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Exploring the Binding Site Crevice of a Family B G Protein-Coupled Receptor, the Type 1 Corticotropin Releasing Factor Receptor

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ABSTRACT

Family B of G protein-coupled receptors (GPCRs) is composed of receptors that bind peptides, such as secretin, glucagon, parathyroid hormone, and corticotropin releasing factor (CRF), which play critical physiological roles. These receptors, like all GPCRs, share a common structural motif of seven membrane-spanning segments, which have been proposed to bind small ligands, such as antalarmin, a nonpeptide antagonist of the type 1 receptor for CRF (CRF_1). This leads to the hypothesis that as for family A GPCRs, the binding sites of small ligands for family B GPCRs are on the surface of a water-accessible crevice, the binding-site crevice, which is formed by the membrane-spanning segments and extends from the extracellular surface of the receptor into the plane of the membrane. To test this hypothesis we have begun to obtain structural information about family B GPCRs, using as a prototype the CRF_1, by determining the ability of sulfhydryl-specific methanethiosulfonate derivatives, such as the methanethiosulfonate-ethylammonium (MTSEA), to react with CRF_1 and thus irreversibly inhibit ^{125}I-Tyr^0-sauvagine binding. We found that MTSEA inhibited ^{125}I-Tyr^0-sauvagine binding to CRF_1 and that antalarmin protected against this irreversible inhibition. To identify the susceptible cysteine(s), we mutated, one at a time, four endogenous cysteines to serine. Mutation to serine of Cys211, Cys233, or Cys364 decreased the susceptibility of sauvagine binding to irreversible inhibition by MTSEA. Thus, Cys211, Cys233, and Cys364 at the cytoplasmic ends of the third, fourth, and seventh membrane-spanning segments, respectively, are exposed in the binding site crevice of CRF_1.

Introduction

Family B of G-protein-coupled receptors (GPCRs) is composed of receptors that bind functionally important peptides, including corticotropin releasing factor (CRF), a 41-amino acid peptide that plays a major physiological role by regulating the activity of the hypothalamic-pituitary-adrenal axis (Vale et al., 1981; Chrousos, 1995; Harmar, 2001).

Sequence analysis of these receptors has revealed seven putative, mostly hydrophobic, plasma membrane-spanning segments connected by alternating intracellular and extracellular loops (Gether, 2000; Grigoriadis et al., 2001; Harmar, 2001). These receptors also have a large extracellular amino-terminal region (N-region) that has been structurally characterized in NMR and crystallographic studies (Grace et al., 2007; Pioszak et al., 2008; Underwood et al., 2010). The N-region and the extracellular loops of family B GPCRs have been shown to play an important role in peptide binding (Holtmann et al., 1996; Liaw et al., 1997; Perrin et al., 1998; Dautzenberg et al., 1999; Unson et al., 2002; Kraetke et al., 2005a; Grace et al., 2007; Assil-Kishawi et al., 2008; Pioszak et al., 2008; Gkountelias et al., 2009).

In contrast to the extracellular regions, little is known about the potential role in ligand binding of the membrane-spanning segments (TMs) of family B GPCRs. Although the TMs have been proposed to bind small nonpeptide ligands, such as antalarmin, an antagonist for the type 1 CRF receptor (CRF_1), the specific interactions have not been identified (Liaw et al., 1997; Hoare et al., 2003). In contrast to the TMs of family A GPCRs, which have been structurally characterized in multiple crystallographic, biophysical, and biochemi-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; CRF, corticotropin-releasing factor; CRF_1, type 1 receptor for the corticotropin releasing factor; TM, transmembrane; MTS, methanethiosulfonate; MTSEA, methanethiosulfonate-ethylammonium; MTSET, methanethiosulfonate-trimethylammonium; MTSES, methanethiosulfonate-ethylsulfonate; WT, wild type; HEK, human embryonic kidney; PBS, phosphate-buffered saline; ANOVA, analysis of variance.

cal studies, there is no structural information about the TMs of family B GPCRs, further complicating a determination of their role in ligand binding. It is noteworthy that family B GPCRs display very little sequence similarity with family A receptors, and their TMs do not share the common structural/functional motifs identified in the latter (Donnelly, 1997; Frimurer and Bywater, 1999; Gether, 2000). All of these factors hinder the construction of accurate molecular models of family B GPCRs. Nevertheless, based on sequence analysis, several models of these receptors have been created (Donnelly, 1997; Frimurer and Bywater, 1999). Despite the assumptions required and the associated uncertainties of the exact boundaries of the TMs and their orientations, the overall TM packing of family B GPCRs has been proposed to be similar to that of family A GPCRs. This, in conjunction with their role in the binding of small nonpeptide ligands, led us to hypothesize that as in the family A GPCRs, the TMs of family B receptors form a water-filled binding-site crevice, which extends from the extracellular surface of the receptor into the plane of the membrane (Javitch et al., 1994). The surface of this crevice is formed by residues that contact ligands and by other residues that may play a structural role and affect binding indirectly.

To test this hypothesis, we sought to obtain structural information for the TMs of family B GPCRs, using as a prototype the CRF_1. Our starting point was to test whether one or more of the endogenous TM cysteines in CRF_1 face the putative binding site crevice by determining their accessibilities for reaction with small, charged, sulfhydryl-specific methanethiosulfonate (MTS) derivatives. These reagents react vastly faster with water-accessible sulfhydryl groups of cysteines than with sulfhydryls buried in the protein or facing the lipid bilayer (Karlin and Akabas, 1998). Using this method, previous studies have revealed the presence of endogenous TM cysteines that face the binding site crevice of various family A GPCRs (Javitch et al., 1994; Deng et al., 2000). Here, we found that the endogenous Cys211, Cys233, and Cys364 in the third, fourth, and seventh membrane-spanning segments of CRF_1 are located on the surface of a binding-site crevice of CRF_1, being accessible to the reaction with charged polar MTS reagents and protected from this reaction by bound antalarmin.

Materials and Methods

Plasmids and Site-Directed Mutagenesis. The cDNA sequence encoding CRF_1 was subcloned into the bicistronic expression vector pcin4, thereby creating the vector pcin4-CRF_1 (Gkountelias et al., 2009). Serine mutations were generated by the polymerase chain reaction-mediated mutagenesis, using *Pfu* polymerase (MBI Fermentas, Hanover, MD) and mutagenic oligonucleotides encoding the desired amino acid substitution. The polymerase chain reaction-generated DNA fragments containing the mutations were subcloned into the pcin4-CRF_1 plasmid, and the mutations were confirmed by DNA sequencing. Mutants are named as (wild-type residue)(residue number)(serine), where the residues are given in the single-letter code.

Cell Culture, Transfection, and Harvesting. Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's modified Eagle's medium/F-12 (1:1) containing 3.15 g/l glucose and 10% bovine calf serum at 37°C and 5% CO_2. Sixty-millimeter dishes of HEK 293 cells at 80 to 90% confluence were transfected with 2 to 3 mg of wild-type (WT) or mutant pcin4-CRF_1 using 9 μl of Lipofectamine and 2 ml of Opti-MEM (both from Invitrogen, Carlsbad, CA). To generate stably transfected pools of cells expressing the receptors 5 to 12 h after transfection, the medium was replaced by Dulbecco's modified Eagle's medium/F-12 (1:1) containing 3.15 g/l glucose, 10% bovine calf serum (Hyclone Laboratories, Logan, UT) and 700 μg/ml G418 (Geneticin), and antibiotic (Invitrogen). The antibiotic was added to select a stably transfected pool of cells. Cells stably expressing WT or CRF_1 mutants, at 100% confluence in 60- or 100-mm dishes, were washed with phosphate-buffered saline (PBS) (4.3 mM $Na_2HPO_4 \cdot 7H_20$, 1.4 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.3–7.4, at 37°C), briefly treated with PBS containing 2 mM EDTA (PBS/EDTA), and then dissociated in PBS/EDTA. Cells suspensions were centrifuged at 50*g* for 2 min at room temperature, and the pellets were resuspended in 1 ml of buffer M (25 mM HEPES containing 5.4 mM KCl, 140 mM NaCl, and 2 mM EDTA, pH 7.2, at 22–25°C) for treatment with MTS reagents or in 1.5 ml of buffer H (20 mM HEPES, containing 10 mM $MgCl_2$, 2 mM EGTA, 0.2 mg/ml bacitracin, and 0.93 μg/ml aprotinin, pH 7.2, at 4°C) for binding assays.

^{125}I-Tyr^0-Sauvagine Binding. For radioligand binding assays, cell suspensions (1.5 ml) in buffer H were homogenized using an Ultra Turrax T25 homogenizer (IKA Janke and Kunkel, Staufen, Germany) at setting ~20 for 10 to 15 s, at 4°C. The homogenates were centrifuged at 16,000*g* for 10 min at 4°C, and the membrane pellets were resuspended in 1 ml of buffer B (buffer H containing 0.1% bovine serum albumin, pH 7.2, at 20°C). The membrane suspensions were diluted in buffer B and used for homologous competition binding studies as described previously (Gkountelias et al., 2009). In brief, aliquots of diluted membrane suspensions (50 μl) were added into low retention tubes (Kisker-Biotech, Steinfurt, Germany), containing buffer B and 20 to 25 pM ^{125}I-Tyr^0-sauvagine with or without increasing concentrations of Tyr^0-sauvagine (American Peptide Co., Sunnyvale, CA). The mixtures were incubated at 20 to 21°C for 120 min and then filtered using a Brandel cell harvester through Whatman 934AH glass fiber filters presoaked for 1 h in 0.3% polyethylenimine at 4°C. The filters were washed three times with 0.5 ml of ice-cold PBS, pH 7.1, containing 0.01% Triton X-100. Filters were assessed for radioactivity in a gamma counter (1275 minigamma, 80% efficiency; LKB Wallac, Chalfont St. Giles, Buckinghamshire, UK). The amount of membrane used was adjusted to ensure that the specific binding was always equal to or less than 10% of the total concentration of the added radioligand. Specific ^{125}I-Tyr^0-sauvagine binding was defined as total binding less nonspecific binding in the presence of 500 to 1000 nM CRF. The K_D values for ^{125}I-Tyr^0-sauvagine binding were determined by analyzing homologous competition data with Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

Reactions with MTS Reagents. For treatment with MTS reagents, aliquots (0.1 ml) of cell suspensions in buffer M were incubated with the MTS reagents MTS-ethylammonium (MTSEA), MTS-ethyltrimethylammonium (MTSET), or MTS-ethylsulfonate (MTSES) at the stated concentrations for 15 s at 22 to 25°C. Cell suspensions were then diluted 140-fold in buffer PBS/EDTA, pH 7.1, at 22 to 25°C containing 10 mM $MgCl_2$, centrifuged at 250*g* for 5 min at 22 to 25°C, and the pellets were resuspended in 1.5 ml of buffer M containing 10 mM $MgCl_2$. Cell suspensions were centrifuged at 250*g* for 5 min at 22 to 25°C, and the pellets were homogenized in 1.5 ml of buffer H, as described above. The homogenates were centrifuged at 16,000*g* for 10 min at 4°C and the membrane pellets were resuspended in 1 ml of buffer B (buffer H containing 0.1% bovine serum albumin, pH 7.2, at 20°C). The membrane suspensions were used to assay for ^{125}I-Tyr^0-sauvagine binding as described above.

Protection experiments were performed by preincubation of aliquots (0.1 ml) of cell suspensions with increasing concentrations of the nonpeptide CRF_1-selective antagonist (1–1000 nM) antalarmin for 30 min at 37°C in a final volume of 1 ml of buffer M. Thereafter, the mixtures were centrifuged at 250*g* for 5 min at 22 to 25°C, and 0.9 ml of supernatant was removed by aspiration. The cell pellets were resuspended in the remaining 0.1 ml of supernatants, and the

mixtures were treated with 2.5 mM MTSEA as described above. Cells were subsequently diluted 140-fold in buffer PBS/EDTA, pH 7.1, at 22 to 25°C containing 10 mM $MgCl_2$, washed twice by centrifugation with buffer M containing 10 mM $MgCl_2$, and membrane homogenates were prepared and used to assay for ^{125}I-Tyr^0-sauvagine binding as described above.

Results

Reaction of MTS Reagents with the CRF_1. To assess for the reaction of MTS reagents with the CRF_1, we treated intact HEK 293 cells stably expressing WT CRF_1 with MTSEA, MTSET, or MTSES and subsequently determined the specific binding of ^{125}I-Tyr^0-sauvagine in membrane homogenates.

Treatment of HEK 293 cells stably expressing CRF_1 with the positively charged MTSEA at a concentration of 2.5 mM significantly decreased the specific binding of ^{125}I-Tyr^0-sauvagine to CRF_1 (Fig. 1). In contrast to MTSEA, the bulkier MTSET (positively charged) and MTSES (negatively charged), at concentrations of 2.5 mM, did not significantly reduce the specific binding of ^{125}I-Tyr^0-sauvagine to CRF_1 (Fig. 1). As shown in Fig. 2, MTSEA treatment decreased the specific ^{125}I-Tyr^0-sauvagine binding in a dose-dependent manner with an IC_{50} of 2.1 mM ($-\log IC_{50}$ = 2.69 ± 0.18), reaching a plateau of approximately 20% residual specific binding. Longer treatment of CRF_1 with 10 mM MTSEA for 2 min demonstrated a similar plateau in radioligand binding (data not shown).

Mechanism of the Inhibitory Effect of MTSEA on Binding. To examine the mechanism of the inhibitory effect of MTSEA on ^{125}I-Tyr^0-sauvagine binding, we determined the binding affinity of ^{125}I-Tyr^0-sauvagine before and after the reaction of MTSEA with CRF_1. As shown in Fig. 3, treatment of WT CRF_1 with either 2.5 or 15 mM MTSEA failed to significantly affect ^{125}I-Tyr^0-sauvagine binding affinity. Thus, inhibition of ^{125}I-Tyr^0-sauvagine binding after the reaction of CRF_1 with MTSEA resulted from a reduction in the apparent number of functional binding sites, consistent with a loss of binding to receptor that was fully derivatized. A possible explanation for the small fraction of residual binding with normal affinity despite treatment with saturating MTSEA could be that a small proportion of receptors was located intracellularly and thus protected from the reagent, whereas the binding experiments were performed with membrane homogenates in which ^{125}I-Tyr^0-sauvagine labeled all receptors.

Protection of CRF_1 Against MTSEA Reaction. To test whether the small nonpeptide CRF_1-selective antagonist an-

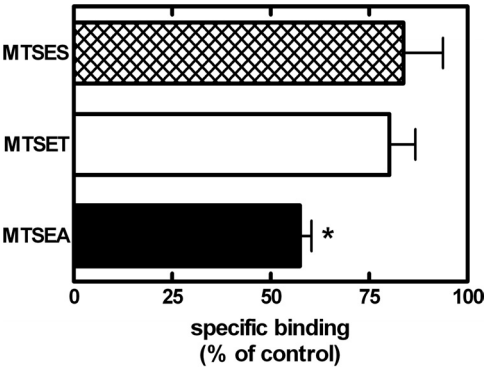

Fig. 1. Effects of MTS reagents on specific ^{125}I-Tyr^0-sauvagine binding to CRF_1. Suspensions of HEK 293 cells stably expressing the wild-type CRF_1 were incubated for 15 s at 22 to 25°C without (control) or with 2.5 mM concentration of the MTS reagents MTSEA, MTSET, or MTSES. Thereafter, the cells were homogenized, and membrane homogenates were assayed for specific binding with ^{125}I-Tyr^0-sauvagine, as described under *Materials and Methods*. The bars represent the specific binding (percentage of control), or residual binding, which is defined as the percentage of ^{125}I-Tyr^0-sauvagine specific binding to MTS-treated receptor divided by the radioligand specific binding to the corresponding untreated receptor. The mean ± S.E. values are from 8 to 40 independent experiments, each performed with duplicate determinations. The asterisk indicates that the MTSEA significantly decreased ^{125}I-Tyr^0-sauvagine-specific binding to CRF_1 treated with MTSEA compared with the untreated receptor ($P < 0.05$, one-way analysis of variance and least significant difference post hoc test).

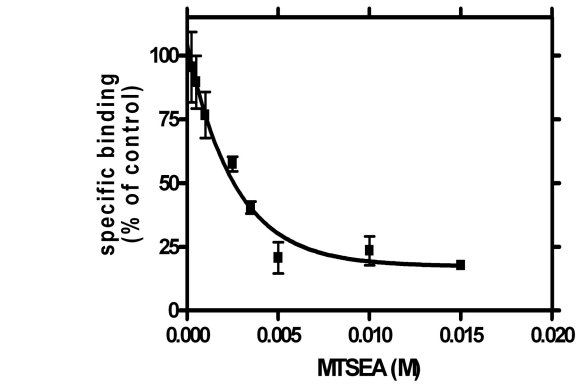

Fig. 2. Dose-dependent effect of MTSEA on specific binding of ^{125}I-Tyr^0-sauvagine to CRF_1. Suspensions of HEK 293 cells stably expressing the wild-type CRF_1 were incubated for 15 s at 22 to 25°C without (control) or with various concentrations of MTSEA. Thereafter, the cells were homogenized and membrane homogenates were assayed for specific binding with ^{125}I-Tyr^0-sauvagine, as described under *Materials and Methods*. Means ± S.E. are shown from 3 to 40 independent experiments, each performed with duplicate determinations.

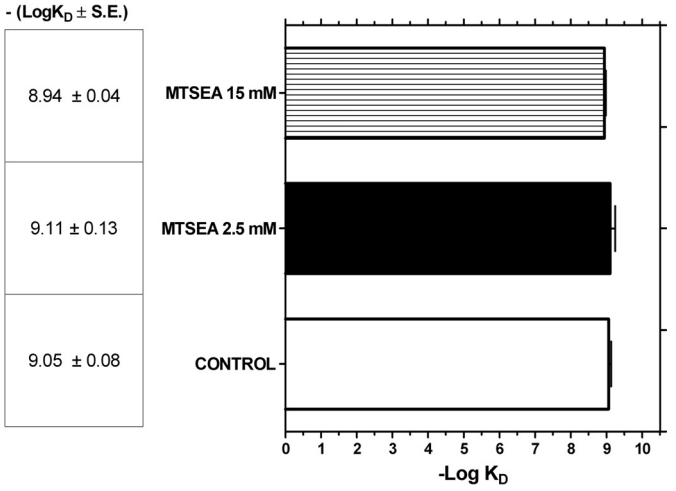

Fig. 3. Effect of MTSEA reaction on the binding properties of CRF_1. Suspensions of HEK 293 cells stably expressing the wild-type CRF_1 were treated without (control) or with 2.5 or 15 mM MTSEA for 15 s at 22 to 25°C. Thereafter, the cells were homogenized, and membrane homogenates were incubated with 20 to 25 pM ^{125}I-Tyr^0-sauvagine in the absence or presence of increasing concentrations of Tyr^0-sauvagine. The data were fit to a one-site competition model by nonlinear regression and $-\log K_D$ values were determined as described under *Materials and Methods*. The bars represent the binding affinity ($-\log K_D$) of ^{125}I-Tyr^0-sauvagine for CRF_1 before or after its treatment with MTSEA. The mean ± S.E. values are from two to six independent experiments. The affinity of radioligand for the untreated receptor was not statistically different from that for the CRF_1 treated with either 2.5 or 15 mM MTSEA (one-way ANOVA followed by least significant difference post hoc test).

talarmin protected the WT CRF_1 against MTSEA reaction, we determined its ability to slow the reaction. We treated CRF_1-expressing cells, incubated with or without antalarmin, with MTSEA (2.5 mM), and after washing, we determined ^{125}I-Tyr^0-sauvagine binding in membrane homogenates from these cells. As shown in Fig. 4, antalarmin protected CRF_1 against MTSEA reaction in a concentration-dependent manner, with complete protection at a high concentration of the ligand.

Mutations of the Endogenous Cysteines of CRF_1. To identify the endogenous cysteine(s) of CRF_1 that reacted with MTSEA to inhibit binding, we mutated to serine, one at a time, the endogenous Cys128, Cys211, Cys233, and Cys364 (thus creating the C128S, C211S, C233S, and C364S mutants, respectively). According to the predicted topology of CRF_1, these cysteines are located in the first (TM1), third (TM3), fourth (TM4), and seventh (TM7) membrane-spanning segments of receptor (Fig. 5) (Grigoriadis et al., 2001).

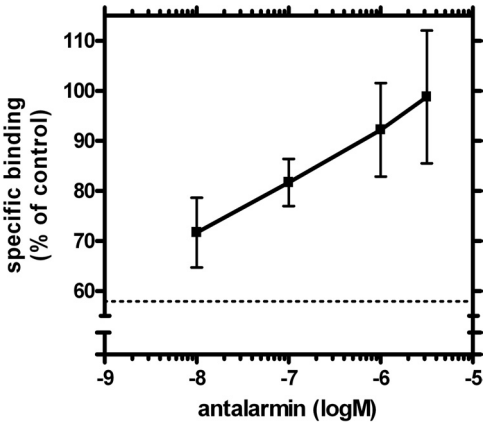

Fig. 4. Protection of ^{125}I-Tyr^0-sauvagine-specific binding by preincubation with varying concentrations of antalarmin. HEK 293 cells stably expressing the wild-type CRF_1 were preincubated with increasing concentrations (1–1000 nM) of the antagonist, antalarmin for 30 min at 37°C and subsequently treated with 2.5 mM MTSEA as described under *Materials and Methods*. Thereafter, the cells were homogenized, and the ability of membrane homogenates to bind ^{125}I-Tyr^0-sauvagine was assayed as described under *Materials and Methods*. The dotted line represents the specific binding after treatment with 2.5 mM MTSEA in the absence of antalarmin. The specific binding (percentage of control) was defined as specific binding of ^{125}I-Tyr^0-sauvagine to MTSEA-treated CRF_1 divided by the specific binding to the untreated receptors (control). Means ± S.E. are shown from six independent experiments, each performed with duplicate determinations.

Before probing the reaction of MTSEA with the C128S, C211S, C233S, and C364S constructs, we tested the effect of the mutations on the functional properties of CRF_1, by determining the binding affinities ($-\log K_D$) of ^{125}I-Tyr^0-sauvagine for WT and the mutant receptors in homologous competition experiments performed under equilibrium conditions in membrane homogenates from HEK 293 cells stably expressing the receptors. Substitution of serine for Cys128, Cys211, Cys233, and Cys364 did not significantly affect the binding affinity of ^{125}I-Tyr^0-sauvagine for CRF_1 (Fig. 6), suggesting that the mutations did not substantially alter the functional properties of the receptor.

Reaction of MTSEA with CRF_1 Mutants. To identify the reactive cysteine(s) of CRF_1, we determined whether the binding of ^{125}I-Tyr^0-sauvagine to C128S, C211S, C233S, and C364S mutants was sensitive to MTSEA. As shown in Fig. 7, mutation of Cys128 to serine did not affect the sensitivity of radioligand binding to MTSEA; the residual ^{125}I-Tyr^0-sauvagine binding to C128S (52.5 ± 3.3%) after MTSEA treatment (2.5 mM) was not significantly different from that to WT CRF_1 (57.4 ± 2.9%). In marked contrast, mutation to serine of Cys211, Cys233, or Cys364 significantly reduced sensitivity to MTSEA; the residual binding of ^{125}I-Tyr^0-sauvagine to C211S (73.3 ± 4.5%), C233S (75.9 ± 9.9%), and C364S (83.0 ± 9.4%) after MTSEA treatment (2.5 mM) was not significantly different, but each was significantly different from that of WT (57.4 ± 2.9%) (Fig. 7).

Because radiolabeled sauvagine has been shown to bind to the extracellular portion of CRF_1 and thus is located at some distance from the endogenous cysteines being derivatized, the effects on binding must be indirect. To explore the mechanism of inhibition, we determined the affinity of ^{125}I-Tyr^0-sauvagine binding to C128S, C211S, C233S and C364S (and to the other mutants tested in this study, as described below) before and after MTSEA reaction. As was the case for WT, MTSEA reaction did not significantly affect the binding affinity of ^{125}I-Tyr^0-sauvagine for C128S, C211S, C233S or C364S (Fig. 6). Thus, as for WT, the decrease of ^{125}I-Tyr^0-sauvagine binding to C128S after MTSEA reaction was due to a reduction of the apparent number of binding sites. In addition, the much smaller affect of MTSEA reaction with C211S, C233S, or C364S on ^{125}I-Tyr^0-sauvagine binding was not due to an MTSEA-associated enhancement of the affinity of residual radioligand binding.

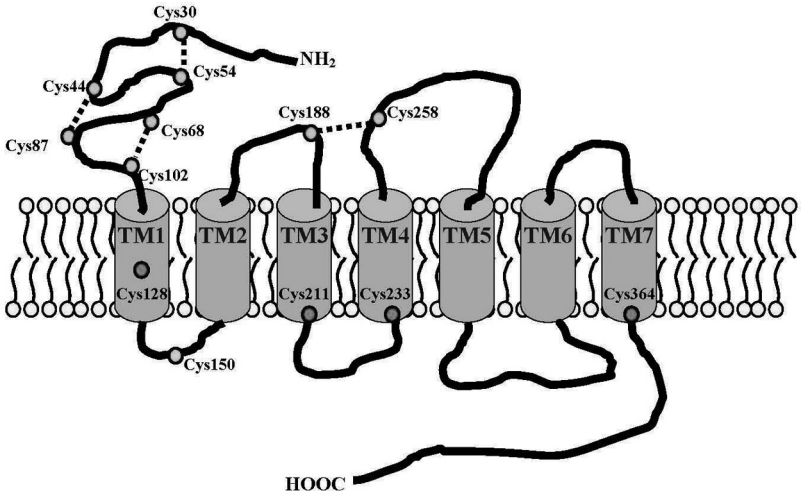

Fig. 5. Snake plot representation of CRF_1 showing the approximate positions of its 13 cysteines. The cylinders represent the membrane-spanning segments (TM1–TM7) of CRF_1. Four endogenous cysteines (Cys128, Cys211, Cys233, and Cys364) are located in the membrane-spanning segments of CRF_1, whereas one is positioned in the first intracellular loop (Cys150) of receptor. Six endogenous cysteines are located in the extracellular N-region (Cys30 and Cys54, Cys44 and Cys87, and Cys68 and Cys102), forming three disulfide bridges (dotted lines) (Pioszak et al., 2008). The resting two endogenous cysteines (Cys188 and Cys258) are positioned in the first and second extracellular loops of CRF_1, and form a disulfide bond (dotted line) (Qi et al., 1997).

MTSEA Reaction with ΔCys Mutants. Based on the finding that substitution of Ser for a single Cys at positions 211, 233 or 364 rendered the receptor much less sensitive to MTSEA, we hypothesized that reaction with MTSEA of only a single one of these Cys, would not reduce sauvagine binding to CRF_1. To test this hypothesis, and even more importantly to create a suitable MTSEA-insensitive background construct for subsequent substituted-cysteine accessibility method studies, we mutated all the endogenous TM Cys (along with the cytoplasmic Cys150) to Ser, thereby creating the mutant ΔCys, which had a binding affinity for ^{125}I-Tyr^0-sauvagine ($-\log K_D$ = 8.60 ± 0.63) similar to that of WT ($-\log K_D$ = 9.05 ± 0.08) (Fig. 8). As anticipated, simultaneous mutation of all the sensitive Cys in CRF_1 to Ser created a receptor less sensitive to 2.5 mM MTSEA; the residual binding of ^{125}I-Tyr^0-sauvagine to ΔCys after MTSEA (2.5 mM) reaction was 90.9 ± 6.1% (Fig. 9). Consistent with our prediction, we found that addition of a single Cys into ΔCys (at positions, 128, 211, 233 or 364, thus creating the mutants ΔCys + 128C, ΔCys + 211C, ΔCys + 233C and ΔCys + 364C, respectively) failed to increase significantly the sensitivity to MTSEA. Thus, the residual binding of ^{125}I-Tyr^0-sauvagine to ΔCys + 128C (83.1 ± 7.6%), ΔCys + 211C (86.0 ± 9.1%), ΔCys + 233C (89.4 ± 7.7%) and ΔCys + 364C (76.7 ± 7.3%) after MTSEA (2.5 mM) reaction was not significantly different from each other or from that of ΔCys (90.9 ± 6.1%), but it was significantly different from the corresponding binding to WT (57.4 ± 2.9%) (Fig. 9). These observations were not due to a mutation-associated or to an MTSEA-induced change of ^{125}I-Tyr^0-sauvagine affinity, because the ΔCys + 128C, ΔCys + 211C, ΔCys + 233C, and ΔCys + 364C mutants, similar to ΔCys, had normal ^{125}I-Tyr^0-sauvagine affinity, which also was unaltered by MTSEA treatment (Fig. 8).

- ($\log K_D$ ± S.E.)

CONTROL	MTSEA
8.59 ± 0.18	8.49 ± 0.16
9.04 ± 0.15	8.67 ± 0.14
8.98 ± 0.07	9.03 ± 0.03
8.45 ± 0.15	8.37 ± 0.20
9.05 ± 0.08	9.11 ± 0.13

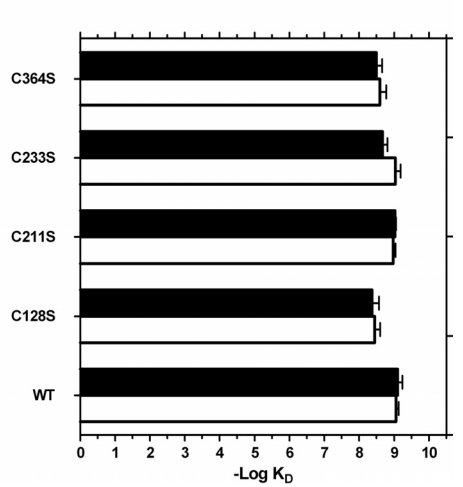

Fig. 6. Binding affinity of Tyr^0-sauvagine for C128S, C211S, C233S, and C364S receptors before and after MTSEA reaction. Suspensions of HEK 293 cells stably expressing WT CRF_1 or the mutants, C128S, C211S, C233S, or C364S were treated without (control) or with 2.5 mM MTSEA for 15 s at 22 to 25°C. Thereafter, the cells were homogenized and membrane homogenates were incubated with ^{125}I-Tyr^0-sauvagine in the absence or presence of increasing concentrations of Tyr^0-sauvagine. The data were fit to a one-site competition model by nonlinear regression and $-\log K_D$ values were determined as described under *Materials and Methods*. The bars represent the binding affinity ($-\log K_D$) of ^{125}I-Tyr^0-sauvagine before (□) or after MTSEA reaction (■). The mean ± S.E. values are from two to six independent experiments. The results were statistically analyzed using one-way ANOVA followed by least significant difference post hoc test. None of the mutations significantly altered ^{125}I-Tyr^0-sauvagine affinity, and MTSEA treatment did not significantly alter radioligand affinity of any of the receptors tested.

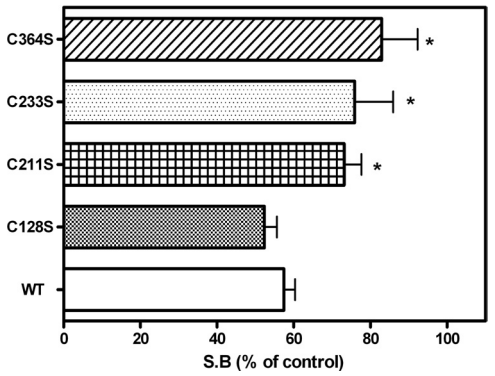

Fig. 7. Effects of MTSEA reaction on specific ^{125}I-Tyr^0-sauvagine binding to C128S, C211S, C233S, or C364S receptors. Suspensions of HEK 293 cells stably expressing WT CRF1 or C128S, C211S, C233S, and C364S mutants were incubated for 15 s at 22 to 25°C with or without (control) 2.5 mM MTSEA. Thereafter, the cells were homogenized, and membrane homogenates were assayed for specific binding with ^{125}I-Tyr^0-sauvagine, as described under *Materials and Methods*. The bars represent the specific binding (percentage of control), or residual binding, which is defined as the percentage of ^{125}I-Tyr^0-sauvagine specific binding to MTSEA-treated receptor divided by the radioligand specific binding to the corresponding untreated receptor. The mean ± S.E. values are from 9 to 40 independent experiments, each performed with duplicate determinations. Asterisk indicates that the residual binding to C211S, C233S, or C364S mutant after MTSEA reaction was significantly different from the corresponding one to WT ($P < 0.05$, one-way analysis of variance and least significant difference post hoc test). In contrast, the residual binding to C211S, C233S, and C364S mutants after MTSEA reaction was not statistically different from each other.

- ($\log K_D$ ± S.E.)

CONTROL	MTSEA
8.68 ± 0.60	8.51 ± 0.89
8.79 ± 0.42	8.53 ± 0.38
8.79 ± 0.50	8.34 ± 0.48
8.52 ± 0.40	8.26 ± 0.18
8.60 ± 0.63	8.60 ± 0.54
9.05 ± 0.08	9.11 ± 0.13

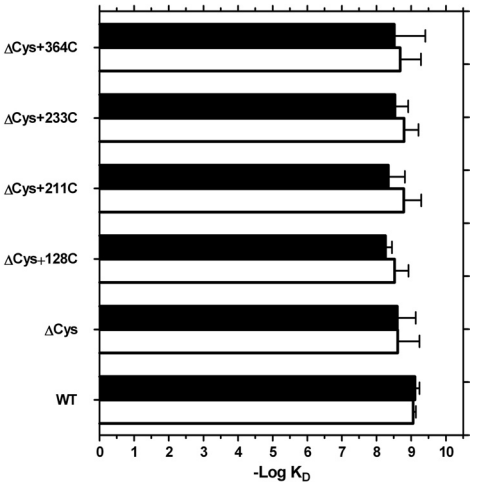

Fig. 8. Binding affinity of Tyr^0-sauvagine for ΔCys, ΔCys + 128C, ΔCys + 211C, ΔCys + 233C, and ΔCys + 364C receptors before and after MTSEA reaction. Suspensions of HEK 293 cells stably expressing WT CRF_1 or the mutants, ΔCys, ΔCys + 128C, ΔCys + 211C, ΔCys + 233C, or ΔCys + 364C were treated without (control) or with 2.5 mM MTSEA for 15 s at 22 to 25°C. Thereafter, the cells were homogenized and membrane homogenates were incubated with ^{125}I-Tyr^0-sauvagine in the absence or presence of increasing concentrations of Tyr^0-sauvagine. The data were fit to a one-site competition model by nonlinear regression, and $-\log K_D$ values were determined as described under *Materials and Methods*. The bars represent the binding affinity ($-\log K_D$) of ^{125}I-Tyr^0-sauvagine before (□) or after MTSEA reaction (■). The mean ± S.E. values are from two to six independent experiments. The results were statistically analyzed using one-way ANOVA followed by a least significant difference post hoc test. None of the mutations considerably altered ^{125}I-Tyr^0-sauvagine affinity and MTSEA treatment of all receptors tested did not significantly alter radioligand affinity.

We next hypothesized that derivatization of all three endogenous cysteines (Cys211, Cys233, and Cys364) is necessary for the reduction of sauvagine binding to CRF_1 after MTSEA reaction. To test this hypothesis, we simultaneously added two or three cysteines (at positions 211, 233, and/or 364) into ΔCys and tested their ability to react with MTSEA. Simultaneous addition of two cysteines into ΔCys, in any combination, thus creating the mutants ΔCys + 233C + 364C, ΔCys + 211C + 364C, and ΔCys + 211C + 233C, did not significantly increase the sensitivity to MTSEA; although the residual binding of ^{125}I-Tyr^0-sauvagine to ΔCys + 233C + 364C (93.6 ± 13.0%), ΔCys + 211C + 364C (101.3 ± 9.9%), and ΔCys + 211C + 233C (96.8 ± 7.8%) after MTSEA (2.5 mM) reaction was not significantly different from each other or from ΔCys (90.9 ± 6.1%), it was significantly different from the corresponding binding to WT (57.4 ± 2.9%) (Fig. 10). In marked contrast, simultaneous addition of the three cysteines into the ΔCys, thus creating the mutant, ΔCys + 211C + 233C + 364C, synergistically increased the sensitivity of the mutant receptor to MTSEA, restoring the wild-type phenotype. The residual binding of ^{125}I-Tyr^0-sauvagine to ΔCys + 211C + 233C + 364C (61.6 ± 5.1%,) after MTSEA treatment (2.5 mM) was not significantly different from the corresponding binding to WT (57.4 ± 2.9%), but it differed significantly from that to ΔCys (90.9 ± 6.1%) (Fig. 10). These findings were not due to a mutation-associated or to an MTSEA-induced change of ^{125}I-Tyr^0-sauvagine affinity, because the mutants had normal ^{125}I-Tyr^0-sauvagine affinity, which also was unaltered by MTSEA treatment (Fig. 11). These results also suggest that, similar to WT, the observed significant decrease of ^{125}I-Tyr^0-sauvagine binding to ΔCys + 211C + 233C + 364C after MTSEA reaction was due to a reduction of the apparent number of binding sites rather than to a decrease of its affinity.

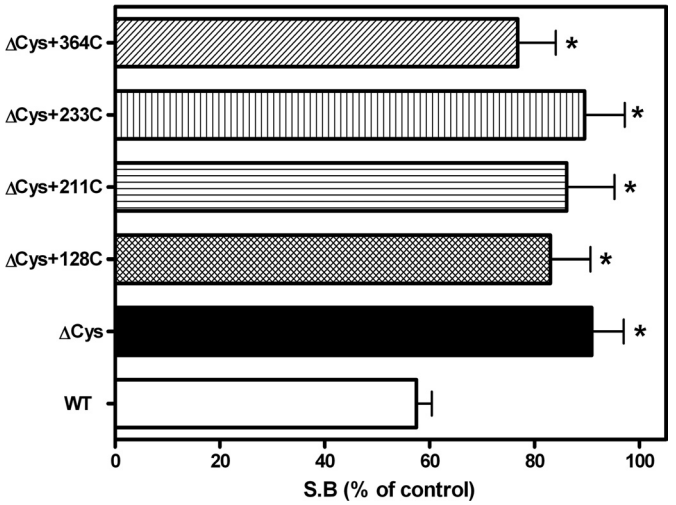

Fig. 9. Effects of MTSEA reaction on specific ^{125}I-Tyr^0-sauvagine binding to ΔCys, ΔCys + 128C, ΔCys + 211C, ΔCys + 233C, or ΔCys + 364C receptors. Suspensions of HEK 293 cells stably expressing WT CRF_1 or ΔCys, ΔCys + 128C, ΔCys + 211C, ΔCys + 233C, or ΔCys + 364C mutants were incubated for 15 s at 22 to 25°C with or without (control) 2.5 mM MTSEA. Thereafter, the cells were homogenized, and membrane homogenates were assayed for specific binding with ^{125}I-Tyr^0-sauvagine, as described under *Materials and Methods*. The bars represent the specific binding (percentage of control), or residual binding, which is defined as the percentage of ^{125}I-Tyr^0-sauvagine specific binding to MTSEA-treated receptor divided by the radioligand specific binding to the corresponding untreated receptor. The mean ± S.E. values are from 8 to 40 independent experiments, each performed with duplicate determinations. Asterisk indicates that the residual binding to ΔCys, ΔCys + 128C, ΔCys + 211C, ΔCys + 233C, or ΔCys + 364C mutant after MTSEA reaction was significantly different from the corresponding one to WT ($P < 0.05$, one-way analysis of variance and least significant difference post hoc test). In contrast, the residual binding to ΔCys, ΔCys + 128C, ΔCys + 211C, ΔCys + 233C, and ΔCys + 364C mutants after MTSEA reaction was not statistically different from each other.

Discussion

Reaction of MTSEA with CRF_1 decreased the specific binding of the radiolabeled agonist ^{125}I-Tyr^0-sauvagine, suggesting that one or more endogenous cysteines was accessible for the reaction with the reagent. CRF_1 contains 13 endogenous cysteines (Fig. 5). Four of these (Cys128, Cys211, Cys233, Cys364) are predicted to be in the TMs of CRF_1 (TM Cys), one is intracellular (Cys150), whereas six (Cys30, Cys44, Cys54, Cys68, Cys87, and Cys102) are located in the extracellular N-region of CRF_1 and form three disulfide bonds (Pioszak et al., 2008). In addition, two cysteines (Cys188, and Cys258) in the first and second extracellular loops, which are highly conserved among the G-protein coupled receptors, probably participate in the formation of a disulfide bond (Qi et al., 1997). Because the MTS reagents do not react with disulfide-bonded cysteines, and have limited access to intracellular cysteines, if added extracellularly to intact cells with high intracellular reducing environment and for a short period of time (15 s in our study) (Javitch et al., 1994, 2002), the

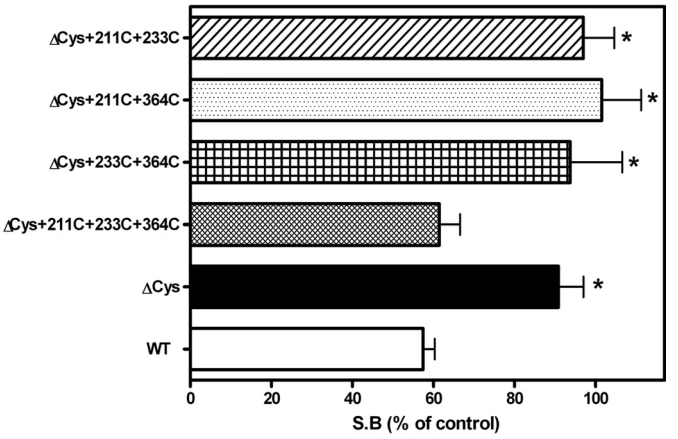

Fig. 10. Effects of MTSEA reaction on specific ^{125}I-Tyr^0-sauvagine binding to ΔCys + 211C + 233C, ΔCys + 211C + 364C, ΔCys + 233C + 364C, or ΔCys + 211C + 233C + 364C receptors. Suspensions of HEK 293 cells stably expressing WT CRF_1 or ΔCys, ΔCys + 211C + 233C, ΔCys + 211C + 364C, ΔCys + 233C + 364C, or ΔCys + 211C + 233C + 364C mutants were incubated for 15 s at 22 to 25°C with or without (control) 2.5 mM MTSEA. Thereafter, the cells were homogenized, and membrane homogenates were assayed for specific binding with ^{125}I-Tyr^0-sauvagine, as described under *Materials and Methods*. The bars represent the specific binding (percentage of control), or residual binding, which is defined as the percentage of ^{125}I-Tyr^0-sauvagine specific binding to MTSEA-treated receptor divided by the radioligand specific binding to the corresponding untreated receptor. The mean ± S.E. values are from 3 to 40 independent experiments, each performed with duplicate determinations. Asterisk indicates that the residual binding to ΔCys, ΔCys + 211C + 233C, ΔCys + 211C + 364C, or ΔCys + 233C + 364C mutant after MTSEA reaction was significantly different from the corresponding one to WT or to ΔCys + 211C + 233C + 364C ($P < 0.05$, one-way analysis of variance and least significant difference post hoc test). In contrast, the residual binding to ΔCys, ΔCys + 211C + 233C, ΔCys + 211C + 364C, or ΔCys + 233C + 364C mutants after MTSEA reaction was not statistically different from each other. In addition, the residual binding to WT and to ΔCys + 211C + 233C + 364C after MTSEA reaction was not statistically different from each other.

endogenous cysteines of CRF_1, which reacted with MTSEA and inhibited ^{125}I-Tyr^0-sauvagine binding, are most likely one or more of the four TM cysteines (Cys128 in the middle of TM1, and Cys211, Cys233, Cys364, which are located near the cytoplasmic ends of TM3, TM4, and TM7, respectively). This suggests that as in family A GPCRs, the TMs of family B GPCRs form a water-accessible crevice with one or more of the endogenous Cys128, Cys211, Cys233, and Cys364 of CRF_1 lying on its surface.

In contrast to MTSEA, MTSET and MTSES did not significantly inhibit ^{125}I-Tyr^0-sauvagine binding to CRF_1. A possible explanation could be that the reactive endogenous cysteines are deep in the crevice such that access of the bulkier MTSET and MTSES is sterically constrained. Likewise, although the cysteines substituted for Ser129 in the cytoplasmic end of TM3 and for Val378 in the cytoplasmic end of TM6 of D2 dopamine receptor were accessible for reaction with MTSEA, they did not react with the bulkier MTSET and MTSES (Javitch et al., 1995, 1998). An alternative explanation for the ability of MTSEA but not MTSET or MTSES to react is that MTSEA might access the site in its uncharged form, which cannot occur for MTSET or MTSES.

To identify the endogenous cysteines that reacted with MTSEA, we mutated Cys128, Cys211, Cys233, and Cys364, one at a time, to serine, thus creating the mutants C128S, C211S, C233S, and C364S. These mutations did not seem to alter significantly the functional and therefore the structural properties of CRF_1. In contrast to C128S, the mutations, C211S, C233S, and C364S rendered the receptor substantially less sensitive to MTSEA. These results suggest that Cys211 in TM3, Cys233 in TM4, and Cys364 in TM7 are exposed in the water-accessible crevice of receptor and reacted with MTSEA to inhibit ^{125}I-Tyr^0-sauvagine binding. It is also conceivable that these amino acids are located in a water-accessible interface formed by the membrane-spanning domains of two or more different CRF_1 molecules, as observed in other GPCRs (Guo et al., 2008). This possibility is also consistent with the reported ability of CRF_1 to oligomerize (Kraetke et al., 2005b).

Simultaneous mutation to serine of the three reactive cysteines as well as Cys128 and Cys150 greatly decreased the sensitivity to MTSEA of the resulting receptor (ΔCys). There was a small amount of inhibition of ΔCys, despite the fact that the remaining cysteines are believed to be disulfide cross-linked and therefore unreactive (Qi et al., 1997; Pioszak et al., 2008). It is possible that there is partially incomplete disulfide bonding when the receptor is expressed heterologously, which could lead to a small inhibition of sauvagine binding to ΔCys by MTSEA. Another possible explanation could be that MTSEA reacts with a CRF_1-associated protein to allosterically inhibit radioligand binding to CRF_1. Curiously, the amino-terminal extracellular region of CRF receptors, which is essential for radioligand binding, forms a Sushi domain, which has been implicated in protein-protein interactions (Perrin et al., 2006).

Substitution of serine for Cys211, Cys233, or Cys364 rendered the receptor significantly less sensitive to MTSEA. This suggests that no single endogenous cysteine was sufficient, after its reaction with MTSEA, to reduce sauvagine binding to CRF_1. Consistent with this interpretation, reaction of MTSEA with the mutants ΔCys + 211C, ΔCys + 233C, and ΔCys + 364C, which contain only one of the reactive endogenous cysteines, failed to inhibit sauvagine binding. It is noteworthy that simultaneous addition of the three Cys (ΔCys + 211C + 233C + 364C), but no combination of two into ΔCys restored the WT sensitivity to MTSEA. This suggests that MTSEA reacted simultaneously with Cys211, Cys233, and Cys364, to disrupt binding of Tyr^0-sauvagine.

The small nonpeptide CRF_1-selective antagonist antalarmin protected CRF_1 against MTSEA reaction. Antalarmin has been proposed to bind to the TMs of CRF_1 (Liaw et al., 1997; Hoare et al., 2003). Given that the reactive Cys211, Cys233, and Cys364 are expected to be deep within the crevice near the cytoplasmic ends of their TMs, it is likely that antalarmin protected them from MTSEA reaction by binding above them and blocking the passage of reagent from the extracellular medium to the cytoplasmic end of the crevice. It is less likely that this nonpeptide CRF analog reached the cytoplasmic ends of TMs of CRF_1 and directly protected Cys211, Cys233, and Cys364 from MTSEA reaction; ligands for different GPCRs and with smaller size than antalarmin, such as epinephrine, dopamine, and acetylcholine, have been shown to bind to residues located approximately in the middle of the TMs of their receptors (Strader et al., 1988; Pollock et al., 1992; Fu et al., 1996; Ward et al., 1999; Liapakis et al., 2000). Likewise, the D2 dopamine receptor antagonist sulpiride protected cysteine substituted for Ser129 at the cytoplasmic end of TM3 and for Val378 at the cytoplasmic end of TM6 from reaction with MTSEA by binding more extracellularly (Javitch et al., 1995, 1998). These findings are

- (LogK_D ± S.E.)	
CONTROL	MTSEA
8.85 ± 0.10	8.80 ± 0.13
9.11 ± 0.05	8.89 ± 0.04
9.11 ± 0.09	8.88 ± 0.08
8.87 ± 0.16	8.80 ± 0.23
8.60 ± 0.63	8.60 ± 0.54
9.05 ± 0.08	9.11 ± 0.13

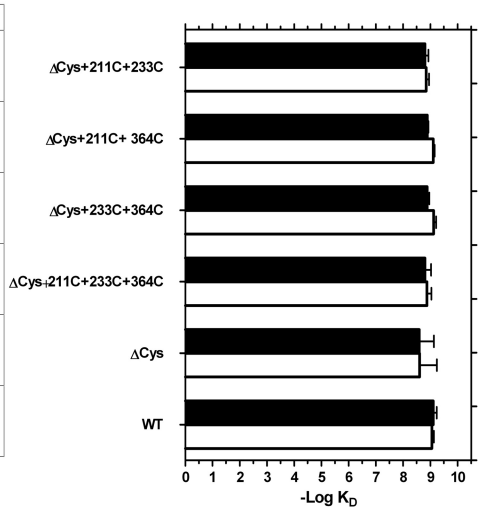

Fig. 11. Binding affinity of Tyr^0-sauvagine for ΔCys + 211C + 233C, ΔCys + 211C + 364C, ΔCys + 233C + 364C, and ΔCys + 211C + 233C + 364C receptors before and after MTSEA reaction. Suspensions of HEK 293 cells stably expressing WT CRF_1 or the mutants ΔCys, ΔCys + 211C + 233C, ΔCys + 211C + 364C, ΔCys + 233C + 364C, or ΔCys + 211C + 233C + 364C were treated without (control) or with 2.5 mM MTSEA for 15 s at 22 to 25°C. Thereafter, the cells were homogenized and membrane homogenates were incubated with ^{125}I-Tyr^0-sauvagine in the absence or presence of increasing concentrations of Tyr^0-sauvagine. The data were fitted to a one-site competition model by nonlinear regression, and $-\log K_D$ values were determined as described under *Materials and Methods*. The bars represent the binding affinity ($-\log K_D$) of ^{125}I-Tyr^0-sauvagine before (□) or after MTSEA reaction (■). The mean ± S.E. values are from two to six independent experiments. The results were statistically analyzed using one-way ANOVA followed by least significant difference post hoc test. None of the mutations considerably altered ^{125}I-Tyr^0-sauvagine affinity and MTSEA treatment did not significantly alter radioligand affinity of the receptors tested.

consistent with the inability of the bulkier MTSET to reach the endogenous TM cysteines of CRF_1. In addition to the possibility of protection through blocking passage of reagent, we cannot rule out an indirect protection through a ligand-mediated propagated structural rearrangement.

In contrast to our results, theoretical arrangements of the seven helices of family B GPCRs deduced from a detailed analysis of their sequences placed the residues of glucagon-like peptide-1 receptor that correspond to Cys211, Cys233, and Cys364 of CRF_1 facing lipid (Donnelly, 1997; Frimurer and Bywater, 1999). Likewise, in the family A GPCRs, D2 dopamine and rhodopsin receptor, residues 3.48 and 4.47, which are predicted to be aligned with Cys211 and Cys233 of CRF_1, respectively (Frimurer and Bywater, 1999), face away from the binding site crevice (Baldwin, 1993; Palczewski et al., 2000; Ballesteros et al., 2001). It is conceivable that these cysteines are located at a water-accessible interface of a CRF_1 oligomeric complex. However, it is also possible that in a single CRF_1 molecule, local distortions, such as those induced by the presence of proline and/or glycine above Cys211 and Cys233 could alter the configuration of TM3 and TM4 such as to position these cysteines facing into the protein interior, in which they are accessible to MTSEA. Consistent with this proposal, proline and glycine are known to modulate α-helical structure (Deupi et al., 2005). Likewise, the presence of a glycine a few residues above Cys364 might alter the conformation of TM7 to position this Cys facing into the protein interior. Residue 7.54 of the β2-adrenergic and D2 dopamine receptor, which are predicted to be aligned with Cys364 of CRF_1, has been shown to be accessible for reaction with MTSEA (Fu et al., 1996; Liapakis and Javitch, 1998). This irregular pattern of accessibility has been proposed to be closely associated with a proline kink at Pro7.50, which is conserved in family A GPCRs, in agreement with the crystal structure of rhodopsin (Fu et al., 1996; Palczewski et al., 2000; Ballesteros et al., 2001). The theoretical models of family B GPCRs, therefore, will need to be refined based on experimental data, including those of the present study. This is further supported by the observed inconsistencies between these theoretical models and the experimental data from a study on parathyroid hormone receptor, which determined the distances between histidine at the cytoplasmic ends of TM3 and TM6 by their ability to form zinc bridges (Sheikh et al., 1999).

Starting with the MTSEA-insensitive ΔCys mutant of CRF_1, we can now systematically replace with cysteine the amino acids of the membrane-spanning segments of CRF_1 and apply the substituted-cysteine accessibility method to characterize the residues lining the binding-site crevice. The resulting data will be used to refine theoretical models of family B GPCRs, ultimately advancing structure-based rational drug design.

Acknowledgments

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References

Assil-Kishawi I, Samra TA, Mierke DF, and Abou-Samra AB (2008) Residue 17 of sauvagine cross-links to the first transmembrane domain of corticotropin-releasing factor receptor 1 (CRFR1). *J Biol Chem* **283:**35644–35651.

Baldwin JM (1993) The probable arrangement of the helices in G protein-coupled receptors. *EMBO J* **12:**1693–1703.

Ballesteros JA, Shi L, and Javitch JA (2001) Structural mimicry in G protein-coupled receptors: implications of the high-resolution structure of rhodopsin for structure-function analysis of rhodopsin-like receptors. *Mol Pharmacol* **60:**1–19.

Chrousos GP (1995) The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. *N Engl J Med* **332:**1351–1362.

Dautzenberg FM, Kilpatrick GJ, Wille S, and Hauger RL (1999) The ligand-selective domains of corticotropin-releasing factor type 1 and type 2 receptor reside in different extracellular domains: generation of chimeric receptors with a novel ligand-selective profile. *J Neurochem* **73:**821–829.

Deng HB, Guang W, and Wang JB (2000) Selected cysteine residues in transmembrane domains of mu-opioid receptor are critical for effects of sulfhydryl reagents. *J Pharmacol Exp Ther* **293:**113–120.

Deupi X, Govaerts C, Shi L, Javitch AJ, Pardo L, and Ballesteros J (2005) Conformational plasticity of GPCR binding sites. Structural basis for evolutionary diversity in ligand recognition, in *The G Protein-Coupled Receptors Handbook* (Devi LA ed) pp 363–388, Humana Press Inc., Totowa, NJ.

Donnelly D (1997) The arrangement of the transmembrane helices in the secretin receptor family of G-protein-coupled receptors. *FEBS Lett* **409:**431–436.

Frimurer TM and Bywater RP (1999) Structure of the integral membrane domain of the GLP1 receptor. *Proteins* **35:**375–386.

Fu D, Ballesteros JA, Weinstein H, Chen J, and Javitch JA (1996) Residues in the seventh membrane-spanning segment of the dopamine D2 receptor accessible in the binding-site crevice. *Biochemistry* **35:**11278–11285.

Gether U (2000) Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocr Rev* **21:**90–113.

Gkountelias K, Tselios T, Venihaki M, Deraos G, Lazaridis I, Rassouli O, Gravanis A, and Liapakis G (2009) Alanine scanning mutagenesis of the second extracellular loop of type 1 corticotropin-releasing factor receptor revealed residues critical for peptide binding. *Mol Pharmacol* **75:**793–800.

Grace CR, Perrin MH, Gulyas J, Digruccio MR, Cantle JP, Rivier JE, Vale WW, and Riek R (2007) Structure of the N-terminal domain of a type B1 G protein-coupled receptor in complex with a peptide ligand. *Proc Natl Acad Sci USA* **104:**4858–4863.

Grigoriadis DE, Haddach M, Ling N, and Saunders J (2001) The CRF receptor: structure, function and potential for therapeutic intervention. *Curr Med Chem Cent Nerv Syst Agents* **1:**63–97.

Guo W, Urizar E, Kralikova M, Mobarec JC, Shi L, Filizola M, and Javitch JA (2008) Dopamine D2 receptors form higher order oligomers at physiological expression levels. *EMBO J* **27:**2293–2304.

Harmar AJ (2001) Family-B G-protein-coupled receptors. *Genome Biol* **2:** REVIEWS3013.

Hoare SR, Sullivan SK, Ling N, Crowe PD, and Grigoriadis DE (2003) Mechanism of corticotropin-releasing factor type I receptor regulation by nonpeptide antagonists. *Mol Pharmacol* **63:**751–765.

Holtmann MH, Ganguli S, Hadac EM, Dolu V, and Miller LJ (1996) Multiple extracellular loop domains contribute critical determinants for agonist binding and activation of the secretin receptor. *J Biol Chem* **271:**14944–14949.

Javitch JA, Ballesteros JA, Weinstein H, and Chen J (1998) A cluster of aromatic residues in the sixth membrane-spanning segment of the dopamine D2 receptor is accessible in the binding-site crevice. *Biochemistry* **37:**998–1006.

Javitch JA, Fu D, Chen J, and Karlin A (1995) Mapping the binding-site crevice of the dopamine D2 receptor by the substituted-cysteine accessibility method. *Neuron* **14:**825–831.

Javitch JA, Li X, Kaback J, and Karlin A (1994) A cysteine residue in the third membrane-spanning segment of the human D2 dopamine receptor is exposed in the binding-site crevice. *Proc Natl Acad Sci USA* **91:**10355–10359.

Javitch JA, Shi L, and Liapakis G (2002) Use of the substituted cysteine accessibility method to study the structure and function of G protein-coupled receptors. *Methods Enzymol* **343:**137–156.

Karlin A and Akabas MH (1998) Substituted-cysteine accessibility method. *Methods Enzymol* **293:**123–145.

Kraetke O, Holeran B, Berger H, Escher E, Bienert M, and Beyermann M (2005a) Photoaffinity cross-linking of the corticotropin-releasing factor receptor type 1 with photoreactive urocortin analogues. *Biochemistry* **44:**15569–15577.

Kraetke O, Wiesner B, Eichhorst J, Furkert J, Bienert M, and Beyermann M (2005b) Dimerization of corticotropin-releasing factor receptor type 1 is not coupled to ligand binding. *J Recept Signal Transduct Res* **25:**251–276.

Liapakis G, Ballesteros JA, Papachristou S, Chan WC, Chen X, and Javitch JA (2000) The forgotten serine. A critical role for Ser-2035.42 in ligand binding to and activation of the beta 2-adrenergic receptor. *J Biol Chem* **275:**37779–37788.

Liapakis G and Javitch JA (1998) Reverting the constitutive activity of a CAM b2 adrenergic receptor by modification of a cysteine in TM7. *Soc Neurosci Abstr* **24:**537.16.

Liaw CW, Grigoriadis DE, Lorang MT, De Souza EB, and Maki RA (1997) Localization of agonist- and antagonist-binding domains of human corticotropin-releasing factor receptors. *Mol Endocrinol* **11:**2048–2053.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, et al. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289:**739–745.

Perrin MH, Grace CR, Riek R, and Vale WW (2006) 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. *Ann NY Acad Sci* **1070:**105–119.

Perrin MH, Sutton S, Bain DL, Berggren WT, and Vale WW (1998) The first extracellular domain of corticotropin releasing factor-R1 contains major binding determinants for urocortin and astressin. *Endocrinology* **139:**566–570.

Pioszak AA, Parker NR, Suino-Powell K, and Xu HE (2008) Molecular recognition of corticotropin-releasing factor by its G-protein-coupled receptor CRFR1. *J Biol Chem* **283:**32900–32912.

Pollock NJ, Manelli AM, Hutchins CW, Steffey ME, MacKenzie RG, and Frail DE (1992) Serine mutations in transmembrane V of the dopamine D1 receptor affect ligand interactions and receptor activation. *J Biol Chem* **267:**17780–17786.

Qi LJ, Leung AT, Xiong Y, Marx KA, and Abou-Samra AB (1997) Extracellular

cysteines of the corticotropin-releasing factor receptor are critical for ligand interaction. *Biochemistry* **36:**12442–12448.
Sheikh SP, Vilardarga JP, Baranski TJ, Lichtarge O, Iiri T, Meng EC, Nissenson RA, and Bourne HR (1999) Similar structures and shared switch mechanisms of the beta2-adrenoceptor and the parathyroid hormone receptor. Zn(II) bridges between helices III and VI block activation. *J Biol Chem* **274:**17033–17041.
Strader CD, Sigal IS, Candelore MR, Rands E, Hill WS, and Dixon RA (1988) Conserved aspartic acid residues 79 and 113 of the beta-adrenergic receptor have different roles in receptor function. *J Biol Chem* **263:**10267–10271.
Underwood CR, Garibay P, Knudsen LB, Hastrup S, Peters GH, Rudolph R, and Reedtz-Runge S (2010) Crystal structure of glucagon-like peptide-1 in complex with the extracellular domain of the glucagon-like peptide-1 receptor. *J Biol Chem* **285:**723–730.
Unson CG, Wu CR, Jiang Y, Yoo B, Cheung C, Sakmar TP, and Merrifield RB (2002) Roles of specific extracellular domains of the glucagon receptor in ligand binding and signaling. *Biochemistry* **41:**11795–11803.
Vale W, Spiess J, Rivier C, and Rivier J (1981) Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* **213:**1394–1397.
Ward SD, Curtis CA, and Hulme EC (1999) Alanine-scanning mutagenesis of transmembrane domain 6 of the M(1) muscarinic acetylcholine receptor suggests that Tyr381 plays key roles in receptor function. *Mol Pharmacol* **56:**1031–1041.

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