Functional rescue of the nephrogenic diabetes insipidus causing vasopressin V_{2} receptor mutants G185C and R202C by a second site suppressor mutation

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SUMMARY

Mutations in the gene of the G protein-coupled vasopressin V2 receptor (V2 receptor) cause X-linked nephrogenic diabetes insipidus (NDI). Most of the missense mutations on the extracellular face of the receptor introduce additional cysteine residues. Several groups have proposed that these residues might disrupt the conserved disulfide bond of the V2 receptor. To test this hypothesis, we first calculated a structure model of the extracellular receptor domains. The model suggests that the additional cysteine residues may form a second disulfide bond with the free, non-conserved extracellular cysteine residue C195 rather than impairing the conserved bond. To address this question experimentally, we used the NDI-causing mutant receptors G185C and R202C. Their C195 residues were replaced by alanine to eliminate the hypothetical second disulfide bonds. This second site mutation led to functional rescue of both NDI-causing mutant receptors, strongly suggesting that the second disulfide bonds are indeed formed. Furthermore we show that residue C195, which is sensitive to "additional C" mutations, is not conserved among the V2 receptors of other species and that the presence of an uneven number of extracellular cysteine residues, as in the human V2 receptor, is rare among class 1 G protein-coupled receptors.
INTRODUCTION

The vasopressin V$_2$ receptor (V$_2$ receptor)\(^1\) belongs to the large family of G protein-coupled receptors (GPCR’s). The receptor is expressed in the basolateral membrane of the principal epithelial cells of the renal collecting duct, where it mediates the antidiuretic action of the hormone AVP (Review: 1). Activation of the V$_2$ receptor leads to stimulation of the G$_s$/adenylyl cyclase system. The subsequent rise in intracellular cAMP induces the fusion of vesicles containing water channels (aquaporin 2) with the apical membrane which is thus rendered water permeable. As a consequence, water is reabsorbed from the lumen of the collecting duct.

Mutations in the gene of the V$_2$ receptor are the cause of X-linked NDI (Review: 2), a disease characterized by the inability of the kidney to concentrate urine despite normal or elevated levels of AVP. Over 170 V$_2$ receptor mutations have been documented, approximately half of which are missense mutations. Most of the missense mutations are clustered within the transmembrane domains of the receptor. These mutations usually affect receptor folding in the bilayer and lead to intracellular retention of the misfolded forms by the quality control system of the ER. In comparison to the numerous mutations found in the transmembrane domains, only few mutations are located on the cytoplasmic or extracellular faces of the receptor. It is striking that most of the extracellular mutations introduce additional cysteine residues. Such mutations include R106C (3), R181C (4, 5), G185C (6), R202C (6, 3, 7) and Y205C (6, 8, 9).

The most obvious explanation for the origin of the defect caused by these additional extracellular cysteine residues is that they impair formation of the single disulfide bond of the V$_2$ receptor which connects residue C112 of the first with C192 of the second extracellular loop and which is conserved in the GPCR family. Several groups have proposed that the mutated residues form an alternative bond with either one of the two conserved cysteine
residues (6, 5, 9, 10). Disease causing mutations which lead to the introduction of additional cysteine residues have been observed for other GPCR’s. The Y178C mutation of rhodopsin was shown to cause retinitis pigmentosa and is suspected to impair the formation of the conserved disulfide bond of this receptor (11). The analysis of the molecular basis of the defect caused by these „additional C„ mutations of GPCR’s is thus of general interest.

In the case of the V2 receptor, recent studies called into question whether the additional cysteine residues impaired formation of the conserved disulfide bond (12): mutation of the conserved cysteine residues led to nonfunctional receptors with a strong transport defect, whereas the NDI-causing mutations G185C and R202C led to binding-defective but transport competent receptors. If the additional cysteines of the NDI-causing mutant receptors were to disrupt the conserved disulfide bond, an impaired intracellular transport of these mutant receptors would also have been expected.

Here we have analyzed the defects of the NDI-causing mutant receptors G185C and R202C in detail. We present molecular modelling and mutagenesis data which strongly suggest that the additional cysteine residues of both mutant receptors participate in a second disulfide bond with residue C195 of the V2 receptor rather than impairing formation of the conserved bond.
MATERIALS AND METHODS

Materials. Restriction enzymes, EndoH and PNGaseF were from New England Biolabs (Schwalbach, Germany). Sulfo-NHS-Biotin and immobilized Neutravidin were from Pierce (Rockford, IL, USA). Trypan blue was from Seromed (Berlin, Germany). Lipofectamine was purchased from Life Technologies (Karlsruhe, Germany). The polyclonal anti-GFP antiserum has been described previously (13), anti-rabbit $^{125}$I-IgG (28-111 TBq/mmol) was from Amersham (Braunschweig, Germany). The Quick Change site-directed mutagenesis kit was from Stratagene (Heidelberg, Germany), and oligonucleotides from Biotez (Berlin, Germany). [${}^3$H]AVP for the binding assay (68.5 Ci/mmol) was from Amersham Pharmacia (Braunschweig, Germany), and [$\alpha^{32}$-P]ATP for the adenylyl cyclase assay (30 Ci/mmol) from NEN (Köln, Germany). All other reagents were from Merck (Darmstadt, Germany) or Sigma (Deisenhofen, Germany). The primate genomic DNA’s were kindly donated by Hans Zischler (Deutsches Primatenzentrum, Göttingen, Germany).

DNA manipulations. Standard DNA preparations and manipulations were carried out according to the handbook of Sambrook and coworkers (14). Nucleotide sequences of DNA constructs were verified using the FS Dye Terminator kit from Perkin Elmer (Weiterstadt, Germany).

Construction of NDI-causing mutant receptors carrying an additional C195A mutation. Plasmid pWT.GFP, encoding a fusion of the red-shifted variant of GFP to residue K367 of the V2 receptor (i.e. to the entire receptor except for the four C-terminal residues) has been described previously (15). The C195A mutation was introduced into pWT.GFP by the use of the Quick Change site-directed mutagenesis kit. A primer with the sequence 5'-
CTGACTGCTGGGCGCGCTTTGCGGAGCCC-3’ and its complementary equivalent were employed. The resulting plasmid C195A.GFP was then used to introduce the NDI-causing mutations G185C and R202C. Primers with the sequences 5’-CAGCGCAACGTGGAATGGCAGCGGGGTCAC-3’ (resulting plasmid pG185C/C195A.GFP) and 5’-CGGAGCCC-TGGGGCTGTCGCACTATGTC-3’ (resulting plasmid pR202C/C195A.GFP) and their complementary equivalents were used.

**PCR amplification and sequencing of primate DNA’s.** A partial sequence of the V2 receptor gene encoding ICL1 to ECL3 was PCR-amplified from the genomic DNA of the following primate species: *Callithrix jacchus* (marmoset = New World monkey), *Macaca mullata* (rhesus monkey = Old World monkey), *Hylobates lar* (white handed gibbon), *Pan troglodytes* (chimpanzee), *Pongo pygmaeus* (orangutan). Primers with the sequences 5’-GCT-GTGGCCCTGAGCAATGGCCTGGTGCTGG-3’ (sense) and 5’-ATGCATAGATCCAGGGTTGGTGCAGCTGTTGAGGC-3’ (antisense) were used for the PCR reaction. The PCR fragments were directly sequenced.

**Cell culture and transfection methods.** All experiments in this study were carried out with transiently transfected HEK 293 cells. Cells were cultured on poly-L-lysine-coated material in Dulbecco's modified Eagle's medium containing 10 % heat-inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) in a 5% CO2 atmosphere. Cells were transfected with lipofectamine according to the supplier's recommendations.

For the [^3H]AVP binding assay, 5x10^4 cells in a 15 mm diameter well (of a 24 well plate) were transfected with 250 ng plasmid DNA and 2 µl lipofectamine. The cell surface biotinylation assay and the isolation of crude membranes for adenylyl cyclase assays and for immunoblots proceeded from confluent cells grown on 60 mm diameter dishes. Here, 5x10^5
cells were transfected with 3.5 µg plasmid DNA and 26.25 µl lipofectamine. The transfection
for laser scanning microscopy was carried out with cells grown in 35 mm diameter dishes
containing glass cover slips. Cells (4x10^4) were transfected with 1 µg plasmid DNA and 7.5
µl lipofectamine. In all cases, cells were further incubated for 48 h after removal of the
transfection reagent.

[^H]AVP binding assay and adenylyl cyclase assay. The[^H]AVP binding assay was car-
ried out with intact, transiently transfected HEK 293 cells as described previously for
COS.M6 cells (16). However, HEK 293 cells were grown in 24 well plates (15 mm
diameter/well) rather than the 35 mm diameter dishes described for COS.M6 cells. The
adenylyl cyclase assay was carried out with nuclei free crude membranes of transiently trans-
fected HEK 293 cells as described previously for stably transfected L^tk- cells (16).

Glycosylation state analysis. Crude membranes of transiently transfected HEK 293 cells
expressing the GFP-tagged V2 receptors were isolated from confluent cells grown on 60 mm
diameter dishes as described previously for COS.M6 cells (17). Membrane proteins were
incubated with or without EndoH or PNGaseF. Receptors were detected by immunoblotting
using a rabbit anti-GFP antiserum and 125I-conjugated anti-rabbit IgG as described previously
(13).

Cell surface biotinylation assay. Transiently transfected HEK 293 cells expressing the GFP-
tagged V2 receptors were grown in a 60 mm diameter dish to confluence. Cells were washed
three times with ice-cold PBS-CM buffer (PBS containing 0.1 mM CaCl2 and 1 mM MgCl2,
PH 7.4). Cell surface proteins were labelled by incubating cells with PBS-CM containing 1
mg/ml Sulfo-NHS-Biotin for 30 min at 4 °C. Labelling reactions were quenched by replacing
the biotin solution with 1 ml NH₄Cl solution (50 mM in PBS-CM). After 10 min, the cells were washed 3 times with ice-cold PBS-CM. One ml ice-cold lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, 150 mM NaCl, 1 mM Na-EDTA, pH 8.0) was added to the dishes and membrane proteins were solubilized for 1 h at 4 °C. Insoluble debris was removed by centrifugation (20 min, 4 °C, 47,000 × g), and biotinylated proteins were recovered from the supernatant by a 1.5 h incubation at 4°C with neutravidin-agarose beads. Beads were sedimented (3 min, 17,000 × g, 4 °C), washed twice with buffer (0.5% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, 50 mM NaCl, 1 mM Na-EDTA, pH 7.4) and once with the same buffer without NaCl. Proteins were solubilized in 50 µl Laemmli buffer (60 mM Tris-HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue, pH 6.8), and biotinylated receptors were detected by immunoblotting using a polyclonal anti-GFP antiserum and ¹²⁵I-conjugated anti-rabbit IgG (13).

Visualization of GFP-tagged receptors by confocal laser scanning microscopy. Transiently transfected HEK 293 cells expressing the GFP-tagged receptors were grown on glass coverslips. Cells were washed twice with PBS (pH 7.4), transferred immediately into a self-made chamber (details on request) and covered with 1 ml PBS (pH 7.4). The GFP fluorescence signals were visualized on a Zeiss 410 invert laser scanning microscope (λ_{exc} = 488 nm, λ_{em} = >515 nm). Subsequently, the cell surface of the same cells was stained with 0.05% trypan blue as described (15). Trypan blue fluorescence (λ_{exc} = 543 nm, λ_{em} = >590 nm) was recorded on a second channel and its overlap with the GFP signals was computed.

V₂ receptor model building. A structure model of the transmembrane and the intracellular domains of the V₂ receptor was described previously (13). For this model, packing of the transmembrane helices was based on electron density maps of frog rhodopsin (18).
The structure model of the extracellular domains was assembled stepwise taking this previously described model as a foundation. The starting conformations of the extracellular domains were obtained by assembling the known conformations of identical or similar fragments of other proteins (3-10 residues) retrieved from the Brookhaven 3D protein databank (Brookhaven National Laboratory, Brookhaven, USA). Overlapping fragments with comparable conformations in several different proteins, indicating a common conformational propensity, are listed in Fig. 1 with their database ID number. The strategy of assembling thoroughly selected fragments with known conformations has the advantage over loop search algorithms in that assignment of the orientation of the hydrophobic and hydrophilic side chains to the hydrophobic and to the water phase respectively is finer. Model components were assembled with the biopolymer module of the Sybyl program package (TRIPOS Inc. St Louis, MO, USA) and minimized by an AMBER 5.0 force field. The stability of the resulting receptor model was finally assessed as described (19). Molecular dynamics simulations maintaining helix stability only by backbone H-bond constraints were performed at 300 K for 200 ps using AMBER 5.0 force field conditions in vacuo. The overall backbone conformations of the extracellular domains remained stable during the molecular dynamics runs; indicating a stable starting conformation.
RESULTS

Construction of a structure model for the extracellular domains of the V_2 receptor. A detailed knowledge of the conformational features of the extracellular domains of the V_2 receptor would indicate whether the additional cysteine residues of the NDI-causing mutant receptors might disrupt the single disulfide bond of the receptor by forming an alternative bridge with either one of the conserved cysteine residues. Since high resolution structural data are not available for the V_2 receptor, we have computed a 3D model with special emphasis on the extracellular domains. Calculations are based on the conformations of identical or similar fragments of other proteins retrieved from the Brookhaven 3D protein databank (see Fig. 1 and Materials and Methods for details of the modelling procedure). The conformations of the protein fragments were assembled stepwise on the foundation of our previously described model of the intracellular and transmembrane domains (13), and the stability of the completed model was assessed by molecular dynamics simulations. Top- and a sideviews of the structure model are shown in Figures 2A and 2B respectively. The positions of the NDI-causing „additional C„, mutations, the conserved cysteine residues C112 and C192 (which form the disulfide bond between ECL1 and ECL2) and the single non-conserved extracellular cysteine residue C195 are indicated. It is thought for the V_2 receptor that AVP binds with its cyclic portion in the large cavity formed by the upper parts of TM3, TM4, TM5 and TM6 and ECL2 and with its C-terminal portion in the smaller cavity formed by TM2, TM3, TM7 and parts of ECL1, ECL2 and ECL3 (20). Similar structure models for the location of the ligand binding domain have been proposed for other GPCR’s with peptide ligands (21). Our model predicts that the entrance of the larger ligand binding cavity is surrounded by the long ECL2 domain which forms a U-like loop (see Fig. 2A). Residue C195 would be located in the centre of the extracellular face of the receptor at the tip of the U-like loop and, more importantly, would be
accessible to each of the cysteine residues introduced by the NDI-causing mutations. Our model thus raises the possibility that the additional cysteine residues form a second disulfide bond with residue C195 rather than disrupting the conserved bond between C112 and C192. This second bond may be formed easily in the case of our previously described mutant receptors G185C and R202C (12), since their additional cysteine residues lie at the surface of the molecule at approximately the same level as residue C195 (see Fig. 2B). Formation of the putative second disulfide bond could cause sealing of the entrance of the larger ligand binding cavity and/or may perturbate this site at a deeper level (see Fig. 2A). In either case, the newly formed disulfide bond should not have a major influence on the overall structure of the receptor.

**A C195A mutation suppresses the defect of the NDI-causing mutations G185C and R202C.** To address the question of whether or not second disulfide bonds are formed upon introduction of the additional extracellular cysteine residues, we have introduced a C195A mutation into our previously described GFP-tagged NDI-causing receptor mutants G185C.GFP and R202C.GFP (12) (resulting receptor mutants G185C/C195A.GFP and R202C/C195A.GFP). If additional bonds are present, mutation of C195 should disrupt them and lead to functional rescue of the NDI-causing mutant receptors. It was previously demonstrated that mutation of residue C195 alone led to receptors with almost wildtype properties, i.e. that this residue is not necessary for V₂ receptor function (22; own unpublished results). The pharmacological properties of the double mutants were characterized in transiently transfected HEK 293 cells by performing [³H]AVP binding assays with intact cells (Fig. 3) and adenylyl cyclase activity assays with crude membrane preparations (Fig. 4). The GFP-tagged wild-type V₂ receptor (WT.GFP) and the NDI-causing single mutants (G185C.GFP and R202C.GFP) were used as controls. As described previously (12), no
significant specific [\(^3\)H]AVP binding was observed for the NDI-causing mutant receptors G185C.GFP and R202C.GFP. The introduction of the C195A mutation, however, led to functional rescue of both mutant receptors. The \(K_D\) values of both double mutants were comparable to that of WT.GFP (5.2 nM for G185C/C195A.GFP vs. 5.4 nM for WT.GFP; 2.1 nM for R202C/C195A.GFP vs. 4.7 nM of WT.GFP). Whereas the \(B_{\text{max}}\) value of G185C/C195A.GFP (0.62 pmol/mg) was also in the range of WT.GFP (0.93 pmol/mg), that of R202C/C195A.GFP (0.24 pmol/mg) was decreased to 25% of that of WT.GFP (0.98 pmol/mg).

The more sensitive adenylyl cyclase assays revealed AVP-dependent cAMP formation for both NDI-causing single mutants with decreased maximal activities and strongly increased \(EC_{50}\) values (159 nM for G185C.GFP vs. 3.4 nM for WT.GFP; 503 nM for R202C.GFP vs. 3.8 nM for WT.GFP). Residual cAMP formation, indicating the presence of low affinity binding sites, was also detected in our previous study for mutant receptor R202C but not for G185C (12). Since untagged receptors were used in the previous study, the adenylyl cyclase-stimulating activity of G185C.GFP is obviously caused by the GFP moiety which may, in this case, cause a stabilization of the mutant receptor. In any case, the adenylyl cyclase activity assays demonstrate the functional rescue of both NDI causing mutant receptors by additional C195A mutations. The dose response curves of both double mutants were left-shifted compared to those of the NDI-causing single mutants (\(K_D\) values: 3.4 nm for G185C/C195A.GFP vs. 159 nM for G185C.GFP; 33,20 nM for R202C/C195A.GFP vs. 503 nM for R202C.GFP). The maximal activities were comparable to that of the wild-type.

The [\(^3\)H]AVP binding assays with intact cells revealed the \(B_{\text{max}}\) of the double mutant R202C/C195A.GFP to be significantly lower than that of WT.GFP (see above). This may be due to decreased expression and/or an impaired cell surface transport caused by the now free cysteine residue at position 202. We therefore analyzed the expression and cellular
distribution of the double mutants by localizing their GFP fluorescence signals in transiently transfected HEK 293 cells, using laser scanning microscopy. After recording the GFP fluorescence signals (Fig. 5, left panels in green), the cell surface of the same cells was identified by the use of trypan blue (Fig. 5, central panels, in red). Trypan blue does not penetrate living cells and its autofluorescence is suitable for visualizing the cell surface (15). Computer overlay of green GFP fluorescence and red trypan blue fluorescence allows identification of receptors which are transported to the cell surface (Fig. 5, right panels, colocalization is indicated by yellow). The GFP signals of the double mutants were compared to that of WT.GFP. The likewise transport competent single mutant receptors G185C.GFP and R202C.GFP (12) were also used as controls.

GFP signals were detected at the cell surface for WT.GFP and the single mutants G185C.GFP and R202C.GFP, as indicated by the overlap with the trypan blue signal. Additional GFP signals were located inside the cells, presumably representing transport intermediates en route to the cell surface or receptors retained within the cell as a consequence of overexpression. The pictures for the double mutants G185C/C195A.GFP and R202C/C195A.GFP were very similar to that of WT.GFP, i.e. the receptors seem to be present in comparable amounts at the cell surface. For the double mutant R202C/C195A.GFP, these results indicate that its decreased $B_{\text{max}}$ is caused neither by decreased expression nor decreased transport to the plasma membrane.

Expression and transport of the double mutants in transiently transfected HEK 293 cells were also analysed by immunoblot detection of total receptors (i.e. intracellular and cell surface receptors) in crude membranes (Fig. 6A) and by monitoring plasma membrane bound receptors with cell surface biotinylation assays (Fig. 6B), respectively. Cells were transfected and grown in 60 mm diameter dishes following an identical protocol prior to the assays.
For the detection of the receptors in crude membranes (Fig. 6A), a glycosylation state analysis was performed to facilitate identification of intracellular forms. Membranes were treated with EndoH to remove high mannose glycosylations (indicative of ER forms), and PNGaseF to remove both high mannose and complex glycosylations (the latter indicative of post-ER forms). WT.GFP was used as a control for the presence of complex glycosylations. The previously described (13) transport deficient GFP-tagged V<sub>2</sub> receptor mutant ΔL62-R64.GFP, which contains a deletion of the sequence 62LAR<sup>54</sup> in ICL1 was used as a negative control for complex glycosylations. Proteins were detected by immunoblotting using an anti-GFP antiserum. For WT.GFP, two immunoreactive protein bands with apparent molecular masses of 60-65 kDa and 75-80 kDa were detected in the untreated membranes as described previously (13). The 60-65 kDa but not the 75-80 kDa bands were sensitive to EndoH, whereas both bands were sensitive to PNGaseF. The 60-65 kDa bands thus represent the high mannose and the 75-80 kDa bands the complex-glycosylated forms (the complex glycosylated forms do not shift to the nonglycosylated 60 kDa form upon PNGase treatment, since O-glycosylations are added to the N-terminus of the V<sub>2</sub> receptor in the Golgi apparatus (23)). In contrast to WT.GFP, only the 60-65 kDa high mannose forms were detectable for mutant ΔL62-R64.GFP, consistent with its previously described ER retention (13). For both double mutants the same pattern and comparable amounts of high mannose and complex glycosylated forms were detected as for WT.GFP. These results show that the decreased B<sub>max</sub> of R202C/C195A.GFP did not result from decreased expression, consistent with the LSM localization study.

For the cell surface biotinylation assay, (Fig. 6B), plasma membrane proteins of intact cells were labelled with biotin. Biotinylated proteins were isolated with neutravidin and subjected to SDS-PAGE. The receptors were detected on immunoblots using an anti-GFP antiserum. Complex glycosylated 75-80 kDa forms were detectable for WT.GFP and both double
mutants, but not in the case of the transport deficient mutant ΔL62-R64.GFP. For WT.GFP and both double mutants, additional faint 60 kDa protein bands were observed. This protein band seems to represent degradation products of the complex glycosylated forms. It was not observed for mutant ΔL62-R64.GFP, demonstrating that it does not represent high mannose forms (which might have become labeled e.g. due to cell lysis). The amount of the complex glycosylated forms of double mutant R202C/C195A.GFP was comparable to that of WT.GFP and the double mutant G185C/C195A.GFP. These results thus show that the decreased \( B_{\text{max}} \) of R202C/C195A.GFP did not result from a decreased transport level, again consistent with the LSM localization study.

In summary, despite the fact that the molecular basis of the decreased \( B_{\text{max}} \) of R202C/C195A.GFP remains elusive, our results clearly show that the NDI-causing mutant receptors G185C and R202C can be functionally rescued by an additional C195A mutation. They thus strongly suggest that the second disulfide bond predicted by the structure model is indeed formed in the mutant receptors.

**The free extracellular cysteine residue C195 of the \( V_2 \) receptor is not conserved throughout evolution.** It was previously shown that residue C195 is not essential for \( V_2 \) receptor function (22). Our results demonstrate that this residue might even be disadvantageous if additional cysteine residues are introduced by a mutation. We therefore examined whether residue C195 is conserved throughout evolution. An alignment of the second extracellular loops of the \( V_2 \) receptors of various species shows that this residue is not conserved among mammals (Fig. 7A). Most mammals have a basic arginine or histidine residue at this position, which protect them from damage caused by mutationally introduced extracellular cysteine residues. We then determined the approximate time point in evolution when this residue was introduced into the \( V_2 \) receptor. We amplified and sequenced the \( V_2 \)
receptor genes of various primates. Surprisingly, residue C195 is not conserved even among primates (Fig. 7B). New and Old World monkeys have arginine and serine residues at this position, similar to other mammals. Residue C195 seems to have appeared roughly 20 to 30 million years ago, since the gibbons, diverging afterwards, already possess this residue.

We then asked how many of the class I GPCR’s containing a conserved disulfide bond have an uneven number of extracellular cysteines, thus predisposing them to the formation of additional disulfide bonds by mutationally introduced cysteine residues. Among a total of 397 receptors only 18 such receptors (4.5%) were found (Fig. 8), representing only 12 different receptors when species variants are ignored. The presence of an uneven number of extracellular cysteine residues is thus rare in this protein family.
DISCUSSION

It was previously demonstrated that residues G185 and R202 are not essential for V₂ receptor function (24) indicating that the defects of the NDI-causing mutants G185C and R202C are mediated by the introduced cysteine residues rather than by the residues replaced. The most obvious explanation for these findings was that the additional cysteine residues impair the formation of the conserved disulfide bond between residues C112 and C192. However, we show here by second site C195A mutations that these residues instead seem to participate in a second disulfide bond with residue C195 of the V₂ receptor.

Another hint in the same direction came recently from studies with the tachykinin NK₁ receptor. Here, introduction of additional cysteine residues into the second extracellular loop did not affect the conserved disulfide bond and it was suggested that the same may apply to the cysteine-introducing, NDI-causing V₂ receptor mutations (25).

From our functional rescue data it appears obvious that two disulfide bonds are indeed formed in the NDI-causing mutant receptors, although we have not demonstrated directly that this second bond is formed between C195 and an additional cysteine residue. The possibility that two bonds are formed, each involving one of the conserved cysteine residues and either C195 or the mutationally introduced residue (e.g. for the R202C mutant the combinations C112-C195 and C192-R202C or C192-C195 and C112-R202C) is, at first sight, also consistent with our functional rescue data. But this possibility is very unlikely when our previous results demonstrating that mutation of the conserved cysteine residues leads to a strong transport defect of the mutant receptors are taken into account (12). Each of these alternative combinations would disrupt the conserved disulfide bond and should also cause a transport defect; however, this was not observed. Formation of a second disulfide bond between the
additional cysteine residues and C195 is also supported by our molecular modelling data. This bond would cause sealing of the entrance of the ligand binding cavity without having a strong influence on overall receptor folding, consistent with the binding defective but transport competent properties of these two mutant receptors (12). The direct demonstration of the second disulfide bonds is nevertheless an important future goal and may be achieved e.g. by receptor isolation, proteolytic digestion and assignment of disulfide bond-connected fragments by mass spectrometry.

For the NDI-causing mutant receptors G185C and R202C, the additional C195A mutation led to receptors with wild-type $K_D$ values for AVP. Whereas the $B_{\text{max}}$ of the double mutant G185C/C195A was also in the range of the wild-type, that of mutant R202C/C195A was significantly reduced. The LSM localization studies, glycosylation state analyses and cell surface biotinylation assays consistently demonstrated that the reduced $B_{\text{max}}$ of this double mutant is attributable to neither low expression levels nor decreased transport to the cell surface. A possible explanation for the reduced $B_{\text{max}}$ is that the cell surface receptors comprise a mixed population of functional and nonfunctional receptors. The liberated cysteine residue at position 202 may cause an unstable folding state leading to the formation of two different conformations: one similar to that of the wild-type; the other leading to a binding-defective but transport-competent receptor.

The mutations G185C and R202C are not unique in causing NDI by the introduction of additional cysteine residues. The mutations R106C, R181C and Y205C belong to the same family. Our structure model also allows predictions of the defects mediated by these mutations. The cysteine residue of mutant receptor R106C would lie at the same level in the molecule as those of the mutant receptors G185C and R202C and may also easily reach residue C195 (see Fig. 2A). The functional properties of mutant receptor R106C have not been described as yet but formation of a second disulfide bond seems to be very likely in this
case. The same may be true for mutant receptor R181C. Although its extra cysteine residue would be located at a deeper level in the molecule it may also be close enough to C195 to form a second disulfide bond (see Fig. 2B). Consistent with this, impaired ligand binding but preserved transport to the plasma membrane was described for this mutant receptor (5). A binding defect was also described for mutant receptor Y205C (9). In this case, a second disulfide bond may also be formed, although the introduced cysteine residue is located more distantly from C195.

Our databank analysis demonstrates that the presence of an uneven number of extracellular cysteine residues is rare within the class I family of GPCR’s containing a conserved disulfide bond. We propose the existence of a strong selection pressure to avoid such a situation because these receptors are predisposed to inactivation by disulfide bond formation following the mutational introduction of extracellular cysteine residues. Interestingly, human rhodopsin was found among the receptors with an uneven number of extracellular cysteines. Here, the Y178C mutation was described as causing retinitis pigmentosa, and, similarly to the corresponding NDI-causing mutations of the V2 receptor, it was proposed that the Y178C mutation impairs the formation of the conserved disulfide bond of rhodopsin (11). Taking our results into account, the introduced cysteine residue may also form a second disulfide bond with the non-conserved, extracellular cysteine residue of rhodopsin. If so, additional mutation of the non-conserved cysteine residue should also suppress the defect of the Y178C mutation.
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REFERENCES


FOOTNOTES

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1 The abbreviations used are: AMBER, Assisted model building with energy refinement; AVP, arginine vasopressin; COS.M6 cells, african green monkey kidney cells; ECL, extracellular loop; ER, endoplasmic reticulum; EndoH, endoglycosidase H; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; HEK cells, human embryonic kidney cells; ICL, intracellular loop; LSM, laser scanning microscopy; NDI, nephrogenic diabetes insipidus; NK₁, neurokinin 1; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PNGaseF, peptide N-glycosidase F; SDS, sodium dodecyl sulfate; TM, transmembrane domain; V₂ receptor, human vasopressin V₂ receptor
LEGENDS TO FIGURES

Fig.1. Assignment of the retrieved protein fragments from the Brookhaven 3D protein database to the extracellular domains of the V₂ receptor. The overlapping sequences are aligned to a partial sequence of the N-terminal tail (Ntt) and to ECL1, ECL2 and ECL3. The V₂ receptor sequences are indicated by a black frame. Identical residues of the fragments are indicated by upper case, similar and non-similar residues by lower case letters. The database accession numbers of the proteins containing the respective fragments sequences are shown on the right. The presence of more than one accession number indicates the occurrence of similar fragments with comparable conformations in different proteins. Only the fragments from the protein represented by the first accession numbers were used for the modelling procedure in these cases.

Fig. 2. Putative conformation of the extracellular domains of the V₂ receptor. (A) Topview of the receptor model. The transmembrane domains are numbered. The conserved extracellular cysteine residues C112 and C192 forming the single disulfide bond and the non-conserved free extracellular cysteine residue C195 are indicated in yellow. The positions where additional cysteine residues are introduced by the NDI-causing mutations (R106, R181, G185, R202, and Y205) are indicated in green. ECL2 (grey) forms a U-like loop which surrounds the entrance of the larger ligand binding cavity. Residue C195 lies at the surface of the receptor approximately in the centre of the molecule. It is accessible to each of the cysteine residues introduced by the NDI-causing mutations. (B) Sideview of the same receptor model. The transmembrane domains are numbered.
Fig. 3. Specific [³H]AVP binding of intact, transiently-transfected HEK 293 cells expressing the double mutants G185C/C195A.GFP and R202C/C195A.GFP. Cells expressing the wild-type GFP-tagged receptors (WT.GFP) and the NDI-causing single mutants (G185C.GFP and R202C.GFP) were used as controls. Data represent mean values of duplicates which differed by less than 10%. Unspecific binding contributed up to 30 % of the total. The results are representative of three independent experiments.

Fig. 4. Adenylyl cyclase assays with crude membranes derived from transiently-transfected HEK 293 cells expressing the double mutants G185C/C195A.GFP and R202C/C195A.GFP. Membranes from cells expressing the wild-type GFP-tagged receptors (WT.GFP) and the NDI-causing single mutants (G185C.GFP and R202C.GFP) were used as controls. Data represent mean values of duplicates which differed by less than 10%. The results are representative of three independent experiments.

Fig. 5. Localization of the double mutants G185C/C195A.GFP and R202C/C195A.GFP in transiently transfected HEK 293 cells by confocal laser scanning microscopy. Cells expressing the wild-type GFP-tagged receptor (WT.GFP) and the likewise transport competent single mutant receptors G185C.GFP and R202C.GFP (12) were used as a control. Cells were analysed with horizontal (xy) scans. Receptor GFP fluorescence signals are shown in green (left panels) and cell surface trypan blue signals of the same cells in red (central panels). GFP and trypan blue fluorescence signals were computer-overlayed (right panels; overlap is indicated by yellow). GFP fluorescence is detectable only in the case of cells that were successfully transfected, whereas cell surface trypan blue fluorescence is detectable for every cell in the field of view. The scans show representative cells. Scale bar, 25 µm. Similar data were obtained in four independent experiments.
Fig. 6. Expression and intracellular transport of the double mutants G185C/C195A.GFP and R202C/C195A.GFP in transiently transfected HEK 293 cells. (A) Glycosylation state analysis. WT.GFP and the transport defective mutant ΔL62-R64.GFP were used as respective positive and negative controls for complex glycosylations. Crude membranes were treated with EndoH (EH, to remove core glycosylations) or PNGaseF (PF, to remove both core and complex glycosylations) or remained untreated (-). Receptors were detected by immunoblotting using a polyclonal anti-GFP antiserum and anti-rabbit 125I-IgG. Untransfected HEK 293 cells (control) were used as a control for antibody specificity. Protein bands described in the text are indicated. The immunoblots are representative of three independent experiments. (B) Cell surface biotinylation assay. WT.GFP and the transport defective mutant ΔL62-R64.GFP were used as respective positive and negative controls for cell surface transport. Plasma membrane proteins of intact cells were labelled with biotin. Biotinylated proteins were isolated with neuravidin and labelled receptors were detected by immunoblotting using a polyclonal anti-GFP antiserum and anti-rabbit 125I-IgG. Untransfected HEK 293 cells (control) were used as a control for antibody specificity. The immunoblots are representative of three independent experiments.

Fig. 7. Conservation of residue C195 throughout evolution (A) Alignment of the ECL2 domains of the V2 receptor of various mammals. The data set was constructed from the SWISS-PROT and EMBL databanks. The conserved cysteine residues at position 192 and the non-conserved cysteine residue at position 195 are indicated by black frames. (B) Conservation of residue C195 among the V2 receptors of primates. V2 receptor sequences encoding ECL2 were PCR-amplified from the genomic DNA of various primates and
subjected to DNA sequence analysis (see the Materials and Methods section for the individual species). The phylogeny of the hominoid genera was adopted from Goodman (26).

**Fig. 8. Alignment of the extracellular domains of class I G protein-coupled receptors with an uneven number of extracellular cysteine residues.** The data set was constructed from the SWISS-PROT and EMBL databanks. The alignment shows only the sequences of the N-terminal tails (Ntt) and the extracellular loops (ECL1, ECL2 and ECL3). The transitions to the transmembrane domains are indicated (T1-T6). Conserved extracellular cysteines are framed in black, non-conserved cysteine residues in grey. The numbers at the end of the receptor abbreviations (clear frame) indicate the total number of extracellular cysteine residues of the corresponding receptors. Receptor abbreviations were adopted from the original SWISS-PROT or EMBL data files: v2r_hum, human vasopressin V2 receptor; opsd_hum, human rhodopsin; opsd_bov, bovine rhodopsin; opsd_rat, rat rhodopsin; d4dr_mou, mouse D4 dopamine receptor; d4dr_rat, human D4 dopamine receptor; rgr_bov, bovine RPE retinal G-protein coupled receptor; rgr_hum, human RPE retinal G-protein coupled receptor; b1ar_mou, mouse beta-1 adrenergic receptor; b1ar_rat, rat beta-1 adrenergic receptor; hh2r_hum, human histamine 2 receptor; 5h2a_rat, human 5-hydroxytryptamine 2a receptor; rat 5h2b_rat, rat 5-hydroxytryptamine 2b receptor; 5h2c_hum, human 5-hydroxytryptamine 2c receptor; 5h5b_mou5, mouse 5-hydroxytryptamine 5b receptor; 5h5b_rat, rat 5-hydroxytryptamine 5b receptor; ssr1_hum, human somatostatin 1 receptor; grhr_pig, pig gonadotropin releasing hormone receptor.
<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Database ID number</th>
</tr>
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| Ntt  
LDTRDPLLAV  
LryRDP  
RDPL  
DPLLv | 1SRY, 1ASZ, 1CAU  
1MMO, 1XND  
1PMY |
| ECL1  
PQANKATDREFGPDALC  
PQLsfr  
WryTDRF  
mvdRvaGP  
FmGPev  
GPvAL | 1ERI  
1MTY, 1MMO  
1SHV  
2LIV  
1EDE, 1BVP, 1OMP, 1PKN, 1XM, 2CCY |
| ECL2  
AQRYVEGSGVTDCWACFAEPWGRRTY  
AQRNV  
VnAGSYV  
VarGSGV  
GelDCW  
fDCW  
WAedidP  
yFAEP  
FAgP  
PWWGTRvY | 1BVP  
2EBN  
1QEB2, 1HKS, 1QOR, 1ST3, 2PGD, 4PFK  
1BDG  
1GMF, 2AAA, 6TA  
1QSO  
2ASR, 2LIG  
1BXS  
1ANS, 1A2V |
| ECL3  
WDPEAPLEGAP  
WDP  
PEAP  
DptApEdqP  
EGAP  
EGpP  
ErpP | 1CDG, 1GOF, 1LOE, 1LTE, 1PRC, 1RIN, 1SBA, 2CP4  
3MDD, 1HME  
1VNC, 1VNE  
1BBT  
1PRC  
1MG1 |
A

WT.GFP
EC<sub>50</sub> = 3.4 nM

G185C/C195A.GFP
EC<sub>50</sub> = 3.4 nM

G185C.GFP
EC<sub>50</sub> = 159 nM

B

WT.GFP
EC<sub>50</sub> = 3.8 nM

R202C/C195A.GFP
EC<sub>50</sub> = 33.2 nM

R202C.GFP
EC<sub>50</sub> = 503 nM

cAMP formed (pmol/min/mg)

AVP (log M)
Additions and Corrections

Vol. 276 (2001) 8384–8392

Functional rescue of the nephrogenic diabetes insipidus-causing vasopressin V<sub>2</sub> receptor mutants G185C and R202C by a second site suppressor mutation.

_Ralf Schülein, Kerstin Zühlke, Gerd Krause, and Walter Rosenthal_

Page 8388: Fig. 5 does not show the appropriate control. The correct Fig. 5 is shown below.

This correction does not affect the interpretation or conclusions of the paper.

**FIG. 5.** Localization of the double mutants G185C/C195A and R202C/C195A.GFP in transiently transfected HEK 293 cells by confocal laser scanning microscopy. Cells expressing the wild-type GFP-tagged receptor (WT.GFP) and the likewise transport competent single mutant receptors G185C.GFP and R202C.GFP (12) were used as a control. Cells were analyzed with horizontal (xy) scans. Receptor GFP fluorescence signals are shown in green (left panels) and cell surface trypan blue signals of the same cells in red (central panels). GFP and trypan blue fluorescence signals were computer-overlaid (right panels; overlap is indicated in yellow). GFP fluorescence is detectable only in the case of cells that were successfully transfected, whereas cell surface trypan blue fluorescence is detectable for every cell in the field of view. The scans show representative cells. Scale bar, 25 μm. Similar data were obtained in four independent experiments.

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Functional rescue of the nephrogenic diabetes insipidus causing vasopressin V2 receptor mutants G185C and R202C by a second site suppressor mutation
Ralf Schülein, Kerstin Zühlke, Gerd Krause and Walter Rosenthal

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