Extracellular Disulfide Bridges Serve Different Purposes in Two Homologous Chemokine Receptors, CCR1 and CCR5

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Received April 8, 2013; accepted June 13, 2013

ABSTRACT

In addition to the 7 transmembrane receptor (7TM)-conserved disulfide bridge between transmembrane (TM) helix 3 and extracellular loop (ECL)-2, chemokine receptors (CCR) contain a disulfide bridge between the N terminus and what previously was believed to be ECL-3. Recent crystal and NMR structures of the CXC chemokine receptors (CXCR) CXCR4 and CXCR1, combined with structural analysis of all endogenous chemokine receptors indicate that this chemokine receptor–conserved bridge in fact connects the N terminus to the top of TM-7. By employing chemokine ligands that mainly target extracellular receptor regions and small-molecule ligands that predominantly interact with residues in the main binding crevice, we show that the 7TM-conserved bridge is essential for all types of ligand-mediated activation, whereas the chemokine-conserved bridge is dispensable for small-molecule activation in CCR1. However, in striking contrast to previous studies in other chemokine receptors, high-affinity CCL3 chemokine binding was maintained in the absence of either bridge. In the highly related CCR5, a completely different dependency was observed as neither activation nor binding of the same chemokines was retained in the absence of either bridge. In contrast, both bridges were dispensable for activation by the same small molecules. This indicates that CCR5 activity is independent of extracellular regions, whereas in CCR1 the preserved folding of ECL-2 is necessary for activation. These results indicate that conserved structural features in a receptor subgroup do not necessarily provide specific traits for the whole subgroup but rather provide unique traits to the single receptors.

Introduction

Chemokine receptors belong to class A 7 transmembrane receptors (7TM), which have a highly conserved disulfide bridge between the extracellular end of transmembrane helix (TM) 3 and extracellular loop (ECL)-2. Recent crystal structures show that this 7TM-conserved bridge forces ECL-2 to form a lid over the binding pocket, and that ECL-2 contains well-defined but rather different secondary structures within the different receptor families (Palczewski et al., 2000; Rasmussen et al., 2007; Jaakola et al., 2008; Scheerer et al., 2008; Warne et al., 2008; Wu et al., 2010; Park et al., 2012). Furthermore, among class A receptors, ECL-2 is on average the longest of the three ECLs and is believed to be critical for proper ligand binding and receptor activation (Peeters et al., 2011).

The presence of additional extracellular disulfide bridges in certain 7TM receptor families are believed to provide further structural constraint of importance for the regulation of receptor function and ligand binding (Peeters et al., 2011).

Such an additional disulfide bridge is present and highly conserved among chemokine receptors, as shown in the structures of the CXC chemokine receptors (CXCR) CXCR1 and CXCR4 (Wu et al., 2010; Park et al., 2012). The chemokine system is essential for the immune system as mediator of cell migration during homeostasis and inflammation, and activation and differentiation of lymphoid cells and functions outside the hematopoietic system (e.g., angiogenesis and cell growth). This large receptor family consists of 10 CC chemokine receptors (CCR1–10), seven CXC chemokines (CXCR1–7), two XC-chemokines 1 and 2, and one CX3C-chemokine receptor, named according to their preferred endogenous ligands. However, the interaction between chemokines and their receptors varies from high selectivity to high promiscuity, where the same chemokine binds several receptors and several chemokines bind to the same receptor (Rossi and Zlotnik, 2000). This redundancy poses a huge challenge in drug development. Nevertheless, due to the central role of the chemokine system in immune control, many small-molecule ligands have been developed. These often share a similar pharmacophore with a positively charged group that anchors to the chemokine receptor–conserved glutamic acid in TM-7 (position VII:06/7.39) (Rosenkilde and
The chemokine receptor–conserved disulfide bridge is located between Cys residues in the N terminus and in what was previously believed to be ECL-3. However, in the structures of CXCR1 and CXCR4, the Cys residue partnering with the N-terminal Cys is located in the top of TM-7, not in ECL-3. This conserved bridge can be formed in all endogenous receptors except for CXCR6, which lacks the N-terminal Cys. The impact of these two disulfide bridges has been studied in a handful of chemokine receptors, where disruption of either bridge was found to reduce receptor surface expression (CXCR4 and CCR5) (Blanpain et al., 1999; Chabot et al., 1999) and/or abolish chemokine binding (CXCR2, CCR5, and CCR6) (Blanpain et al., 1999; Al and Liao, 2002; Limatola et al., 2005). This large impact on chemokine interaction is not surprising, as chemokines, due to their large size, primarily interact with the extracellular parts of the receptor (Schwarz and Wells, 2002; Allen et al., 2007), making them critically dependent on correct folding of these regions.

In the present study, we focus on the impact of the disulfide bridges for receptor activity and ligand binding in two closely related CC-chemokine receptors: CCR1 and CCR5. Initial bioinformatic analyses of all endogenous chemokine receptors revealed that despite a large diversity in the extracellular domains, the positions of the four Cys residues are extremely conserved (Fig. 1). CCR1 was chosen due to the availability of both peptide and nonpeptide agonists and antagonists. Thus, the two chemokine agonists CCL3 and CCL5 were included together with the CCR1-selective nonpeptide antagonist BXX471 (Horuk, 2005) as well as two chemically different series of small-molecule agonists: a novel carbamid-based series (Jensen et al., 2012; Petersen et al., 2013) and metal-ion chelators (Jensen et al., 2008; Thiele et al., 2012). As CCR5 is the closest homolog to CCR1 and overlaps in ligand-binding profiles with CCR1 (Murphy et al., 2000; Jensen et al., 2008; Thiele et al., 2011, 2012), similar studies were performed on CCR5.

Despite structural and functional homology between the receptors, our experiments revealed opposite impacts of the disulfide bridges on ligand binding and receptor function. This indicates that even though a structural feature (such as the disulfide bridges in chemokine receptors) is extremely conserved among a large subgroup of receptors, it does not necessarily provide a specific functional trait for the given subgroup but rather unique traits to single receptors within the subgroup.

**Materials and Methods**

CCL3 was purchased from R&D Systems (Minneapolis, MN). The cDNA for wild-type (WT) human CCR1 was kindly provided by Tim Wells (Serovon Pharmaceutilcal Research Institute, Geneva, Switzerland), and the WT CCR5 was cloned in house from a spleen cDNA library. 3H-myio-inositol (PT6-271) and 125I-CCL3 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The promiscuous chimeric G protein Go/Go64myr, was kindly provided by Evi Kostenis (University of Bonn, Germany).

**Site-Directed Mutagenesis.** Point mutations were introduced in the receptor by use of the polymerase chain reaction overlap extension technique with human chemokine receptors as a template. All reactions were performed with Pfu polymerase (Stratagene, La Jolla, CA). The mutant receptors were cloned into the eukaryotic expression vector pcDNA3.1+ and were expressed in Escherichia coli cells. The mutations were verified by DNA sequencing by Eurofins MWG Operon (Ebersberg, Germany).

**Transfections and Tissue Culture.** COS-7 cells were grown in 10% CO2 at 37°C in Dulbecco’s modified Eagle’s medium with glutamax (cat. no. 21885-025; Invitrogen/GIBCO, Long Island, NY) adjusted with 10% fetal bovine serum, 180 μg/ml penicillin, and 45 μg/ml streptomycin. Transfection of COS-7 cells was performed by the calcium phosphate precipitation method. In 75 cm2 flasks, 10 μg receptor DNA and 15 μg GoΔ64q4myr were mixed with 30 μl 2 mM CaCl2 and Tris-EDTA buffer for a total volume of 240 μl. We added 240 μl 2x Hanks’ buffered salt solution, and the mixture was given 45 minutes for precipitation before it was added to the cells along with 2 mg/ml of chloroquine. The flasks were incubated for 5 hours at 37°C before the media were replaced with 10 ml fresh COS-7 media.

**Inositol-Phosphate Assay (IP Turnover).** COS-7 cells were transfected as described previously. The cotransfection with GoΔ64q4myr turns the Gs signal into a Gi coupled signal, making it possible to measure the phospholipase C (PLC) activation as inositol-phosphate (IP) turnover. One day after transfection, the cells were seeded in 24-well plates (1.5 × 105 cells/well) and incubated with 2 μCi of 3H-myio-inositol in 0.3 ml growth medium for 24 hours. Cells were washed twice with Hanks’ balanced salt solution supplemented with CaCl2 and MgCl2 (GIBCO14025) and afterward were incubated for 15 minutes in 0.2 ml of buffer supplemented with 10 mM LiCl before the ligand addition followed by 90 minutes of incubation. Cells were extracted by addition of 1 ml of 10 mM formic acid followed by incubation on ice for 30 minutes. The generated 3Hinositol phosphate was purified on AG 1X8 anion exchange resin. Determinations were made in duplicate.

**Binding Experiments.** COS-7 cells were transiently transfected, then transfected to culture plates and left overnight. The number of cells seeded per well was determined by the apparent expression efficiency of the receptor and was aimed to obtain 5 to 10% specific binding of the added radioactive ligand. The competition binding was carried out for 3 hours’ incubation at 4°C using 10–15 pM 125I-CCL3 along with the unlabeled ligand in 0.3 ml 50 mM HEPES buffer pH 7.4 supplemented with 1 mM CaCl2, 5 mM MgCl2, and 0.5% bovine serum albumin (BSA). After incubation, the cells were washed twice with 10 mM CaCl2 binding buffer supplemented with 0.5 M NaCl. Nonspecific binding was determined as the binding in the presence of 0.1 μM unlabeled CCL1, CCL3, or CCL5, respectively. Determinations were made in duplicate. Bmax values were calculated based on the homologous competition binding experiments by the following formula: [Top of curve in counts per minute (CPM) – Bottom of curve in CPM] * IC50 (M) * Assay Volume (L) * 1015/[(Total radioactivity in CPM) * 106] cells = fmoI/106 cells.

**Surface Enzyme-Linked Immunosorbent Assay.** COS-7 cells were transiently transfected with N-terminal M1-FLAG tagged receptor mutant receptors, and seeded in 96-well plates (35,000 cells/well). The cells were washed once in Tris-buffered saline (TBS) (50 mM Tris-base, 150 mM NaCl, pH 7.4) and subsequently fixed in 150 μl 4% formaldehyde for 15 minutes. After three washes in TBS, the cells were blocked in TBS containing 2% BSA for 30 minutes. Subsequently, the cells were incubated with mouse M1 anti-FLAG antibody 2 μg/ml in TBS containing 1% BSA and 1 mM CaCl2 for 2 hours. After three washes in TBS with 1 mM CaCl2, the cells were incubated with goat anti-mouse horseradish peroxidase-conjugated IgG antibody diluted 1:1000 in TBS containing 2% BSA and 1 mM CaCl2 for 1 hour. After three washes in TBS 1 mM CaCl2 the immune reactivity was revealed by addition of horseradish peroxidase substrate according to the manufacturer’s instructions.
-Arrestin Recruitment. Recruitment of β-arrestin was measured using the PathHunter β-arrestin assay (DiscoverX, Fremont, CA). The CCR1 and CCR5 wild-type receptors were fused with the ProLink pk1-tag (a small fragment of the enzyme β-galactosidase) and cloned into a pCMV-vector. Mutations were made with QuikChange polymerase chain reaction (Agilent Technologies, Santa

**Fig. 1.** Positional conservation of the cysteines in the extracellular disulfide bridges. Serpentine models of a chemokine receptor emphasizing the position of the partnering cysteines relative to the conserved transmembrane residues in the class A 7TM-conserved disulfide bridge (A) and in the chemokine receptor-conserved disulfide bridge (D). A graphical presentation of the number of amino acids from a conserved extracellular cysteine to a conserved transmembrane residue for the class A 7TM-conserved disulfide bridge (B and C) and for the chemokine receptor-conserved disulfide bridge (E and F). CXCR6 is highlighted in red to emphasize lack of the chemokine-conserved disulfide bridge.
Clara, CA). Assays were performed in a Chinese hamster ovary K1 enzyme acceptor-arrestin cell line stably expressing β-arrestin1 or β-arrestin2 coupled to the large β-galactosidase fragment. Cells were seeded out in 96-well plates, 20,000 cells/well, and transfected the following day with 50 ng DNA using FuGENE6 (Promega, Madison, WI) reagent (0.15 μl/well). Then, 24 hours after transfection, the medium was removed, and 100 μl Opti-MEM I (GIBCO) was added. The next day, the cells were stimulated with varying concentrations of CCL3 or CCL5 for 90 minutes at 37°C. The detection reagent solution was added before the 60-minute incubation at room temperature, and the β-arrestin recruitment was measured as chemiluminescence using the PerkinElmer EnVision 2104 Multilabel Reader (PerkinElmer, Waltham, MA).

Bioinformatics. The nucleotide sequences of the chemokine receptors were acquired from GenBank of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The alignment was performed in Geneious 6.0.5 (Biomatters Ltd., Auckland, New Zealand) using MAFFT v6.814b (http://mafft.cbrc.jp/alignment/software/). The BLOSUM62 matrix was applied with gap open penalty and offset value of 1.53 and 0.123, respectively. The sequence logo was generated using the Internet-based program WebLogo (http://weblogo.berkeley.edu).

Results

Highly Conserved Localization of the Disulfide Bridges and Length of Extracellular Regions in Chemokine Receptors. When we compare the positions of the four extracellular cysteines involved in disulfide bridge formation in chemokine receptors, it becomes evident that there is a high degree of positional conservation (Fig. 1). The relative position of the highly conserved cysteine at the top of TM-3 (position III:01/3.25) is very constant (Fig. 1B, black symbols) as expected from its presence in all class A 7TM receptors (Mizadegan et al., 2003). However, also the length of ECL-1 is surprisingly conserved, with the majority of receptors containing 25 amino acids between CysIII:01 and the conserved Asp in TM-2 (II:10/2.50) (Fig. 1B, white symbols). The localization of the partnering Cys in ECL-2 of the 7TM-conserved disulfide bridge is less conserved, as reflected by a larger variability in the length of ECL-2, although the length of ECL-2a (the part prior to the Cys) is a bit more preserved than ECL-2b (the part from the Cys to TM-5) (Fig. 1C). When examining the positions of the cysteine residues in the chemokine receptor-conserved disulfide bridge, there is a huge diversity in the length of the region preceding the N-terminal Cys, reflecting that the main chemokine binding motifs are localized in this region (Allen et al., 2007) (Fig. 1E). However, at the same time there is a striking degree of positional conservation in the N-terminal Cys when considering the distance to the conserved AsnI:18/1.50, as 16 out of 20 receptors have 28 amino acids in this region, whereas the rest have 27 or 31 amino acids. The relative position of the partnering Cys in TM-7 is also highly conserved. Thus, in all chemokine receptors there are exactly 25 amino acids to the ProVII:17/7.50 (Fig. 1F, black symbols); when counted from ProVI:15/6.50, there are either 19 or 20 (Fig. 1F, white symbols). Taken together, this highly preserved positioning of not only the 7TM-conserved disulfide bridge but also the chemokine receptor-conserved disulfide bridge gives testament to the importance of structural conservation for the overall function of chemokine receptors.

Reposition of One of the Cysteines in the Chemokine-Conserved Disulfide Bridge from ECL-3 to the Top of TM-7. The high positional conservation of the four extracellular cysteines specifies that the overall number of residues and thus the lengths of the extracellular loops (and of the region between the N-terminal Cys and TM-1) are very similar within the chemokine family. When assessing the published structures of CXCR1 and CXCR4, the same tendencies are observed (Wu et al., 2010; Park et al., 2012). We therefore looked at the primary structures in these regions of all endogenous chemokine receptors. As shown in Supplemental Fig. 1, there is generally low amino acid conservation in the extracellular receptor parts compared with the transmembrane regions, reflecting that chemokine-recognition relies on extracellular receptor regions (Allen et al., 2007). However, there is a high degree of similarity in certain smaller extracellular areas, such as the WxF motif in ECL-1 and the aromatic residue 4 amino acids after the Cys in ECL-2 in 13 out of the 20 receptors. Importantly, the Cys, in what was previously believed to be ECL-3, aligns to the top of TM-7, as confirmed by the structures of CXCR1 and CXCR4 (Wu et al., 2010; Park et al., 2012). This indicates that the chemokine receptor-conserved disulfide bridge links the N terminus to the top of TM-7 and thereby confers less flexibility to this bridge.

Both Bridges Are Essential for Chemokine-Mediated Activation, but Not for Binding. To clarify the importance of the overall structure of the extracellular regions for ligand binding and receptor activation in CCR1, we constructed single Ala substitutions of the four extracellular Cys residues, thereby disrupting the 7TM-conserved bridge (between C106 in TM-3 and C183 in ECL-2) and the chemokine-conserved bridge (between the N-terminal C24 and C273 in TM-7). The mutated receptors were tested for their ability to be activated by CCL3 and CCL5. Thus, COS-7 cells were transiently transfected with plasmids containing any of the four Ala-substituted receptors along with a chimeric G protein (Gqi4myr) that is recognized as a Gαq subunit but transduces a Gαq signal. Consequently, G protein–mediated activation was measured by IP3 accumulation. As seen in Fig. 2, A and B, and Table 1, CCL3- and CCL5-mediated activation was highly impaired and for some mutations completely absent, but they acted as previously described on CCR1 WT (Neote et al., 1993; Jensen et al., 2008). Enzyme-linked immunosorbent assay-based determination of surface expression uncovered WT-like expression of [C24A]- and [C273A]-CCR1, but that of [C106A]- and [C183A]-CCR1 (with disrupted 7TM-conserved bridge) was reduced up to 6-fold compared with CCR1 WT (Table 1).

Given the preserved receptor expression, the impaired activation (Fig. 2, A and B) could be due to either impaired ligand binding or the mutations interfering with the overall ability of the receptors to be activated. The CCL3 binding was therefore determined by homologous competition binding, and intriguingly all mutated receptors retained WT-like high-affinity CCL3 binding (Fig. 2, C and D; Table 1), although with reduced maximum binding (Bmax) (Table 1). This high-affinity CCL3 binding without triggering a G protein–mediated activation prompted us to explore whether non-G protein–dependent pathways were affected as well. This was indeed the case, as the β-arrestin recruitment was completely abolished in all four mutations, whereas CCL3 induced high-potency β-arrestin recruitment in CCR1 WT (Fig. 3).
Disruption of the 7TM-Conserved Bridge also Impairs Activation by Small-Molecule Agonists, but These Act Independently of the Chemokine-Conserved Bridge.

Given their small size and thus more restricted binding mode compared with the endogenous ligands (Handel and Lau, 2004; Jensen et al., 2008), two series of small-molecule agonists (metal-ion chelators and carbamide-based agonists) served as valuable tools for investigating whether the impaired activation by CCL3 was due to a general impaired receptor activation or to an altered CCL3 targeting to CCR1. Upon disruption of the 7TM-conserved bridge, the activation of the metal-ion chelator complexes (Cu in complex with bipyridine and phenanthroline) were severely impaired, but these ligands acted with WT-like potencies and efficacies in the absence of the chemokine-conserved bridge (Fig. 4, A and B; Table 2). The same pattern was observed for the more potent carbamide-based CCR1 agonist 4-(3-phenoxy-benzyl)homopiperazine-1-benzyl-carboxamide (TUG-584) (Supplemental Fig. 2, A and B), thus demonstrating that the chemokine-conserved bridge—in contrast to the 7TM-conserved bridge—is not essential for activation as such, only for activation mediated by the chemokines.

Positive Allosteric Modulators Shift toward Competitive Antagonism in the Absence of the 7TM-Conserved Bridge.

Due to the maintained ligand-binding but differential impact of the two disulfide bridges on ligand-mediated activation, the 7TM-conserved bridge is not essential for activation as such, only for activation mediated by the chemokines. This is in contrast to the chemokine-conserved bridge, which is essential for activation mediated by the chemokines but not for activation mediated by the metal-ion chelator complexes.

**TABLE 1**

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<td>% of CCR1 WT</td>
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activation, we explored whether the positive allosteric properties, previously reported for the metal-ion chelators in CCR1 WT (Jensen et al., 2008; Thiele et al., 2012), were affected. Consistent with the preserved activity of these small-molecule agonists (Fig. 4B), the positive allosteric enhancement was maintained with WT-like affinities in the absence of the chemokine-conserved bridge (Fig. 4D). In contrast, the metal-ion chelator complexes lost their ability to enhance 125I-CCL3 binding upon disruption of the 7TM-conserved disulfide bridge; in fact, they displayed an entirely different binding pattern.

Fig. 3. b-arrestin recruitment depends on maintained disulfide bridges. CCL3 induced b-arrestin recruitment of (A) CCR1 WT ( ), [C106A]-CCR1 ( ), and [C183A]-CCR1 ( ) and (B) CCR1 WT ( ), [C24A]-CCR1 ( ), and [C273A]-CCR1 ( ). All curves are normalized against CCL3 induced b-arrestin recruitment at CCR1 WT (n=3).

Fig. 4. Small molecule activation in CCR1 is impaired and shift toward competitive antagonism when the 7TM-conserved bridge is disrupted. (A and B) IP-accumulation experiments of Cu2+ phenanthroline (CuPhe) on CCR1 WT ( ), [C24A]-CCR1 ( ), [C106A]-CCR1 ( ), [C183A]-CCR1 ( ), and [C273A]-CCR1 ( ). All results have been normalized to CCR1 WT (n=3-14). C and D, [C183A]-CCR1 represents disruption of the 7TM-conserved bridge (C) and [C273A]-CCR1 represents disruption of the chemokine-conserved bridge (D). Heterologous binding of CuPhe on CCR1 WT ( ), and mutant receptors ( ). All curves are normalized to CCR1 WT (n=3-7).

Table 2

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<th>IP Accumulation: CuBip</th>
<th>IP Accumulation: CuPhe</th>
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CuBip, Cu2+bipyridine; CuPhe, Cu2+phenanthroline.

Results display allosteric displacement (in contrast to allosteric enhancement).
they shifted toward being competitive ligands for $^{125}$I-CCL3 binding (Fig. 4C). Consequently, these binding data support the dependency of the 7TM-conserved disulfide bridge for the agonistic properties of these small molecules (Figs. 4, A and B). Besides showing that the metal-ion chelators in fact bind to the receptors with high affinity, these results intriguingly show how disruption of the 7TM-conserved bridge alters the ligand-binding mode.

**Endogenous Ligand Binding Is Altered upon Disruption of the 7TM-Conserved Bridge, Whereas Small-Molecule Interaction Is Retained.** To further explore the altered ligand-binding mode, we constructed CCR1 receptors lacking the conserved GluVII:067.39 in the absence of the 7TM-conserved bridge [C106A-E287A]-CCR1 and [C183A-E287A]-CCR1. This Glu residue acts as an anchor point for the metal-ion chelators, whereas the interaction of CCL3 with CCR1 is independent of this residue (Jensen et al., 2008). Despite the maintained surface expression, no specific $^{125}$I-CCL3 binding was observed in any of the two double-mutant receptors (Table 1). This suggests that CCL3—in the absence of the 7TM-conserved bridge—is dependent on E287 for proper CCR1 interaction, and that the altered competitive nature observed for the small-molecule agonists (Fig. 4C) could be centered on a mutual interaction with this specific residue.

To ensure proper folding of the CCR1 mutant receptors, we performed heterologous competition binding experiments with the CCR1-specific small-molecule antagonist BX471. This ligand has a well-established binding mode consisting primarily of aromatic interactions in the main binding crevice, where it spans the whole binding pocket interacting with the CCR1-specific small-molecule antagonist BX471. This Glu residue acts as an anchor point for the metal-ion chelators, whereas the interaction of CCL3 with CCR1 is independent of this residue (Jensen et al., 2008). Despite the maintained surface expression, no specific $^{125}$I-CCL3 binding was observed in any of the two double-mutant receptors (Table 1). This suggests that CCL3—in the absence of the 7TM-conserved bridge—is dependent on E287 for proper CCR1 interaction, and that the altered competitive nature observed for the small-molecule agonists (Fig. 4C) could be centered on a mutual interaction with this specific residue.

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**Different Roles of the Extracellular Areas in CCR5 and CCR1.** Because CCR5 ligand-binding profile overlaps with that of CCR1 (Murphy et al., 2000; Jensen et al., 2008; Thiele et al., 2011, 2012), we decided to construct the corresponding Ala-substitutions: [C20A], [C101A], [C178A], and [C269A]-CCR5. Similar to CCR1, chemokine-induced G protein activation could not be obtained for any of these mutations (Fig. 5, A and B; Table 3). This was not because of a lack of receptor surface expression, as all four Cys-to-Ala substitutions displayed >50% of WT expression (Table 3). Like for CCR1, the chemokine-induced β-arrestin recruitment was absent in the mutant receptors (unpublished data). However, in contrast to CCR1, and despite maintained surface expression, no specific binding of $^{125}$I-CCL3 was observed in any of the CCR5 mutations (Fig. 5, C and D).

**Maintained Small-Molecule Activity in the Absence of Disulfide Bridges in CCR5.** Another noticeable difference from CCR1 was observed when studying the small molecules. Thus, in contrast to the highly impaired CCR1 lacking the 7TM-conserved bridge (Figs. 2 and 4), the similar disruption in CCR5 was insignificant for small molecule activation. Here, the metal-ion chelators as well as the carbamide-based ligand TUG-588 acted with WT-like potencies in both [C101A]- and [C178A]-CCR5 (Fig. 6A; Supplemenatal Fig. 2, E and F; Table 3); however, compared with the metal-ion chelator efficacies on CCR5 WT, we observed a 50% decrease (Table 3). The same independency was observed upon disruption of the chemokine-conserved bridge in CCR5 (Fig. 6B; Table 3), as was the case in CCR1 (Fig. 4, C and D). Thus, there was no difference between CCR1 and CCR5 with regard to the impact of the chemokine-conserved bridge for small-molecule agonist action, whereas in CCR5 the small-molecule activation was maintained despite disruption of the 7TM-conserved bridge.

**Discussion**

We have assessed the importance of the conserved disulfide bridges for CCR1 function and compared it with CCR5. The strong positional conservation for the implicated cysteine residues and thereby also the length conservation of the involved extracellular regions are striking observations (Fig. 1). By employing chemokines that mainly target extracellular regions and small-molecule agonists that target the main binding crevice, we show that the disulfide bridges have different impacts, despite the overlapping ligand-binding profiles and structural similarities for CCR1 and CCR5 (summarized in Fig. 7).

**The Disulfide Bridge between TM-3 and ECL-2 Is a Conserved Structural Trait Among 7TM Receptors.** The presence of disulfide bridges in the extracellular parts of 7TM receptors are believed to be important for the receptor
TABLE 3
Molecular interaction of endogenous and small-molecule ligands with CCR5 alleles. Ala substitutions of extracellular Cys residues were screened for surface expression in IP3-accumulation and homologous-binding experiments. Surface expression is given as a percentage of CCR5 WT expression. The IP3-accumulation data display the potency as log EC50 and EC50 (nM). The differences in potency of a given ligand compared with the WT receptor are given as fold. 

<table>
<thead>
<tr>
<th>CCR5 WT</th>
<th>C20A</th>
<th>C101A</th>
<th>C178A</th>
<th>C269A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface Expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of CCR5 WT</td>
<td>100</td>
<td>81</td>
<td>52</td>
<td>61</td>
</tr>
<tr>
<td>CuBip (WT)</td>
<td>10</td>
<td>&gt;100</td>
<td>NA</td>
<td>&lt;100</td>
</tr>
<tr>
<td>CuPhe (C178A)</td>
<td>16</td>
<td>16</td>
<td>NA</td>
<td>16</td>
</tr>
<tr>
<td>CuBip (C269A)</td>
<td>61</td>
<td>61</td>
<td>NA</td>
<td>61</td>
</tr>
<tr>
<td>CuPhe (WT)</td>
<td>60</td>
<td>60</td>
<td>NA</td>
<td>60</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Potency</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuBip</td>
<td>6.00</td>
</tr>
<tr>
<td>CuPhe</td>
<td>9.8</td>
</tr>
</tbody>
</table>

The differences in potency between these results are likely explained by differences in cell lines and transfection procedures. In CXCR2, Ala substitution of Cys residues hardly affected surface expression yet the binding of CXCL8 was completely disrupted, suggesting improper conformation of the extracellular parts of the receptor (Limatola et al., 2005). As in CXCR4, a study of CCR6 found that substitution of the 7TM-conserved bridge retained the receptor in the cell (Ai and Liao, 2002).

Intriguingly, ECL-2 is the largest (although absent in certain class A receptors such as the metal-ion chelator receptors (Benneds-Jensen et al., 2011) and the most divergent of all three extracellular loops, yet among chemokine receptors we here show that the length of ECL-2 is quite...
Despite the conserved disulfide bridge, ECL-2 adopts very different structures, ranging from a β-sheet that dives deep into the ligand-binding pocket (rhodopsin) to a more open conformation with an α-helix (β-adrenergic receptors) as evident from recent crystal structures (Palczewski et al., 2000; Rasmussen et al., 2007; Hanson et al., 2008; Jaakola et al., 2008; Scheerer et al., 2008; Warne et al., 2008; Wu et al., 2010; Peeters et al., 2011). These features are believed to confer important roles for ligand selectivity among receptors (Peeters et al., 2011). Furthermore, ECL-2 has been speculated to adopt different conformations during ligand binding and receptor activation, first to allow the ligand to enter the binding crevice and second to stabilize the ligand-induced receptor conformations (Avlani et al., 2007; Peeters et al., 2011).

A Disulfide Bridge between the N Terminus and TM-7 Is Conserved in Chemokine Receptors. Additional extracellular disulfide bridges can be found in subgroups of class A receptors either within a certain loop (adenosine A2A receptors) or between the loops and/or the N terminus (e.g., chemokine receptors, gonadotropin-releasing hormone receptor, bradykinin receptor, and GPR39), and are believed to rigidify the extracellular domains further, thereby providing important mechanisms of regulating receptor activity and ligand binding (Jaakola et al., 2008; Peeters et al., 2011). An additional disulfide bridge between the N terminus and the top of TM-7 (and not ECL-3 as previously anticipated) is thus believed to be present in the majority of endogenous chemokine receptors, as confirmed by the recent structures of CXCR4 and CXCR1 (Wu et al., 2010; Park et al., 2012). In fact, only CXCR6 lacks the possibility to form such a disulfide bridge. Our study clearly supports its presence in CCR1 and CCR5 (Figs. 2 and 5), as also suggested by previous studies in CCR5, CCR1, CCR2, and CCR4 (Leong et al., 1994; Blanpain et al., 1999; Chabot et al., 1999; Limatola et al., 2005). Yet this may not hold true in all chemokine receptors, as a study in CCR6 has suggested that this bridge is absent here (Ai and Liao, 2002).

Previous studies in CCR5 have demonstrated that the N terminus and ECL-2 are important areas for chemokine as well as gp120 binding (Lee et al., 1999) and that even minor alterations in these areas destroy ligand recognition (Samson et al., 2004).
et al., 1997; Dragic et al., 1998). Furthermore, consistent with our findings in CCR5, Blanpain et al. (1999) have shown that extracellular conformation-dependent antibodies failed to recognize CCR5 when one or the other disulfide bridge was destroyed. Taken together with the overall extracellular binding mode of chemokines (Pease et al., 1998; Xanthou et al., 2003), this explains why CCL3 and CCL5 were not able to bind to CCR5. However, the maintained small molecule activation in CCR5 (Fig. 6; Supplemental Fig. 2) confirms proper folding of the transmembrane and intracellular regions in the absence of the disulfide bridges (Fig. 6).

Chemokines and Small-Molecule Ligands Interact Differently with Chemokine Receptors. According to the so-called two-step model initially proposed for the C5a receptor (Siciliano et al., 1994) and later suggested for the chemokine system by Pease et al. (1998) and Xanthou et al. (2003), the chemokine initially interacts with the receptor N terminus, followed by an interaction with the extracellular loops. As a last step, the chemokine N terminus initiates receptor activation, either by docking into the main binding crevice or via interaction with the extracellular parts (Schwarz and Wells, 2002). This model was based on investigations of chemokine interactions with chimeric receptors of CXCR1:3 (Xanthou et al., 2003) and CCR1:3 (Pease et al., 1998). Also supporting this, CXβCL1 depends on residues in the N terminus as well as ECL-3 of CXβC chemokine receptor 1 (Chen et al., 2006), and CXCL16 depends on all extracellular regions of CXCβ6 (Petit et al., 2008). Likewise, we and others have reported that the chemokine-induced activation is critically dependent upon the receptor N terminus in CCR1, CCR2, and CCR8 (Samson et al., 1997; Jensen et al., 2008, 2012).

Due to the large size of the chemokines, small-molecule ligands are usually allosteric, with binding sites primary located in the main binding crevice of the receptors (Berkhout et al., 2003; de Mendonca et al., 2005; Maeda et al., 2006; Vaidehi et al., 2006; Rosenkilde et al., 2007; Jensen et al., 2007, 2008; Thiele et al., 2011). A common pharmacophore of most small molecules is an elongated structure with a more or less centered, positively charged group flanked by aromatic side chains. Several studies have indicated that the positively charged group anchors to the chemokine receptor-conserved GluVII:06 and that the aromatic groups interact with the aromatic environment in the two parts of the main binding crevice (Rosenkilde and Schwartz, 2006; Rosenkilde et al., 2010). Yet in many studies the participation of the extracellular domains has been rather neglected. However, as shown in the crystal structure of CXCR4 (and of the adenosine 2A, rhodopsin, the β1- and the β2-adrenergic receptors) residues in ECL-2 may participate directly in the binding of ligands targeted to the main binding crevices (Palczewski et al., 2000; Cherezov et al., 2007; Jaakola et al., 2008; Warne et al., 2008; Wu et al., 2010). Likewise, mutational studies have revealed participation of ECL-2 in small-molecule ligand binding in CCR1, CCR5, and CCR8 (Maeda et al., 2006; Thiele et al., 2011; Jensen et al., 2012).

Disruption of conserved disulfide bridges is likely to interfere with the active receptor conformations that are induced by/recognized and stabilized by agonists. Thus, disulfide bridge-disruption could shift the equilibrium of active/inactive receptor populations in either direction or open up for possible ligand interaction to otherwise nonaccessible conformations. These considerations could explain the alterations in efficacy and $B_{\text{max}}$ values of both small-molecule and chemokine agonists. Thus, a shift in receptor population toward inactive conformations upon disruption of the chemokine-conserved disulfide bridge could result in proper receptor/ligand interaction (retained affinity and surface expression but severely reduced $B_{\text{max}}$ values) though at a concentration too low to observe activation.

Concerning the TTM-conserved disulfide bridge, we find that proper constraint of ECL-2 is important for the action but not the binding of chemokine and small-molecule agonists in CCR1 (Figs. 2, 4, and 5). The shift from ago-allosteric action on CCR1 WT to competitive displacement of $^{125}$I-CCL3 upon disruption of this disulfide bridge (Fig. 5) indicates an altered binding mode, which along with the introduced sensitivity of CCL3 for GluVII:06 suggests a competition centered on this residue. In contrast to the findings in CCR1, the TTM-conserved bridge was not essential for CCR5 activity, as the small molecules were still able to act in the absence of C101 or C169, whereas no binding (or action) was observed for chemokines.

Despite fully conserved positioning of the two disulfide bridges in CCR1 and CCR5, and a generally conserved positioning of these among all chemokine receptors (Fig. 1), the bridges have a very different impact on ligand-mediated receptor activation. As these observations include two highly homologous receptors that even share ligand-binding profiles, it is very likely that disulfide bridges serve different purposes in not only other chemokine receptors, but in class A receptors in general.

Acknowledgments

The authors thank Tine A. Kristensen for synthesizing BX471 and Randi Thøgersen for skilled technical assistance.

Authorship Contributions

Participated in research design: Rummel, Rosenkilde.

Conducted experiments: Rummel, Thiele, Hansen.

Contributed new reagents or analytic tools: Petersen, Ulven.

Performed data analysis: Rummel, Thiele, Hansen, Sparre-Ulrich.

Wrote or contributed to the writing of the manuscript: Rummel, Sparre-Ulrich, Rosenkilde.

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combination with granulocyte colony-stimulating factor to mobilize hematopoietic stem cells to the peripheral blood for collection and subsequent autologous transplantation.


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