A Second Disulfide Bridge from the N-Terminal Domain to Extracellular Loop 2 Dampens Receptor Activity in GPR39[†]

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ABSTRACT: A highly conserved feature across all families of 7TM receptors is a disulfide bridge between a Cys residue located at the extracellular end of transmembrane segment III (TM-III) and one in extracellular loop 2 (ECL-2). The zinc sensor GPR39 contains four Cys residues in the extracellular domains. By using mutagenesis, treatment with the reducing agent TCEP, and a labeling procedure for free sulfhydryl groups, we identify the pairing of these Cys residues in two disulfide bridges: the prototypical bridge between Cys^{108} in TM-III and Cys^{210} in ECL-2 and a second disulfide bridge connecting Cys^{11} in the N-terminal domain with Cys^{191} in ECL-2. Disruption of the conserved disulfide bond by mutagenesis greatly reduced the level of cell surface expression and eliminated agonist-induced increases in inositol phosphate production but surprisingly enhanced constitutive signaling. Disruption of the nonconserved disulfide bridge by mutagenesis led to an increase in the Zn^{2+} potency. This phenotype, with an approximate 10-fold increase in agonist potency and a slight increase in E_{max}, was mimicked by treatment of the wild-type receptor with TCEP at low concentrations, which had no effect on the receptor already lacking the second disulfide bridge and already displaying a high Zn^{2+} potency. We conclude that the second disulfide bridge, which according to the β2-adrenergic structure will form a covalent link across the entrance to the main ligand binding pocket, serves to dampen GPR39 activation. We suggest that formation of extra disulfide bridges may be an important general mechanism for regulating the activity of 7TM receptors.

A putative disulfide bridge from a Cys residue at the extracellular end of transmembrane segment III (TM-III) to a Cys residue in extracellular loop 2 (ECL-2),[1] usually in the middle, is recognized as essentially the only common structural feature across all families of 7TM G protein-coupled receptors, aside from the seven hydrophobic transmembrane segments (*1*). Within the large rhodopsin-like family A of receptors, 92% have these conserved Cys residues, and those which do not, for example, the melanocortin receptors, usually have instead a very short ECL-2 which directly connects TM-IV to TM-V (*1*). It was originally proposed that the conserved disulfide bond was required to stabilize the correct structure of the receptors (*2–4*). However, it was subsequently found that, for example, rhodopsin mutants in which these conserved Cys residues were replaced with Ala residues were able to fold properly but were unable to signal and had reduced stability (*5, 6*).

GPR39 is a 7TM receptor of the Ghrelin family, which comprises a number of receptors mainly involved in food intake and gastrointestinal functions and includes receptors for ghrelin, motilin, neuromedin U, and neurotensin. GPR39 is constitutively active and is activated by what are believed to be physiological concentrations of zinc ions (*7, 8*). Obestatin was reported to be the endogenous ligand for GPR39, but this could not be reproduced by other groups (*9–14*). On the other hand, it is becoming increasingly accepted that GPR39 could be a physiological Zn^{2+} sensor. For example, when Yasuda and colleagues were characterizing the GPR39-activating component from fetal bovine serum, they ultimately identified Zn^{2+} as this factor (*8*). Thus, while another endogenous ligand may yet be found, it is entirely possible that Zn^{2+} is a physiologically relevant agonist for GPR39. Surprisingly, the binding site for Zn^{2+} is apparently located in the extracellular domain of GPR39 between His^{17} and His^{19} in the N-terminal extension and possibly Asp^{313} in ECL-3 (*15*).

With respect to potential disulfide bridges, human GPR39 is particularly interesting as it contains four Cys residues in the extracellular domains. That is, besides the conserved Cys residue at the extracellular end of TM-III (Cys^{108}), there is one Cys residue located in the N-terminal domain (Cys^{11}) and two in ECL-2 (Cys^{191} and Cys^{210}) (Figure 1). Thus, it is unclear which of the Cys residues in ECL-2 is the disulfide bridge partner for Cys^{108} in TM-III. In the Swiss-Prot entry for GPR39, Cys^{210} is identified as the disulfide partner by homology, but a recent bioinformatics multianalysis of

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[1] Abbreviations: TCEP, tris(2-carboxyethyl)phosphine; GPR39, G protein-coupled receptor 39; 7TM, seven-transmembrane domain; ECL, extracellular loop; IP, inositol phosphate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum.

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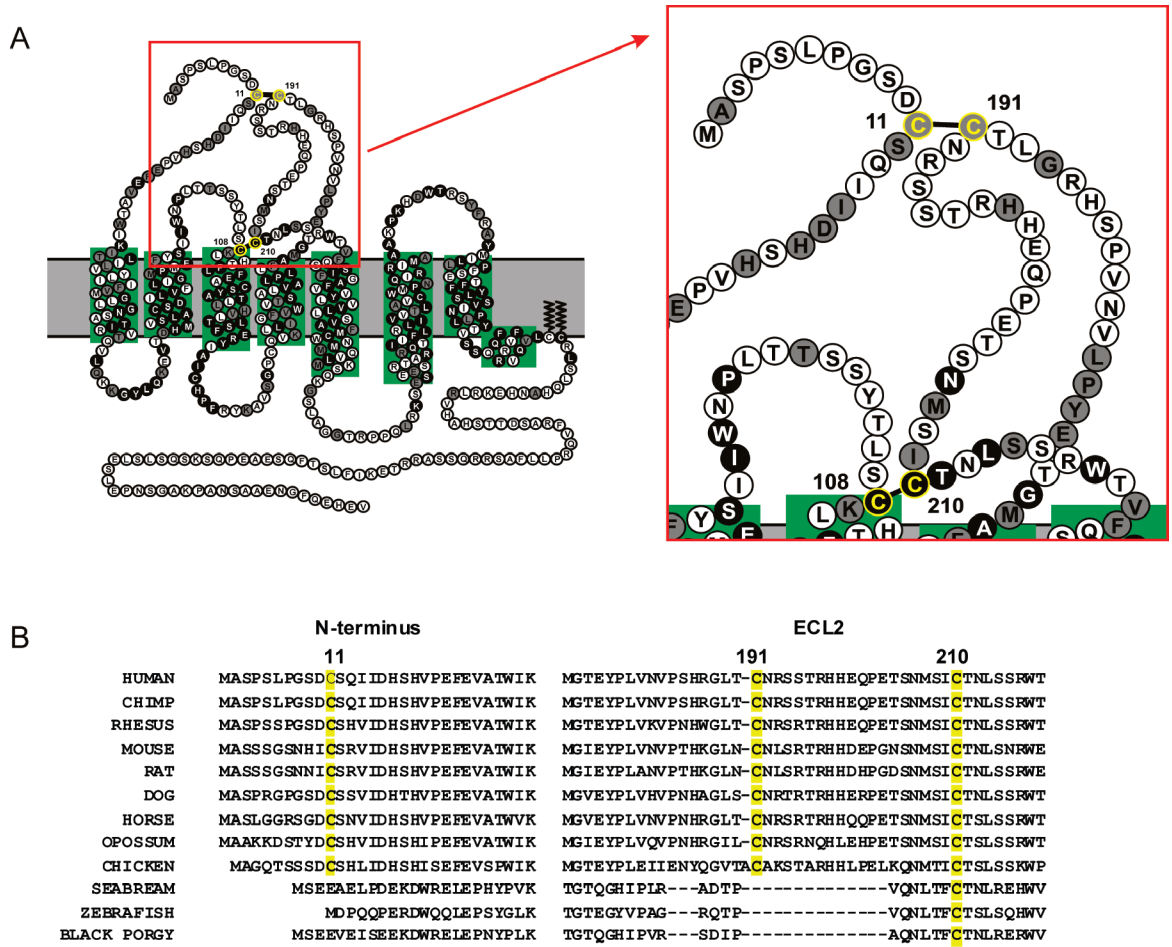

FIGURE 1: Amino acid sequence and features of GPR39. (A) Serpentine diagram of human GPR39. The four extracellular cysteine residues are numbered, and the disulfide pairings identified in this paper are indicated. Amino acid residues conserved across all 12 species are shown as white letters on black circles; those conserved in all but the fish species are shown as black letters on gray circles. (B) Interspecies alignment of the N-terminal domain and the second extracellular loop of GPR39. Seabream, zebrafish, and black porgy are all fish species. For brevity, Cys^{108}, conserved across all species, is not pictured.

ECL-2 sequences of 7TM receptors proposed the other ECL-2 Cys residue (Cys^{191}) as the partner (*16*). It is also unclear whether the two "remaining" Cys residues in the extracellular domain of GPR39 will form a second disulfide bridge between the N-terminal domain and ECL-2. This issue has become particularly important in view of the newly reported high-resolution structure of the β_2-adrenergic receptor, in which it is clear that, in contrast to rhodopsin, there is direct access to the deep main ligand-binding pocket from the extracellular space due, in part, to a second disulfide bridge in this receptor (*17*). Thus, an "extra" disulfide bridge from the N-terminal domain to ECL-2 would covalently join these two extracellular segments across the entrance to the binding pocket. This could potentially affect ligand binding and receptor activation.

This study addresses the questions of disulfide bridge formation in GPR39 and assesses their roles in both ligand-mediated and constitutive activity of the receptor, using both mutagenesis and controlled chemical reduction of disulfides. Moreover, we attempt to put this in perspective with regard to the new knowledge concerning the overall structure of extracellular domains in 7TM receptors activated by diffusible ligands as shown by the new structure of the β_2-adrenergic receptor.

EXPERIMENTAL PROCEDURES

Materials. Bond-Breaker tris(2-carboxyethyl)phosphine (TCEP) and immobilized avidin beads were from Pierce. *myo*-[2-^{3}H]Inositol (13 Ci/mmol) and polylysine yttrium silicate scintillation proximity assay (YSi-SPA) beads (1 g reconstituted in 10 mL of H_2O and stored at 4 °C for up to 1 month) were from GE Healthcare. *N*α-(3-Maleimidylpropionyl)biocytin, a sulfhydryl-reactive biotin moiety, was from Invitrogen.

Sequence Comparison. Clustal W was used to align the following GenPept sequences: XP_515800 (chimpanzee), XP_001116212 (rhesus monkey), AAH_85285 (mouse), NP_001094413 (rat), XP_853332 (dog), XP_001489024 (horse), XP_001369493 (opossum), NP_001073574 (chicken), ABU53899 (seabream), ABU897 (zebrafish), and ABU53895 (black porgy).

Receptor Construction. The cDNA of the human GPR39 in a mammalian expression vector was provided by K. Hansen (7TM Pharma A/S) and corresponds to GenPept entry NP_001499. The M2 FLAG epitope (DYKDDDDK) was inserted at the N-terminus following the start codon. Mutant receptors were constructed using the PCR overlap extension method (*18*) and were verified by DNA sequence analysis.

Cell Culture. Human embryonic kidney cells (HEK-293) were grown at 37 °C in 5% CO_2 and 95% humidity in DMEM with Glutamax, 10% fetal bovine serum, 100 units/mL penicillin G, and 100 μg/mL streptomycin.

Inositol Phosphate Accumulation Assay. Twenty thousand cells per well were seeded overnight (without antibiotics) in poly-D-lysine-coated 96-well plates, and then transient transfections were performed using 30 ng of receptor DNA/well with Effectene reagent (Qiagen). Fresh medium containing 10 μCi/mL of *myo*-[2-^{3}H]inositol was added the next day. Following incubation for ∼24 h, cells were washed with HBSS, and compounds were added to cells in HBSS with 10 mM LiCl (100 μL total volume) and incubated for 45 min at 37 °C. Subsequently, cells were lysed in 50 μL of 10 mM formic acid on ice for ≥30 min. Ysi-SPA beads were diluted 8-fold in H_2O immediately before use, and 80 μL was combined with 20 μL of cell lysate in white 96-well plates, shaken vigorously for 5−30 min, centrifuged at 400*g* for 5 min, and incubated at room temperature for ≥8 h and radioactivity (counts per minute) measured in a TopCount-NXT scintillation counter (Packard). Each experiment was performed in duplicate or triplicate.

Cell Surface ELISA. Approximately forty-eight hours post-transfection (as above), cells were washed with PBS, fixed for 10 min in 3.7% formaldehyde, washed (3 × 10 min) with PBS, blocked for 30 min in PBS with 3% milk powder and 50 mM Tris-HCL (pH 7.5), incubated with a 1/1000 dilution of anti-FLAG antibody (Sigma catalog no. 1804) in blocking buffer for 1−2 h, washed, incubated in a 1/1250 dilution of goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Pierce) in blocking buffer for 1 h, washed, and visualized by addition of 100 μL of TMB Plus substrate (Kem-En-Tec), and the reaction was stopped with 100 μL of 0.2 M H_2SO_4. One hundred microliters from each well was transferred to a clear plate and absorbance measured at 450 nm for 1 s on a Wallac Victor2 instrument (Perkin-Elmer). Four to six replicates per experiment were performed.

Sulfhydryl Labeling Western Blot. HEK-293 cells (4 × 10^6) were plated overnight on poly-D-lysine-coated 100 mm dishes and transfected using the calcium phosphate precipitation method with 30 μg of plasmid DNA and incubated for ∼24 h. For labeling, 4 mL of each solution was used per dish. All steps were performed at room temperature, unless otherwise noted. After an initial wash with TBS (pH 7.5), cells were incubated for 15 min in 10 mM TCEP in TBS to reduce disulfide bonds as a control ("+TCEP") or TBS alone ("−TCEP" samples) to maintain disulfide bonds. Solutions were removed, and 500 μM *N*α-(3-maleimidylpropionyl)-biocytin in PBS (pH 7.5) was added for 30 min to label the sulfhydryl groups of free (i.e., non-disulfide-bonded) Cys residues. After aspiration, freshly prepared 10 mM sodium 2-mercaptoethanesulfonate (MeSNA) in TBS was added for 5 min. This solution was removed, and cells were harvested using a cell scraper in PBS, 10 mM EDTA, and 5 mM *N*-ethylmaleimide (NEM) (freshly prepared). Samples were subsequently kept on ice. Cells were pelleted; then supernatants were removed and stored at −80 °C, or the membrane protein was immediately isolated as follows. Pellets were resuspended in 20 mM HEPES, 10 mM EDTA, and 5 mM NEM with protease inhibitors and homogenized using an Ultrathorax at setting 4.5 for 30 s. Lysates were centrifuged at 4 °C for 45 min at 15000*g*. Supernatants were discarded and pellets resuspended in 20 mM HEPES, 0.1 mM EDTA, and 5 mM NEM with protease inhibitors and centrifuged as described above. Supernatants were discarded and pellets resuspended in lysis buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA, and 5 mM NEM with protease inhibitors). After resuspension, Triton X-100 was added to a final concentration of 1%, and tubes were incubated overnight at 4 °C with end-over-end rotation. Tubes were centrifuged at 4 °C for 20 min at 15000*g*. Supernatants containing the solubilized proteins were transferred to new tubes, and protein concentrations were determined.

Immunoprecipitations were performed in lysis buffer supplemented with 1% Triton X-100. For the −TCEP samples, 200 μg of membrane protein was immunoprecipitated, while for +TCEP samples, 50 μg of membrane protein was immunoprecipitated by addition of 175 μL of avidin beads and overnight incubation with end-over-end rotation. Beads were washed four times in lysis buffer supplemented with 1% Triton X-100 with centrifugation at 4000 rpm for 3 min. After removal of the last wash, beads were resuspended in 60 μL (for −TCEP samples) or 120 μL (for +TCEP samples) of loading buffer (Laemmli buffer, 10 mM NEM, 50 mM DTT, and protease inhibitors), incubated for 10 min at 95 °C, and centrifuged at 4000 rpm for 3 min, and eluates were moved to new tubes for storage (−20 °C) or directly loaded onto 8 to 12% SDS−PAGE gels and run under reducing conditions. Proteins were transferred to PVDF membranes and blotted according to the protocol provided with the α-FLAG primary antibody (Sigma catalog no. F7425) diluted 1/1000. The secondary antibody (Pierce, goat α-rabbit HRP) was used at a 1/200000 dilution. Detection was performed using SuperSignal Pico West (Pierce), and blots were exposed to X-ray film and developed.

Disulfide Bridge Patterns in 7TM Receptors. The hypothesized disulfide bonds in various receptors as depicted in Figure 6 were compiled from published functional studies and/or through analysis of the conservation of cysteine residues across multiple species using Swiss-Prot (S-P), including NMU1 (S-P), GnRH (*19, 20*), DP_1, EP_1 and IP_1 (*21*), AT_1 (*22*), AT_2 (*23, 24*), Y_1 and Y_4 (S-P), $P2Y_1$ (*25*), the chemokine family (*26*), β_2-AR (*17, 27*), β_1-AR and β_3-AR (S-P), the olfactory family (*28*), MC_{1-5} (*29*), EDG_{1-5} (S-P), and CB_1 and CB_2 (S-P) receptors.

Data Analysis. Concentration−response curves were generated, EC_{50} values determined, and statistical analyses performed using Prism (version 5, GraphPad Software) and fitting normalized data by nonlinear regression to a four-component logistic equation [log(agonist) vs response − variable slope], unless otherwise noted.

RESULTS

Comparison of Cysteine Residues in GPR39 across Species. Sequence alignment of GPR39 from 12 species reveals that among the three Cys residues in the extracellular domains, only Cys^{210} (human numbering) is completely conserved from humans to fish besides Cys^{108} at the top of TM-III (Figure 1B). This suggests that Cys^{210} might be the residue that makes the conserved disulfide bridge to Cys^{108} at the extracellular end of TM-III (Figure 1A). Cys^{191}, i.e., the other Cys residue in ECL-2, is conserved between mammals and chicken but not in fish (Figure 1B). In the

Table 1: Pharmacological Characterization of Cysteine to Alanine GPR39 Mutants[a]

	EC_{50}					
	−log (M)	μM	*x*-fold improvement[b]	N[c]	E_{max}	expression
WT	4.54 ± 0.05	28.6	1.0	24	100	100
C11A	4.86 ± 0.12 (n.s.)	13.8	2.1	4	110 ± 13 (ns)	85 ± 9 (ns)
C191A	5.26 ± 0.71 (n.s.)	5.5	5.2	6	84 ± 5 (*)	88 ± 7 (ns)
C11A/C191A	5.36 ± 0.06 (***)	4.4	6.5	7	103 ± 8 (ns)	123 ± 12 (ns)
C108A	>3	−	−	5	−	22 ± 5 (***)
C210A	>3	−	−	6	−	13 ± 2 (***)
C11A/C108A	>3	−	−	3	−	11 ± 2 (***)
C11A/C210A	>3	−	−	3	−	20 ± 4 (**)
C108A/C191A	>3	−	−	3	−	40 ± 9 (*)
C108A/C210A	>3	−	−	3	−	16 ± 2 (***)
C191A/C210A	>3	−	−	3	−	7 ± 1 (***)
C11A/C108A/C191A	>3	−	−	3	−	7 ± 1 (***)

[a] Constructs transiently transfected in HEK-293 cells were analyzed for function in inositol phosphate accumulation assays in response to 10-point zinc concentrations and for cell surface expression in an ELISA. Values include the standard error of the mean, where applicable. The statistical significance of difference in values for each mutant as compared to that of WT was determined using a one-sample *t* test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant). [b] Determined by EC_{50}(WT)/EC_{50}(mutant). [c] *N* represents the number of separate experiments.

N-terminal domain, the amino acid sequence between Cys^{11} and the start of TM-I is surprisingly well conserved between mammals and chicken, including Cys^{11}, but again not in fish. Thus, the presence of the second Cys residue in ECL-2 is strictly correlated with the presence of a Cys residue in the N-terminal domain (Figure 1B). This suggests that these two Cys residues would in fact form a second disulfide bridge in GPR39.

Functional Analysis of Cysteine Replacement Mutants. Mutant receptors with Ala substitutions for each of the four extracellular Cys residues were transiently expressed in HEK-293 cells. Subsequently, these were analyzed for their ability to mediate Zn^{2+}-induced increases in inositol phosphate (IP) production as well as for cell surface expression as determined by an ELISA. Notably, a clear pattern appeared in the cell surface expression of these mutant receptors. That is, Ala substitution of Cys^{108} at the extracellular end of TM-III decreased the level of expression to 22% of that of the wild type (WT), which is similar to the reduction observed for Ala substitution of Cys^{210} in ECL-2 to 13% of that of WT (Table 1). In contrast, the C11A and C191A mutants were well expressed at similar levels, 85 and 88% of that of WT, respectively. Thus, the expression levels alone would indicate that Cys^{108} and Cys^{210} are "coupled", as are Cys^{11} and Cys^{191}.

For the Cys^{108} and Cys^{210} pair, this pattern of similarity was also observed with respect to signaling. Both C108A and C210A exhibited an increased level of constitutive signaling, most pronounced for C108A, despite the reduced level of cell surface expression (Figure 3 and Table 1). Neither C108A nor C210A responded to Zn^{2+} even at concentrations of up to 1 mM. Surprisingly, on the basis of the assumption that these two residues should form a disulfide bridge, C11A and C191A single mutants exhibited considerably different signaling phenotypes. In fact, little effect was observed for the Ala substitution of Cys^{11}. In the C11A mutant, the constitutive activity was unaltered and the efficacy of Zn^{2+} was also similar to that of WT (Figure 2). However, the potency of Zn^{2+} was improved approximately 2-fold, from 29 to 14 μM (Table 1). The C191A mutant exhibited increased constitutive activity but could still be stimulated by Zn^{2+} to almost the same E_{max} that was observed for the WT receptor. Although the potency, as in the C11A mutant, was improved (from 29 to 5.5 μM), the incremental increase in IP production in response to Zn^{2+} was small due to the high constitutive activity (Figure 2).

A series of systematic double mutations were introduced to improve our understanding of the pairings of the Cys residues. All of the double mutants involving either Cys^{108} or Cys^{210} had an expression and signaling phenotype similar to what was observed for the C108A and C210A single mutants, i.e., weakened surface expression, increased constitutive signaling (in some), and lack of a Zn^{2+} response (Table 1 and Figure 3). Interestingly, the C11A/C191A double mutant, which expressed well (123% compared to that of WT), displayed somewhat decreased constitutive activity but responded well to Zn^{2+}, i.e., reached an E_{max} similar to that of WT GPR39 and displayed a 6.5-fold improved Zn^{2+} potency (Figure 2 and Table 1).

Sulfhydryl Labeling of Cysteine Residues in GPR39. To determine whether the Cys residues have free thiol groups or are involved in disulfide bonds in GPR39, we employed a cell surface sulfhydryl labeling protocol (*30*). In this procedure, transfected cells are incubated with the membrane-impermeant, sulfhydryl-reactive reagent, *N*α-(3-maleimidylpropionyl)biocytin, which attaches a biotin moiety to the sulfhydryl group of unbonded Cys residues but cannot react with disulfide-bonded Cys residues. Subsequently, biotin-labeled receptors are immunoprecipitated with avidin beads, and GPR39 is then visualized on Western blots using an anti-FLAG antibody. No sulfhydryl labeling could be detected in the WT receptor in the absence of reducing agent (Figure 4), indicating that no free Cys residues are present in the extracellular domains of GPR39. In contrast, in the presence of the reducing agent TCEP, strong labeling was observed in the wild-type receptor. As another control, the C11A mutant, which has an uneven number of three Cys residues remaining in the extracellular domain, was probed in a similar way and exhibited strong labeling in the absence of TCEP (Figure 4). Similar results were obtained with the C191A mutant (data not shown). This indicates that Cys^{11} and Cys^{191} at least do not form intermolecular disulfide bridges with their counterparts in the dimer partner as that would have eliminated the free sulfhydryl group which is observed to be labeled. All together, these results demonstrate that the four Cys residues found in the extracellular domains of WT GPR39 are not free to be labeled and consequently most likely form two intramolecular disulfide bonds.

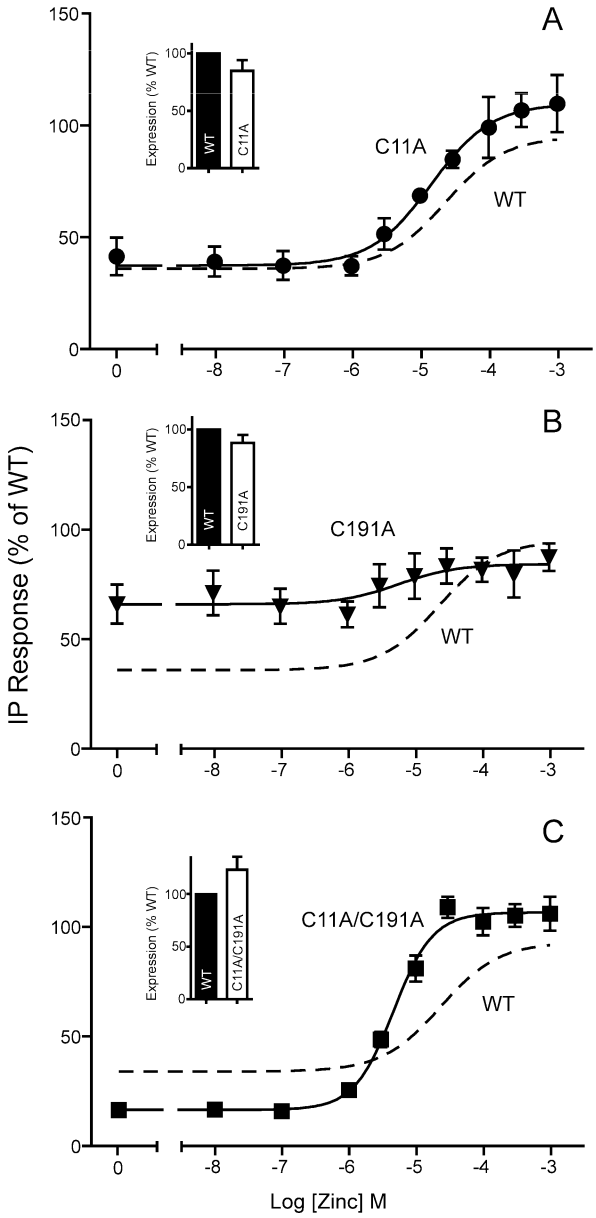

FIGURE 2: Activation of WT and mutant GPR39. Inositol phosphate accumulation assays in response to zinc for the mutant receptors (A) C11A, (B) C191A, and (C) C11A/C191A. The dashed line represents data for wild-type GPR39 for comparison. Data are means ± the standard error of the mean of at least three separate experiments. See Experimental Procedures for details of data analysis. The slope of the C191A mutant data was fixed at 1 to improve curve fitting.

Effect of Reducing Agents on Signaling of GPR39 and Mutant Forms. As presented above, we observed an increase in potency for Zn^{2+} in all three mutants in which the putative disulfide bridge between Cys^{11} and Cys^{191} was selectively broken (Table 1). Previously, we observed that artificial disulfide bridges in the extracellular domains of the tachykinin NK_1 receptor were more susceptible to breakage than the conceivably more buried, conserved disulfide bridge from the extracellular end of TM-III to ECL-2 (*31*). Therefore, we attempted to selectively break the nonconserved disulfide bridge from Cys^{11} to Cys^{191} with reducing agents and examine any affects on signaling.

On the WT receptor, addition of 0.1 mM TCEP improved the potency of Zn^{2+} in IP accumulation assays from 29 to 3.7 μM (log $EC_{50} = -4.6 \pm 0.03$ without TCEP vs $-5.4 \pm$ 0.11 with 0.1 mM TCEP; $p = 0.016$). Addition of 1 mM TCEP further enhanced the potency of zinc ($EC_{50} = 2.3$ μM; log $EC_{50} = -5.6$; $p = 0.011$ compared to that without TCEP), as well as the efficacy (Figure 5A). This increase in Zn^{2+} potency is similar to what we observed, in the absence of reducing agent, in the C11A/C191A double mutant as compared to WT (Figure 2C and Table 1). The reducing agent has no significant effect on the C11A/C191A mutant (log $EC_{50} = -5.4 \pm 0.03$ without TCEP, -5.6 ± 0.08 with 0.1 mM TCEP, and -5.4 ± 0.09 with 1 mM TCEP; no statistically significant differences between these values), which lacks the disulfide bridge linking the N-terminal domain and ECL-2 (Figure 5B).

In the opposite situation, treatment with increasing concentrations of TCEP potentiates WT GPR39 signaling in the presence of Zn^{2+} with an EC_{50} of approximately 50 μM, and this is again not observed in the C11A/C191A double mutant (Figure 5C,D). At high concentrations of TCEP, the level

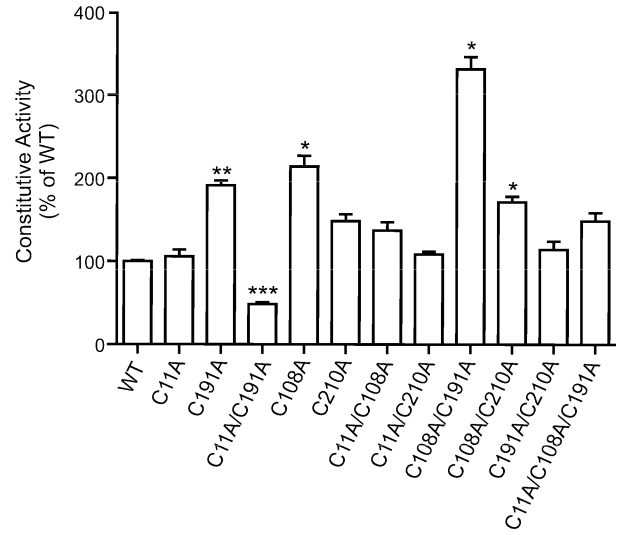

FIGURE 3: Constitutive activity of the WT and mutant forms of GPR39. Basal activity in the inositol phosphate accumulation assay is expressed as a percentage of WT basal activity. Data are not normalized for apparent expression to prevent overestimation of the constitutive activity of the mutant receptors with an apparent very low level of expression (Table 1) and are consequently conservative estimates. Data are means ± the standard error of the mean of at least three separate experiments. The statistical significance of difference in constitutive activity for each mutant as compared to WT was determined using a one-sample *t* test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

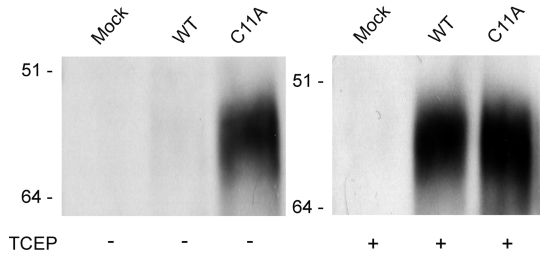

FIGURE 4: Sulfhydryl labeling Western blots. Free cysteine residues are labeled with a sulfhydryl-reactive biotin, while disulfide-bonded cysteine residues cannot be labeled in the absence of the reducing agent TCEP (−TCEP) but will be labeled if the disulfide bridges are first reduced with TCEP (+TCEP). Immunoprecipitations were performed with avidin beads (which will pull down only biotin-labeled receptors) and immunoblotted using an anti-FLAG antibody to detect GPR39. The blots shown are representative of several performed.

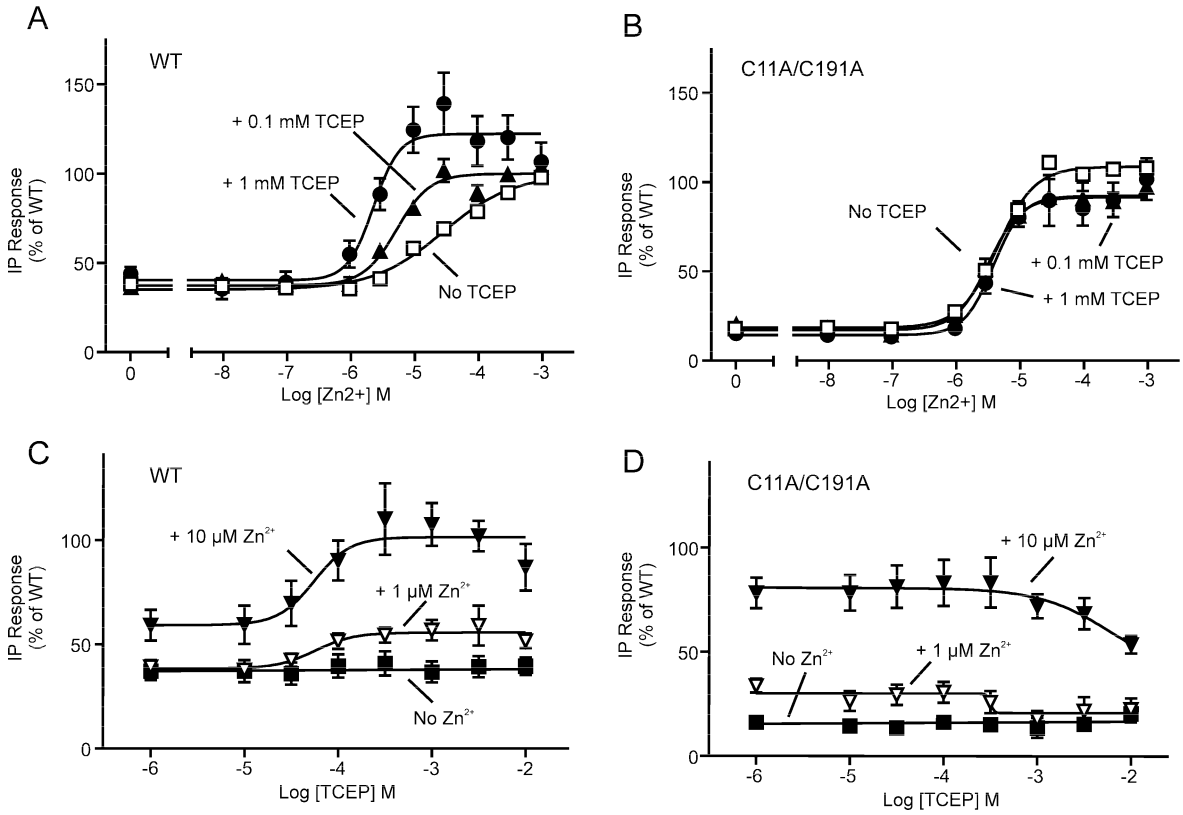

FIGURE 5: Inositol phosphate accumulation assays in the presence of reducing agents. (A) Zinc concentration−response curves (CRC) for wild-type GPR39 in the presence of 0, 0.1, or 1 mM TCEP. (B) Zinc CRC for the C11A/C191A mutant in the presence of 0, 0.1, or 1 mM TCEP. (C) TCEP CRC for wild-type GPR39 in the presence of 0, 1, or 10 μM zinc. (D) TCEP CRC for the C11A/C191A mutant in the presence of 0, 1, or 10 μM zinc. Data are means ± the standard error of the mean of at least three separate experiments.

of signaling is reduced in both the WT and the C11A/C191A double mutant, which probably indicates that the conserved disulfide bridge is being broken at these high concentrations (Figure 5C,D).

We conclude that breakage of the nonconserved disulfide bridge between Cys^{11} in the N-terminal domain and Cys^{191} in ECL-2, either through mutagenesis or by controlled treatment with a reducing agent, increases the potency of the agonist Zn^{2+} for GPR39. Thus, it appears that the extracellular domains are dampening the signaling of GPR39 and the nonconserved disulfide bridge is important for this function.

DISCUSSION

In this report, GPR39 is demonstrated to have two disulfide bridges with distinct functions in its extracellular domain. The conserved disulfide bridge between Cys^{108} at the extracellular end of TM-III and Cys^{210} in ECL-2 is required for proper cell surface expression and for agonist-mediated activation. The second disulfide bridge, linking Cys^{11} in the N-terminal domain to Cys^{191} in ECL-2, is not required for expression or receptor activation but appears instead to be important for a dampening effect of the extracellular domains on ligand-activated receptor signaling. Thus, receptors in which this disulfide bridge is broken (through mutations or by treatment with reducing agents) exhibit higher potencies and somewhat higher efficacies for the agonist, Zn^{2+}.

Conserved TM-III to ECL-2 Disulfide Bridge. Surprisingly, in several of the constructs where this disulfide bridge was broken, we observed increased constitutive activity of the receptor despite the fact that the level of cell surface expression of these, as determined by an ELISA, was decreased, most clearly illustrated by the simple C108A mutant (Figure 3 and Table 1). Attempts to increase the level of receptor expression of the C108A mutant, either by adding more plasmid DNA to the transfections or by using an inducible expression system, failed to increase the level of cell surface expression (data not shown). Furthermore, Western blotting of the C108A mutant receptor revealed that the majority of the protein was unglycosylated (data not shown), indicating that the mutant protein is largely retained in the endoplasmic reticulum (ER). That receptors with a low level of cell surface expression are signaling-competent may be explained in several ways. It might, in part, reflect a high receptor reserve of WT GPR39 such that even low levels of constitutively active mutants are sufficient to produce strong IP responses. This has been suggested to explain the high efficacy displayed by M_3 muscarinic receptor mutants in which Ala substitutions of conserved Cys residues resulted in poor cell surface expression (*32*). Alternatively, it may reflect an extremely high activity from the small number of receptors that reach the cell membrane. The receptor structure that promotes this constitutive activity might be a result of the formation of an unnatural disulfide bridge in these mutants. It is also possible that these signals arise from an intracellular location, such as within the ER, which has been proposed for the 7TM estrogen receptor, though this is a controversial issue (*33–37*).

Extra Disulfide Bridge Connecting the N-Terminal Domain with ECL-2. The two Cys residues forming this disulfide

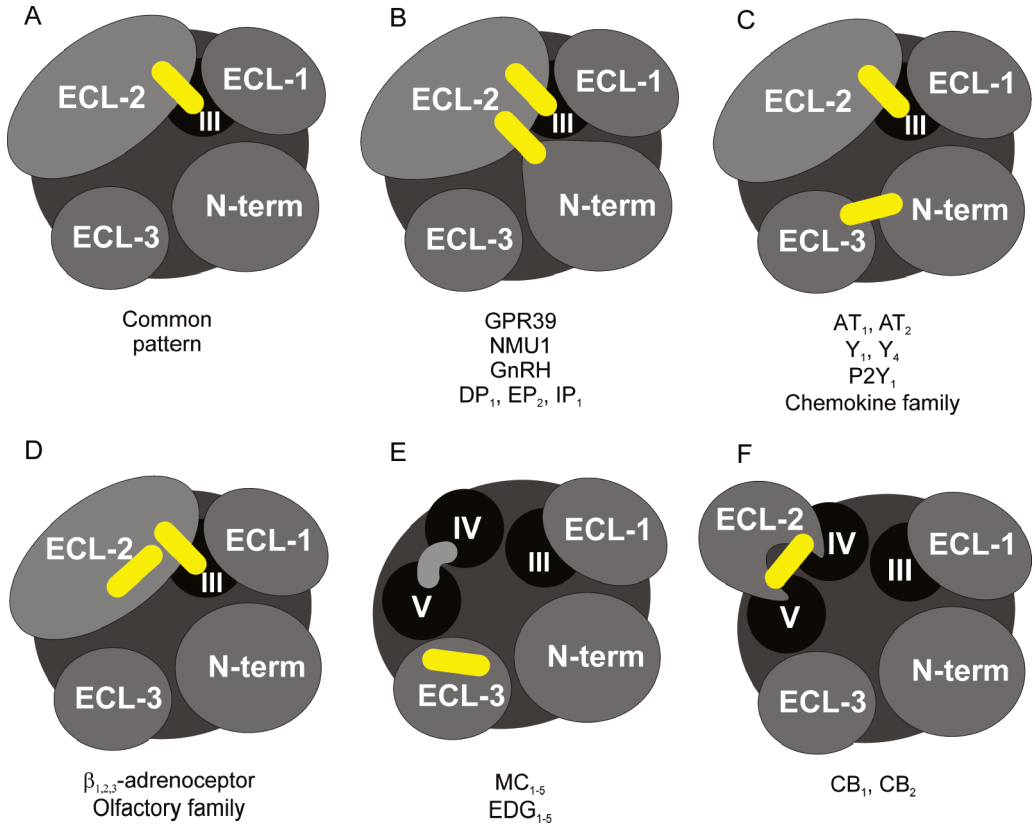

FIGURE 6: Disulfide bridge patterns in family A 7TM receptors. Schematic drawing depicting the various configurations of disulfide bridges found in this family of 7TM receptors as seen from the extracellular side. Selected examples of the receptors hypothesized to contain these patterns are listed below (nomenclature according to IUPHAR), as deduced from the literature or through bioinformatics analyses of receptor sequences (see Experimental Procedures for details). The yellow bars represent the disulfide bridges. Roman numerals III, IV, and V denote those transmembrane domains. Note that combinations of these patterns are also suggested by sequence conservation for some 7TM receptors.

bridge in GPR39, Cys^{11} and Cys^{191}, are conserved among all mammalian sequences available as well as in chicken, but not in fish (Figure 1). Interestingly, disruption of this covalent lock between the N-terminal domain and ECL-2 increased agonist potency and to some extent also E_{max}, as demonstrated by both Ala substitution mutagenesis and the application of reducing agent. In light of the new β_2-adrenergic receptor structure showing an open entrance to the relatively deeply located ligand-binding pocket (*17, 38, 39*), we propose that a covalent link between the N-terminal domain and ECL-2, located on opposite "edges" of this entrance, could constitute a structural barrier for the entrance of ligand into the funnel leading down to the receptor core (Figure 6). Breakage of this barrier would then make it easier for the ligand to gain access to the actual binding site. How this applies to the small Zn^{2+} ligand is unclear, but it could, for example, be related to the electrostatic potential in the funnel. However, though breakage of the second disulfide bridge increases agonist potency, it does not lead to increased constitutive activity of the GPR39 receptor as illustrated by the C11A and C11A/C191A mutants.

For β-adrenergic receptors, it has previously been noted that exposure to reducing agents produces first functional activation and then inactivation (*40–43*), leading to the hypothesis that cleavage of a disulfide bond is part of the activation process of 7TM receptors, though the idea that breakage of the conserved disulfide bridge could lead to activation is supported by very little evidence (*44–48*). The β_2-adrenergic receptor (β_2-AR) has four extracellular cysteine residues, though none of these are accessible to alkylation, suggesting that all participate in disulfide bonds (*49*). The cysteine residue pairs involved in these two bonds were subsequently identified, and both were shown to be required for high-affinity binding (*27*). Direct confirmation of the existence of two disulfide bridges, one the conserved bridge between TM-III and ECL-2 and a second bridge linking two additional ECL-2 cysteine residues, has recently been obtained from the high-resolution crystal structure of human β_2-AR (*17*). Given that the β_2-adrenergic receptor, like GPR39, contains a second disulfide bridge, albeit in this case within ECL-2, it is tempting to speculate that the activation by reducing agents is due to cleavage of the extra, nonconserved disulfide bond. In the β_2-adrenergic receptor, the extra disulfide bridge appears to be stabilizing the α-helical structure of ECL-2. One could speculate that ECL-2 in the β-adrenergic receptors normally functions as a "lid" or "damper" by occupying the entrance to the main ligand-binding pocket and that disruption of the helical structure of ECL-2 impairs this function.

The angiotensin AT_2 receptor can also be activated by reducing agents, a phenomenon which appears to depend upon the putative second disulfide bridge between the N-terminal domain and, in this case, ECL-3 (Figure 6) (*23, 50, 51*). Unlike the AT_2 receptor, the AT_1 receptor is inactivated by reducing agents, despite having the same possible arrangement of disulfide bridges. However, a recent report showed that mutation of one or both of the Cys residues involved in the putative second disulfide bridge

resulted in increased constitutive activity of the AT_1 receptor (*52*). Thus, similar to this report for GPR39, additional disulfide bridges in 7TM receptors appear to dampen receptor activity.

In GPR39, we observe a different signaling phenotype that depends upon which of the two Cys residues involved in the extra disulfide bridge is mutated. Thus, the C11A mutant receptor has nearly WT properties, in contrast to the C191A mutant, which more resembles the mutants in which the conserved disulfide bridge is disrupted. We speculate that the free sulfhydryl group at Cys^{11} in the N-terminal domain (found in the C191A mutant) might affect the formation of the conserved disulfide bond, while the free sulfhydryl of Cys^{191} (found in the C11A mutant) does not do that. Disulfide bond exchange has been shown to occur in rhodopsin, under certain experimental conditions, such that a normally unbonded Cys residue reacts with a Cys residue in the conserved disulfide bridge and forms an unnatural disulfide bridge (*53*). Furthermore, an analysis of family A 7TM receptors containing an odd number of extracellular Cys residues, and thus necessarily containing a free sulfhydryl group, shows that this is rare (occurring in 4.5% of 397 receptors examined) (*54*).

General Disulfide Patterns in 7TM Receptors. In the majority of family A receptors, only the prototypical, conserved bridge from the extracellular end of TM-III to ECL-2 is found, as shown schematically in Figure 6A. The pattern described for GPR39 in this study, with Cys residues positioned to form a second bridge between the N-terminal domain and ECL-2, is also found in the closely related neuromedin U type 1 (NMU1) receptor, as well as in the gonadotropin-releasing hormone (GnRH) receptor, and in three prostaglandin receptors (Figure 6B). The two angiotensin receptors discussed earlier have the potential to form a second disulfide bridge between the N-terminal domain and ECL-3, thus "closing the ring" by making a covalent connection between the extracellular ends of TM-I and TM-VII (Figure 6C). This pattern is also found in the neuropeptide Y_1 receptor, in the pancreatic polypeptide Y_4 receptor, in the $P2Y_1$ receptor, and in a number of chemokine receptors (Figure 6C). The configuration presented in the high-resolution structure of the β_2-adrenergic receptor with a second disulfide bridge within ECL-2 is also found in the β_1- and β_3-adrenergic receptors, and potentially in a large number of olfactory receptors (Figure 6D).

Disruption of an extra (i.e., not the conserved) disulfide bridge appears to result in an increased level of receptor signaling (constitutive or agonist-induced), not only in GPR39 as demonstrated in this report but also in β-adrenergic receptors and angiotensin receptors, the other patterns which contain two disulfide bridges, as shown in panels B−D of Figure 6. This indicates a theme within 7TM receptors in which extracellular domains in certain receptors serve a function as a damper on receptor signaling. However, there are receptors in which the extracellular domains have the opposite purpose. For example, in the ghrelin and melanocortin MC_4 receptors, the extracellular domain can function as a tethered agonist, as suggested by point mutations and SNPs which selectively eliminate the otherwise high constitutive signaling (*55, 56*).

The disulfide patterns shown in panels E and F of Figure 6 represent a special family of receptors which lack the otherwise obligatory disulfide bridge from the extracellular end of TM-III to ECL-2, and which also lack the otherwise highly conserved ProV:16 in TM-V (*1, 57*). Instead, the melanocortin family of receptors, for example, keeps TM-IV and TM-V close to each other by having an ultrashort ECL-2 comprising only a few residues (Figure 6E). Interestingly, within this family, the structure of ECL-3 is unique and highly conserved with a particular pattern of Pro residues and three Cys residues, of which two likely form an intradomain disulfide bridge (Figure 6E) (*57*).

Disruption of native and/or creation of novel disulfide bridges in 7TM receptors is known to be a major cause of rare diseases involving these receptors, such as retinitis pigmentosa (rhodopsin), diabetes insipidus (V_2 vasopressin receptor), and obesity (MC_4 melanocortin receptor) (*54, 58–60*). This underscores the importance of disulfide bridges for the proper function of 7TM receptors.

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