The role of conserved extracellular cysteine residues in vasopressin V2 receptor function and properties of two naturally occurring mutant receptors with additional extracellular cysteine residues

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Abstract The G protein-coupled vasopressin V2 receptor (V2 receptor) contains a pair of conserved cysteine residues (C112 and C192) which are thought to form a disulfide bond between the first and second extracellular loops. The conserved cysteine residues were found to be important for the correct formation of the ligand binding domain of some G protein-coupled receptors. Here we have assessed the properties of the V2 receptor after site-directed mutagenesis of its conserved cysteine residues in transiently transfected human embryonic kidney (HEK 293) cells. Mutant receptors (C112S, C112A and C192S, C192A) were non-functional and located mostly in the cell's interior. The conserved cysteine residues of the V2 receptor are thus not only important for the structure of the ligand binding domain but also for efficient intracellular receptor transport. In addition to the functional significance of the conserved cysteine residues, we have also analyzed the defects of two mutant V2 receptors which cause X-linked nephrogenic diabetes insipidus (NDI) by the introduction of additional cysteine residues into the second extracellular loop (mutants G185C, R202C). These mutations are assumed to impair normal disulfide bond formation. Mutant receptor G185C and R202C were efficiently transported to the plasma membrane but were defective in ligand binding. Only in the case of the mutant receptor R202C, the more sensitive adenylyl cyclase activity assay revealed vasopressin-stimulated cAMP formation with a 35-fold increased EC_{50} value and with a reduced EC_{max}, indicating that ligand binding is not completely abolished. Taking the unaffected intracellular transport of both NDI-causing mutant receptors into account, our results indicate that the observed impairment of ligand binding by the additional cysteine residues is not due to the prevention of disulfide bond formation between the conserved cysteine residues.

Key words: Vasopressin V2 receptor; Nephrogenic diabetes insipidus; Disulfide bond; Green fluorescent protein fusion protein

1. Introduction

The antidiuretic action of the hormone arginine vasopressin (AVP) in renal collecting duct epithelial cells is mediated by the vasopressin V2 receptor, a member of the large family of G protein-coupled receptors (GPCRs). Alignment of the primary sequences of GPCRs revealed that many contain a pair of conserved cysteine residues, one in the first and the other in the second extracellular loop. It is thought that these residues are covalently linked by a disulfide bond which ensures correct folding of these loops and, as a consequence, the establishment of the ligand binding domain. The requirement of the disulfide bond for ligand binding was demonstrated for the m_1 muscarinic acetylcholine receptor [1] and the gonadotropin releasing hormone (GnRH) receptor [2]. For the β_2 adrenergic receptor, four extracellular cysteine residues (the two conserved residues and two additional non-conserved ones) were shown to be critical for high affinity binding sites [3]. Recent data indicate that two disulfide bonds are formed, surprisingly between conserved and non-conserved cysteine residues in this receptor [4]. In contrast to the GPCRs described above, the disulfide bond between the conserved cysteine residues of rhodopsin seems not to be required for correct receptor folding [5].

The V2 receptor also contains the two conserved cysteine residues (C112 and C192) in the first and second extracellular loops, whose functional significance are not known. Here we have assessed the properties of the receptor after site-directed mutagenesis of these two residues. The investigation of the putative disulfide bond of the V2 receptor is of particular interest since it may have clinical implications: mutations in the gene of the V2 receptor cause X-linked nephrogenic diabetes insipidus (NDI) [6], a disease which is characterized by the inability of the kidney to concentrate urine despite normal or elevated levels of AVP. Interestingly, the majority of NDI-causing mutations (review: [7]) in the second extracellular loop introduce additional cysteine residues and may impair receptor function by altering disulfide bonding. Here we have analyzed the defects of two NDI-causing mutations belonging to this group, namely the mutants G185C [8], and R202C [8–10].

2. Materials and methods

2.1. Materials

Lipofectamine was purchased from Life Technologies (Eggenstein,

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Abbreviations: AVP, arginine vasopressin; ER, endoplasmic reticulum; GFP, green fluorescent protein; GnRH, gonadotropin releasing hormone; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; NDI, nephrogenic diabetes insipidus; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; V2 receptor, human vasopressin V2 receptor

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Germany) and restriction enzymes from New England Biolabs (Schwalbach, Germany). Trypan blue was from Seromed (Berlin, 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 (64.8 Ci/mmol) and [α-^{32}P]ATP for the adenylyl cyclase assay (30 Ci/mmol) were from NEN (Boston, MA, USA). All other reagents were from Merck (Darmstadt, Germany). Plasmids pRCDN2, encoding the wild-type V2 receptor cDNA [11], and plasmid pWT.GFP, encoding a fusion of the red-shifted variant of the green fluorescent protein (GFP) to residue K367 of the V2 receptor, were described previously [12].

2.2. DNA manipulations

Standard protocols were used for DNA preparations and manipulations. The nucleotide sequences of DNA fragments were verified using the FS Dye Terminator kit from Perkin Elmer (Weiterstadt, Germany).

2.3. Construction of mutant V2 receptors

Mutations C112S, C112A, C192S, C192A, G185C, and R202C were introduced either by conventional PCR mutagenesis or by the use of the Quick Change site-directed mutagenesis kit into the cDNA of the untagged V2 receptor (plasmid pRCDN2 [11]) and of the GFP-tagged V2 receptor (plasmid pWT.GFP [12]). Primer sequences were: 5′-GGCCAGATGCCCTGAGTCGGGCCGTG-3′ (C112S, resulting plasmids pC112S and pC112S.GFP); 5′-GGCCAGATGCCCTGGCTCGGGCCGTG-3′ (C112A, resulting plasmids pC112A and pC112A.GFP); 5′-GGGGTCACTGACAGCTGGGCCTGCTTTG-3′ (C192S, resulting plasmids pC192S and pC192S.GFP); 5′-GCGGGGTCACTGACGCCTGGGCCTGCTTTG-3′ (C192A, resulting plasmids pC192A and pC192A.GFP); 5′-GACTGCTGGGCCAGCTTTGCGGAGCC-3′ (C195S, resulting plasmids pC195S and pC195S.GFP); 5′-CAGCGCAACGTGGAATGCGGCAGCGGGGTCAC-3′ (G185C, resulting plasmids pG185C and pG185C.GFP); 5′-CGGAGCCCTGGGGCTGTCGCACCTATGTC-3′ (R202C, resulting plasmids pR202C and pR202C.GFP).

2.4. Cell culture and transfection methods

HEK 293 cells were cultured at 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were grown on poly-L-lysine-coated plastic material to improve adherence. Cells were transfected with lipofectamine according to the supplier's recommendations. For a 15 mm diameter well of a 24 well plate, 5×10^4 HEK 293 cells were transfected with 250 ng plasmid DNA and 2 μl lipofectamine (for laser scanning microscopy lower cell densities were used; see below). After removal of the transfection reagent, cells were incubated for 48 h.

2.5. [^{3}H]AVP binding assay and adenylyl cyclase assay

The [^{3}H]AVP binding assay was carried out with intact, transiently transfected HEK 293 cells as described previously for African green monkey kidney (COS.M6) cells [11]. However, HEK 293 cells were grown in 24 well plates (15 mm diameter/well) instead of the 35 mm diameter dishes described for COS.M6 cells. The adenylyl cyclase assay was carried out with nucleus-free crude membranes of transiently transfected HEK 293 cells as described previously for stably transfected L^{tk-} cells [11].

2.6. Visualization of GFP-tagged receptors and cell surface staining in living transiently transfected HEK 293 cells

HEK 293 cells (4×10^4) in a 35 mm diameter dish containing a coverslip were transfected with 500 ng plasmid DNA and 7.5 μl lipofectamine according to the supplier's recommendations. After removal of the transfection reagent, cells were incubated for 48 h and the coverslips with the adherent cells were washed twice with phosphate-buffered saline (PBS, pH 7.0) and transferred immediately into a self-made chamber (details on request). Cells were covered with 1 ml PBS (pH 7.0), and GFP fluorescence was 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 [12]. Trypan blue fluorescence (λ_{exc} = 543 nm, λ_{em} = > 590 nm) was recorded on a second channel and overlap with the GFP signals was computed.

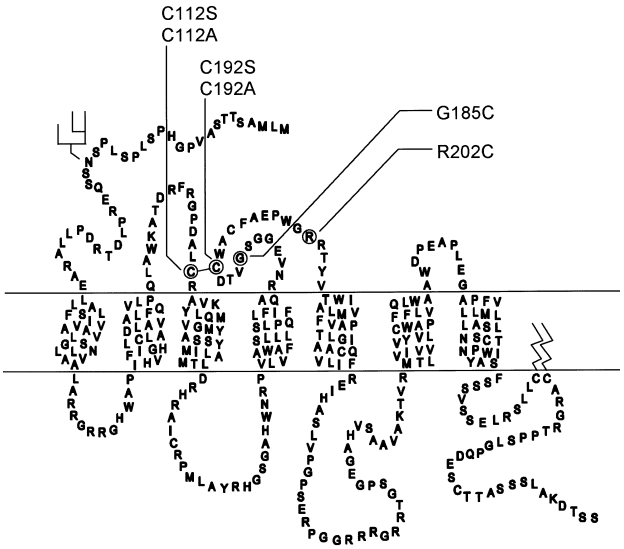

Fig. 1. Topological model of the human V2 receptor. The one-letter code for amino acids is used. The following putative post-translational modifications are shown: glycosylation at asparagine 22, palmitoylation at cysteine residues 341 and 342. The amino acid replacements for the conserved cysteine residues (C112S, C112A, C192S, C192A) and for the two NDI-causing mutations (G185C, R202C) are indicated. The putative disulfide bridge is depicted between the conserved extracellular cysteine residues C112 and C192.

3. Results

3.1. Construction of mutant V2 receptors

To assess the significance of the conserved cysteine residues for V2 receptor function, they were exchanged for serine residues by site-directed mutagenesis yielding mutant receptors C112S and C192S (Fig. 1). Since it was shown for rhodopsin that replacement of the conserved cysteine residues by serine residues may impair receptor folding and that this problem is avoided by alanine substitutions [5], alanine replacements were also constructed (mutant receptors C112A and C192A). To analyze the defects of V2 receptor mutants causing X-linked NDI by the introduction of additional cysteine residues in the second extracellular loop, mutant receptors G185C and R202C (Fig. 1) were constructed.

3.2. Pharmacological properties of the mutant V2 receptors

To analyze the functional properties of the mutant V2 receptors, HEK 293 cells were transiently transfected and [^{3}H]AVP binding profiles to intact cells were recorded (Fig. 2). For the wild-type V2 receptor, a typical binding curve was obtained with a K_D of 4.6 nM, in agreement with numerous previous reports. In contrast, no significant binding of [^{3}H]AVP to intact cells was detected when either of the conserved cysteine residues was replaced by serine or alanine residues (mutant receptors C112S, C112A, C192S and C192A). The same was true for the two NDI-causing mutant receptors G185C and R202C.

[^{3}H]AVP binding to intact cells is not suited to detect functional mutant receptors which are retained intracellularly. Low affinity binding sites at the cell surface may also not be detected due to the limited sensitivity of the assay. Therefore, total membrane fractions of transiently transfected HEK 293 cells were stimulated with AVP and adenylyl cyclase activity assays were performed (Fig. 3). Efficient adenylyl cyclase stim-

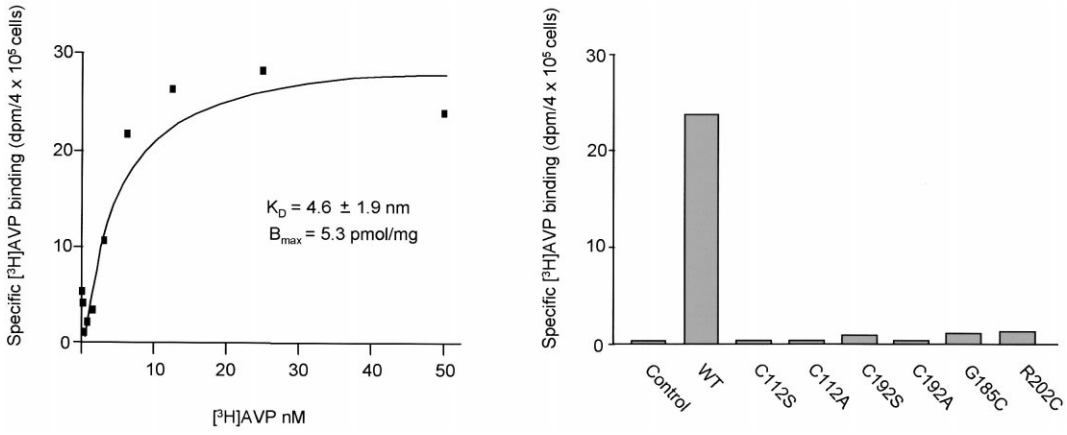

Fig. 2. Specific [^{3}H]AVP binding of intact, transiently transfected HEK 293 cells expressing wild-type and mutant V2 receptors. Left panel: binding isotherm for cells expressing the wild-type V2 receptor. Data represent mean values of duplicates which differed by less than 5%. The binding isotherms of mutant receptors C112S, C112A, C192S, C192A, G185C and R202C overlapped the baseline and are thus not shown. For clarity, specific [^{3}H]AVP binding of cells expressing these mutant receptors is shown in the right panel only at a saturating concentration (50 nM). Columns represent mean values of duplicates, which differed by less than 5%. In all binding experiments, unspecific binding contributed up to 30% of total binding. Untransfected HEK 293 cells were used as a control. The results are representative of three individual experiments.

ulation (EC_{50} = 1.9 nM) was only observed in membranes containing the wild-type V2 receptor. For mutant receptor R202C, AVP-sensitive adenylyl cyclase activity was detectable. However, the dose–response curve was right-shifted. The EC_{50} value (65.3 nM) was about 35-fold higher than that of the wild-type receptor. The EC_{max} was also reduced (65% of wild-type). Low affinity binding sites must thus be present in the case of this mutant receptor. Adenylyl cyclase stimulation was not detectable in membranes containing mutant receptors C112S, C112A, C192S, C192A and G185C indicating that these receptors were indeed not functional. Loss of function may result from misfolding, i.e. from a gross alteration of the overall receptor structure, or from a more defined defect in a functional domain. Misfolded membrane proteins are normally recognized by the quality control system of the endoplasmic reticulum (ER) and, as a consequence, are retained in the ER. Receptors with a more defined defect in a functional domain may, however, still be transport-competent. To assess these two possibilities, we have also analyzed the intracellular transport of the mutant receptors.

3.3. Intracellular transport of the mutant receptors

We have previously shown that C-terminally GFP-tagged V2 receptors exhibit pharmacological properties similar to those of the untagged receptors and that these GFP fusions are suited to localize mutant receptors in the cellular membrane system [12]. Equivalent C-terminal GFP fusions were constructed to analyze the intracellular transport of the mutant receptors. Mutant receptors C112S.GFP, C112A.GFP, C192S.GFP, C192A.GFP, G185C.GFP and R202C.GFP were expressed in transiently transfected HEK 293 cells, and the GFP fluorescence signals of the receptors were monitored by laser scanning microscopy in living cells (Fig. 4, left panels, in green). The cell surface of the same cells was identified by the use of trypan blue (Fig. 4, central panels, in red; trypan blue does not penetrate living cells and its autofluorescence is

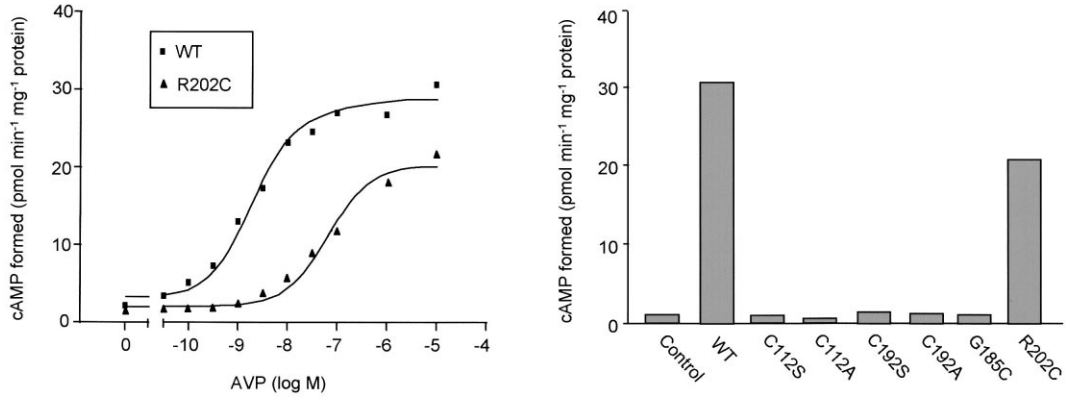

Fig. 3. Adenylyl cyclase activity assay with crude membranes of transiently transfected HEK 293 cells expressing wild-type and mutant V2 receptors. Left panel: AVP dose–response curves of membranes containing the wild-type receptor (WT) and mutant receptor R202C. Data represent mean values of two independent experiments each performed in duplicate. Duplicates differed by less than 10%. The calculated EC_{50} values were 1.9 nM (WT) and 65.3 nM (R202C). The dose–response curves of mutant receptors C112S, C112A, C192S, C192A and G185C overlapped the baseline and are thus not shown. For clarity, the adenylyl cyclase activity of membranes expressing these mutant receptors is shown in the right panel only after maximal stimulation with 10 μM AVP. Columns represent mean values of two independent experiments each performed in duplicate. Duplicates differed by less than 10%. Untransfected HEK 293 cells were used as a control.

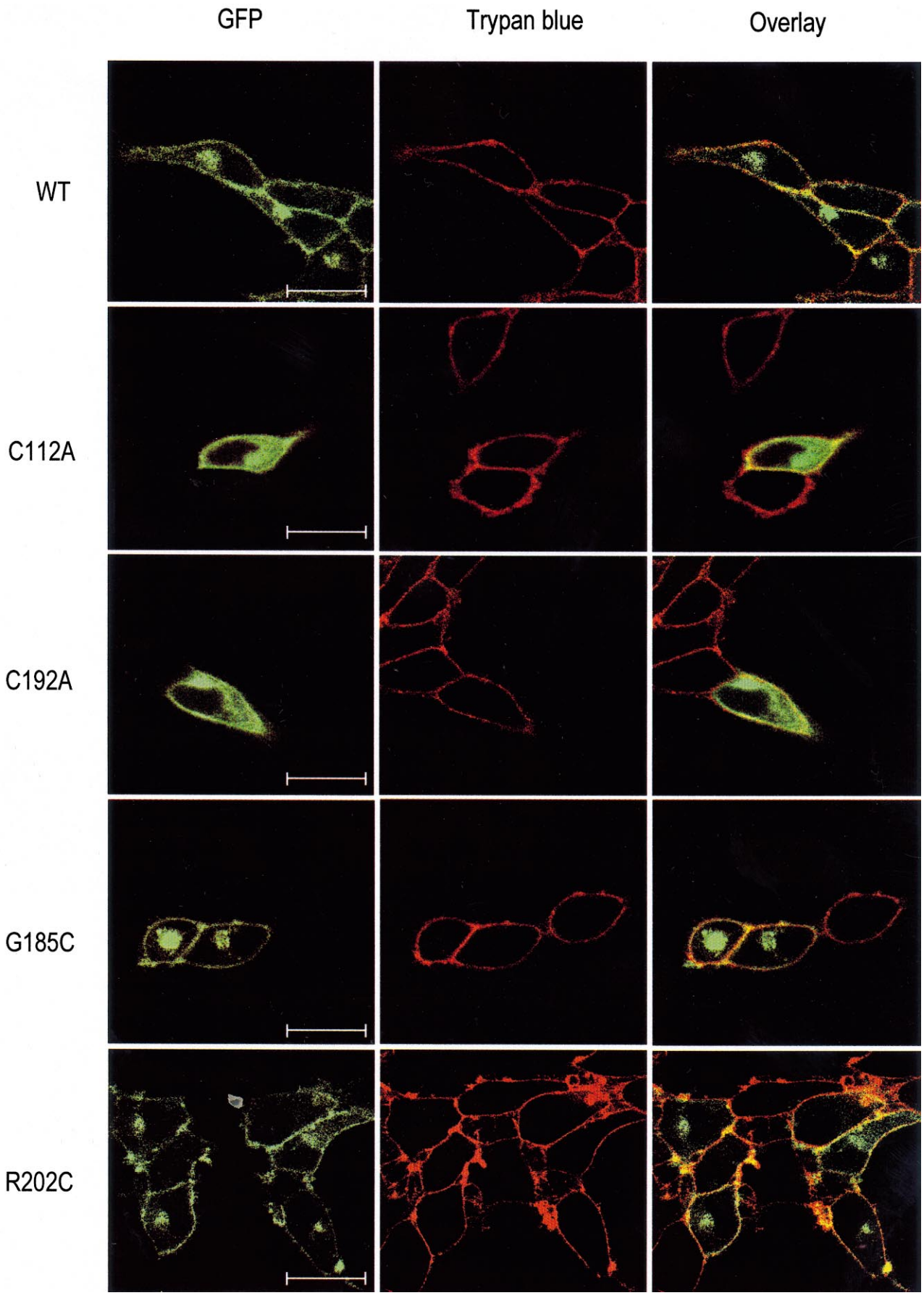
GFP
Trypan blue
Overlay
WT
C112A
C192A
G185C
R202C

Fig. 4. Localization of GFP-tagged wild-type and mutant V2 receptors in living transiently transfected HEK 293 cells. Cells expressing the wild-type V2 receptor (WT.GFP) and receptor mutants C112A.GFP, C192A.GFP, G185C.GFP and R202C.GFP were analyzed by confocal laser scanning microscopy 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 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 present in the field of view. The scans show representative cells. Scale bar, 25 μm. Similar data were obtained in four independent experiments.
←

suitable for visualizing the cell surface [12]). Computer overlay of green GFP fluorescence and red trypan blue fluorescence allows identification of receptors which are transported to the cell surface (Fig. 4, right panels, colocalization is indicated by yellow).

For the wild-type receptor (WT.GFP), GFP signals were detected at the cell surface, as indicated by the substantial 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 which are abundant as a consequence of overexpression. In contrast, the GFP signals of the receptors carrying alanine substitutions of the conserved extracellular cysteine residues (C112A.GFP and C192A.GFP) were detected predominantly intracellularly. The GFP signals diffusely filled the cell's interior with the exception of the nucleus and only small amounts were located at the cell surface, indicated by faint yellow stains at the cell surface. For the mutant receptors carrying the corresponding serine substitutions (C112S.GFP and C192S.GFP), identical results were obtained (data not shown). The conserved extracellular cysteine residues are thus essential for the efficient intracellular transport of the V2 receptor. When the data obtained by the functional assays ([^{3}H]AVP binding and adenylyl cyclase assay) are taken into account, it is likely that the available cell surface receptors are unable to bind the ligand and that the conserved extracellular cysteine residues are thus also essential for ligand binding similar as described for other GPCRs. [^{3}H]AVP binding of the wild-type V2 receptor was also sensitive to dithiothreitol (data not shown), which is consistent with the view that disulfide bond formation is a prerequisite for a functional ligand binding domain.

For the NDI-causing mutant receptors G185C.GFP and R202C.GFP, the distribution of the GFP signals was equivalent to that of WT.GFP, i.e. large amounts were detectable at the cell surface, indicated by their overlap with the trypan blue signal. These mutant receptors are thus transport-competent and, taking the functional data into account, characterized only by a ligand binding defect.

4. Discussion

We have shown here that the conserved extracellular cysteine residues in the first and second extracellular loops of the V2 receptor are essential for the efficient intracellular transport and most likely also for the ligand binding properties of the receptor. The significance of these residues for ligand binding has also been reported for other GPCRs, namely for the rat m_1 muscarinic acetylcholine receptor [1] and the GnRH receptor [2]. Similar results were obtained for the conserved extracellular cysteine residues of the β_2-adrenergic receptor, although these residues contribute to two disulfide bonds, each of which is formed between a conserved and a non-conserved residue [4]. For all these GPCRs it was postulated that connection of the first and second extracellular loops by a disulfide bond is a prerequisite for correct formation of the ligand binding domain. The same seems to be true for the V2 receptor: although small portions of the mutant receptors C112S, C112A, C192S and C192A were detectable at the cell surface, the binding assay with intact cells and the adenylyl cyclase assay with membranes did not reveal any functional activity.

The reason why mutation of the conserved extracellular cysteine residues led to receptors with an impaired cell surface transport is not clear. The observed transport deficiencies may result if the loss of the disulfide bond causes receptor conformations which provoke a prolonged association with the chaperones of the quality control system of the ER (review: [13]). It is unlikely that the conserved extracellular cysteine residues and the putative disulfide bond formed between them contribute to a signal which allows sorting into transport vesicles. Although these signals are likely to occur in membrane proteins [14,15], they should be located in the cytoplasmic domains to interact with vesicular coat proteins rather than in luminal (extracellular) domains, as is the case for the conserved cysteine residues.

It was previously speculated that NDI-causing mutations which lead to V2 receptors with additional extracellular cysteine residues may affect the disulfide bond of the V2 receptor [8,16,17]. Here we show that two such mutations cause receptor defects different from those observed for the receptors with mutations of the conserved cysteine residues. This suggests that the disulfide bond between the two conserved cysteine residues may not be affected by these two mutations. The NDI-causing mutant receptor G185C was efficiently transported to the cell surface but was completely binding-defective. A G185N substitution at this position did not affect AVP binding, demonstrating that the replaced residue G185 itself is not critical for the ligand binding properties of the V2 receptor [18]. It can be concluded that the observed ligand binding defect results from the properties of the introduced cysteine residue. An explanation which is consistent with the preservation of the conserved disulfide bond, but with a defect resulting nevertheless from the properties of the introduced cysteine residue, would be that the additional cysteine residue forms a second disulfide bond with the non-conserved residue C195 of the second extracellular loop lying in the vicinity. This additional bond may then impair ligand binding. This hypothesis may also apply to some of the other NDI-causing mutant receptors which have additional cysteine residues in the second extracellular loop. For mutant receptor R202C, however, the results can also be explained without the formation of an additional disulfide bond (see below).

The NDI-causing mutant receptor R202C was previously characterized by immunofluorescence microscopy and [^{3}H]AVP binding assays as being transport-competent but

binding-defective [10]. The ability of this mutant receptor to couple to the G_s/adenylyl cyclase system was not determined in this study. In agreement with this previous work, receptor GFP fluorescence signals were observed at the cell surface and we failed to detect specific [^{3}H]AVP binding sites. Using the more sensitive adenylyl cyclase assay, however, we found that this mutant receptor can activate the G_s/adenylyl cyclase system, although the EC_{50} is increased 35-fold and the E_{max} is reduced. In an extension to the previous study it can be concluded that AVP binding is not completely abolished for this mutant receptor. Furthermore, although it is clear that the adenylyl cyclase assay is more sensitive than the [^{3}H]AVP binding assay, the extent of adenylyl cyclase stimulation (E_{max} = 65% of wild-type) by the mutant receptor R202C is surprising. Assuming a 35-fold decrease in ligand binding activity (i.e. a decrease equivalent to the increase of the EC_{50}), specific binding should be detectable for this mutant receptor, at least when concentrations $\geq$50 nM [^{3}H]AVP are used. A low affinity binding conformation may, in this case, be accompanied by a conformation which allows a more efficient G protein coupling.

As to the mechanism which might lead to the low affinity binding properties of mutant receptor R202C, recent data indicating that residue R202 controls the ligand binding selectivity of the rat vs. the human receptor are of particular interest [18]. In the rat receptor, a leucine residue at this position facilitates ligand binding whereas the arginine of the human receptor is unfavorable. It was postulated that this position is part of an exclusion mechanism, i.e. that it constitutes a negative determinant which restricts ligand–receptor interactions. Taking these data into account, the cysteine residue of the R202C receptor mutation may simply strengthen this negative determinancy and promote the exclusion further. In addition to the formation of a second disulfide bond, the defect of mutant receptor R202C may thus also be explained by the replacement of a residue which is directly relevant for ligand binding.

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