Cys-27 Variant of Human δ-Opioid Receptor Modulates Maturation and Cell Surface Delivery of Phe-27 Variant via Heteromerization

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Background: Human δ-opioid receptor (hδOR) carries F27C polymorphism in its extracellular domain. Results: HδOR-Cys-27 with inherently impaired maturation/intracellular trafficking forms heteromers with hδOR-Phe-27 in the endoplasmic reticulum and enhances its targeting to degradation. Conclusion: HδOR-Cys-27 impairs hδOR-Phe-27 cell surface delivery in a dominant negative manner. Significance: Homo/heteromerization early in the biosynthetic pathway governs the levels of functionally active receptors at the cell surface.

The important role of G protein-coupled receptor homo/heteromerization in receptor folding, maturation, trafficking, and cell surface expression has become increasingly evident. Here we investigated whether the human δ-opioid receptor (hδOR) Cys-27 variant that shows inherent compromised maturation has an effect on the behavior of the more common Phe-27 variant in the early secretory pathway. We demonstrate that hδOR-Cys-27 acts in a dominant negative manner and impairs cell surface delivery of the co-expressed hδOR-Phe-27 and impairs conversion of precursors to the mature form. This was demonstrated by metabolic labeling, Western blotting, flow cytometry, and confocal microscopy in HEK293 and human SH-SY5Y neuroblastoma cells using differentially epitope-tagged variants. The hδOR-Phe-27 precursors that were redirected to the endoplasmic reticulum-associated degradation were, however, rescued by a pharmacological chaperone, the opioid antagonist naltrexone. Co-immunoprecipitation of metabolically labeled variants revealed that both endoplasmic reticulum-localized precursors and mature receptors exist as homo/heteromers. The existence of homo/heteromers was confirmed in living cells by bioluminescence resonance energy transfer measurements, showing that the variants have a similar propensity to form homo/heteromers. By forming both homomers and heteromers, the hδOR-Cys-27 variant may thus regulate the levels of receptors at the cell surface, possibly leading to altered responsiveness to opioid ligands in individuals carrying the Cys-27 variant.

The three opioid receptors, δ, κ, and μ, have a vital function in pain perception/modulation and analgesia (1). They belong to the family A G protein-coupled receptors (GPCRs) and have a membrane topography that characterizes all GPCRs consisting of seven membrane-spanning domains with an extracellular N terminus and an intracellular C terminus. The human δ-opioid receptor (hδOR) has a common single-nucleotide polymorphism (T80G) that results in the replacement of phenylalanine (Phe) with cysteine (Cys) at the amino acid position 27 in the N-terminal domain of the receptor. The allelic frequency of the less common Cys-27 variant varies depending on the ethnic background and is around 10% in Caucasians (2, 3). Recently, we demonstrated that the Cys-27 variant shows an altered trafficking profile when expressed in HEK293 and CHO cells (4). The Cys-27 variant, but not the Phe-27 variant, shows compromised maturation and cell surface delivery. The receptor precursors, which are incapable of endoplasmic reticulum (ER) export, accumulate and are eventually targeted to ER-associated degradation (ERAD) (4). The ER-retained receptors, however, are not permanently misfolded, as they can be rescued to the plasma membrane by membrane-permeable opioid receptor pharmacological chaperones (5, 6).

In recent years it has become increasingly evident that opioid receptors do not exist only as monomers but form homomers and also heteromeric complexes with other opioid receptor subtypes and even with other GPCRs. This has been demonstrated using a number of biochemical and biophysical approaches such as co-immunoprecipitation and various resonance energy transfer techniques (see e.g. Refs. 7–16). These approaches utilizing heterologous expression systems have

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5 The abbreviations used are: GPCR, G protein-coupled receptor; BRET, bioluminescence resonance energy transfer; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GABA_A, R2, type 2b γ-aminobutyric acid receptor; hδOR, human δ-opioid receptor; LHR, luteinizing hormone receptor; rLHR, rat LHR; RLuc, R. reniformis luciferase; IP, immunoprecipitation; Ab, antibody.
been recently complemented by in vivo studies. For example, the existence of δ-κ and δ-μ heteromers has been demonstrated in CNS of rodents using heteromer-specific opioid ligands and antibodies, respectively (13, 17).

Opioid receptor heteromerization has been found to lead to pharmacological and functional diversity, as heteromers show altered ligand binding, signaling, and trafficking properties compared with the corresponding mono/homomers (for review, see Refs. 18 and 19). In contrast, much less is known about the functional significance of homomeric complexes. The family C, GPCRs, such as GABAB receptors, exist as obligatory homo/heteromers that are formed shortly after synthesis in the ER (20). This notion has now been extended to family A GPCRs, and it has been suggested that homo/heteromerization might constitute a quality control step in the ER, predicting that proper receptor-receptor interactions are prerequisite for ER export and delivery to the plasma membrane (21). In support of this idea are the observations for an increasing number of naturally occurring GPCR slice variants and mutant forms as well as engineered receptor mutants that have been shown to behave as dominant-negatives of their corresponding wild-type forms by preventing their expression at the cell surface (e.g. rhodopsin (22) and D3 dopamine (23), D2 dopamine (24), gonadotropin-releasing hormone (25, 26), melanocortin-1 (27, 28), thyroid-stimulating hormone (29), luteinizing hormone (30), CCR5 chemokine (31, 32), and V2 vasopressin (33) receptors). On the other hand, a few family A GPCRs have been shown to promote cell surface delivery of other receptors. For instance, it was shown that the e11δ-adrenergic receptor requires interaction with the α1n-adrenergic receptor for targeting to the cell surface (34). Even more direct evidence for a connection between receptor homo/heteromerization and ER export has emerged from recent studies in which homomerization-compromised receptor mutants were found to be retained intracellularly (35, 36). Whether homomerization (or heteromerization) is required for ER exit of opioid receptors as well is still an open question. Whereas a few studies suggest that opioid receptors form homo/heteromers intracellularly (11, 15), others argue that this takes place only at the cell surface (37).

Based on the divergent trafficking properties of the two hδOR variants (4), we set out to investigate whether the Cys-27 variant might alter trafficking of the Phe-27 variant in the early secretory pathway, possibly by forming heteromeric complexes in the ER. We show that the Cys-27 variant indeed acts in a dominant negative manner and impairs maturation and cell surface delivery of the Phe-27 variant. Furthermore, using co-immunoprecipitation of differentially tagged variants, the ER-localized precursors were found to exist as heteromers and the bioluminescence resonance energy transfer (BRET) technique revealed that the variants had a similar ability to form homo and heteromers. The F27C polymorphism may thus govern the levels of functionally active hδOR homo/heteromers at the cell surface.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—The hδOR<sup>Cys-27</sup>, hδOR<sup>Phe-27</sup>, and the rat luteinizing hormone receptor (rLHR) constructs in the pFT-SMMF vector with a cleavable influenza HA signal peptide, an N-terminal Myc-tag (EQKLISEEDL), and a C-terminal FLAG-tag (DYKDDDDK) have been described previously (4, 6, 38). The constructs encoding hδOR<sup>Cys-27</sup> and hδOR<sup>Phe-27</sup> with a HA signal peptide and an N-terminal HA-tag (YPYDVPDYA) in pcDNA3.1 and the corresponding hδOR<sup>Cys-27</sup> construct in pFT-SMMF have been described in Refs. 39 and 4, respectively.

Expression vectors encoding hδOR<sup>Cys-27</sup> and hδOR<sup>Phe-27</sup> fused to either the Venus variant of the enhanced YFP (40) or *Renilla reniformis* luciferase (Rluc) were prepared for the BRET<sup>3</sup> experiments. In short, the HA-tagged hδOR<sup>Cys-27</sup> (4) was subcloned into a modified pcDNA3.1/Zeo<sup>+Rluc</sup> vector using restriction enzymes Nhel and AvrII in a way that the 3' end of the hδOR<sup>Cys-27</sup> sequence was fused onto the 5' end of Venus. This resulted in an in-frame fusion of hδOR<sup>Cys-27</sup> with the Venus tag separated by a seven-amino acid linker. The hδOR<sup>Cys-27</sup>-Rluc-pcDNA3.1/Zeo<sup>+</sup> construct has been described previously (14). The corresponding vectors encoding hδOR<sup>Phe-27</sup> were created by the QuickChange site-directed mutagenesis kit (Stratagene) as described (4). The construct encoding the HA- and Venus-tagged type-2b GABA<sub>B</sub> receptor (GABA<sub>B</sub><sub>R2</sub>-R2) that was used as a control has been described earlier (41).

**Cell Culture and Transfections**—Cells were maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin (complete DMEM) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. If needed, the medium contained the appropriate selection antibiotics. The tetracycline inducible HEK293 cell line (HEK293<sub>t</sub>) expressing the Myc- and FLAG-tagged hδOR<sup>Cys-27</sup> (6) was transfected with hδOR<sup>Phe-27</sup>-pcDNA3.1 encoding the HA-tagged hδOR<sup>Phe-27</sup> with the Lipofectamine 2000 transfection reagent (Invitrogen) under Blasticidin S (4 μg/ml; InvivoGen), hygromycin (400 μg/ml; InvivoGen), and Geneticin (400 μg/ml; InvivoGen) selection. Two clones were isolated and selected expressing hδOR<sup>Phe-27</sup> at ~2 and 19 pmol/mg of membrane protein in the absence of tetracycline induced expression of hδOR<sup>Cys-27</sup>. The latter clone was used for co-immunoprecipitation experiments (Fig. 6). The cells were plated on culture flasks or plates (5–6 × 10<sup>6</sup> cells to 75-cm<sup>2</sup> culture flasks or 100-mm plates or 2 × 10<sup>6</sup> cells to 25-cm<sup>2</sup> culture flasks) for experiments and cultured for 3 days in complete DMEM. The hδOR<sup>Cys-27</sup> expression was induced by adding tetracycline (0–500 ng/ml; Invitrogen) to the culture medium for various periods of time as indicated in the figure legends. The proteasomal inhibitor lactacystin (10 μM; Enzo Life Sciences) was added to the culture medium 6 h before cells were harvested (Fig. 3B). For Western blotting and pulse-chase labeling experiments, cells were incubated in PBS, 20 mM N-ethylmaleimide for 10 min before harvesting, quick-frozen in liquid nitrogen, and stored at −70 °C.

The Flp-in-293 cells (Invitrogen) and human SH-SY5Y neuroblastoma cells (a kind gift of Dr. Mikko Hiltunen, University of Eastern Finland, Kuopio, Finland) were plated on 100-mm (2–4.5 × 10<sup>6</sup> cells) or 6-well (3 × 10<sup>5</sup> cells) culture plates and cultured for 24 h before transfection with hδOR or rLHR constructs. The medium was changed to Opti-MEM (Invitrogen), 4% (w/v) FBS 3 h before transfection. The DNA constructs (1–8 μg) and the Lipofectamine 2000 transfection reagent were incubated in Opti-MEM for 5 min, mixed (the mixture had a
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final DNA:Lipofectamine ratio of 1:3), and incubated for another 20 min before adding the mixture to the cells (Figs. 4 and 5, D–F, supplemental Figs. 1A and 2). An alternative method was occasionally used for immunofluorescence microscopy to increase the number of transfected cells expressing only one receptor construct. For this purpose, each DNA construct was mixed with the transfection reagent separately before adding the mixtures to the cells (supplemental Fig. 1, B–D). In some cases cells transfected with the two methods were mixed before plating them to coverslips (Fig. 5, A–C). The amount of total DNA was kept constant with the appropriate vector DNA in experiments with varying amounts of constructs. The medium was changed to complete DMEM after 4 h, and cells were harvested or fixed 20 h later.

The HEK293 cells that were used for BRET1 experiments were plated on 6-well plates (3 × 105 cells), cultured for 24 h in complete DMEM without selection antibiotics, and transfected with increasing amounts of receptor-RLuc and receptor-Venus constructs. Polyethyleneimine (Polysciences Inc.) was used as the transfection reagent in a ratio of 3:1 (DNA (μg):polyethyleneimine). The amount of total DNA (2.1 μg) was kept constant with the appropriate vector DNA. After 24 h, the medium was changed for complete DMEM, and the cells were harvested 24 h later.

BRET1 Assay—The transfected cells were washed with PBS containing 5 mM EDTA, detached, and resuspended in PBS. After quantifying the amount of protein by the Bio-Rad DC protein assay, the cells (2 μg) were distributed on 96-well plates (gray plates; PerkinElmer Life Sciences), and the total fluorescence was measured using the FlexStation IV ( Molecular Devices) with excitation and emission filters set at 485 and 538 nm, respectively. The cells were then transfected to white Devices) with excitation and emission filters set at 485 and 538 nm (gray plates; PerkinElmer Life Sciences), and the total fluorescence was measured using the Mithras LB 940 instrument (Berthold Life Sciences). The cells were washed twice with the chase medium (DMEM supplemented with 5 mM methionine) and chased for different periods of time as indicated in the figures. The opioid antagonist naltrexone (10 μM; Torcios) was added to the medium at the beginning of induction, and lactacystin (10 μM) at the beginning of depletion and were maintained thereafter.

Preparation of Whole Cell Extracts and Immunoprecipitation of Solubilized Receptors—Total cellular lysates were prepared in 0.5% (v/v) n-dodecyl-β-d-maltoside (Enzo Life Sciences), 25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 2 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 10 μg/ml benzamidine, and the solubilized receptors were immunoprecipitated by one or two-step immunoprecipitation using the FLAG M2 or HA antibody affinity resins (both from Sigma) and eluted in SDS-sample buffer as described previously (6, 42). Mouse IgG (Sigma) was used as a control in the second immunoprecipitation step. Transferrin receptors were purified with the human transferrin receptor antibody (1:100; Zymed Laboratories Inc.) by two-step immunoprecipitation following a technique described earlier (42). The purified transferrin receptors in SDS-sample buffer (50 μl) were reduced with 10 μl of 0.2 M DTT (95 °C, 5 min) and alkylated with 10 μl of 0.5 M iodoacetamide (37 °C, 30 min).

Deglycosylation of Immunoprecipitated Receptors—Deglycosylation of immunoprecipitated receptors was performed using endo-β-N-acetylgalactosaminidase H or peptide N-glycosidase F (both from Roche Applied Science) as described (6).

SDS-PAGE and Western Blotting—Samples were separated on SDS-PAGE using 7 or 10% SDS-polyacrylamide gels (6). The separated proteins were electroblotted, and the blots were probed with FLAG M2 (0.5 μg/ml, Sigma), HA (HA-7, 0.12 μg/ml, Sigma), and ubiquitin (1:1000, Enzo Life Sciences) antibodies (6). The SDS-PAGE gels containing radioactively labeled proteins were treated for fluorography (42), and films were scanned with the Umax PowerLook 1120 color scanner and the Image Master 2D Platinum 6.0 software. Data were quantified and analyzed as described (38).

Flow Cytometry—Cell surface receptors expressed in stably transfected HEK293, or in transiently transfected SH-SYSY cells were analyzed by flow cytometry as described earlier (43) using c-Myc 9E10 (2 μg/ml; Santa Cruz) or HA (HA-7, 4 μg/ml; Sigma) antibodies and the phycoerythrin-conjugated rat antimouse IgG, secondary antibody (2 μg/ml; BD Biosciences). The fluorescence of live cells was measured with the BD Biosciences FACSCalibur flow cytometer, and the data were analyzed with CellQuestPro 6.0 as described (44). For the analysis of transiently transfected cells, the mean fluorescence of live cells minus the mean fluorescence of cells stained only with the secondary antibody was used for calculations.

Immunofluorescence—SH-SYSY and Flp-in-293 cells were transiently transfected as described above, and after 4 h of...
transfection, 2.5 × 10⁶ cells were plated onto poly-L-lysine-coated coverslips in 12-well plates and cultured for 20 h in complete DMEM. Cells were fixed, permeabilized, and stained as described elsewhere (4). The primary antibodies were anti-HA (HA-7, 5 μg/ml; Y-11, 2 μg/ml, Santa Cruz), anti-cMyc (A-14, 1 μg/ml, Santa Cruz), anti-calreticulin (1:1000; Enzo Life Sciences), anti-Sec61β (1:500; Millipore), anti-ER-Golgi intermediate compartment (ERGIC)-53 (1:500; Alexix), and anti-GM130 (1:500; BD Biosciences). Alexa Fluor® 488 goat antimouse and Alexa Fluor® 568 goat anti-rabbit (1:250, Invitrogen) were used as secondary antibodies. Additionally, the nuclei were labeled with TO-PRO®-3 iodide (1:800, Invitrogen) and fluorography. As seen in Fig. 2A, lanes 5–8 and fluorography. As seen in Fig. 2A, lanes 5–8 and 43-kDa receptors were detectable with the FLAG antibody (Fig. 1, A and C). Thus, these results show that the hORPhe-27 variant has a dominant negative effect on expression of the co-expressed hORPhe-27 variant, causing a decrease in the number of mature cell surface receptors.

The Co-expressed hORCys-27 Interferes with Maturation of hORPhe-27 Variant—The observed changes in the steady-state biosynthesis of the Phe-27 form was impaired by the co-expressed Cys-27 variant. To test this possibility more directly, metabolic pulse-chase labeling experiments were performed (Fig. 2). Cells were first treated with tetracycline to induce expression of the Cys-27 variant for 16 h or were left untreated. Thereafter, they were labeled with [35S]methionine/cysteine for 40 min and chased for 0–4 h. Receptors were then purified by immunoprecipitation and analyzed by SDS-PAGE and fluorography. As seen in Fig. 2A, the major form of the Phe-27 variant at the end of the pulse was the 43-kDa precursor, which in time disappeared and was converted to the mature 55-kDa form. The processing of the precursors was, however, impaired in cells that co-expressed the Cys-27 variant. Whereas
almost all precursors had disappeared within 4 h in the non-induced cells, a substantial amount was still apparent in the induced cells (3.1 ± 1.3 and 10.8 ± 1.7 % were left in the non-induced and induced cells, respectively, \( p = 0.023, n = 3 \). Concomitantly, less mature receptors were detected (compare lanes 4 and 8 in Fig. 2A). Quantification of the fluorograms of the 2-h chase samples revealed that co-expression of the two variants led to a significant decrease in the mature receptor/precursor ratio (Fig. 2E). These changes were dependent on the tetracycline concentration used to induce expression of the Cys-27 variant and were detectable already at the lowest concentration tested, 10 ng/ml (Fig. 2B). Although the maturation efficiency of h\( \alpha \)OR-Phe-27 was decreased, the kinetics of maturation appeared to be normal (Fig. 2D), suggesting that the h\( \alpha \)OR-Phe-27 precursors that are eventually able to leave the ER in the co-transfected cells mature normally. The transferrin receptor that is endogenously expressed in HEK293 cells showed no impaired maturation, as the ratio of the two immu-
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**A**

| h50R(Phc27) | IP: HA Ab | Chase (h) |
|-------------|-----------|-----------|
| 0           | 0.5       | 2        | 4   |
| 1           | 2        | 3        | 4   |

Tetracycline (500 ng/ml)

**B**

| Mature/precursor |
|-----------------|
| 0   | 2  |
| 1  | 2 |
| 2  | 2 |
| 3  | 2 |
| 4  | 2 |
| 5  | - |

**C**

TR (IP: TR Ab, Chase (h) = 97)

**D**

h50R(Phc27) (Tetracycline (500 ng/ml))

**E**

Mature/precursor

**F**

h50R(Phc27) (IP: HA Ab) vs. h50R(Phc27) (IP: FLAG Ab)

Tetracycline (500 ng/ml)

TNTX

noprecipitated receptor forms, the precursor and mature forms (45), was not changed significantly by h50R(Cys-27) expression in the 2-h chase samples (Figs. 2, C and E). This indicates that h50R(Cys-27) expression does not lead to general impairment in membrane protein biosynthesis. Furthermore, the co-expression did not lead to ER stress, as no differences in expression of the ER molecular chaperones BiP, ERP72, or protein-disulfide isomerase were detected, in contrast to what was observed for cells treated with tunicamycin (data not shown), a well known inducer of ER stress and the unfolded protein response.

Interestingly, the relative amount of Phe-27 variant glycoforms carrying only one N-glycan were increased in the induced versus the non-induced cells (Fig. 2A). The change was detected for both receptor precursor and mature forms. This suggests impairment in glycosylation of the nascent h50R(Phe-27), i.e. less efficient co-translational addition of N-glycan to Asn-33 (44) when the two variants are co-expressed. In addition, the mature h50R(Phe-27), whether carrying one or two N-glycans, migrated as less distinct bands on SDS-PAGE in cells co-expressing the Cys-27 variant (Fig. 2A), suggesting more heterogeneous processing of receptor-bound glycans in the Golgi. The changes in h50R(Phe-27) glycosylation were specific and did not result from a general impairment in protein glycosylation as no apparent changes were detected for the transferrin receptor (Fig. 2C), which is O-glycosylated and carries three N-glycans (45, 46).

Opioid Receptor Pharmacological Chaperone Enhances Maturation of Co-expressed h50R Variants—Earlier studies have shown that membrane-permeable opioid antagonists can be used as pharmacological chaperones to enhance maturation of h50R(Cys-27) as well as h50R(Phe-27) when the variants are expressed individually (4–6). These ligands are able to bind to ER-localized precursors and enhance their folding and transport to the cell surface (6). Thus, we assessed whether the Cys-27 variant-mediated impairment in maturation of the Phe-27 variant could be overcome with a pharmacological chaperone. In a positive case, this would give further support to the notion that the Cys-27 variant alters the behavior of the Phe-27 variant early in the secretory pathway. The stably transfected HEK293 cells were induced to express Myc-h50R(Cys-27)-FLAG and were subjected to metabolic labeling in the absence or presence of 10 μM naltrexone, an opioid receptor-specific antagonist. The treatment led to a decrease in the amount of precursors and a concomitant increase in the amount of mature receptors (Fig. 2F). The latter change, however, was clearly apparent only for the Cys-27 variant. The increase in the amount of the mature h50R(Cys-27) carrying only one N-glycan was particularly evident, in line with our previous report (4).

Co-expression of h50R Variants Leads to Increased Targeting of h50R(Phe-27) Precursors to ERAD—As the processing of h50R(Phe-27) precursors was compromised after co-expression of the Cys-27 variant, we next tested the possibility that the precursors that were not exported out of the ER are targeted to ERAD. To this end, non-induced and induced stably transfected HEK293 cells were treated with a specific proteasomal inhibitor lactacystin or vehicle and subjected to metabolic labeling. After a 2-h chase, the cells were harvested, and receptors were purified and analyzed. The lactacystin treatment stabilized the Myc-h50R(Cys-27)-FLAG precursors (compare lanes...
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A similar stabilization of hδOR-Phe-27 precursors was observed, but this was clearly seen only in cells expressing both variants (compare lanes 3 and 4 to lanes 1 and 2 in Fig. 3A). This suggests that hδOR-Phe-27 co-expression leads to targeting of some of the hδOR-Phe-27 precursors to ERAD. This conclusion was also supported by the finding that a slowly migrating high molecular weight smear at the top of the SDS-PAGE gels became apparent in the lactacystin-treated cells (Fig. 3A). Importantly, the high molecular weight polyubiquitinated forms of the hδOR-Phe-27 variant were more apparent in induced versus non-induced cells even without the lactacystin treatment (Fig. 3A). Such diffuse high molecular weight species were also seen in the pulse-chase experiments shown in Fig. 2A.

hδOR-Phe-27 Interferes with Maturation and Trafficking of hδOR-Phe-27 in Human SH-SY5Y Neuroblastoma Cells—The results presented above suggest that hδOR-Phe-27 that shows inherently impaired ER export, redirects some of the co-expressed hδOR-Phe-27 precursors to ERAD, and thus lowers their maturation efficiency in co-transfected HEK293 cells. To rule out the possibility that these results were a peculiarity of HEK293 cells or the isolated stable cell line, we co-expressed the two variants in neuronal cells, human neuroblastoma SH-SY5Y cells that are known to express δORs endogenously (47). A Western blot analysis of immunoprecipitated receptors from cells transfected separately with Myc-hδOR-Cys-27-FLAG and HA-hδOR-Phe-27 cDNA showed that the two variants were expressed as precursor and mature receptor forms in a similar manner as in the stably transfected HEK293 cells (Fig. 4A, lanes 1 and 10). The difference in the mature receptor/precursor ratio between the two variants was clearly evident, being even more apparent than in the HEK293 cells. This confirms that the low maturation efficiency of the Cys-27 variant is an inherent property of this variant and is clearly detected also in cells endogenously expressing the receptor. To assess the consequences of co-expression, the cells were transfected with a constant low amount (1 μg) of HA-hδOR-Phe-27 cDNA and increasing amounts of Myc-hδOR-Cys-27-FLAG cDNA, reaching a ratio of 1:8. Receptor expression was then studied by immunoprecipitation and Western blotting or, alternatively, by flow cytometry. As seen in Fig. 4A, the amount of both mature receptors and precursors of hδOR-Phe-27 decreased upon co-expression, already at a 1:1 plasmid ratio. A concomitant significant decrease in the number of cell surface receptors was detected by flow cytometry (Fig. 4B). No such changes were observed when Myc-rLHR-FLAG was co-expressed with HA-hδOR-Phe-27 (Fig. 4, C and D), confirming that the changes observed were not due to general membrane protein overexpression. LHR is another family A GPCR that shows compromised ER export and maturation (38).

The Cys-27 variant-mediated impairment in maturation and cell surface expression of the Phe-27 variant appeared to be more apparent in the transiently transfected SH-SY5Y cells than in the stably transfected HEK293 cells. To verify that this was because of a different transfection system and not a cell line-specific phenomenon, Flip-in-293 cells were transiently co-transfected with Myc-hδOR-Cys-27-FLAG and HA-hδOR-Phe-27 in a similar manner as in the case of the neuroblastoma cells. As expected, the Western blot analysis of the expressed receptors revealed results that were comparable with those obtained using the SH-SY5Y cells (supplemental Fig. 1A). Finally, we investigated whether the distinct epitope tags added to the two variants might have an effect on the observed changes in receptor behavior. For this purpose, we transfected the SH-SY5Y cells with receptor constructs harboring reciprocal epitope tags: N-terminal Myc-tagged and C-terminal FLAG-tagged hδOR-Phe-27 and N-terminal HA-tagged hδOR-Cys-27. Again, the number of cell surface receptors of the hδOR-Phe-27 variant was decreased after co-expression of the hδOR-Cys-27 variant (data not shown).

The co-transfected SH-SY5Y cells were then subjected to immunofluorescence analysis to assess the subcellular localization of the two hδOR variants. As seen in Fig. 5, the Phe-27 variant was localized mainly at the cell surface, whereas the Cys-27 variant clearly showed more extensive intracellular staining. In the co-transfected cells, both variants accumulated...
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FIGURE 4. hδOR<sup>Cys-27</sup> impairs maturation and cell surface expression of the hδOR<sup>Phe-27</sup> variant in transiently co-transfected SH-SY5Y neuroblastoma cells. SH-SY5Y cells were co-transfected with HA-hδOR<sup>Phe-27</sup> and Myc-hδOR<sup>Cys-27</sup>-FLAG for 24 h using various cDNA ratios as indicated (A and B). As a control, HA-hδOR<sup>Phe-27</sup> was co-transfected with Myc-rLHR-FLAG (hOR/LHR, 1:8) (C and D). After 24 h, cells were analyzed by flow cytometry (B and C) or were lysed and subjected to immunoprecipitation, SDS-PAGE, and Western blotting (WB) using the indicated antibodies (48). These results indicate that the variants have a similar propensity to form homo/heteromers. Identical results were obtained using Flp-in-293 cells (supplemental Fig. 2).

in a perinuclear compartment that is likely to correspond to receptor precursors that are eventually targeted to ERAD. Similar results were obtained using Flp-in-293 cells (supplemental Fig. 1, B–G). In the co-transfected SH-SY5Y cells, the accumulating Phe-27 variant co-localized weakly with ER markers calreticulin and Sec61β and more strongly with ER-Golgi intermediate compartment and Golgi markers, ERGIC-53 and GM130, respectively (supplemental Fig. 2).

Both Precursor and Mature Forms of hδOR<sup>Cys-27</sup> and hδOR<sup>Phe-27</sup> Form Heteromers—The altered behavior of hδOR<sup>Phe-27</sup> precursors upon co-expression of the Cys-27 variant would be consistent with the idea that the variants form heteromers early in the secretory pathway. To more directly test this hypothesis, a co-immunoprecipitation assay was applied using the stably transfected HEK293 cells. The cells were induced to express the Cys-27 variant, labeled with [35S]methionine/cysteine and harvested either immediately after the pulse or, alternatively, after a 6-h chase. This allowed assessing directly whether precursors or mature forms or both are capable of forming heteromers, as the former are the major receptor forms detected after the pulse (see Fig. 2A), whereas only mature receptors are detectable after the 6-h chase (4). The HA-hδOR<sup>Phe-27</sup> and the Myc-hδOR<sup>Cys-27</sup>-FLAG variants were immunoprecipitated from the cellular lysates using HA and FLAG antibodies, respectively, and after denaturation the co-immunoprecipitated receptors were pulled down in a second immunoprecipitation step using the appropriate antibodies. As a control, equal aliquots of the first immunoprecipitates were subjected to immunoprecipitation with the immobilized mouse IgG. As seen in Fig. 6A, the FLAG antibody pulled down the Cys-27 variant precursor forms, carrying either one or two N-glycans, from the HA antibody immunoprecipitate (compare lanes 3 and 5), and similarly, the HA antibody precipitated the 43-kDa Phe-27 precursor carrying two N-glycans from the FLAG antibody immunoprecipitate (compare lanes 2 and 6). No specific bands were found in the second immunoprecipitate if the mouse IgG was used (Fig. 6A, lanes 1 and 4). These results confirm that the two variants can heteromerize already in the ER and also suggest that both precursor glycoforms are capable of forming homo/heteromers. The results obtained using the 6-h chased samples were comparable with those obtained using the pulse-labeled ones (Fig. 6B), confirming that the mature variants also exit as homo/heteromers.

Finally, the homo/heteromerization of the hδOR variants was verified with a BRET assay using intact cells (Fig. 6C). This allowed demonstrating the specificity of the oligomerization in vivo without the detergent solubilization step that was required for the co-immunoprecipitation experiments. For the BRET assay, the hδOR variants were tagged at their C termini with Renilla luciferase (Rluc) or a variant of the enhanced YFP (Venus). The Venus- and Rluc-tagged receptors were then transiently co-transfected in HEK293 cells, and BRET titration experiments were performed. As seen in Fig. 6C, the titration curves for the different donor and acceptor pairs were best fitted to a hyperbolic function, and the calculated BRET<sub>50</sub> values that reflect the relative affinity between the donor and acceptor (48) were similar. This indicates that the variants have a similar propensity to form homomers and heteromers. Identical
results were obtained for the reciprocal constructs (data not shown), indicating that the position of the BRET donor and acceptor had no effect on the results. In addition, the specificity of the interaction was demonstrated by the lack of significant energy transfer under similar conditions when HA-hA-h9254 ORphe-27-Rlus was co-expressed with an unrelated receptor, the HA- and Venus-tagged glutamate receptor family member, GABA_A-R2 (Fig. 6C).

DISCUSSION

In recent years several studies have suggested that homo/heteromerization of GPCRs occurs constitutively before the receptors are inserted to the plasma membrane. This notion was originally thought to characterize only family C receptors but has now been extended to the largest GPCR subfamily, family A (also known as rhodopsin type GPCRs). The results shown in this study are in full agreement with this idea. We demonstrate that the hA-h9254 OR Cys-27 variant that shows inherently compromised ER export impairs maturation and cell surface delivery of the co-expressed Phe-27 variant, leading to its enhanced targeting to ERAD. The ER-localized precursors, as well as the cell surface mature variants, were found to exist in homo/heteromers and no differences were observed in their ability to form these oligomeric complexes. The cellular expression level of each of the two variants thus governs the ratio of homo/heteromers. The hA-h9254 OR F27C polymorphism may thus have functional consequences in heterozygous individuals that carry both variant forms, possibly relating to altered receptor expression levels and cellular responsiveness to the opioid ligand exposure.

A number of naturally occurring mutants and splice variants of family A GPCRs have previously been shown to cause ER retention of the corresponding wild-type receptors upon co-expression. For example, several rhodopsin mutants that cause autosomal dominant retinitis pigmentosa, redirect the wild-type protein to degradation when co-expressed in HEK293 cells (22). In this study, the impairment of hA-h9254 OR Phe-27 maturation and ER export in cells co-expressing the two hA-h9254 OR variants was shown to be specifically mediated by the Cys-27 variant and was not because of overloading the capacity of the cells to handle overexpressed membrane proteins. No induction of the unfolded protein response was observed and no impairment in maturation of the endogenously expressed transferrin receptor

![Subcellular localization of hA-h9254 OR Cys-27 and hA-h9254 OR Phe-27 in transiently co-transfected SH-SYSY neuroblastoma cells.](image-url)
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Co-expression of Human δ-Opioid Receptor F27C Variants

have relatively subtle difficulties in folding/ER export can act in
a dominant negative manner and lead to a decreased cell sur-
face expression of the corresponding "wild-type" receptors. 
This underscores the importance of the stringent ER quality
control mechanisms that scrutinize newly synthesized proteins 
and, furthermore, raises the question whether a similar domi-
nant negative effect is a more general characteristic of common
GPCR polymorphic variants than has been anticipated.

The dominant negative effects of mutant receptors on their
wild-type counterparts have been taken as evidence that GPCR
homo/heteromerization occurs intracellularly. This study pro-
vides ample evidence that this is the case for δOR when consid-
ering both the physical interactions between hδORPhe-27 and
hδORCys-27 and the influence of their co-expression on the mat-
uration profile of hδORPhe-27. Co-expression of the two recep-
tor variants in either the HEK293 or SH-SY5Y cells led to an
intracellular accumulation of the hδORPhe-27 precursors with
a concomitant decrease in the number of mature receptors at the
cell surface. This was shown by metabolic labeling, Western
blotting, and flow cytometry as well as by confocal microscopy.
In addition, the co-expression led to an enhanced targeting of
hδORPhe-27 precursors to ERAD, as they were stabilized in cells
 treated with a proteasomal inhibitor lactacystin. The treatment
also resulted in an increase in the amount of polyubiquitinated
receptors. Furthermore, maturation of the ER-retained precur-
sors was rescued by a membrane-permeable opioid antagonist
naltrexone that is known to act intracellularly as a pharmaco-
logical chaperone for hδOR (4, 5).

More direct evidence supporting the notion that the hδOR
oligomeric complexes form shortly after synthesis was pro-
vided by the sequential co-immunoprecipitation experiments.
Using metabolically labeled cells and differentially epitope-
tagged variants, we were able to demonstrate that the receptor
precursors exist as heteromers. The use of metabolically labeled
cells was a clear advantage because it allowed the independent
investigation of ER-localized precursors and mature cell sur-
face receptors and the use of sequential immunoprecipitation.
Previous co-immunoprecipitation studies on opioid receptors
have relied mostly on Western blotting techniques (8, 9, 49),
which do not equivocally allow discrimination between intra-
cellular biosynthetic intermediates and cell surface proteins.
Furthermore, the two-step immunoprecipitation used in this
study was performed in more stringent conditions than the
one-step co-immunoprecipitations previously used for West-
ern blot assays. The BRET measurements performed utilized
intact cells and could, therefore, not distinguish, in which
cellular compartment the variant homo/heteromers were
located. Previously, this BRET technique has been success-
fully combined with subcellular fractionation, demonstrating
that GPCR homo/heteromerization is detectable in ER
membrane fractions (11, 50, 51). Similarly, specific BRET
signals between β2-adrenergic receptors were detected in
cells that were treated with brefeldin A (36), a drug that
collapses the Golgi and impairs ER export of newly synthe-
-sized proteins. Furthermore, mutations in the β2-adrenergic
receptors that impair dimerization were found to lead recep-
tor retention in the ER (36).

was detected in the co-transfected HEK293 cells. Furthermore,
overexpression of the rat LHR that shows similar impaired ER
export as hδORCys-27 (38) had no effect on hδORPhe-27
matura-
tion in the co-transfected human SH-SY5Y neuroblastoma 
cells. These results thus indicate that not only fully folding
incompetent mutant receptors but also receptor forms that

FIGURE 6. Both precursor and mature receptor forms of hδORCys-27 and
hδORPhe-27 variants form homo/heteromers. A and B, co-immunoprecipi-
tation. HEK293, cells constitutively expressing HA-hδORPhe-27 were induced
to express Myc-hδORCys-27-FLAG, pulse-labeled with [35S]methionine/cys-
teine for 40 min, and harvested immediately (A) or chased for 6 h (B). Recep-
tors were purified from cellular lysates by sequential immunoprecipitation,
performing the first step in native conditions with the FLAG M2 or HA anti-
body. Proteins were eluted with SDS-containing buffer, and re-immunopre-
cipitation was performed from the diluted denatured eluates with the FLAG
M2 or HA antibodies or the mouse IgG, as indicated. Only one-eighth and
one-fourth of the samples was loaded on lanes 2 and 5 in panel A and on lanes
2 and 6 in panel B, respectively. In panel A, the outlined squared area of lane 6
is shown with enhanced contrast. In panel B, the lanes 4 and 8 represent
shorter exposures of lanes 2 and 6, respectively. The precursor and mature
receptor forms are indicated with open and closed symbols, respectively, as
specified in Fig. 1. The difference in migration of the 2-N-glycan precursors of
the two variants (A) is because of the distinct N- and C-terminal tags. C, BRET
measurements. HEK293 cells were transiently co-transfected with different
amounts of energy donors (hδORCys-27-RLuc, hδORPhe-27-RLuc) and energy
acceptors (HA-hδORCys-27-Venus, HA-hδORPhe-27-Venus, HA-GABaR2-R2-Venus)
as indicated. After 48 h, BRET signals were measured after the addition of the
luciferase substrate coelenterazine H. The results shown are plotted as a func-
tion of the ratio of total fluorescence (Venus) and total luminescence (RLuc)
and are representative of two independent experiments carried out in duplic-
ate. The curves were fitted using a non-linear regression analysis with a single
binding site.
**Co-expression of Human δ-Opioid Receptor F27C Variants**

The quantitative changes in the expression of hδOR\textsuperscript{Phe-27} precursors and mature forms that were observed in the cotransfected HEK293 cells were accompanied by alterations in the appearance of different receptor glycoforms. We have shown previously that \(N\)-glycosylation of Asn-33 in the N-terminal domain of hδOR\textsuperscript{Cys-27} is inefficient, leading to expression of two glycoforms carrying either one or two \(N\)-glycans attached to Asn-18 or Asn-18/Asn-33, respectively (44). In contrast, the Phe-27 variant, when expressed alone, is \(N\)-glycosylated efficiently at both sites (4). The co-expression, surprisingly, led to an increase in the relative amount of hδOR\textsuperscript{Phe-27} precursors and mature forms carrying only one \(N\)-glycan. No apparent changes were observed in glycosylation of the endogenously expressed transferring receptor. These observations support the assumption that homo/heteromerization of the hδOR variants occurs very early after synthesis, even co-translationally. It can be hypothesized that the specific changes in \(N\)-glycosylation of the Phe-27 variant result from steric hindrance, possibly by a protein that interacts with the hδOR\textsuperscript{Cys-27} N-terminal domain during or shortly after synthesis. We have suggested previously that such an interaction is likely to be the cause for ER accumulation of the Cys-27 variant after long term expression (4).

Importantly, the qualitative changes observed for the receptor glycoforms were accompanied by changes in apparent heterogeneity of the mature hδOR\textsuperscript{Phe-27} forms on SDS-PAGE when the two variants were co-expressed. The most likely explanation for this observation is an altered processing of receptor \(N\)- and \(O\)-glycans in the Golgi, suggesting that the newly synthesized receptors, once having formed homo/heteromeric complexes in the ER, transit the Golgi to the plasma membrane in the oligomeric form. It can be hypothesized that the close proximity of receptor N termini after homo/heteromerization might sterically hinder the Golgi-localized glycosyltransferases. The existence of cell surface homo/heteromers of the hδOR variants was verified in the co-immunoprecipitation experiments, showing that not only the precursors but also the mature receptors existed as homo/heteromers. Whether these oligomeric complexes that reach the cell surface are stable or transient in nature remains to be investigated in the future.

The dynamic nature of cell surface opioid receptor oligomeric complexes is presently under extensive research, and no definitive consensus has been reached whether there is specific regulation of the homo/heteromers relating to receptor activation and internalization (for review, see Ref. 18). Studies on the possible functional consequences of the hδOR variant heteromers on receptor internalization will be especially important. The two variants show altered trafficking properties not only in the secretory pathway but also at the cell surface, as the Cys-27 variant appears to be unstable at the cell surface and is very prone to constitutive internalization (4). It is likely to be subject to cell surface quality control that disposes apparent membrane proteins to lysosomal degradation (52). In our recent study we demonstrated that hδOR F27C polymorphism might represent a risk factor for Alzheimer disease as heterozygotes were overrepresented among the Alzheimer disease patients in two independent study populations. We also observed that overexpression of the Cys-27 variant, but not that of the Phe-27 one, caused major changes in processing of the exogenously or endogenously expressed amyloid precursor protein in SH-SY5Y and HEK293 cells (3). This occurred most likely via a mechanism that relates to the enhanced constitutive internalization of hδOR\textsuperscript{Cys-27} (4). These results underscore the possibility that even common GPCR polymorphisms that are characterized by altered receptor trafficking may have unexpected consequences in vivo.

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