Biased and Constitutive Signaling in the CC-chemokine Receptor CCR5 by Manipulating the Interface between Transmembrane Helices 6 and 7*

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Background: Information about the structure-function relationship and activation mechanism of 7TM receptors is needed.

Results: Single mutations in CCR5 induce biased signaling with increased activation through Ga13 but decreased β-arrestin recruitment.

Conclusion: The TM6/7 interface controls the G protein-dependent and -independent activity state of CCR5.

Significance: Knowledge about specific 7TM receptor regions targeted by pathway-selective (biased) ligands is vital for future drug design.

The equilibrium state of CCR5 is manipulated here toward either activation or inactivation by introduction of single amino acid substitutions in the transmembrane domains (TMs) 6 and 7. Insertion of a steric hindrance mutation in the center of TM7 (G286F in position VII:09/7.42) resulted in biased signaling. Thus, β-arrestin recruitment was eliminated, whereas constitutive activity was observed in Ga13-mediated signaling. Furthermore, the CCR5 antagonist aplaviroc was converted to a full agonist (a so-called efficacy switch). Computational modeling revealed that the position of the 7TM receptor-conserved Trp in TM6 (Trp-248 in position VI:13/6.48, part of the CWXP motif) was influenced by the G286F mutation, causing Trp-248 to change orientation away from TM7. The essential role of Trp-248 in CCR5 activation was supported by complete inactivity of W248A-CCR5 despite maintaining chemokine binding. Furthermore, replacing Trp-248 with a smaller aromatic amino acid (Tyr/Phe) impaired the β-arrestin recruitment, yet with maintained G protein activity (biased signaling); also, here aplaviroc switched to a full agonist. Thus, the altered positioning of Trp-248, induced by G286F, led to a constraint of G protein signaling (NMR) spectroscopy in 2012 (12).

Chemokine receptors are critical mediators of leukocyte trafficking and thereby regulation and development of the immune system. They belong to class A of the seven transmembrane spanning (7TM) receptors, which are the largest family of membrane proteins in the human genome and are activated by highly diverse ligands (1). In accordance with the large variety of endogenous agonists, 7TM receptors are involved in regulating most aspects of normal physiology and pathophysiology. Consequently, they have enormous potential as drug targets, and studying their activation process is of great importance. Despite the chemical diversity among endogenous ligands, it is generally believed that all 7TM receptors share the overall same activation mechanism (2). Recently, crystal structures of fully activated β2-adrenergic receptors (receptors in complex with both agonist and G protein) were published (3, 4). Compared with the crystal structures of inactive receptors (5–11), the most pronounced structural changes are at the cytosolic face. Here, transmembrane helix 5 (TM5) and TM6 move outward away from the center of the receptor, whereas TM3 and TM7 move slightly inward (3, 9, 10). Three-dimensional structures of inactive chemokine receptors have also been published; that is, crystal structures of CXCR4 in complex with a peptide and a small-molecule compound as the first in 2010 (11) followed by a structure of CXCR1 determined by nuclear magnetic resonance (NMR) spectroscopy in 2012 (12).

Even though we have insights into the overall movements during receptor activation, the details of the structural events have not been clearly established. Importantly, the difference in receptor structure depending on which effector molecule is bound (e.g. G protein versus β-arrestin) has gained a lot of attention recently. Ligands that bind to a receptor and can elicit different responses in different pathways (termed “biased signaling” or “functional selectivity”) have been recognized for their therapeutic potential (13). Thus, selective targeting of signaling events that contribute to disease while preserving other functions would reduce the negative effects of some therapeu-

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2 The abbreviations used are: 7TM, 7-transmembrane helix; TM, transmembrane domain; PI, phosphatidylinositol.
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etics. Ever since the chemokine receptor CCR5 was discovered for its pivotal role in HIV entry into host cells (14, 15), several CCR5-targeting compounds have been developed as anti-HIV treatment. Among these, biased ligands have been described; one of the first was an N-terminal-modified form of the endogenous ligand RANTES (or CCL5), AOP-RANTES (16). AOP-RANTES was shown to cause CCR5 internalization in peripheral blood mononuclear cells (17) but was incapable of inducing chemotaxis of monocytes (16). Another N-terminal-modified form of CCL5 (5P14-RANTES) induced significant amounts of CCR5 internalization in activated CD4⁺ T cells but no G protein-mediated signaling, assessed in HeLa cells (18). Both ligands effectively inhibited HIV entry, very likely because of their ability to sequester CCR5 inside the cell. Meanwhile, the lack of G protein-mediated signaling could decrease the risk of unwanted effects (for review, see Ref. 19).

Biased signaling has also been described in several other 7TM receptors, e.g. CXCR7 (20), the herpesvirus-encoded CXC-chemokine receptor ECRF3 (21), the β₂-adrenergic receptor (22), and the nicotinic receptor GPR109A (23).

Here we describe how manipulation of the interface between TM6 and TM7 in CCR5 can be used to determine receptor areas important for G protein signaling relative to β-arrestin recruitment. Whereas most of the single amino acid mutations shift the equilibrium toward a more active state of CCR5 in G protein-coupled signaling (i.e. increase in basal activity and “efficacy switch” of an antagonist to agonist), β-arrestin binding is impaired. Computational modeling of CCR5 showed that a likely mechanism could be an interaction between two residues; one in TM7 (Gly-286, VII:09/7.42, which was mutated to a Phe) and one in TM6 (Trp-248, VI:13/6.48). Together these results show that the interface of TM6 and TM7 is very important for the activation state of CCR5 and that compounds targeting this area very likely could have biased properties. The results also show that small local alterations can lead to large overall changes, i.e. biased and constitutive signaling, without alteration in ligand binding.

EXPERIMENTAL PROCEDURES

Materials—The human chemokines CCL3 and CCL5 were purchased from Peprotech. The human CCR5 WT cDNA was cloned from a spleen-derived cDNA library. The small molecule CCR5 antagonists Merck, SCH-C, TAK-779, and aplaviroc were kindly provided by Gary Bridger (AnoMED, Langley, British Columbia, Canada). Iodinated CCL3 was purchased from PerkinElmer Life Sciences. The promiscuous chimeric G protein Gq₆₅₄myr (Gq₄₄myr), which converts Goq signal into a Gq₆₅ signal, making it possible to measure the chemokine receptor activation as PI-turnover (24, 25). One day after transfection, the cells were seeded in 24-well plates (1.5 × 10⁵ cells/well) and incubated with 2 μCi of myo-[³²P]inositol in 0.3 ml of growth medium for 24 h. Cells were washed twice with Hanks’ buffered salt solution supplemented with CaCl₂ and MgCl₂ and afterward incubated for 15 min in 0.3 ml of buffer supplemented with 10 mM LiCl before ligand addition followed by 90 min of incubation. When used, the antagonists were added 10 min before the agonist. The generated [³²P]inositol phosphates were purified on AG 1×8 anion exchange resin. Determinations were made in duplicate.

cAMP Assay—CCS7 cells (3.5 × 10⁴ cells/well) were seeded in 96-well plates 1 day before transfection with receptor DNA. Two days after transfection the cells were washed twice with Heps buffered saline (HBS) buffer and incubated with HBS and 1 mM 3-isobutyl-1-methylxanthine for 30 min at 37 °C. Forskolin (Sigma) was added in various concentrations, and the cells were incubated for 30 min at 37 °C. The HitHunter™ cAMP XS+ assay (DiscoveRx) was carried out according to the manufacturer’s instructions. Determinations were made in triplicate.

β-Arrestin Recruitment Assay—PathHunter U2OS β-arrestin cells were seeded in 96-well plates (2.0 × 10⁴ cells/well). Overnight transient transfection with PK1-tagged receptor DNA was started the following day and stopped a day before the assay. PathHunter™ β-arrestin GPCR assay (DiscoveRx) were carried out according to the manufacturer’s instructions.

125I-CCL3 Competition Binding Assay—CCS7 cells were seeded in wells 1 day after transfection with receptor DNA with the number of cells seeded per well aimed at obtaining 5–10% specific binding of the added radioactive ligand (3–15 × 10⁵ cells/well for the different CCR5 constructs). Two days after transfection, cells were assayed by competition binding for 3 h at 4 °C using 20–70 pm 125I-CCL3 as well as unlabeled ligand in 50 mM Hepes buffer, pH 7.4, supplemented with 1 mM CaCl₂, 5
mm MgCl₂, and 0.5% (w/v) bovine serum albumin (BSA). After incubation, cells were washed twice in ice-cold binding buffer supplemented with 0.5 M NaCl. Nonspecific binding was determined as the binding in the presence of 0.1 μM unlabeled CCL3. Determinations were made in duplicate.

Cell Surface Expression Measurement (ELISA)—COS-7 cells were transfected with FLAG-tagged (M1) receptor DNA in 96-well plates (3.5 × 10⁴ cells/well). Two days after transfection, cells were washed 3 times in Tris-buffered saline (TBS), fixed in 3.7% formaldehyde for 15 min at room temperature, washed, and incubated in blocking solution (TBS supplemented with 2% BSA) for 30 min. Cells were kept at room temperature for subsequent steps. Cells were incubated for 2 h with anti-FLAG (M1) antibody (Sigma) at 2 μg/ml in TBS with 1 mm CaCl₂ and 1% BSA. After 3 washes with TBS/CaCl₂/BSA, the cells were incubated with goat anti-mouse horseradish peroxidase-conjugated antibody (Abcam, Cambridge, UK) at 1:1000 dilution. After extensive washing, the immunoreactivity was revealed by the addition of TMB Plus substrate (Kem-En-Tec, Taastrup, Denmark), and the reaction was stopped with 0.2 M H₂SO₄. Absorbance was measured at 450 nm on a Wallac Victor2 platerreader (PerkinElmer Life Sciences).

Calculations—IC₅₀, E₅₀, and Kᵢ values were determined by nonlinear regression, and Bₘₐₓ max values were calculated using the GraphPad Prism 4.0 software (GraphPad, San Diego, CA).

CCR5 Comparative Models and Docking of Aplaviroc—A pair-wise sequence alignment and the construction of a comparative homology model of the human CCR5 receptor was produced in the Internal Coordinate Mechanics software package (Molsoft LLC, La Jolla, CA) using the human CXCR4 (11) (PDB entry 3ODU) receptor as the structural template. In brief, manual adjustment of the alignment was necessary to ensure proper alignment of the loop regions and eliminate gaps in the TM regions. During the model construction, a disulfide bridge between Cys-101 (III:01/3.25) and Cys-197 (in ECL2) together with a disulfide bridge between Cys-20 (in the N terminus) and Cys-269 (in ECL3) was applied as a structural constraint. The developed CCR5 models were subjected to full-atom structure relaxation using the ROSETTA membrane force field (28) in Rosetta 3.2.1 (29). A total of 100 models were generated, and a set of 10 representative low energy CCR5 receptor models was selected as a receptor ensemble for the subsequent docking of aplaviroc. Full flexible ligand docking was performed using the biased probability Monte Carlo docking routine in ICM under softened van der Waals conditions using 4D grids represented by six grid potentials of 0.5 Å spacing, including three van der Waals grid potentials for a carbon probe, large atom probe, or hydrogen probe, a hydrogen bonding grid potential, an electrostatic grid potential, and a hydrophobic grid potential ICM (30, 31). The docking grids were defined to encompass a binding pocket described by all corresponding receptor residues within 4.5 Å of the IT1t ligand (11) in the CXCR4 template structure when superimposed onto the stack of generated CCR5 models. The final docking grid was extended ~10 Å toward TM5 to allow aplaviroc to interact with pocket formed between TM3, -4, -5, and -6. Individual best scored docking poses were subsequently optimized using a combined Monte Carlo and minimization procedure (using the MMFF94 force field), keeping ligand and surrounding protein residues (in an 8 Å radius from the starting position) flexible. All backbone coordinates were held fixed. Two rounds of optimization were performed: an initial refinement under a softened van der Waals potential and a second refinement with the full van der Waals potential. A final stack of 50 conformations was generated that was saved and manually analyzed to identify the complexes between aplaviroc and CCR5.

Conformational Sampling and Statistics of Side Chain Rotamer States—In brief, the G286F-CCR5 receptor variant was constructed from the initial CCR5 WT model using the residue substitution function in the Rosetta 3.2.1 (29). CCR5 WT as well as G286F-CCR5 was subjected to full-atom structure relaxation using the ROSETTA membrane force field (28) in Rosetta 3.2.1 (29) to optimize the structures and repack the side chain packing. A total of 1000 models were generated of both CCR5 WT and G286F-CCR5. Statistics on side chain rotamer states for both receptors were analyzed using customized scripts and function in the CCP4 software package (32).

RESULTS

Regulation of Basal Receptor Activity by a Space-filling Substitution in the Center of TM7—Approximately half of the amino acids at position VII:09/7.42 in class A 7TM receptors are small, whereas this number is much higher among chemokine receptors (89%) (Fig. 1). In CCR5, a Gly (Gly-286) occupies position VII:09. To explore the role of this small amino acid, Gly-286 was mutated to a Phe, G286F (i.e. introduction of a
stERIC hindrance). The mutant was tested in competition binding with 125I-CCL3 and in G protein-mediated signaling assays; that is, inhibition of forskolin-induced cAMP accumulation and PI-turnover (phosphatidylinositol) measurements, where co-transfection with the chimeric G protein Gaq\textsubscript{emy} ensures transmission of a Ga\textsubscript{q}-coupled receptor activity into a Ga\textsubscript{q} readout (24, 25). The level of cAMP accumulation in G286F-CCR5 induced by forskolin was decreased to 20% that of the WT level, indicating constitutive activity via Ga\textsubscript{q}. In contrast, no constitutive activity was observed in CCR5 WT (Fig. 2A). Moreover, G286F displayed a 30-fold increase in basal activity in PI-turnover (Fig. 2B and Table 1). As in cAMP accumulation, virtually no agonist-independent (i.e. constitutive) PI-turnover was observed for CCR5 WT. Importantly, the enhanced basal signaling was not a consequence of enhanced receptor surface expression, as the expression of G286F, determined by ELISA against an N-terminal M1 tag, was ~50% decreased compared with WT (Fig. 2C). The potency of CCL3 was basically unaltered, and the potency of CCL5 was decreased 7-fold compared with WT (Table 1). Despite unaltered CCL3 potency, the affinity determined by homologous competition binding experiments was increased 3.5-fold compared with WT (Table 2). Thus, the presence of a large aromatic side chain in VII:09/7.42 in CCR5 induces constitutive signaling via Ga\textsubscript{q}.

The Constitutively Active G286F Promotes Efficacy Switch of Aplaviroc—Due to the agonist-prone nature of G286F, we analyzed its influence on binding and action of four small-molecule antagonists: SCH-C, TAK-779, aplaviroc, and a ligand structurally similar to compounds previously described by Merck (33) (Fig. 3). All four have previously been reported to inhibit CCR5 activation and HIV cell-entry via CCR5 with nanomolar potencies (33–37). Consistently, they all completely inhibited CCL3-induced PI-turnover in CCR5 WT with EC\textsubscript{50} values between 41 and 104 nm and displayed no intrinsic activities (Fig. 3, A and B, and Table 3). The potencies were matched by similar affinities as determined in heterologous competition binding experiments against 125I-CCL3 (Table 2). In contrast to the complete inhibition in CCR5 WT, the CCL3 activity was only partially inhibited in G286F by SCH-C, TAK-779, and the Merck-derived compound, which acted with EC\textsubscript{50} values in the same range as on WT (SCH-C) or slightly increased (Merck and TAK-779) (Fig. 3C, Table 3). Aplaviroc, however, did not antagonize CCL3 at all, but instead potentiated it with a potency drastically increased compared with its antagonistic counterpart on CCR5 WT (~30-fold increase, Table 3 and Fig. 3C). The affinities of the antagonists did not differ more than 3.5-fold from WT, and intriguingly, despite its radically different efficacy profile, the affinity of aplaviroc was basically unaltered (Table 2). The partial inhibition by SCH-C, TAK-779, and the Merck-derived compound as well as the CCL3-potentiation by aplaviroc prompted us to determine their effects alone. Interestingly, none of the antagonists acted as inverse agonists on G286F (Fig. 3D). Instead, aplaviroc activated the receptor with the same efficacy as CCL3, i.e. it was converted from a full antagonist on WT to a full agonist on this mutant (Fig. 3D). The potency of aplaviroc when acting solo was increased ~3-fold compared with its (antagonistic) potency on WT (Table 3).

Molecular Modeling of CCR5 WT and G286F-CCR5—To further explore the mechanism behind the increased basal activity and efficacy switch on G286F-CCR5, docking of aplaviroc in CCR5 WT was performed (Fig. 4, A and C, and Fig. 5). Aplaviroc was subsequently introduced (Fig. 4, B and D). The docking of aplaviroc suggested three different poses all in contact with residues shown to be important based on previous mutational mapping (38). One was selected, but the other poses were equally likely. We focused on the dynamics of aromatic amino acids in the vicinity of position 286: three in TM6 (Tyr-244 (VI:09/6.44), Trp-248 (VI:13/6.48), and Tyr-251 (VI:16/6.51)) and one in TM3 (Phe-112 (III:12/3.36)). To create an overview of potential discrepancies in the positions of these amino acids between WT and G286F, the χ₁ angle was computed in the

![FIGURE 2. Activation of G286F in G protein-coupled signaling.](image-url)

The data are normalized to the level obtained in untransfected cells (A) or E\textsubscript{max} of CCL3 activation on CCR5 WT and G286F (B). The surface expression of the receptors was estimated in ELISA in COS-7 cells using an N-terminal FLAG tag, and the data are normalized to CCR5 WT (C).
TABLE 2
Affinity of CCL3 and four small-molecule antagonists on CCR5 WT and mutants.

| Residue | CCR5 WT | CCL5 BIASED (1000 models) | SCH-C | Merck | TAK-779 |
|---------|---------|--------------------------|-------|-------|---------|
|        | $K_d$ (nM) | $K_i$ (nM) | $K_d$ (nM) | $K_i$ (nM) | $K_d$ (nM) | $K_i$ (nM) |
|        | log($K_d$) | log($K_i$) | log($K_d$) | log($K_i$) | log($K_d$) | log($K_i$) |
| Trp-248 | 89.7 ± 1.2 | 5.0 | 89.7 ± 1.2 | 5.0 | 89.7 ± 1.2 | 5.0 |
|        | 89.7 ± 1.2 | 5.0 | 89.7 ± 1.2 | 5.0 | 89.7 ± 1.2 | 5.0 |
|        | ND | ND | ND | ND | ND | ND |

1000 models from the Rosetta protocol, and the numerical value was categorized into either of two preferred rotamer states, $g^+$ or $trans$ (the performed normalization was not more than 35 degrees in any case). Fig. 4C illustrates the position of the side chains of the five amino acids in CCR5 WT, with the corresponding distribution of the $\chi$ angles in this model illustrated in Fig. 4E. In CCR5 WT only the side chain of Phe-112 varied slightly between the models. In G286F, the distribution of the $\chi$ angles of Tyr-244, Tyr-251, and Phe-112 did not differ from CCR5 WT. However, the rotamer position of Trp-248 was changed in 10% of the models (Fig. 4, D and F). Thus, in CCR5 WT and 90% of the models of G286F, the side chain of Trp-248 was oriented toward TM7, corresponding to $g^+$ (shown in magenta). However, in the 10% models of the G286F model, where the side chain of Trp-248 was rotated to $trans$ (shown in orange), the residue pointed toward TM5.

**Trp-248 in the CWXP Motif in TM6 Is Important for CCR5 Signaling**—The computational modeling of G286F-CCR5 suggested that the side chain of Trp-248 changes to an alternative rotamer state in 10% of the models (Fig. 4). During the history of 7TM receptor activation much focus has been put on this conserved position (39–41). Therefore, Trp-248 was mutated to Ala, which resulted in complete elimination of chemokine-induced activity (Fig. 5, A and B, and Table 1). However, CCL3 binding was still maintained (Table 2) despite a low cell surface expression (Table 1).

To further determine the role of the particular characteristics of Trp as opposed to those of aromatic amino acids in general, Trp-248 was substituted with Phe and Tyr (W248F/Y). This resulted in WT-like efficacy and potency of CCL3-induced PI-turnover and no constitutive activity, whereas the potency of CCL5 was increased up to 3.5-fold (Table 1 and Fig. 5, A and B). Furthermore, the affinity of CCL3 measured in homologous competition binding was increased up to 4-fold as compared with WT (Table 2). Thus, substitution to a non-aromatic residue (Ala) resulted in maintained chemokine binding but completely blocked activation. In contrast, substitution with other aromatic residues (Tyr and Phe) resulted in WT-like properties both with respect of agonist binding and activation (Table 2 and Fig. 5, A and B).

Among the four antagonists, TAK-779 completely inhibited CCL3-induced activity on W248F/Y with WT-like potencies, whereas both the Merck-derived compound and SCH-C only inhibited to $\sim$50% (Fig. 5C). Moreover, they displayed up to $\sim$20-fold decreased potencies as compared with WT (Table 3). Intriguingly, aplaviroc was unable to inhibit CCL3-induced activation (Fig. 5C), and furthermore, in the absence of CCL3 it induced activation to 70% that of the CCL3-induced PI-turnover (Fig. 5D and Table 3). No intrinsic activity was observed for the Merck compound and SCH-C despite the partial inhibition. Thus, aplaviroc displayed the same efficacy switch from antagonist to agonist on W248F/Y as seen on G286F with an agonistic potency equal to the antagonistic potency at WT (Table 3). Despite huge changes in efficacies, the affinities of these four compounds did not differ more than 2.5-fold from WT (Table 2).

**Impact of Aromatic Residues Above and Below Trp-248**—In addition to Trp-248, TM6 in CCR5 contains two other aro-
matic amino acids in the proximity of Gly-286, namely Tyr-244 and Tyr-251 located below and above Trp-248, respectively (Fig. 1). Both residues have been implicated in 7TM receptor activation previously (39). Although the computational modeling did not show side chain alterations of these in the G286F mutation (Fig. 4), they were both substituted with Ala. CCL3 was able to bind both mutant receptors, albeit with reduced affinity (11–29-fold) (Table 2). With regard to the signaling, Y251A resulted in a 2–5-fold decrease in potency of chemokine-induced PI-turnover, whereas Y244A completely abolished this (Table 1). The small molecule antagonists acted as full antagonists on Y251A-CCR5, with potencies similar to WT (TAK-779 and the Merck-derived compound) or slightly increased (SCH-C and aplaviroc, Table 3).

**Biased Signaling Impaired β-Arrestin Recruitment Despite Constitutive Goi Activity and Agonist-prone Nature**—Lately much attention has been drawn to the signaling pathways beyond the ones coupled to G proteins, e.g. recruitment and signaling via β-arrestins. We, therefore, investigated whether the signaling mediated by β-arrestin was similar to the G protein-coupled pathway. Fig. 6A shows that CCR5 WT recruited β-arrestin even in absence of chemokines, with a basal level of 30% of E_{max}. The potency of CCL3 and CCL5 (EC_{50} of 8.0 and 2.2 nM, respectively) was in the same range as the one obtained in PI-turnover (Table 1). Surprisingly, despite the enhanced signaling through Goi, G286F-CCR5 resulted in a complete loss of the basal β-arrestin recruitment. Furthermore, it was completely unable to be activated by the chemokines (Fig. 6A). In accordance with the lack of Goi activity in W248A, no β-arrestin recruitment was observed here (Fig. 6B). Ala substitution of the other two aromatic amino acids in TM6 (Y251A and Y244A) also completely eliminated chemokine-induced β-arrestin recruitment (data not shown) despite maintained Goi activity for Y251A. Furthermore, the efficacy of W248F and W248Y was highly impaired with regard to β-arrestin recruitment, as an 80% (CCL5-mediated) to 90% (CCL3) decrease was observed compared with CCR5 WT (Fig. 6C). The potency of CCL3 was decreased ~4-fold, whereas the potency of CCL5 actually was increased 10-fold, the same tendency as seen in PI-turnover (Fig. 6). However, unlike the efficacy switch of apla-
viroc observed for W248(F/Y) in the G protein-coupled pathway (Fig. 5, C and D), no aplaviroc-induced β-arrestin recruitment was observed. Instead, when added together with CCL3, aplaviroc acted as an antagonist with a potency similar to that on WT (Fig. 6D). No aplaviroc-induced β-arrestin recruitment was observed on G286F either (data not shown). Thus, all of the mutations constructed in TM6 and TM7 either completely eliminated or heavily impaired β-arrestin recruitment.

DISCUSSION

By single point mutagenesis we show here that the interplay between TM6 and TM7 is critical for CCR5 activation, in particular for the balance between G protein-dependent and -independent pathways (summarized in Fig. 7).

Manipulation with the TM6/7 Interface Induces Biased Agonism—Lately, the concept of biased signaling has gained a lot of attention because of its potential in drug development (42). A drug that selectively targets receptor endocytosis without affecting signaling or the opposite would benefit from the lack of side effects determined by the altered pharmacodynamics. For example, it has been shown that the GPR109A agonist, FIGURE 5. Effect of the elimination or alteration of the aromatic side chain of Trp-248 in PI-turnover. Shown are CCL3- (A) and CCL5-induced (B) PI turnover in COS-7 cells for CCR5 WT (dashed lines), W248A (○), W248F (△), and W248Y (□). The four small molecule antagonists, Merck (▼/▼), and SCH-C (〇/〇), and aplaviroc (O/O) were tested in the presence (white symbols, □) and absence (black, △) of 10 nM CCL3 (for simplicity, only results for W248Y are shown). For comparison, the similar action of these on CCR5 WT (from Fig. 3A) is indicated as dashed lines in C and D.

FIGURE 6. β-Arrestin recruitment in CCR5 WT and mutations. The receptors were tested in enzyme fragment complementation-based β-arrestin recruitment in U2OS cells. CCL3 (white symbols)- and CCL5 (black symbols)-induced β-arrestin recruitment in G286F (A), W248A (B), and W248(F/Y) (C) with corresponding CCR5 WT dose-response curves (dashed lines) are shown (A–C). D, aplaviroc inhibition of CCL3-induced β-arrestin recruitment on W248F (▼) and Y (∙) is shown. The curves are normalized to maximum CCL3-induced β-arrestin recruitment on each receptor.
MK-0354, selectively signals via G\(\alpha_i\), which mediates beneficial anti-lipolytic effects in \textit{vivo} but does not affect the \(\beta\)-arrestin pathway and thus does not cause the unwanted side effect of flushing that normally accompanies GPR109A agonists (43, 44). Since the discovery of CCR5 and CXCR4 as co-receptors for HIV entry (14, 15), much focus has been drawn toward development of receptor antagonists (45, 46). Yet, agonists are also able to inhibit HIV cell entry by blocking the interaction between gp120 and CCR5/CXCR4, and by inducing receptor internalization they thereby obliterate the gate for virus cell entry. A biased drug with agonism toward \(\beta\)-arrestin recruitment and not G protein signaling would be superior as functional antagonist for HIV cell entry. Such a biased drug would cause receptor internalization, thereby preventing virus interaction with CCR5 and a lack of potential side effects after unwanted cellular signaling. Importantly, these properties were recently presented in ESN-196, a novel small-molecule CCR5-targeting ligand that induced CCR5 sequestration without promoting chemotaxis (47). Theoretically, targeting human components involved in the HIV infection cycle instead of the viral proteins would decrease the risk of resistance (48, 49). Currently, the CCR5 antagonist maraviroc is the only entry inhibitor targeting a chemokine receptor on the market. Despite its human target, some HIV clones have developed resistance by altered CCR5 recognition in the presence of maraviroc (48, 50).

MK-0354 has a small side chain (Ala, Gly, or Ser) is found in 73% of all 7TM receptors in position 1100, which mediate beneficial anti-lipolytic effects in vivo but does not affect the \(\beta\)-arrestin pathway and thus does not cause the unwanted side effect of flushing that normally accompanies GPR109A agonists (43, 44). Since the discovery of CCR5 and CXCR4 as co-receptors for HIV entry (14, 15), much focus has been drawn toward development of receptor antagonists (45, 46). Yet, agonists are also able to inhibit HIV cell entry by blocking the interaction between gp120 and CCR5/CXCR4, and by inducing receptor internalization they thereby obliterate the gate for virus cell entry. A biased drug with agonism toward \(\beta\)-arrestin recruitment and not G protein signaling would be superior as functional antagonist for HIV cell entry. Such a biased drug would cause receptor internalization, thereby preventing virus interaction with CCR5 and a lack of potential side effects after unwanted cellular signaling. Importantly, these properties were recently presented in ESN-196, a novel small-molecule CCR5-targeting ligand that induced CCR5 sequestration without promoting chemotaxis (47). Theoretically, targeting human components involved in the HIV infection cycle instead of the viral proteins would decrease the risk of resistance (48, 49). Currently, the CCR5 antagonist maraviroc is the only entry inhibitor targeting a chemokine receptor on the market. Despite its human target, some HIV clones have developed resistance by altered CCR5 recognition in the presence of maraviroc (48, 50).

It is conceivable that a biased ligand inducing receptor internalization and thereby decreasing the availability of receptors on the cell surface would decrease viral resistance. Individuals homozygous for a deletion in CCR5 (\(\Delta 32\) CCR5) do not express CCR5 on cell surfaces and are highly resistant to HIV, proving that this will be an effective treatment strategy (51–54).

To develop biased ligands it is vital to know which areas of the receptors are involved in activation of, for example, \(\beta\)-arrestin but not G protein or the opposite. Our results clearly show a bias of G286F-CCR5 toward G protein coupled signaling as opposed to \(\beta\)-arrestin recruitment. The same bias was observed for Y251A, whereas W248A and Y244A were overall silent. The activation of W248(F/Y)-CCR5 induced by CCL3 was highly biased toward G protein signaling. On the other hand, the CCL5 potency was increased compared with WT in \(\beta\)-arrestin recruitment. Thus, tampering with the TM6/7 interface had a huge impact on \(\beta\)-arrestin recruitment. The biased activity was seen with both endogenous ligands, CCL3 and CCL5, despite different binding patterns (55). In accordance with our results, recently published structural data from an NMR study of the \(\beta_2\)-adrenergic receptor (56) and from crystal structures of the avian \(\beta_1\)-adrenergic receptor in complex with a biased as well as unbiased ligands (57) showed that \(\beta\)-arrestin biased ligands are more prone to interact with residues in especially TM7 than non-biased ligands. It is important to stress that a crystal structure of a 7TM receptor in complex with \(\beta\)-arrestin has yet to be published, and therefore, we do not know the conformation of a receptor in such complex. However, our results combined with structural data like those obtained from the \(\beta_1\)- and \(\beta_2\)-adrenergic receptors (56, 57) suggest that TM6 and -7 play a pivotal role both in the G protein signaling and \(\beta\)-arrestin recruitment.

**G286F Affects the Rotameric State of Trp-248**—It was already pointed out by Holst \textit{et al.} in 2004 (58) that a small side chain (Ala, Gly, or Ser) is found in 73% of all 7TM receptors in position \(\chi_1\) and \(\chi_2\) (59), and mutational analysis in members of the ghrelin receptor family revealed that a large aromatic Phe or Tyr naturally occurring at this position was associated with high constitutive activity (58). In 2010, the same group showed that insertion of a Val instead of an Ala in the bombesin receptor BB \(_3\) increased the level of basal activity (60). In line with these results, we here show that mutation of this residue (Gly to Phe, G286F) in CCR5, which is otherwise not constitutively active through G\(\alpha_i\), raised the basal activity through G\(\alpha_i\) to 30% that of maximum CCL3 activity. In 10% of the models obtained by the computational simulation protocol, the side chain of Trp-248 had an alternative rotamer state in G286F compared with CCR5 WT (\textit{trans} versus \textit{g+}). Confirming the importance of this residue, a complete elimination of chemokine activity was observed upon mutation to Ala despite maintained high affinity chemokine binding. In several different 7TM receptors it has been shown that elimination of this conserved aromatic side chain (84% aromatic, of which 64% is a Trp) has a negative impact on the G protein activation (e.g. the 5-HT\(_4\) and the \(\beta_2\)-adrenergic receptor (40, 41) among others). Before crystal structures of active 7TM receptors were published, Schwartz and co-workers (2, 39) speculated that the side chain of this Trp serves as a rotamer switch, which is important for the overall activation mechanism of the receptor. In fact, when the \(\chi_1\) angle was \textit{trans}, the Trp was proposed to be in the active con-
formation, which correlates well with the increase in activity of G286F in G protein signaling. However, despite major helical movements of TM6, none of the agonist-bound crystal structures confirmed this (3, 4, 61–64). Even though we have seen structures of what, by definition are truly activated receptors (a ternary complex of an agonist, the receptor itself, and a G protein or equivalent), these structures do not provide an overview of the dynamics in receptor activation. We observed a change in the side chain of Trp-248 in 10% of the cases, i.e. a relatively low fraction that could explain why no side chain rotations is seen in the two crystal structures of truly active receptors (3, 4). Another possibility could be that the conformational change of the conserved Trp differs between receptors. Indeed, one-third of the chemokine receptors carry a Gln in VI:13/6.48, and accordingly, a different mechanism may be in play here. Alteration of TM kink properties or displacement of potential water molecules could also contribute. However, in CCR5 we clearly see a linkage between the G286F mutation and a rotation of the side chain of Trp-248, which could contribute to the altered conformational equilibrium in Goq-mediated signaling.

Identification of an Efficacy Switch Region for a Small Molecule CCR5 Antagonist—In this study four different well known small molecule CCR5 antagonists were included. Changing a single amino acid (G286F or W248(F/Y)) completely reversed the efficacy of one (aplaviroc) from full antagonist to full agonist (Table 2). Thus, presumably the transmembrane domain, aplaviroc is also dependent on the extracellular loop 2 (37), in accordance with a previously suggested binding mode (38). Thus, among these four, aplaviroc is the only one that depends upon residues in the extracellular regions, which could be the reason why it alone acquires agonistic properties in the present study.

The present study indicates that the interface between TM6 and -7 in CCR5 is highly important for the activation state both when coupling to G protein and β-arrestin. It is generally acknowledged that 7TM receptors share a common activation mechanism, and thus, it is possible that parallels could be drawn between the results presented here for CCR5 and other chemokine receptors, even to other 7TM receptors.

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REFERENCES

1. Sharman, J. L., Mpamhanga, C. P., Spedding, M., Germain, P., Staels, B., Dacquet, C., Lauzet, V., Harmar, A. J., and NC-IUPHAR (2011) IUPHAR DB. New receptors and tools for easy searching and visualization of pharmacological data. Nucleic Acids Res. 39, D534–D538

2. Nygaard, R., Frimurer, T. M., Holst, B., Rosenkilde, M. M., and Schwartz, T. W. (2009) Ligand binding and micro-switches in 7TM receptor structures. Trends Pharmacol. Sci. 30, 249–259

3. Rasmussen, S. G., DeVree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Kobikila, T. S., Thian, F. S., Chae, P. S., Pardon, E., Calinski, D., Mathiesen, J. M., Shah, T. S., Lyons, J. A., Caffrey, M., Gellman, S. H., Steyaert, I., Skiniotis, G., Weis, W. I., Sunahara, R. K., and Kobikila, B. K. (2011) Crystal structure of the β2 adrenergic receptor-G protein complex. Nature 477, 549–555

4. Rasmussen, S. G., Choi, H.-J., Jung, J. P., Pardon, E., Casarosa, P., Chae, P. S., Devree, B. T., Rosenbaum, D. M., Thian, F. S., Kobikila, T. S., Schnapp, A., Konetzki, I., Sunahara, R. K., Gellman, S. H., Pautsch, A., Steyaert, I., Weis, W. I., and Kobikila, B. K. (2011) Structure of a nanobody-stabilized active state of the β2 adrenoceptor. Nature 469, 175–180

5. Palczewski, K., Kumasaka, T., Hori, T., Behnkne, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Crystal structure of rhodopsin. A G protein-coupled receptor. Science 289, 739–745

6. Cherezov, V., Rosenbaum, D. M., Hansson, M. A., Rasmussen, S. G., Thian, F. S., Kobikila, T. S., Choi, H.-J., Kuhn, P., Weis, W. I., Kobikila, B. K., and Stevens, R. C. (2007) High-resolution crystal structure of an engineered human β2-adrenergic G protein-coupled receptor. Science 318, 1258–1265

7. Rasmussen, S. G., Choi, H.-J., Rosenbaum, D. M., Kobikila, T. S., Thian, F. S., Edwards, P. C., Burghammer, M., Ratnaike, V. R., Sanishvili, R., Fischetti, R. F., Schertler, G. F., Weis, W. I., and Kobikila, B. K. (2007) Crystal structure of the human β2 adrenergic G protein-coupled receptor. Nature 450, 383–387

8. Warner, T., Serrano-Vega, M. J., Baker, J. G., Moukhametzianov, R., Edwards, P. C., Henderson, R., Leslie, A. G., Tate, C. G., and Schertler, G. F. (2008) Structure of a β1-adrenergic G protein-coupled receptor. Nature 454, 486–491

9. Iakola, V.-P., Griffith, M. T., Hansson, M. A., Cherezov, V., Chien, E. Y., Lane, I. R., Ijzerman, A. P., and Stevens, R. C. (2008) The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. Science 322, 1211–1217

10. Jardón-Valadez, A., Ulloa-Aguirre, A., and Piñeiro, A. (2008) Modeling and molecular dynamics simulation of the human gonadotropin-releasing hormone receptor in a lipid bilayer. J. Phys. Chem. B 112, 10704–10713

11. Wu, B., Chien, E. Y., Mol, C. D., Fenalti, G., Liu, W., Katrich, V., Abagyan,
Biased and Constitutive Signaling in CCR5

R., Brooun, A., Wells, P., Bi, F. C., Hamel, D. J., Kuhn, P., Handel, T. M., Cherezov, V., and Stevens, R. C. (2010) Structures of the CXCR4 chemo- kine GPCR with small-molecule and cyclic peptide antagonists. Science 330, 1066–1071.

12. Park, S. H., Das, B. B., Casagrande, F., Tian, Y., Nothnagel, H., Chavarria, J., Cihak, J., Simmons, G., Clapham, P. R., Keegan, R. M., Krissinel, E., Lesk, M. E., Ho, D. D., Trkola, A., Stoddart, C. A., Moore, J. P., Reyes, G. R., and Baroudy, B. M. (2001) SCH-C(SCH351125), an orally bioavailable, small molecule antagonist of the chemokine receptor CCR5, is a potent inhibitor of HIV-1 infection. Proc. Natl. Acad. Sci. U.S.A. 98, 12718–12723.

13. Rokni, K., Nakata, H., Koh, Y., Miyakawa, T., Ogata, H., Taoka, Y., Shibayama, S., Sagawa, K., Fukushima, D., Moravek, J., Koyanagi, Y., and Mitsuya, H. (2004) Spirodiketopiperazine-based CCR5 inhibitor which preserves CC-chemokine/CCR5 interactions and exerts potent activity against R5 human immunodeficiency virus type 1 in vitro. J. Virol. 78, 8654–8662.

14. Thiele, S., Steen, A., Jensen, P. C., Mokrosinski, J., Frimmer, T. M., and Rokni, K., Nakata, H., Koh, Y., Miyakawa, T., Ogata, H., Taoka, Y., Shibayama, S., Sagawa, K., Fukushima, D., Moravek, J., Koyanagi, Y., and Mitsuya, H. (2004) Spirodiketopiperazine-based CCR5 inhibitor which preserves CC-chemokine/CCR5 interactions and exerts potent activity against R5 human immunodeficiency virus type 1 in vitro. J. Virol. 78, 8654–8662.
Biased and Constitutive Signaling in CCR5

42. Rajagopal, S., Rajagopal, K., and Lefkowitz, R. J. (2010) Teaching old receptors new tricks: biasing seven-transmembrane receptors. Nat. Rev. Drug Discov. 9, 373–386

43. Richman, J. G., Kanemitsu-Parks, M., Gaidarov, I., Cameron, J. S., Griffin, P., Zheng, H., Guerra, N. C., Cham, L., Maciejewski-Lenko, D., Behan, D. P., Boatman, D., Chen, R., Skinner, P., Ornelas, P., Waters, M. G., Wright, S. D., Semple, G., and Connolly, D. T. (2007) Nicotinic acid receptor agonists differentially activate downstream effectors. J. Biol. Chem. 282, 18028–18036

44. Semple, G., Skinner, P. J., Gharbaoui, T., Chen, W., Zhan, P., De Clercq, E., and Liu, X. (2012) Recent progress in small molecule CCR5 antagonists as potential HIV-1 entry inhibitors. Part I. Chemokine receptor antagonists. 2004 - 2010. Expert Opin. Ther. Pat. 21, 227–269

45. Chen, W., Zhan, P., De Clercq, E., and Liu, X. (2012) Recent progress in small molecule CCR5 antagonists as potential HIV-1 entry inhibitors. Curr. Pharm. Des. 18, 100–112

46. Ferain, T., Hoveyda, H., Ooms, F., Schols, D., Bernard, J., and Fraser, G. (2011) Agonist-induced internalization of CC chemokine receptor 5 as a mechanism to inhibit HIV replication. J. Pharmacol. Exp. Ther. 337, 655–662

47. Tilton, J. C., Wilen, C. B., Didigu, C. A., Sinha, R., Harrison, J. E., Agrawal-Gamse, C., Henning, E. A., Bushman, F. D., Martin, J. N., Deeks, S. G., and Doms, R. W. (2010) A maraviroc-resistant HIV-1 with narrow cross-resistance to other CCR5 antagonists depends on both N-terminal and extracellular loop domains of drug-bound CCR5. J. Virol. 84, 10863–10876

48. A. K., Cai, T.-Q., Carballo-Jane, E., Behan, D. P., Connolly, D. T., and Tate, C. G. (2011) Agonist-bound adenosine A2A receptor structures reveal common features of GPCR activation. Nature 474, 521–525

49. Lebon, G., Varne, T., Edwards, P. C., Bennett, K., Langmead, C. J., Leslie, A. G., and Tate, C. G. (2011) Agonist-bound adenosine A2A receptor structures reveal common features of GPCR activation. Nature 474, 521–525

50. Holst, B., Mokrosinski, J., Lang, M., Brandt, E., Nygaard, R., Frimurer, T. M., Beck-Sieking, A. G., and Schwartz, T. W. (2007) Identification of an efficacy switch region in the ghrelin receptor responsible for interconversion between agonism and inverse agonism. J. Biol. Chem. 282, 15799–15811

51. Cho, W., Taylor, L. P., and Akil, H. (1996) Mutagenesis of residues adjacent to transmembrane prolines alters D1 dopamine receptor binding and signal transduction. Mol. Pharmacol. 50, 1338–1345

52. Marie, J., Koch, C., Prunen, D., Paquet, J. L., Groblewski, T., Larguier, R., Lombard, C., Deslauriers, B., Maigret, B., and Bonaffous, J. C. (1999) Constitutive activation of the human bradykinin B2 receptor induced by mutations in transmembrane helices III and IV. Mol. Pharmacol. 55, 92–101

53. Dragic, T., Trkola, A., Thompson, D. A., Cormier, E. G., Kajumo, F. A., Maxwell, E., Lin, S. W., Ying, W., Smith, S. O., Sakmar, T. P., and Moore, J. P. (2000) A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. Proc. Natl. Acad. Sci. U.S.A. 97, 5639–5644

54. Tsami, F., Gavrilo, S., Kajumo, F., Seibert, C., Kuhmann, S., Ketas, T., Trkola, A., Palani, A., Clader, J. W., and Tagat, J. R. (2003) Analysis of the mechanism by which the small-molecule CCR5 antagonists SCH-351125 and SCH-350581 inhibit human immunodeficiency virus type 1 entry. J. Virol. 77, 5201–5208

55. Baldwin, J. M. (1993) The probable arrangement of the helices in G protein-coupled receptors. EMBO J. 12, 1693–1703

56. Schwartz, T. W. (1994) Locating ligand-binding sites in 7TM receptors by protein engineering. Curr. Opin. Biotechnol. 5, 434–444

57. Ballesteros, J. A., and Weinstein, H. (1995) Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. Methods Neurosci. 25, 366–428