By screening patients with severe early onset obesity for mutations within the melanocortin 4 receptor (MC4R) gene, we have identified a missense mutation (C271R) that occurs homozygous in two siblings with obesity. In-depth functional characterization of C271R revealed a right-shifted concentration response curve due to lower affinity to natural and synthetic MC4R agonists and a reduced cell surface expression. Cys-271 is located in the third extracellular loop. Here, we provide evidence that Cys-271 forms an intra-loop disulfide bond with Cys-277. Unexpectedly, we found that loss of receptor function is not only caused by the disruption of this disulfide bond. Our data strongly support a new mechanism in which the receptor malfunction in the C271R mutant is induced by formation of a functionally disastrous disulfide bridge between Cys-277 and a third Cys residue at position 279. Mutational and chemical disruption of this improper disulfide bond was able to restore normal receptor potency. By demonstrating that a loss of a disulfide bond-participating Cys residue can favor a functionally disastrous disulfide bond, we now add a new mechanism of how Cys residues can be involved in G-protein-coupled receptor malfunction.

Within the last few years, the knowledge of the molecular pathogenesis of obesity is increasing rapidly. Mutations in genes mostly involved in the leptin-melanocortin pathway were identified in a rare case of extreme obesity (1-4). The target receptors for melanocortins belong to the large superfamily of G-protein-coupled receptors (GPCR).1 Mutations in GPCR genes are responsible for an increasing number of human diseases including an inherited form of obesity, which is caused by inactivating mutations in the melanocortin receptor type 4 (MC4R) (5). Approximately 3-5% of early onset obese children studied so far contain mutations in the MC4R (6, 7). In contrast to inactivating mutations in other obesity-related genes that are inherited recessively, mutations in MC4R are often diagnosed in the heterozygous state. To date, only two homozygous MC4R mutations were reported, resulting in a complete or a partial loss of receptor function (7, 8).

The MC4R is activated by the pro-opiomelanocortin-derived peptides α-MSH and β-MSH. At the molecular level, MC4R stimulation increases intracellular cAMP via Gs/adenyl cyclase activation (9), finally resulting in a reduction of food intake and an increase in energy expenditure with the consequence of body weight reduction (10).

Herein, we describe the identification of a homozygous mutation in the MC4R in siblings with severe early onset obesity. The mutation C271R was located in the third extracellular loop (EL3). Functional characterization revealed a partial loss-of-function. Cys residues in EL3 are highly conserved among melanocortin receptors. Extracellular Cys residues often participate in disulfide bond formation. To gain insight into the molecular mechanism of this inactivating mutation, the functional relevance of Cys-271 was further characterized by systematic site-directed mutagenesis. By introduction of an additional Cys residue (e.g. P272C) into EL3, we were able to rescue the function of C271R. These data strongly support a participation of Cys-271 in a disulfide bond. In an attempt to identify the partner in disulfide bridge formation, the conserved Cys-40 (N terminus) and Cys-196 (EL2/transmembrane domain 5 junction) were excluded. Two other possible disulfide bond partners for Cys-271 (Cys-277 and Cys-279) are located in EL3. Mutagenesis studies and functional characterization of various mutants revealed that Cys-277 is most probably the disulfide partner of Cys-271. In case of C271R, an improper disulfide bond is formed between Cys-277 and Cys-279, which interferes with efficient cell surface expression and ligand binding.

Our data clearly indicate that intramolecular disulfide bonds can occur within a single loop of a GPCR. Disruption of this intra-loop bridge gives rise to an alternative intra-loop disulfide bond, which induces a functionally disastrous receptor folding defect. This new findings extends our knowledge of mechanism of clinically relevant GPCR malfunction and may provide starting points for novel therapeutic approaches in curing such diseases.

**EXPERIMENTAL PROCEDURES**

**Mutation Detection and Construction of Wild-type and Mutant Receptors**—Genomic DNA was prepared from peripheral white blood cells using a commercial kit (Blood amp kit, Qiagen, Hilden, Germany). The entire coding region of the MC4R gene was amplified in one fragment using the following primers: forward 5'-TGA GAC GAC TCC TGG ACC CAG G-3' and reverse 5'-CCT ACA CGG AAG AAG ATG-3' and sequenced in three overlapping fragments using the Big Dye Termina-
tor Cycle sequencing ready reaction kit (Applied Biosystems) and an automatic sequencer (ABI 373, Applied Biosystems, Foster City, CA). The wild-type MC4R was amplified from a healthy control and cloned in the pcDps expression vector. Genetic analyses were conducted in accordance with the guidelines proposed in the Declaration of Helsinki, and informed consent was obtained from all family members.

Mutant MC4R were generated using standard mutagenesis techniques and the WT MC4R-pcDps as template. PCR fragments containing the various mutations were cloned into the EcoRI/Bsu36I or EcoRI/SpeI sites of the MC4R-pcDps vector. For receptor quantification in cell surface ELISA studies, an N-terminal hemagglutinin (HA) epitope-tag was inserted. The correctness of all of the PCR-derived sequences were proven by automatic sequencing.

Cell Culture, Transfection, and Functional Characterization—Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO2 incubator. COS-7 cells were seeded into 12-well plates (2 × 105 cells/well) for investigation of agonist-stimulated cAMP accumulation. 72 h after transfection, cells were stimulated with increasing concentrations of NDP-α-MSH. Intracellular cAMP was measured as described under “Experimental Procedures.” B, shown is the result of three independent experiments. For binding assay, cells were seeded into 48-well plates and transfected. Displacement studies with 125I-NDP-α-MSH as radioligand were performed as described under “Experimental Procedures.” Data are presented as the means ± S.E. of four independent experiments, each carried out in duplicate.

Fig. 1. Pedigree of the family with the MC4R-C271R mutation. Mutational screening of early onset obese patients revealed a homozygous MC4R mutation, C271R, located in EL3 of the MC4R. This mutation was found in two extremely obese and hyperphagic siblings (filled circles). All of the other family members were heterozygous for C271R with the phenotype of obese females (half-filled circles) and normal weight males (half-filled squares). The age (in years (yrs)) together with the body mass index are given for each family member.

FIG. 2. Functional characterization of MC4R-C271R. A, for functional characterization, the WT and the mutant (C271R) MC4R were cloned into the expression vector pcDps and tested for agonist-induced cAMP accumulation. 72 h after transfection, cells were stimulated with increasing concentrations of NDP-α-MSH. Intracellular cAMP was measured as described under “Experimental Procedures.” B, shown is the result of three independent experiments. Data are presented as the means ± S.E. of four independent experiments, each carried out in duplicate.
day later, cAMP accumulation assays were performed. Cells were washed once and incubated in serum-free Dulbecco’s modified Eagle’s medium containing 1 mM 3-isobutyl-1-methylxanthine (Sigma) in the absence or in increasing amount of agonist NDP-α-MSH (Sigma), α-MSH, and β-MSH (kindly provided by Dr. P. Henklein) for 1 h at 37 °C. The reactions were stopped by aspiration of medium, and intracellular cAMP was released by the incubation of 1 ml of 5% trichloric acid, which was measured by anion-exchange chromatography as described previously (11). Cyclic AMP accumulation data were analyzed by the GraphPad Prism program (GraphPad Software, San Diego, CA).

Radioligand Binding Assay and ELISA for Measurement of the Cell Surface Expression—For cell surface binding studies, cells (2 × 10⁶ cells/10-cm dish) were transfected with 5 μg of plasmid DNA/dish and LipofectAMINE Plus. One day later, cells were trypsinized and seeded in 48-well plates. 48 h later, cells were incubated overnight in the presence of 125-I-NDP-α-MSH (specific activity 2000 Ci/mmol, 120,000 cpm/well, Amersham Biosciences) with increasing amounts of agonist NDP-α-MSH. After washing, specifically bound NDP-α-MSH was measured. Bₘₐₓ values were calculated from displacement curves by the method of Cheng and Prusoff (12).

To investigate cell surface expression, WT and mutants receptors were N-terminally HA-tagged and cell surface ELISAs were performed. Cells (5 × 10⁴/well) were seeded in 48-well plates and transfected with LipofectAMINE Plus. Three days later, cells were washed, paraformaldehyde-fixed, and probed with a biotin-labeled anti-HA antibody (Roche Applied Science). Bound anti-HA antibody was detected by peroxidase-labeled streptavidin (Dianova, Hamburg, Germany) and a substrate/chromogen reaction (13).

RESULTS

Identification of MC4R-C271R and Functional Characterization—By screening for MC4R mutations in early onset obese children, we identified a homozygous MC4R mutation, C271R, located in EL3 of the MC4R. The mutation was found in two extremely obese and hyperphagic siblings. All other family members investigated were heterozygous for C271R, presenting the phenotype of obese females and normal weight males (Fig. 1). For functional characterization, the C271R mutation was introduced in an eucaryotic expression vector containing the cDNA of the MC4R (MC4R-pCDps).

WT and mutant receptors were transiently transfected into COS-7 cells and characterized in cAMP accumulation assays, radioligand binding studies, and cell surface ELISA studies. As shown in Fig. 2A and Table I, stimulation of the WT MC4R with the superpotent and enzymatically resistant α-MSH analog NDP-α-MSH resulted in a robust increase in cAMP levels with an Eₘₐₓ value of 11.3-fold over basal and an EC₅₀ value of 0.5 nM. In contrast, C271R displayed a reduced efficiency (Eₘₐₓ 8.1, EC₅₀ 0.5) when compared with the WT-MC4R (data not shown). Values of specific binding sites, all attempts failed to exactly determine the Kᵣ value of C271R (even at the maximal possible concentrations of 125-I-NDP-α-MSH), probably because of the combination of low receptor expression and a reduced 125I-NDP-α-MSH affinity. To circumvent this problem, we established an indirect cell surface ELISA for determination of cell surface expression independently from receptor binding abilities. In concert with the results of cAMP and radioligand binding studies, C271R showed significantly reduced cell surface expression levels (40.7 ± 2.9%) when compared with the WT-MC4R (data not shown).

Search for a Putative Partner of Cys-271 for Disulfide Bridge Formation—Cys residue in extracellular loops often participates in intramolecular disulfide bond formation, and mutations of these Cys residues are often found in patients with diseases caused by inactivating mutations in GPCRs (5). In melanocortin receptors, four extracellularly located Cys residues including Cys-271 are highly conserved among subtypes and species (Fig. 3). It should be noted that melanocortin receptor do not process the highly conserved Cys residue pair, connecting EL1 and EL2 in most GPCRs of family 1.

Because all of the melanocortin receptors cloned so far contain a Cys residue in their N termini in close proximity to TM1, we addressed this putative interaction partner for Cys-271 by site-directed mutagenesis. Assuming a disulfide bond between Cys-40 and Cys-271, we speculated that the mutation of Cys-40 should present the same functional phenotype as observed for
C271R. Therefore, we created C40R according to the patient’s mutation. Substitution of Cys-40 to Arg affected neither the agonist-stimulated cAMP accumulation nor binding properties (see Table I and Fig. 4A). To exclude the possibility that Cys-40 is somehow involved in the molecular mechanism of C271R dysfunction, the double mutant (C40R/C271R) was tested in functional assays. Our data clearly indicate that the substitution of Cys-40 did not significantly affect WT and C271R function (see Table I).

As a next putative disulfide bridge-forming candidate, the Cys-196 residue at the EL2/TMD5 transition was targeted by site-directed mutagenesis. According to Cys-40, C196R and a double mutant C196R/C271R were generated and functionally investigated. Substitution of Cys-196 by Arg almost completely abolished receptor function as reflected by a 400-fold increase of EC50 value and an extreme reduced cell surface expression (see Table I). The double mutant C196R/C271R was completely inactive (see Table I). At this point, Cys-196 could be considered as a potential candidate for disulfide bond formation with Cys-271.

To complete the systematic search for disulfide bond partners, we focused on two Cys residues located in the EL3. It was recently shown that in the MC1R Cys-265 (corresponding to Cys-271 in MC4R) and Cys-273 (corresponding to Cys-279 in MC4R) form a disulfide bridge within the EL3 (14). Similarly, we mutated Cys-279 to Ala and tested C279A in COS-7 cells. In contrast to the MC1R, substitution of Cys-279 had no effect on MC4R function (Fig. 4A and Table I). C279A was introduced next into C271R, resulting in the double mutant C271R/C279A. Surprisingly, the introduction of C279A partially rescued the function of C271R but cell surface expression was reduced. For control purposes, we replaced the second Cys residue at position 277 with Ala. Cell surface expression was dramatically reduced, whereas EC50 value was unchanged compared with the WT MC4R (see Fig. 4A and Table I). Strikingly, the intro-

![Fig. 3. Structural conservation of cysteine residues in melanocortin receptors.](image)

![Fig. 4. cAMP accumulation of mutated extracellular Cys residues and influence of DTT.](image)
COS-7 cells were transfected with the WT or MC4R mutants. EC50 and Emax values were obtained from concentration-response curves (from 10 pm to 10 μM α-MSH and β-MSH) using the computer program GraphPad Prism. The mean Emax value of the WT MC4R was 2522 ± 109 cpm/well for α-MSH and 3184 ± 33 cpm/well for β-MSH. Data are indicated as the means ± S.E. of three independent experiments each performed in duplicates.

### Rescue of Mutant MC4R

| Construct            | α-MSH | β-MSH |
|----------------------|-------|-------|
|                      | Emax/EmaxWT | EC50 | %     |       |       |
| MC4R WT              | 100   | 3.9 ± 2.8 | 100 | 26.1 ± 8.7 |
| C271R                | 48 ± 3 | 209 ± 5 | 22.5 ± 4.5 | 1174 ± 648 |
| C277A                | 101 ± 2.5 | 14.8 ± 7 | 80 ± 19 | 86 ± 7.5 |
| C271R/C277A          | 95 ± 7.5 | 78 ± 40 | 72 ± 3 | 263 ± 86 |
| C279A                | 97 ± 1 | 3.6 ± 2.3 | 98.5 ± 5.5 | 36.5 ± 3.5 |
| C277A/C279A          | 45 ± 13 | 145 ± 63 | 39 ± 19 | 390 ± 113 |
| C277A/C279A          | 85.5 ± 2.5 | 21 ± 9 | 77 ± 3 | 271 ± 25 |
| C271R/P272C          | 84 ± 10 | 232 ± 37 | 53 ± 1 | 200 ± 1.5 |

### Discussion

- **Functional Rescue of C271R Dysfunction by Introduction of an Additional Cys Residue**
  - We next tested the functional consequence of additional Cys residues introduced into EL3 of the WT MC4R and C271R. Thus, residues in close proximity to position 271 were mutated, yielding the mutant MC4Rs S270C, P272C, Q273C, N274C, and P275C and the corresponding double mutants S270C/C271R, C271R/P272C, C271R/Q273C, C271R/N274C, and C271R/P275C. All of the mutants were tested in cAMP accumulation assays and ligand binding and ELISA studies.
  - Introduction of an additional Cys residue at five different positions into EL3 had no significant impact on functional properties of the WT receptor (see Table I). However, an additional Cys residue at position 272 (P272C) can fully restore the signaling and binding abilities of C271R. Similarly, the double mutants S270C/C271R, C271R/Q273C, and C271R/N274C regained normal signal transduction abilities but displayed reduced cell surface expression level (Table I).

- **EL3 Is Involved in Binding the Natural MC4R Agonists α-MSH and β-MSH**
  - Most functional studies on MC4R are performed with NDP-α-MSH because of its higher potency and stability compared with the natural ligand α-MSH and β-MSH. To evaluate the impact of the C271R mutation on the functional properties of both natural agonists, the EC50 and Emax values of α-MSH and β-MSH were determined. As shown in Table II, α-MSH is more potent on the WT MC4R when compared with β-MSH. The introduction of C271R resulted in a more pronounced right-shifted concentration response for both agonists when compared with NDP-α-MSH (≈50-fold, respectively). C277A and C279A were tested next for functional relevance and their ability to rescue the phenotype of C271R. In contrast to the NDP-α-MSH studies, the mutation of Cys-277 to Ala interfered significantly with the signal transduction abilities of both agonists, whereas the C279A had no influence on receptor function (Table II). However, both mutations were able to partially rescue the malfunction of C271R.
  - We have shown that introduction of an additional Cys (e.g., P272C) into C271R can fully restore NDP-α-MSH-induced receptor signaling (see above). To test whether this holds true also in case of the natural agonists, the double mutant C271R/P272C was characterized. Integration of additional cysteine residues did not (α-MSH) or only partially (β-MSH) partially rescued receptor function (Table II).

- **Restoring the function of C271R by DTT**
  - All of our previous experimental data suggested that C271R is somehow involved in disulfide bridge formation. The resulting malfunction can be rescued by either an introduction of an additional cysteine residue (P272C) or by destroying potential candidates for an improperly formed disulfide bond (C277A, C279A). We addressed the question of whether an improperly formed disulfide bond is involved in the molecular mechanism of C271R malfunction by application of a disulfide bond-reducing agent.
  - Stimulation of the WT receptor in the presence of 10 mM DTT resulted in a reduction of the Emax value without a severe alteration of the EC50 value. Incubation of C271R with 10 mM DTT restored the receptor function to the same level as observed for the WT receptor in the presence of 10 mM DTT (Fig. 4B). These findings further support a scenario in which the malfunction of C271R is caused by the formation of an improper disulfide bond rather than due to the loss of a disulfide bridge in the absence of Cys-271.

### Loss and Gain of Cys Residues Are Frequently Found as Molecular Basis of GPCR Inactivation

Mutational screening revealed a frequency of 3–5% mutations in the MC4R of severely early onset obese children (6, 7, 15–19). Herein, we identified a homozygous missense mutation, C271R, which is located in EL3. This position appears to be critical for MC4R function because C271Y and C271R have been found recently in two other obese families (7).

Mutationally, the introduction as well as the loss of Cys residues are often found in patients with GPCR-related diseases. It has been extensively shown that posttranslational formation of one or more extracellular disulfide bonds is essential for receptor trafficking and high affinity ligand binding (5).

- Disease-causing mutations of the highly conserved disulfide bonds connecting EL1 and EL2 have been identified in the AVPR2 (20) and rhodopsin (21, 22). In principle, mutational disruption of this disulfide bond results in a loss of high affinity binding, suggesting a pivotal role of EL1- and EL2-connecting disulfide bridges for proper receptor assembly. On the other hand, many of the mutations in the loops found in patients with nephrogenic diabetes insipidus and retinitis pigmentosa are characterized by substitutions of various amino acid residues to Cys residues (23).

The molecular mechanism of additional Cys residues within extracellular loops is more complex. The presence of an extra Cys residue caused by mutations may offer alternatives in disulfide bond formation between EL1 and EL2. A recent study by Schüllein et al. (24) provided an additional explanation for the mechanism of AVPR2 dysfunction caused by Cys substitutions. It was shown that additional Cys mutations in EL2 of the AVPR2 (G185C, R202C) probably form a new disulfide bond with a free Cys (Cys-195) residue also located in EL2 residue interfering with high affinity binding.
Mutation of this endogenous Cys residue (C195A) could rescue the function of G185C and R202C. Such a mechanism may also account for Cys mutations in rhodopsin, leading to an uneven number of Cys residues in the extracellular loops (24). Similarly, in MC1R, MC2R, and MC4R both types, the loss and introduction of Cys residues have been identified as the cause of the disease (7, 25, 26). However, the exact molecular mechanism has not been determined yet.

An Intra-loop Disulfide Bond Is Formed between Cys-271 and Cys-277—Understanding the molecular mechanisms of mutationally induced receptor dysfunction is a major task in the development of new receptor ligands and novel therapeutic strategies. Although the waste majority of over 40 mutations in the MC4R were functionally characterized and were shown to result in a loss of function, little knowledge exists regarding the detailed structure-function relationships of this receptor. To provide insights into the molecular mechanism of C271R, we first speculated that Cys-271 may participate in disulfide bridge formation and disruption of this disulfide bond is the cause of receptor malfunction. This assumption was supported by the fact that the mutation of Cys-271, independently from the nature of the substitution (Ala, Tyr, and Arg), resulted in a loss-of-function phenotype in MC4R and MC1R (see Table I) (6, 14). Based on these data, it was reasonable to assume that mutation of the counterpart Cys residue may also result in a loss of receptor function.
In an attempt to identify the disulfide bond partner for C271R by mutating putative candidates (Cys-40, Cys-196, Cys-277, Cys-279), only the C196R mutation at the EL2/TMD5 interface and the C277A in EL3 displayed an altered receptor function. Previous studies with the MC1R and the MC4R have already addressed the importance of Cys-196 (or Cys-189 in MC1R) and consistently found that mutation to Ala had only little effect on ligand binding and signal transduction (14, 27). Additionally, the MC2R contains a Thr residue at the corresponding position at the EL2/TMD5 transition (see Fig. 3B). These facts indicate that the loss of function found in C196R is more likely induced by a rather specific effect of the polar and bulky Arg substitution at position 196 and not by a general effect on disulfide bridge formation. However, in contrast to C196A, the signal transduction of C277A is strongly altered mainly in respect to their binding abilities and also to the potency of natural agonists (Table I and Table II). Our data favor Cys-277 as a potential interaction partner for Cys-271 (Fig. 5). This assumption is supported by the findings of the MC1R, which also suggest an intra-loop disulfide bond (14). It was speculated that this disulfide bond in the MC1R forms between Cys-265 and Cys-273 within EL3, which correspond with Cys-271 and Cys-279 in MC4R, respectively. These findings were based on the observation that the substitution of Cys-265A and C273A in the MC1R resulted in a loss of α-MSH and Zn(II)-induced cAMP formation, whereas C271A resulted only in a loss of Zn(II) but not α-MSH-induced cAMP accumulation (14). In contrast to findings in the MC1R, our mutagenesis experiments with the MC4R provide strong evidence for the existence of an intra-loop bond between Cys-271 and Cys-277 and exclude Cys-279 as a potential partner.

Malfunction of C271R Is Caused by an Improperly Formed Disulfide Bond within the EL3—Because our data favor a scenario in which Cys-271 normally forms a disulfide bond with Cys-277, we asked the question whether the function of C271R can be restored by inserting an alternative Cys residue into C271R. Introduction of an extra Cys (P272C) in close proximity to position 271 almost completely rescued the function in C271R, indicating that an exact positioning of a Cys residue is not required for functional compensation of C271R. However, further N- or C-terminal movement of this Cys was not fully tolerated, reflecting a limited spatial freedom of such compensational Cys residue within EL3. These data strongly suggested at this point that the loss of a Cys residue in the C271R mutant, which contributes to an intra-loop disulfide bridge, mainly contributes to the loss of function found for the mutant MC4R. However, this mechanistic view changed, at least partially, at the point when double mutants C271R/C277A and C271R/C279A were tested. Strikingly, both double mutants regained potencies to NDP-α-MSH as found for the WT MC4R. Similarly, α- and β-MSH potency increased after the introduction of C277A or C279A into C271R (see Tables I and II). Because there are no partners for disulfide bridge formation within EL3 in all of the double mutants, the relevance of an intraloop disulfide bond for high affinity agonist binding of NDP-α-MSH is questionable. The functional relevance of an intra-loop disulfide bond is further challenged by the fact that a double mutant (C277A/C279A) in which both potential interaction partners for Cys-271 are mutated displays almost WT receptor signal transduction abilities. However, the intra-loop bond might be more important for proper receptor function for the natural agonists α- and β-MSH as shown by altered EC_{50} values (Tables I and II). Moreover, a proper EL3 structure based on an intra-loop bond might be crucial for MC4R trafficking because all of the double mutants (C271R/C277A, C271R/C279A, C277A/C279A) are partially retained in the cell interior.

Since the loss of a disulfide bridge can not completely account for the loss-of-function in C271R, what other mechanism may be the reason for this mutationally induced receptor inactivation? There are three Cys residues in the EL3 that are only able to form one disulfide bridge at the same time (Fig. 5A). In the WT MC4R, the bond forms probably between Cys-271 and Cys-277. In case of the C271R, there is still the chance of forming a disulfide bridge between Cys-277 and Cys-279 (see Fig. 5B). One may ask whether a disulfide bond can form between two Cys residues that are apart just by one residue in-between. There are several examples in which disulfide bridge forms naturally between two Cys in close proximity, e.g. disulfide bridge-forming Cys residues separated by two amino acid residues in DasB (28), tryptophan-in-I (29), and Cmgrp—reducing oxidoreductase (30). A disulfide bridge between Cys residues that are only one residue apart is seldom but was found in the yeast oxidoreductase Erv2p (31). Clearly, bridging Cys-277 and Cys-279 is entropically not the preferred structure and may disturb the loop structure. We could demonstrate that Ala substitution of one of these Cys residues or chemical reduction of Cys bridges by DTT as well as introduction of an entropically preferred additional Cys residue (P272C) prevent the formation of this disastrous disulfide bond (see Fig. 5, C-E). Based on these data, we could demonstrate for the first time that a loss of a disulfide bond-participating Cys residue can favor a functionally disastrous disulfide bond, resulting in the loss of function of the receptor. These results add an additional mechanism of how Cys residues can be involved in GPCR malfunction.

Taken together, our mutagenesis studies clearly provide evidence for a disulfide bond between Cys-271 and Cys-277. Furthermore, there is strong evidence that receptor malfunction in C271R is not the result of a mutational loss of this disulfide bond. A new disulfide bond within the EL3 probably leads to an improperly formed loop. These results may initiate the development of therapeutically relevant MC4R agonists, which can also activate mutant MC4R independently from the EL3 structure or specifically stabilizing EL3 in its natural conformation.

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