels that were 73% and 74% of control, respectively (Table 2). C188 and C258 in the first and second extracellular loops, respectively, are noteworthy because cysteines at the corresponding positions are present in all GPCRs (Dohlman et al., 1990). Moreover, it was recently reported that C98 and C179 in the TRH receptor, which corresponds to C188 and C258 of the CRF receptor, respectively, are involved in disulfide bond formation (Perlman et al., 1995; Cook et al., 1996). In our study, the C188S and C258A mutant receptors had low CRF binding, although a high level of cell surface expression (117% and 72.7% of control, respectively) was observed (Table 2). Similarly, the combined C188S/C258A mutation had good expression (74.1% of control) and a low CRF binding (Table 2).

In contrast to its high level of expression, the C188S receptor mutant had very low response to CRF: maximal stimulation was only 23% of control, and the EC_{50} was higher than 1000 nM (data not shown). The C258A mutation was more tolerated with a maximal stimulation that was 35% of control and an EC_{50} that was 2 nM (Table 2). Interestingly, the adverse effects of the C188S mutation were reversed by the C258A mutation in the C188S/C258A double mutant receptor: maximal stimulation was 53.3% of control, and the EC_{50} was 2 nM (Table 2).

Properties of Cysteine to Serine Mutations within the Transmembrane and Intracellular Domains. Receptors having cysteine to serine mutations within the transmembrane and cytoplasmic regions had higher expression levels than those of the receptors carrying cysteine to serine mutations in the extracellular domains (Table 3). Since cysteines within these regions are conserved among CRF receptor subtypes, it was speculated that these cysteine residues may play an important role in CRF binding and signaling. Surprisingly, all the cytoplasmic and intracellular cysteine mutations showed binding properties that were indistinguishable from those of the ECR (Table 3). The low CRF binding observed in the C233S mutation is concordant with its low surface expression (Table 3). Furthermore, cysteine mutation within these regions did not cause significant changes in the sensitivity of CRF-stimulated cAMP accumulation. CRF receptors carrying C128S, C150S, C211S, or C364S mutations showed CRF-stimulated cAMP accumulation that was similar to that of ECR with similar maximum and EC_{50} (Table 3). C233S had a low maximal cAMP response to CRF (70% of control) that was concordant with its expression and CRF binding levels (64% and 70% of control values, respectively, Table 3).

DISCUSSION

Disulfide bonds are essential for maintaining the secondary/tertiary structure of many membrane proteins and cytokines and growth factor receptors (Sorokin et al., 1994). A disulfide bond that connects the first and second extracellular loops has been shown to occur in rhodopsin (Karnik & Khorana, 1990) and has been suggested by mutational analysis of many other G protein-coupled receptors (Fraser, 1989; Dohlman et al., 1990; Savarese et al., 1992; Perlman et al., 1995). Our data with paired mutation of the cysteine residues in the first and second extracellular loops of the CRFR do not contradict the hypothesis that the first and the second extracellular loops of the CRFR are also connected together with a disulfide bond.

The amino-terminal extracellular extension of the R7G2 receptors contains two structural features that distinguish the R7G2 receptor family from all other G protein-coupled receptors. The amino terminus is intermediate in length when compared to the extensive extracellular termini of the glycoprotein hormone receptors or to the very short amino termini of the catecholamine receptors. Additionally, the amino terminus of the R7G2 receptors contains six cysteine residues that are highly conserved among all the members of this receptor family. Interestingly, all the ligands of the R7G2 receptors are polypeptides of an intermediate length. Therefore, ligand−receptor interaction may require a common structural organization of the extracellular domains that involve the highly conserved cysteines.

Our data that reduction of the disulfide bonds with DTT decreased CRF binding in a dose-dependent manner clearly indicate an important role for disulfide bridges in ligand interaction. The decrease in ligand binding capacity without changing ligand binding affinity suggests that DTT causes a dose-dependent appearance of a population of "reduced" CRF receptors that completely lose their ligand binding capacity whereas the remaining receptor population maintains normal ligand binding properties. In agreement with our data, Karpf et al. (1991) have shown that treatment of native PTH/PTHrP receptors with DTT decreased ligand binding without modifying the apparent binding affinity. These data indicate that intramolecular disulfide bonds are critical for ligand recognition in the CRF/PTH receptor family. For comparison, treatment of the β_2-adrenergic receptor (Dohlman et al., 1990) and thyrotropin-releasing hormone receptor (Perlman et al., 1995; Cook et al., 1996) with DTT decreased both ligand binding and apparent binding affinity. Taken together, these data indicate that disulfide bonds may have distinct functional roles in different G protein-coupled receptor families.

All the cysteine residues found in the extracellular domains of the CRFR are well conserved in this receptor family. Lee et al. (1994) used site-directed mutagenesis to study the role of the disulfide bonds in the function of the PTH/PTHrP receptor, a member of the CRF receptor family (Juppner et al., 1991; Abou-Samra et al., 1992). All six cysteine to serine mutations in the amino-terminal domain of the PTH/PTHrP receptor caused a dramatic impairment of receptor expression. The reduced expression of these mutants has limited the utility of receptor mutagenesis in understanding the role of disulfide bonds in the functioning of the PTH/PTHrP receptor. In contrast, the cysteine to serine (or to alanine) CRF receptor mutants in our study were relatively well expressed. With the exception of C30S and C54S, all cysteine to serine mutations caused a dramatic reduction in CRF binding and CRF-stimulated cAMP accumulation. These data are consistent with those obtained with DTT treatment and further support the hypothesis that certain disulfide bonds within the extracellular domains of CRF receptor are essential for ligand recognition.

With few exceptions, such as the cannabinoid receptor (Matsuda et al., 1990), G protein-coupled receptors have two highly conserved extracellular cysteines, which are located in the first and second extracellular loops, respectively. Mutagenesis and biochemical studies have indicated that these cysteines are linked by a disulfide bond in β_2AR (Dohlman et al., 1990), TRHR (Perlman et al., 1995), m1 AChR (Savarese et al., 1992), and rhodopsin (Karnik & Khorana, 1990). In the PTH/PTHrP receptor, double mutation of these two cysteines slightly increased ligand binding as compared to single mutations at these sites (Lee et al., 1994). These data are in agreement with ours where a single C188S mutation dramatically decreased CRF-stimulated cAMP accumulation despite a high expression level, while the combined mutations, C188S/C258A, rescued the CRF effect. These data suggest that, similar to other G protein-coupled receptors, the two cysteine residues, in the first and second extracellular loops, may be connected by a disulfide bond. However, the functional phenotypes of C188S and C258A single mutations were not similar. This difference could not be due to one single cause such as the loss of an S−S bridge; instead, it may be due to a local effects of the mutations. In such a case C188 may be important for receptor function in its reduced form.

Single and double mutations of C30 and C54 resulted in receptors that are well expressed on the cell surface, that bind the CRF radioligand, and that increase cAMP accumulation after a challenge with CRF. Therefore, cysteines at these positions may not form a disulfide bond, or alternatively the disulfide bond at this position is not critical for receptor expression or ligand recognition. These two cysteines, however, may play a role in receptor activation. For example, the C30S mutant receptor had a highly improved EC_{50} for CRF stimulation of cAMP accumulation although it had reduced cell surface expression and ligand binding levels.

Single mutations of any of the other cysteines in the extracellular domains markedly decreased CRF binding. These mutational studies combined with our DTT experiments strongly suggest that the side chain thiol groups of some extracellular cysteines are likely to form disulfide bonds that maintain the secondary/tertiary structure of the extracellular domain of the CRF receptor. In support of this interpretation, receptor mutants carrying double mutations of C44S and C102S, C68S and C87S, had an improved EC_{50} for CRF-stimulated cAMP accumulation as compared with receptor carrying the respective single mutations (Table 1). Mutation of any one of the disulfide bond-paired cysteines would presumably result in the thio group of the other cysteine forming a new disulfide bond with the free thio groups of other cysteines such as C30 and C54, which worsens the effect of receptor structure disruption. However, removal of the second cysteine of the pair prevents the formation of aberrant disulfide bonds, and the disruption of the receptor protein structure would be minimal. Therefore, our results suggest that C44 and C102, and C68 and C87 may form disulfide bonds that are critical for ligand binding and signaling. The cysteine residues are located in the

amino-terminal domain of the receptor, a region that has been shown to be critical for ligand binding in the PTH/PTHrP receptor system (Lee et al., 1994, 1995).

Except for mutations at positions 30 and 54, all other single or paired extracellular cysteine mutations resulted in receptor mutants that have no detectable or weak radioligand binding. These receptor mutants, however, mediate an increase in cAMP accumulation after a CRF challenge. Therefore, these receptor mutants may have very low binding affinity that does not permit detectable radioligand binding. However, due to signal amplification by the receptor G protein cascade, CRF receptor interaction could be readily assessed by measuring CRF-stimulated cAMP accumulation. This property allowed us to determine the effects of paired cysteine mutations on CRF receptor interactions. Furthermore, our functional data suggest a model in which C44 and C102, C68 and C87, and C188 and C258 are interconnected with S−S bonds. This model requires confirmation with other biochemical methods.

Substitutions of the cysteines with serines in the cytoplasmic and transmembrane regions resulted in receptors with normal expression, ligand binding, and signaling properties. In other GPCRs, cysteines at these regions had been implicated in G protein coupling. In α_2AR (Eason et al., 1994) and β_2AR (O'Dowd et al., 1989), the cysteine near the carboxy-terminal tail is palmitoylated and found to be critical for G protein coupling and agonist-induced desensitization. The CRF receptor does not contain any cysteine residue in its carboxy-terminal tail. The CRF receptor contains five cysteine residues in its transmembrane and cytoplasmic region; three of them, C128, C211, and C233, are conserved in the type 1 and type 2 CRF receptor family in all species thus far characterized. Our results indicate that neither of these cysteines is essential for receptor function. Therefore, the cysteines in the transmembrane and cytoplasmic regions are not likely to be involved in the formation of disulfide bonds or participate critically in ligand binding and receptor signaling.

In summary, our results show that four cysteines in the amino-terminal domains and two cysteines in the extracellular loops are required for proper functioning of the CRF receptor. These cysteines are likely to be involved in the formation of disulfide bond(s) that is (are) critical for ligand interactions.

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