A Membrane-proximal, C-terminal α-Helix Is Required for Plasma Membrane Localization and Function of the G Protein-coupled Receptor (GPCR) TGR5*

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Background: TGR5 is a G protein-coupled bile acid receptor that modulates the immune response, glucose homeostasis, and liver regeneration.

Results: Secondary structure of the receptor C terminus determines plasma membrane trafficking.

Conclusion: TGR5 plasma membrane content and responsiveness to extracellular ligands depends on C-terminal α-helix formation.

Significance: This provides insights into the structure-function relationship of TGR5, which is a potential drug target for metabolic diseases.

The C terminus of G protein-coupled receptors (GPCRs) is important for G protein-coupling and activation; in addition, sorting motifs have been identified in the C termini of several GPCRs that facilitate correct trafficking from the endoplasmic reticulum to the plasma membrane. The C terminus of the GPCR TGR5 lacks any known sorting motif such that other factors must determine its trafficking. Here, we investigate deletion and substitution variants of the membrane-proximal C terminus of TGR5 with respect to plasma membrane localization and function using immunofluorescence staining, flow cytometry, and luciferase assays. Peptides of the membrane-proximal C-terminal variants are subjected to molecular dynamics simulations and analyzed with respect to their secondary structure. Our results reveal that TGR5 plasma membrane localization and responsiveness to extracellular ligands is fostered by a long (9 residues) α-helical stretch at the C terminus, whereas the presence of β-strands or only a short α-helical stretch leads to retention in the endoplasmic reticulum and a loss of function. As a proof-of-principle, chimeras of TGR5 containing the membrane-proximal amino acids of the β2 adrenergic receptor (β2AR), the sphingosine 1-phosphate receptor-1 (S1P1), or the κ-type opioid receptor (κOR) were generated. These TGR5β2AR, TGR5S1P1, or TGR5κOR chimeras were correctly sorted to the plasma membrane. As the exchanged amino acids of the β2AR, the S1P1, or the κOR form α-helices in crystal structures but lack significant sequence identity to the respective TGR5 sequence, we conclude that the secondary structure of the TGR5 membrane-proximal C terminus is the determining factor for plasma membrane localization and responsiveness towards extracellular ligands.

TGR5 (Gpbar-1, M-Bar) is a G protein-coupled receptor (GPCR) that is expressed almost ubiquitously in humans and rodents (1–4). The receptor is coupled to a stimulatory G protein. Both unconjugated and conjugated bile acids as well as various steroid hormones have been identified as potent TGR5 agonists (5, 6). In the liver, TGR5 is localized in sinusoidal endothelial cells, Kupffer cells, cholangiocytes, gallbladder epithelial cells, and gallbladder smooth muscle cells (7–13). Here, TGR5 modulates hepatic microcirculation, exerts anti-inflammatory, anti-apoptotic and choleretic effects, and promotes gallbladder filling (7, 12–15). In the intestine, TGR5 is expressed in enteroendocrine L-cells, in immune cells as well as in neurons and astrocytes of the enteric nervous system (16–18). Although the latter suggests a role for TGR5 in intestinal motility, activation of TGR5 in L-cells has been linked to increased glucagon-like peptide-1 secretion and the regulation of glucose homeostasis (18). In animal models administration of TGR5 agonists improved glucose tolerance and reduced liver inflammation and steatosis as well as atherosclerotic plaque formation (14, 18, 19). Thus, TGR5 is a promising drug target for the treatment of metabolic disorders, such as type II diabetes, obesity, atherosclerosis, and non-alcoholic steatohepatitis (7, 19).

Although TGR5 functions in different organs are progressively elucidated, the regulation of the receptor expression, localization, and function has not been studied so far. We previously identified a naturally occurring TGR5 mutation (Q296X) that leads to the truncation of the 35 C-terminal

The abbreviations used are: GPCR, G protein-coupled receptor; aa, amino acids; AR, adrenergic receptor; MD, molecular dynamics; PM, plasma membrane; TLC, tauroliothocholic acid; WT, TGR5 wild type; ER, endoplasmic reticulum; S1P1, sphingosine 1-phosphate receptor-1; κOR, κ-type opioid receptor; β2AR, β2-adrenergic receptor.
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amino acids (20). In comparison to the wild type protein the Q296X variant failed to activate adenylate cyclase after stimulation with the agonistic bile acid tauroliothocholic acid (TLC). Immunofluorescence staining revealed that the truncated protein was retained in the endoplasmic reticulum (ER) of transfected HEK293 and Madin-Darby canine kidney cells (20). Similar findings, i.e. a reduced functionality and retention in the ER, have been demonstrated for other GPCRs with a truncated membrane-proximal, intracellular C terminus, such as the luteinizing hormone/chorionic gonadotropin receptor (21), the vasopression 2 receptor (22), and the A1 adenosine receptor (23). This indicates that the cellular signaling response is determined by the amount of functionally active receptor in the plasma membrane (PM) (24). However, the underlying molecular mechanisms for ER retention varied for different GPCRs. Although the C terminus of some receptors contained an ER export signal composed of specific amino acid residues (25, 26), the C terminus of other receptors required a sequence of hydrophobic amino acids to form a putative helix as a prerequisite for proper folding and anterograde trafficking to the PM (27, 28) (for a recent review, see Ref. 24).

Based on the naturally occurring TGR5 truncation mutation Q296X and guided by secondary structure predictions from molecular dynamics (MD) simulations, we generated further deletion and substitution variants within the membrane-proximal C terminus to identify the amino acid motifs/structural determinants that facilitate PM localization of this bile acid receptor. Using these variants and three chimeras of TGR5 with the membrane proximal C terminus of the β2 adrenergic receptor (β2AR), the sphinogine 1-phosphate receptor-1 (SIP1), or the κ-type opioid receptor (κOR), we demonstrate that the formation of a membrane-proximal α-helix (helix 8) is essential for anterograde trafficking of TGR5 from the ER to the PM and thus for receptor function.

**EXPERIMENTAL PROCEDURES**

Cell culture reagents were from PAA (Coelbe, Germany). Fetal calf serum (FCS) was from Biochrom (Berlin, Germany). TLC and forskolin (Forsk) were from Sigma and Calbiochem, respectively.

**Cloning of TGR5 Variants and TGR5 Chimera**—Human TGR5 was cloned as described (10, 20). TGR5 mutations were introduced into two different human TGR5 cDNA constructs. One construct contained part of the 5′-UTR and the complete coding sequence of human TGR5 including the stop codon and was cloned into the pcDNA3.1+ vector (Clontech, Palo Alto, CA) (20). The second construct, FLAG–TGR5–YFP, was cloned into the pEYFP-N1 vector (Clontech, Palo Alto, CA) (10) and contained the coding sequence with an N-terminal FLAG tag and a C-terminal yellow fluorescent protein (YFP) tag as described (20). Mutations were generated using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA). Deletion variants as well as the substitution variants 285–297A and 291–297P were generated using PCR-based cloning strategies. The receptor chimera TGR5β2AR was cloned with primers, in which amino acids 285–297 (QRYTAPWRAAAQR) of the TGR5 C terminus were replaced with alanine acids 330–342 (PDFRIAFQELLCL) of the β2AR. The first PCR was carried out using the forward primer of the respective TGR5 pcDNA3.1+/pEYFP constructs (10, 20) and a reverse primer containing part of the sequence of the β2AR. The second PCR was performed with a forward primer containing the β2AR sequence fragment as well as the respective reverse primers of the TGR5 pcDNA3.1+/pEYFP constructs (10, 20). The third PCR used the PCR products of the first two PCRs as template and contained the forward and reverse cloning primers of the TGR5 pcDNA3.1+/pEYFP constructs (10, 20). The receptor chimera TGR5S1P1 was cloned accordingly with primers in which amino acids 285–294 (QRYTAPWRAA) of the TGR5 C terminus were replaced with amino acids 316–325 (KEMRRA-FIRI) of the SIP1. The receptor chimera TGR5κOR was cloned with primers in which amino acids 285–296 (QRYTAP-WRAAQ) of the TGR5 C terminus were replaced with amino acids 335–346 (ENFKRCAFDFCF) of the κOR. All mutagenesis primer sequences and cloning strategies can be obtained upon request. Successful cloning and mutagenesis was verified by sequencing (GenBank™ accession numbers: TGR5, NM_001077191.1; β2AR, NM_000024.5; SIP1, NM_001400.4; κOR, NM_000912.3).

**Cell Culture and Transfection**—Human embryonic kidney 293 (HEK293) cells were cultured in DMEM with 10% FCS and were kept at 37 °C and 5% CO2. Cells were transiently transfected with cDNA plasmids using Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions. Cells were used for immunofluorescence staining, FACS, or luciferase assays 48 h after transfection.

**Immunofluorescence and Confocal Laser Scanning Microscopy**—HEK293 cells, grown on glass coverslips, were transfected with the different TGR5 cDNA constructs. After 48 h cells were fixed in 100% ethanol (−20 °C) for 30 s and incubated with the following antibody dilutions (2% FCS in PBS without calcium chloride and magnesium chloride (PBS−/−)): anti-TGR5 amino acids 298–318 (RVLR2) (10), 1:500; anti-FLAG-M2 (Sigma), 1:250; sodium/potassium (Na+/K+) ATPase (Sigma), 1:100; protein disulfide isomerase (Thermo Fisher Scientific Inc., Rockford, IL), 1:150. Fluorescent-labeled secondary antibodies (fluorescein, 1:100; cyanine-3, 1:500), were purchased from Dianova (Hamburg, Germany). Nuclei were stained with Hoechst 34580 (1:20,000; Invitrogen). Images were analyzed on a Zeiss LSM510META confocal microscope using a multitracking modus. A 63× objective and a scanning resolution of 1024×1024 pixels was used for all samples.

**Flow Cytometry**—To quantify PM localization, flow cytometry was performed as described (20). In brief, transiently transfected HEK293 cells were washed in PBS−/−, detached with FACS buffer (PBS−/−, 0.5 mM EDTA, 2% FCS) and centrifuged (2000×g, 3 min, 4 °C). Cells were resuspended in FACS buffer and washed once by centrifugation and resuspension in FACS buffer (2000×g, 3 min, 4 °C). The FLAG tag was detected with the anti-FLAG M2-antibody using the Zenon PacificBlue Label-Kit (Invitrogen) for 30 min (4 °C; dilution 1:250). Subsequently, cells were washed in FACS buffer and measured for forward scatter (FCS), side scatter (SCS), YFP, and PacificBlue fluorescence using a FACS-CANTO-II (BD Biosciences). TGR5 PM localization was determined by the amount of FLAG tag-positive cells divided by the total amount.
of TGR5 positive cells as detected by YFP fluorescence. Control experiments were carried out using unlabeled non-transfected (no fluorescence) cells, unlabeled FLAG-TGR5-YFP transfected (only YFP fluorescence) cells, and permeabilized cells (20).

**Measurement of TGR5 Activity Using a cAMP-responsive Luciferase Assay**—HEK293 cells, grown on 6-wells, were co-transfected with a cAMP-sensitive reporter gene construct (Bayer AG; Leverkusen, Germany; 1.6 μg), a Renilla expression vector (Promega; Madison, WI; 0.1 μg), and human TGR5 variants in pcDNA3.1+ (0.5 μg diluted with 1.1 μg of pEFYP-N1 vector). The cAMP reporter construct contains five cAMP-responsive elements (CREs) in front of the luciferase gene. Luciferase activity was normalized to transfection efficacy. Cell lysis and luciferase assays were performed using the dual-luciferase kit (Promega) according to the manufacturer’s instructions. Control experiments were performed with the pcDNA3.1+ vector (20). Luciferase activity after stimulation with DMSO (vehicle) served as control and was set to 1.0, and forskolin and TLC-dependent luciferase activity is represented as multiple thereof.

**Molecular Dynamics Simulations of Peptidic Variants of the C Terminus**—Peptidic deletion and substitution variants of the C terminus of TGR5 as well as of the TGR5β2AR chimeras were subjected to MD simulations. Starting structures of all peptides were built using the leap program from the Amber 11 suite of molecular simulation programs (29). Each structure was generated in an extended conformation to reduce any bias by the starting structure on the subsequent MD simulations.

MD simulations were performed with Amber 11 (29) using the force field by Cornell et al. (30) with modifications suggested by Simmerling et al. (31). Each peptide was placed into an octahedral periodic box of TIP3P water molecules (32) and, if required, neutralized by Cl− ions. The distance between the edges of the water box and the closest atom of the peptide was at least 11 Å, resulting in system sizes of ~16,000 atoms for the smallest Δ291–297 and ~41,000 atoms for the largest system 285–290A. The particle mesh Ewald method (33) was used to treat long-range electrostatic interactions. Bond lengths involving bonds to hydrogen atoms were constrained using SHAKE (34). The time-step for all MD simulations was 2 fs, with a direct-space, non-bonded cutoff of 8 Å. The starting structures were initially minimized by 2500 steps of steepest decent and conjugate gradient minimization applying harmonic restraints with force constants of 5 kcal mol−1 Å−2 to all solute atoms. Then NVT-MD, i.e. MD simulation with a constant number of particles, volume, and temperature, was carried out for 50 ps during which the system was heated from 100 K to 300 K. Subsequent NPT-MD, i.e. MD simulation with a constant number of particles, pressure, and temperature, was used for 50 ps to adjust the solvent density. As the last step of the thermalization procedure, we performed NVT-MD for 200 ps. Of the following 600 ns of NVT-MD at 300 K, the last 500 ns were used for analysis with conformations extracted every 40 ps.

Two additional MD simulations were performed for the wild type (WT), the 285–290A, and the 291–297A and -G variants. When pooled alongside the original simulations, a similar distribution across clusters of secondary structure sequence (see below) was found (data not shown), thus indicating the convergence of the simulations.

**Clustering According to Secondary Structure Sequence**—For each conformation the secondary structure was assigned to each amino acid using the “continuous extension version” of the DSSP algorithm (35). This resulted in a string consisting of the letters B, E, G, H, T, and “–”. To overcome the problem of sparse secondary structure types, we assigned all helical-like secondary structure elements (letter G (3–10-helix), letter T (Turn)) the letter H (α-helix). We also assigned the secondary structure element β-bridge (letter B) the letter E (β-sheet). Amino acids with unassigned secondary structure are marked by “–”. Based on these assignments, a similarity matrix of all conformations was computed considering only those assignments that occur at least 20 times per amino acid in each mutant. Missing amino acids in deletion variants were substituted with the letter X. The elements of the similarity matrix were computed by summing one for each exact letter match between respective string positions and zero otherwise. A hierarchical clustering was then performed on the similarity matrix applying Ward’s method as implemented in the program R (36).

**Statistical Analysis**—Experiments were performed independently at least three times. Results are expressed as the means ± S.E. and analyzed using the two-sided student t test. A p < 0.01 was considered statistically significant.

**RESULTS**

**PM Localization and Function of Membrane-proximal Deletion and Alanine Substitution Variants of the TGR5 C Terminus**—A previous study had demonstrated that the deletion of the 35 C-terminal amino acids (aa) of human TGR5 (Gpbar-1) results in a retention of the mutated protein in the ER and a complete loss of function (20). To determine the role of the TGR5 C terminus for receptor localization and function, several C-terminal truncation variants were generated. Using the TMHMM program, Asp-284 was identified as the first amino acid of the intracellular C terminus (37, 38). Thus, premature stop codons were introduced into the TGR5 cDNA constructs (TGR5 pcDNA3.1+, FLAG-TGR5-YFP) by site-directed mutagenesis at the following residues: Asp-284, Pro-290, Arg-297, Gln-300, and Ser-310 (Fig. 1). Localization of the mutated proteins was studied in transiently transfected HEK293 cells by immunofluorescence staining with an anti-FLAG antibody and confocal laser scanning microscopy as described (20). Immunofluorescence staining of the truncation variants D284X, P290X, and R297X showed a reticular, intracellular fluorescence pattern (Fig. 2A) that was identified as the ER by double-labeling with an antibody against the ER marker protein disulfide isomerase (data not shown). The mutated protein Q300X was detected both in the ER and in the PM, whereas the S310X variant was mainly localized in the PM (Fig. 2A).

Subsequently, TGR5 responsiveness was analyzed in HEK293 cells co-transfected with the respective TGR5 constructs and a cAMP-responsive luciferase reporter plasmid as described (8, 20). Luciferase activity served as a measure for the rise in intracellular cAMP after TGR5 activation by TLC, which is the most potent endogenous agonist for this receptor (1, 2, 6). Forskolin elevates cAMP levels independently of TGR5 and was used as a
positive control. As described previously, stimulation of TGR5 WT with TLC showed a dose-dependent increase in luciferase activity (8, 20) (Fig. 2B). The truncation of the TGR5 C terminus at amino acids 284 and 290 completely abolished the responsiveness of the receptor towards TLC, whereas the forskolin-induced luciferase activity was unaffected (Fig. 2B). Truncation at amino acid 297 led to a significant reduction in TLC-dependent luciferase activity with a remaining increase of 2.6 ± 0.2-fold (n = 10) at 10 μM TLC (Fig. 2C). Although the Q300X variant showed a similar TLC responsiveness as the WT at concentrations >2.5 μM, no significant rise in luciferase activity was detected after stimulation with 0.1 μM TLC. The loss of the last 20 amino acids in the TGR5 variant S310X had no effect on receptor responsiveness towards TLC (Fig. 2C). These findings are in line with previous studies demonstrating that the intracellular signaling response of a GPCR is determined by the amount of functionally active receptor in the PM (24). Furthermore, these results suggest that amino acids 284–297 are essential for localization of TGR5 in the PM. To elucidate the role of these residues in more detail, additional cDNA constructs were generated: deletion of amino acids 285–290, 285–297, and 291–297 and alanine substitution of amino acids 285–290 and 291–297 (Figs. 1, 3, and 4).
The truncated proteins Q300X and S310X were not detected in the PM but were localized intracellularly within the ER. Although deletion of amino acids 291–297 (Δ291–297) resulted in a significantly reduced responsiveness towards low TLC concentrations (0.1 and 0.5 μM) compared with the WT protein. However, stimulation of Δ285–290 and Δ285–297 with 10 μM TLC induced a significant rise in luciferase activity by 3.3 ± 0.2- (n = 8) and 5.5 ± 0.7-fold (n = 10), respectively (Fig. 3B). Although deletion of amino acids 291–297 (Δ291–297) resulted in a significantly reduced responsiveness towards low TLC concentrations (0.1 and 0.5 μM) compared with the WT, incubation with higher TLC concentrations (2.5 and 10 μM) led to a similar rise in luciferase activity as observed with WT (Fig. 3B).

The substitution of amino acids 285–290 with alanine (285–290A) led to an accumulation of the mutated protein in the ER, whereas the 291–297A variant had no effect on receptor localization as demonstrated by the colocalization of the mutant protein with the PM marker Na⁺/K⁺ ATPase (Fig. 4A). In line

**FIGURE 2. Analysis of TGR5 truncated variants in HEK293 cells.** A, HEK293 cells were transiently transfected with the different FLAG-TGR5-YFP truncation variants. The FLAG tag was made visible using an anti-FLAG-M2 antibody (red). In contrast to the WT, the truncation variants D284X, P290X, and R297X were not detected in the PM but were localized intracellularly within the ER. The truncated proteins Q300X and S310X were localized both in the PM and the ER. Nuclei were stained with Hoechst (blue). Bars = 10 μm. **B** and **C**, HEK293 cells were co-transfected with TGR5 (pcDNA3.1+), a cAMP-responsive luciferase reporter construct, and a Renilla expression vector (8). Luciferase activity served as a measure of the rise in intracellular cAMP after activation of TGR5. Forskolin (Forsk, 10 μM) was used as a TGR5-independent positive control. The truncation variants D284X and P290X completely lost responsiveness to stimulation with the TGR5 agonistic bile acid TLC (n = 9 each; B). The R297X variant showed a 2.6-fold increase after incubation with 10 μM TLC, whereas the Q300X mutation was equally responsive to 10 μM TLC as the WT (n = 10 each; C). The variant S310X did not affect receptor responsiveness to TLC (n = 12; C). Results are expressed as the mean ± S.E. * and # = significantly different (p < 0.01) from DMSO (Control) and TGR5 WT, respectively.

**FIGURE 3. Analysis of TGR5 deletion variants in HEK293 cells.** HEK293 cells were transiently transfected with TGR5 WT and the deletion TGR5 (pcDNA3.1+) variants. A, the TGR5 protein was made visible using an anti-TGR5 antibody (RVLR2, red). WT TGR5 was almost completely targeted to the PM as demonstrated by the yellow coloring in the overlay image with the PM marker protein sodium/potassium (Na⁺/K⁺) ATPase (green). Although the deletion variant Δ285–290 was mainly retained in the ER, the variant Δ285–297 was present both in the ER and the PM, and the Δ291–297 variant was located predominately in the PM. Nuclei were stained with Hoechst (blue). Bars = 10 μm. B, receptor responsiveness towards TLC was measured using a cAMP-responsive luciferase construct. The deletion variants Δ285–290 and Δ285–297 showed a significantly reduced responsiveness towards TLC stimulation as compared with the TGR5 WT. The Δ291–297 variant showed normal receptor activity after stimulation with 2.5 and 10 μM TLC and reduced activity in response to lower TLC concentrations (0.1 μM, 0.5 μM). Results (Δ285–290, Δ291–297 n = 8; Δ285–297 n = 10; WT n = 23) are expressed as the mean ± S.E. * and # = significantly different (p < 0.01) from DMSO (Control) and TGR5 WT, respectively.

**FIGURE 4. A C-terminal α-Helix Determines TGR5 Localization and Function.**
Interestingly, stimulation of the 291–297A variant with 10 μM TLC caused a significant increase in luciferase activity as compared with the TGR5 WT. At a concentration of 10 μM TLC the rise in luciferase activity measured for 291–297A (18.7 ± 2.4; n = 13) was even significantly higher than the luciferase activity obtained with the WT (10.1 ± 0.7; n = 43) (Fig. 4B).

**Secondary Structure Content of Membrane-proximal C-terminal Peptides Predicted from Molecular Dynamics Simulations**—Because the membrane-proximal part of the TGR5 C terminus does not contain a known ER exit signal (di-acidic motif, FX_6LL or EX_5LL motifs) (24–26, 39) and because the deletion variants as well as the 291–297A variant were able to reach the PM, we hypothesized that it is the secondary structure of the membrane-proximal C terminus rather than a specific amino acid sequence that determines the localization and function of the receptor. To investigate the secondary structure content of the membrane-proximal C terminus of the WT and the variants, peptides excised from the C termini were subjected to MD simulations in explicit solvent over 600 ns of simulation time, of which the last 500 ns were used for analysis. The WT and the mutant peptides had a length of 18 aa, whereas the Δ285–290 and Δ291–297 variants had a length of 12 and 11 aa, respectively. As a starting structure, a straight peptide conformation was used, in that way avoiding any structural bias in the setup of the simulations. Fig. 4, C and D, show the secondary structure content over the course of the MD simulations for WT and 285–290A, respectively, which was retained in the ER. For the WT, an α-helix content of, on average, 10.2 ± 2.1% (mean ± S.E.; n = 3) with a maximum of 37.8% was observed, with only a low tendency (0.5 ± 0.2%; mean ± S.E.; n = 3) to form antiparallel β-sheets (Fig. 4C). In contrast, for 285–290A, a predominant β-sheet formation during 120 ns of the MD simulation (maximum content of 51.0%) was observed, with only an overall low tendency to form α-helices (3.6 ± 0.5%; n = 3) (Fig. 4D). These results suggest that trafficking of TGR5 from the ER to the PM requires the formation of a membrane-proximal α-helix.

**PM Localization and Function of Membrane-proximal C Termini with Glycine and Proline Substitution**—To further investigate the influence of the secondary structure content of the membrane-proximal TGR5 C terminus on PM localization and function of the receptor, substitution variants were generated where amino acids 285–290 and 291–297 were replaced by glycine (285–290G) and proline, which is a potenter helix breaker (41, 42), respectively. The 285–290P variant accumulated in the ER, whereas the 285–290G variant almost completely lost responsiveness towards TLC as measured by luciferase activity (Fig. 5B). Only when stimulated with 10 μM TLC did the 285–290P and 285–290G variants show a small but significant increase in luciferase activity by 1.5 ± 0.1-(n = 8) and 3.3 ± 0.3-fold (n = 8), respectively (Fig. 5B). Thus, the exchange of amino acids 291–297 to glycine (291–297G) and proline (291–297P) resulted in retention of the mutated proteins in the ER and abolished their responsiveness towards TLC stimulation (Fig. 5B).
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FIGURE 5. Analysis of TGR5 glycine and proline substitution variants in HEK293 cells. HEK293 cells were transiently transfected with the different TGR5 (pcDNA3.1+) glycine and proline substitution variants at aa 285–290 and 291–297. A, the TGR5 protein was detected with the anti-TGR5 antibody (RVR2, red). An antibody against Na+/K+ ATPase (green) was used to stain the plasma membrane. The variants 291–297G, 285–290P, and 291–297P were mainly localized in the ER, whereas the 285–290G variant was also present in the PM as demonstrated by the yellow coloring in the overlay image with the PM marker protein Na+/K+ ATPase (blue). Bars = 10 μm. B, receptor responsiveness towards TLC was measured using a cAMP-responsive luciferase assay. All four substitution variants showed a significantly reduced responsiveness towards TLC. Although stimulation with 10 μM TLC resulted in a 3.3 ± 0.3-fold increase in luciferase activity by the 285–290G variant, the variants 285–290P, 291–297G, and 291–297P caused an almost complete loss of receptor function. Results (285–290G, 285–290P, 291–297P n = 8; 291–297G n = 9; WT n = 43) are expressed as the mean ± S.E. * and # significantly different (p < 0.01) from DMSO (Control) and TGR5 WT, respectively.

Clusterling of MD-generated Conformations According to the Secondary Structure Sequence—To identify similarities and differences in the secondary structure of the TGR5 WT and the variants (Fig. 1) on a per-residue level, conformations of the last 500 ns of all trajectories were pooled and then hierarchically clustered according to their secondary structure sequence (Fig. 6A). Five main clusters were identified: conformations in cluster 1 mostly show an α-helical content at the beginning and the end of the peptides with a β-bend in between (Fig. 6B); conformations in cluster 2 show a short β-sheet at the beginning and the end of the peptides with a short α-helical stretch in between (Fig. 6C); conformations of clusters 3 and 4 show almost no defined secondary structure except for an α-helical turn of three amino acids length at the C-terminal end (cluster 4); finally, in cluster 5, most of the conformations are dominated by an α-helix formation over nine amino acids in the C-terminal region along with a turn at the N terminus (Fig. 6D). Forming a subcluster, conformations of variant 291–297P share the turn at the N terminus with the other members of cluster 5 but are unstructured in the C-terminal region (Fig. 6E).

Regarding the composition of the clusters in terms of conformations of the TGR5 variants, a distinct pattern is obvious that correlates with receptor PM localization and function (Table 1). The WT and 291–297A variants appear predominantly in clusters 1 and 5 (~60% and 20%, respectively); both variants also show the highest PM localization as determined by flow cytometry and the highest responsiveness towards TLC as measured by luciferase activity. Intermediate levels of PM localization and responsiveness towards TLC are found for the variants 285–290G, Δ285–290, and Δ291–297; these variants constitute predominantly cluster 3 (~38%), with conformations of 285–290G and Δ291–297 also found in clusters 1 and 5. In contrast, conformations of the 285–290A and 291–297G variants occur predominantly in cluster 2 (~76%); both variants show low PM localizations and an almost complete loss of responsiveness towards TLC. Variants 285–290P and 291–297P were similarly retained in the ER and were non-functional; they constitute predominantly cluster 4 (~30%), with conformations also found in cluster 1 and 5.

PM Localization and Function of TGR5β2AR, TGR5S1P1, and TGR5xOR Chimeras—To investigate whether indeed mere secondary structure content rather than a specific amino acid sequence determines PM localization and function, a chimeric receptor was constructed with the 13 membrane-proximal amino acids of the C terminus of the β2AR (aa 330–342) replacing the respective amino acids in TGR5 (aa 285–297). These β2AR residues were chosen because (i) they form an α-helix in the β2AR crystal structure (PDB code 3D4S) (43, 44) and (ii) the sequence identity between the exchanged amino acids from TGR5 and β2AR is 0%, whereas the sequence similarity is 33%.

In transiently transfected HEK293 cells the TGR5β2AR chimaera was localized in the PM, as demonstrated by the colocalization with the PM marker protein Na+/K+ ATPase as well as in the ER, as shown by the colocalization with the ER marker protein disulfide isomerase (Fig. 7, A and B). Using flow cytometry analysis, the percentage of transfected HEK293 cells with the chimeric receptor in the PM was calculated to be 87.4 ± 1.2% (n = 21), which is similar to the WT (91.3 ± 0.8%; n = 30) (Fig. 7C). MD simulations of the membrane-proximal 18 amino acids of the C terminus of the TGR5β2AR chimaera revealed an α-helix content of, on average, 24.2% (Table 2) with a maximum of 77.7%, which is higher than what has been found for the respective amino acids of the TGR5 WT. Hence, the most frequently occurring conformation of the TGR5β2AR chimaera is...
an α-helix (Fig. 8D). Functional analysis with 10 μM TLC showed a receptor function of the TGR5β2AR chimera that is similar to the one of WT. However, when TGR5β2AR was stimulated with lower TLC concentrations, the rise in luciferase activity was significantly lower than in the case of WT (Fig. 7D). Because the exchanged region of β2AR contains an FX3LL motif, previously identified as a sorting motif essential for the trafficking of the β2AR from the ER to the PM (45), chimera variants containing mutations in this motif were evaluated (Fig. 8A). Mutation of the phenylalanine 287 to tyrosine (F287Y)
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TABLE 1
Results of clustering according to secondary structure sequence, function, and protein localization of the TGR5 membrane-proximal C terminus

| Variant      | Cluster | Function | Membrane localization |
|--------------|---------|----------|-----------------------|
| TGR5WT       | 58      | 1 2 3 4 5 | 100.0 ± 7 90.9 ± 0     |
| 285-290A     | 8       | 8       | 1 2                   | 13.3 ± 1 52.8 ± 3     |
| 285-290G     | 57      | 1 4 5   | 0 0                   | 30.3 ± 3 69.7 ± 4     |
| 285-290P     | 86      | 1 6 7   | 3 4                   | 14.4 ± 1 55.0 ± 3     |
| Δ285-290     | 16      | 1 2     | 1 3                   | 36.8 ± 2 70.8 ± 3     |
| 291-297A     | 23      | 0 2     | 0 3                   | 174.1 ± 23 80.4 ± 3   |
| 291-297G     | 13      | 1 5     | 1 5                   | 11.9 ± 1 48.1 ± 4     |
| 291-297P     | 0       | 12      | 2 3                   | 10.5 ± 1 40.7 ± 4     |
| Δ291-297     | 6       | 38      | 25 31                 | 107.5 ± 6 70.3 ± 2    |

1 Percentage of the cluster distribution for each variant.
2 Function at 10 μM TLC as percent of wildtype ± S.E.
3 Percentage of cell membrane localization determined by FACS analysis ± S.E.

FIGURE 7. Analysis of TGR5β2AR chimera in HEK293 cells. HEK293 cells were transiently transfected with the TGR5β2AR pcDNA3.1– (A, B, and D) and FLAG–TGR5β2AR–YFP (C) chimera. A and B, TGR5 was stained with the anti-TGR5 (RVLRL2, red) antibody. The TGR5β2AR chimera was demonstrated in the PM as demonstrated by the colocalization with the PM marker Na+/K + ATPase (A, green) as well as in the ER, as shown by the colocalization with the ER marker protein disulfide isomerase (PDI) (B, green). Nuclei were stained with Hoechst (blue). Bars = 10 μm. C, quantification of PM localization of WT and TGR5β2AR chimera proteins as determined by flow cytometry of unpermeabilized HEK293 cells. The amount of FLAG–TGR5–YFP within the PM corresponds to the amount of FLAG tag-positive labeling (extracellular labeling) divided by the total amount of YFP fluorescence. 91.3% of the WT and 87.4% of the TGR5β2AR chimera were detected in the PM. Results (WT, n = 30; TGR5β2AR, n = 21) are expressed as mean ± S.E. D, TGR5 receptor activity was determined by the cAMP-responsive Luciferase assay. The TGR5β2AR chimera showed a similar rise in Luciferase activity in response to 10 μM TLC as the WT. However, at lower TLC concentrations (0.1, 0.5, 2.5 μM) the Luciferase activity was significantly reduced compared with the WT. Results (WT n = 29; TGR5β2AR n = 21) are expressed as mean ± S.E. * and # = significantly different (p < 0.001) from DMSO (Co) and WT, respectively.

TABLE 2
α-Helicity, β-sheet content, function, and protein localization of the TGR5β2AR mutant membrane-proximal C terminus

| Chimera variant | α-Helix1 | β-Sheet2 | Function3 | Membrane localization3 |
|-----------------|----------|----------|-----------|------------------------|
| hTGR5β2AR       | 24.2     | 0.0      | 100.0 ± 13| 87.4 ± 1               |
| F287Y           | 23.0     | 0.5      | 94.5 ± 15 | 86.8 ± 1               |
| L294V           | 10.4     | 0.2      | 125.5 ± 25| 88.0 ± 1               |
| L295V           | 4.5      | 0.6      | 86.1 ± 15 | 88.1 ± 1               |
| L294V/L295V     | 1.9      | 6.2      | 83.5 ± 15 | 84.2 ± 1               |
| F287Y/L294V/L295V| 11.2   | 0.3      | 123.9 ± 15| 89.2 ± 3               |
| L294A/L295A     | 0.8      | 4.1      | 85.8 ± 10 | 78.2 ± 1               |
| F287AL294A/L295A| 8.0     | 4.6      | 91.6 ± 5  | 74.2 ± 4               |
| F291AL294A/L295A| 3.9     | 1.6      | 91.6 ± 9  | 85.1 ± 3               |

1 Secondary structure content averaged over all residues throughout the MD simulations; in %.
2 Function at 10 μM TLC as percent of TGR5β2AR ± S.E.
3 Percentage of cell membrane localization determined by FACS analysis ± S.E.

The results of clustering according to secondary structure sequence, function, and protein localization (Table 1) revealed that the TGR5β2AR chimera showed a similar rise in Luciferase activity in response to 10 μM TLC as the WT. However, at lower TLC concentrations (0.1, 0.5, 2.5 μM), the Luciferase activity was significantly reduced compared with the WT. Results (WT n = 29; TGR5β2AR n = 21) are expressed as mean ± S.E. * and # = significantly different (p < 0.001) from DMSO (Co) and WT, respectively.

The results of clustering according to secondary structure sequence, function, and protein localization (Table 2) showed that the TGR5β2AR chimera demonstrated a high α-helical content (4.6–8.0%) for the F287Y/L294V/L295V chimera that showed PM localization levels and functional responsiveness towards 10 μM TLC indistinguishable from the WT. However, at lower TLC concentrations (0.1, 0.5, 2.5 μM), the Luciferase activity was significantly reduced compared with the WT. Results (WT n = 29; TGR5β2AR n = 21) are expressed as mean ± S.E. * and # = significantly different (p < 0.001) from DMSO (Co) and WT, respectively.

Exchange of both leucines to valine (L294V/L295V) or alanine (L294A/L295A) reduced plasma membrane localization to 84.2 ± 1.1% (n = 23) and 79.7 ± 2.0% (n = 16), respectively (Fig. 8C; Table 2). Mutation of the phenylalanine and the leucine residues in the FX6LL motif to alanine (A6LL) resulted in a PM localization of 86.8 ± 1.1% (n = 19), which was significantly less than the membrane content of TGR5 WT (91.3 ± 0.8%; n = 30) but was indistinguishable to the cell surface levels of the TGR5β2AR chimera (87.4 ± 1.2%; n = 21) (Fig. 8B). This is supported by the MD simulation of the F287Y variant showing α-helix (23.0%) and β-sheet (0.5%) contents almost identical to the ones of TGR5β2AR. Mutation of either leucine 294 or 295 to valine (L294V or L295V) did not affect PM localization or Luciferase activity after stimulation with 10 μM TLC. Mutation of the FX6LL motif to YX6VV resulted in a TGR5β2AR chimera (F287Y/L294V/L295V) chimera that showed PM localization levels and functional responsiveness towards 10 μM TLC indistinguishable from the WT and the TGR5β2AR chimera (Table 2). MD simulations demonstrate α-helix contents between 4.5 and 11.2% for the F287Y/L294V/L295V, L294V, and L295V variants but a low β-sheet content (< 0.6%) as well.

Visual inspection of the conformations of the L294A/L295A variant with high β-sheet content revealed that the β-sheet is stabilized by a hydrophobic contact between phenylalanine 291 and alanine 295. This suggested the replacement of phenylalanine 291 with alanine to break this contact. Accordingly, MD simulations predicted a low α-helical stretch (Fig. 8F). Thus, we expected that with this variant PM localization and functional activity will be restored. Indeed, the F291A/L294A/L295A variant showed a PM localization level and Luciferase activity in
To further support the hypothesis that the secondary structure formation of the membrane-proximal C terminus is essential for TGR5 ER to PM trafficking, we generated two additional receptor chimeras in which 12 or 10 amino acids of TGR5 (aa 285–296, aa 285–294) were replaced by membrane-proximal amino acids of the OR isoform-1 (aa 335–346) or the S1P1 (aa 316–325), respectively. These OR and S1P1 residues were chosen because (i) they form an α-helix in the crystal structures (PDB codes 4DJH and 3V2Y, respectively) (46, 47), (ii) the sequence identity (similarity) between the exchanged amino acids from TGR5 and those from OR is 8% (33%) and those from S1P1 is 0% (30%), and (iii) they lack the FX6LL sorting motif.

In transiently transfected HEK293 cells the TGR5OR and TGR5S1P1 chimeras were sorted to the PM as shown by the colocalization with the PM marker protein Na+/K+ ATPase (Fig. 9, A and B). Using flow cytometry analysis, the percentage of transfected HEK293 cells with chimeric TGR5OR and TGR5S1P1 receptors in the PM were 91.1 ± 1.5% (n = 15) and 86.3 ± 1.7% (n = 10), respectively (Fig. 9C). Functional analysis with 10 μM TLC showed a receptor function of the TGR5OR and TGR5S1P1 chimeras that was similar to the one of TGR5 WT. However, when TGR5OR was stimulated with lower TLC concentrations, the rise in luciferase activity was significantly lower than in the case of TGR5 WT (Fig. 9D). In contrast, luciferase activity of the TGR5S1P1 chimera was indistinguishable from the WT when stimulated with 0.1–10 μM of TLC (Fig. 9E). Taken together, these results confirm the hypothesis that an α-helical structure in the proximal part of the C terminus is required for TGR5 PM localization and responsiveness toward extracellular ligands.

DISCUSSION

The molecular mechanisms involved in the trafficking of GPCRs from the ER to the PM are incompletely understood (24). Furthermore, the determinants required for ER exit seem to vary between different GPCRs (24). A naturally occurring truncation variant in the TGR5 C terminus (Q296X) led to ER

FIGURE 8. Effect of mutations in the FX6LL motif on the localization, function, and structure of TGR5β2AR chimera. A, sequence alignment of the membrane-proximal C terminus regions of TGR5 WT, β2AR, and TGR5β2AR chimera. Mutations of the FX6LL motif within the TGR5β2AR chimera are marked in red. B and C, localization of the mutated TGR5β2AR chimeras in transiently transfected HEK293 cells was revealed by detecting TGR5 with the RVLR2 antibody (red). Conservative amino acid mutations (Phe to Tyr and Leu to Val) have only minor effects on receptor plasma membrane localization. However, substitution of Leu to Ala in L294A/L295A and Phe to Ala as well as Leu to Ala in F287A/L294A/L295A impaired trafficking of the mutated proteins, which can be detected in both the ER as well as the plasma membrane of transfected cells. Nuclei were stained with Hoechst (blue). Bars = 10 μm. D, most frequently occurring conformation of the TGR5β2AR chimera C terminus as found by MD simulations. E, most frequently occurring conformation of the C terminus of the L294A/L295A variant as found by MD simulations. F, most frequently occurring conformation of the C terminus of the F291A/L294A/L295A mutant. D–F, the coloring indicates the sequence from the N terminus (blue) to the C terminus (red).
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FIGURE 9. Analysis of TGR5xOR and TGR5S1P1 chimeras in HEK293 cells. HEK293 cells were transiently transfected with the TGR5xOR pcDNA3.1+ and TGR5S1P1 pcDNA3.1+ chimeras (A, B, D, E) or with the FLAG-TGR5xOR-YFP and FLAG-TGR5S1P1-YFP chimeras (C). A and B, the TGR5 chimeric proteins were detected with the anti-TGR5 antibody (RVLR2, red). An antibody against Na+/K+ ATPase (green) was used to stain the PM. Both chimeras TGR5xOR (A) and TGR5S1P1 (B) were localized in the PM as demonstrated by the yellow coloring in the overlay image with the PM marker protein Na+/K+ ATPase. C, the PM localization of WT and TGR5 chimeras was quantified as described under "Experimental Procedures" using flow cytometry. TGR5xOR and TGR5S1P1 were detected on the cell surface in 91.1 and 86.3% of the transfected cells, which was similar to the TGR5 WT with 91.3% (WT, n = 30; TGR5xOR, n = 15; TGR5S1P1, n = 10). D and E, receptor responsiveness towards TLC was measured using a cAMP-responsive luciferase assay. Although the TGR5xOR chimeras showed a significantly reduced responsiveness toward lower TLC concentrations (0.1, 0.5, 2.5 μM) and no difference at 10 μM TLC compared with the WT (D, WT n = 8; TGR5xOR n = 7), the TGR5S1P1 chimera showed luciferase activity indistinguishable from the WT (E, WT n = 6; TGR5S1P1 n = 8). Results are expressed as mean ± S.E. * #, significantly different (p < 0.01) from DMSO (Co) and TGR5 WT, respectively.

retention and abolished ligand responsiveness (20), indicating that the C terminus of TGR5 is required for PM localization and function of this bile acid receptor. Using different substitution and deletion variants within the TGR5 C terminus, the present study demonstrates that the secondary structure of the membrane-proximal amino acids 285–297 has a strong influence on ER to PM trafficking of the TGR5 receptor.

Employing MD simulations that are 3-fold longer than the time required for α-helix formation as determined from experiments (48–50), we showed that the wild type peptide encompassing residues 285–297 preferentially forms an α-helix. In contrast, the 285–290A variant, which was retained in the ER, showed an exclusive β-sheet formation within the first 120 ns of the simulation. This finding was unexpected because alanine has a high helix propensity (40). However, the β-sheet formation seemed to be favored in this case by interactions between the alanine residues in positions 285–290 with naturally occurring hydrophobic alamines in positions 294–296. These initial analyses suggested that a high β-sheet content in the membrane-proximal C terminus prevents ER to PM trafficking of TGR5. In contrast, formation of an α-helix, located in TGR5 at a position equivalent to helix 8 in crystallographically determined GPCR structures (44, 51, 52), facilitates it.

To further support our findings, the localization, functional, and MD simulation analyses were extended to additional mutation and deletion variants in the C terminus of TGR5. As the most outstanding result, a clear correlation between the secondary structure sequence of the respective peptide and the localization of TGR5 and, thus, its function emerged. As such, the variant 291–297A most strongly resembles the WT with respect to the secondary structure sequence, as determined by cluster analysis of all MD-generated conformations; both preferentially occur in clusters 1 and 5 (Table 1). 291–297A is also correctly sorted to the PM and shows a functional activity that is even larger than that of the WT when stimulated with 10 μM TLC. As a possible explanation for the increased activity, ~1/3 of the conformations of 291–297A occur in cluster 5, which largely contains conformations of other highly active variants, too. This suggests that the 291–297A variant has a particularly high probability to form a favorable conformation. Likewise, about 1/3 of all generated conformations of the Δ291–297 variant, which shows a similarly high PM localization and functional activity as 291–297A, are found in cluster 5. The conformations in clusters 1 and 5 are dominated by long α-helical stretches at the C termini of the peptides.

A second major fraction of the Δ291–297 conformations is found in cluster 3; conformations in cluster 3 are largely unstructured. Likewise, major fractions of the conformations of the Δ285–290 and 285–290G variants are found in this cluster, with other major fractions found in cluster 1. This reduced conformational preference to form an α-helix of the Δ285–290 and 285–290G variants goes along with experimental findings of a reduced PM localization and functional activity.

Finally, of the least functional variants that also showed the highest retention in the ER, 291–297G mostly strongly resembles the variant 285–290A in terms of the formation of a β-sheet (appearance of the major fraction of the conformations in cluster 2). In contrast, a major fraction of the conformations of the variant 291–297P show a short stretch of α-helix in the C-terminal region (cluster 4), which is apparently not sufficient to lead to ER to PM trafficking. To our surprise, the largest fraction of the conformations of the 291–297P variant was found in cluster 5. Yet these conformations form a subcluster there and are characterized by an N-terminal TTTS motif but lack the long α-helical stretch at the C terminus, in agreement with experimental findings that the 291–297P variant is largely retained in the ER. As to the last variant, most of the conformations of 285–290P are found in cluster 4, too, again in agreement with the findings that this variant is retained in the ER. However, another major fraction is found in cluster 1, which would lead to the false prediction that the 285–290P variant will be sorted to the PM. In that respect, 285–290P is the sole variant investigated that does not fit to the relationship between the secondary structure sequence of a respective peptide and the localization of TGR5.

Taken together, these results reveal that PM localization and, thus, function of the receptor is fostered by a long (~9 residues)
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α-helical stretch at the C terminus of the variants, whereas the presence of β-strands or only a short α-helical stretch leads to ER retention and a loss of function.

As a proof-of-principle, a chimera of TGR5 containing the membrane-proximal amino acids of the β2AR was generated. The respective amino acid sequence of β2AR forms an α-helix as shown in high resolution crystal structures (43, 44). MD simulations of the membrane-proximal 18 amino acids of the C terminus of the TGR5β2AR chimera, which contains 13 amino acids of the membrane-proximal C terminus of β2AR, reproduced this α-helix character (Fig. 8D), which demonstrates the quality of the setup of our simulations. We presumed that the TGR5β2AR chimera would be mainly localized in the PM due to the α-helical structure of its C terminus. The TGR5β2AR chimera was indeed correctly sorted to the PM and showed similar functional activity in response to 10 μM TLC as WT TGR5.

However, the membrane-proximal part of the β2AR contains an FX6LL motif that has previously been identified as an important GPCR ER export motif facilitating interaction of the β2AR with the GTPase Rab1, that way promoting ER to PM trafficking (45, 53). To assess whether the FX6LL motif promotes membrane trafficking of the TGR5β2AR chimeric receptor, too, we evaluated chimera variants containing mutations in this motif. This again revealed a strong correlation between the secondary structure content of the respective variant and its localization and function. First, the conservative replacement of phenylalanine by tyrosine in the F287Y variant does not change the α-helical content with respect to the WT; in agreement, this variant also showed a high level of membrane localization and retained functional activity. Second, variants with conservative mutations at positions 294 and 295 revealed a reduced α-helical content compared with the WT and negligible β-sheet content (F287Y/L294V/L295V, L294V, and L295V) and showed intermediate levels of membrane localization and functional activity. Third, the alanine mutants F287A/L294A/L295A and L294A/L295A and the valine double mutant L294V/L295V revealed the lowest α-helical contents but pronounced β-sheet formation. As in the case of the TGR5 variants, these chimera variants showed marked retention in the ER. Finally, we created the F291A/L294A/L295A variant guided by insights from structural analysis and MD simulations that this variant has a lower β-sheet and a higher α-helical content. The variant indeed showed PM localization level and luciferase activity in response to 10 μM TLC indistinguishable from the TGR5β2AR chimera.

Two additional chimeras in which 12 or 10 amino acids of TGR5 were replaced by membrane-proximal amino acids of κOR or S1P1, respectively, showed PM localization and functional activity very similar to the TGR5 WT and the TGR5β2AR chimera. Although there is only a very low or even no sequence identity between the exchanged residues, respectively, the κOR or S1P1 residues do form an α-helix in the respective crystal structures (46, 47).

In summary, these results demonstrate that PM trafficking and, thus, function of the TGR5 chimera are determined by the secondary structure of the membrane-proximal C terminus. As found for variants of TGR5 itself, a high α-helix content of this region fosters PM localization, whereas a high β-sheet content affects PM localization adversely. Furthermore, the modifications of the FX6LL motif in the TGR5β2AR chimera demonstrated that trafficking to the PM occurs as along as the modifications retain a high α-helix content in the membrane-proximal C terminus. Using the PDBMotif webservice (54) the frequency of occurrence of the FX6LL motif in secondary structures was analyzed in all non-redundant proteins within the Protein Data Bank. This revealed that the FX6LL motif occurs in helices in 71% of the cases, whereas it is found in sheets and loops in 5 and 24% of cases, respectively. Taken together our results suggest that the FX6LL motif itself has an important role for α-helix formation but not as a sequence-specific sorting motif in the TGR5β2AR chimera.

Similar findings have been described for the human cannabinoid receptor 1 (27, 55, 56). Using overexpression, purification, and circular dichroism spectroscopy analysis of the full-length CB1 C terminus as well as different substitution variants, it was demonstrated that it is the level of hydrophobicity rather than specific amino acids that is critical for helix formation and, thus, targeting to the PM of this GPCR (27, 55, 56). Another example that hydrophobic amino acid residues in the membrane-proximal C terminus allowing α-helix formation are essential for ER exit and PM localization has been described for the human vasopressin II receptor (28). However, in contrast to our finding with the TGR5β2AR chimera, replacement of the C-terminal tail of the vasopressin II receptor with amino acids 327–413 of the human β2AR resulted in a chimera that failed to reach the PM (22). It is unclear whether this discrepancy is dependent on special amino acid residues in the membrane proximal C terminus or on the different cloning strategies for the chimera. In the TGR5β2AR chimera, 13 amino acids (aa 330–342) of the cytoplasmic C-tail of the β2AR replaced the respective 13 amino acids of TGR5 C terminus (aa 285–297). In contrast, in the vasopressin 2 receptor β2AR chimera, the complete C-terminal tail of the β2AR (aa 327–413) was fused to alanine 325 of the human vasopressin II receptor, which is predicted to be part of transmembrane domain 7 (22).

Although an important role of the C terminus anterograde trafficking from the ER to the PM has been described for many different GPCRs, it remained unclear whether specific amino acid signals or, rather, a certain conformation are essential for successful ER export (24). Most likely the structural or amino acid sequence determinants required for correct sorting to the PM vary for different GPCRs. For TGR5, which lacks any of the previously described ER exit motifs (di-acidic motif, FX6LL or EX6LL motifs) (24–26, 39), our study demonstrates that the secondary structure of the membrane-proximal C terminus, which forms an α-helix according to the MD simulations, is essential for PM localization and, thus, function of this bile acid receptor.

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