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TITLE PAGE:

Extracellular disulfide bridges serve different purposes in two homologous chemokine receptors, CCR1 and CCR5.

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RUNNING TITLE: The role of disulfide bridges in homologous chemokine receptors

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List of abbreviations: 7TM : 7 transmembrane receptor

CCR : CC chemokine receptor

CuBip : Cu^{2+} Bipyridine

CuPhe : Cu^{2+} Phenanthroline

CXCR : CXC chemokine receptor

CX_3CR : CX_3C chemokine receptor

ECL : Extracellular loop

ELISA : Enzyme-Linked Immunosorbent Assay

IP : Inositol Phosphate

MC : Metal-ion chelator

TM : Transmembrane

XCR : XC chemokine receptor

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ABSTRACT

In addition to the 7TM receptor-conserved disulfide bridge between transmembrane helix (TM) 3 and extracellular loop (ECL) 2, chemokine receptors contain a disulfide bridge between the N-terminus and what previously was believed to be ECL-3. Recent crystal- and NMR-structures of 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 where dispensable for activation by the same small-molecules. This indicates that CCR5 activity is independent of extracellular regions, whereas in CCR1, preserved folding of ECL2 is necessary for activation. These results indicate that conserved structural features in a receptor subgroup, does not necessarily provide specific traits for the whole subgroup, but rather provides unique traits to the single receptors.

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INTRODUCTION

Chemokine receptors belong to class A 7TM receptors, 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 (Rasmussen et al., 2007;Warne et al., 2008;Jaakola VP et al., 2008;Palczewski et al., 2000;Scheerer et al., 2008;Park et al., 2012;Wu et al., 2010). 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 CXCR1 and CXCR4 (Park et al., 2012;Wu et al., 2010). The chemokine system is essential for the immune system as mediator of cell migration during homeostasis and inflammation, 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), 7 CXC-chemokine (CXCR1-7), 2 XC-chemokine (XCR1 and 2) and 1 CX_3C-chemokine receptor (CX_3CR1), 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 A., 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

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conserved glutamic acid in TM7 (position VII:06/7.39[1]) (Rosenkilde and Schwartz, 2006)). However, despite strong efforts only two small-molecule ligands, maraviroc (CCR5) and mozobil (CXCR4), have reached the market (Maeda et al., 2012;Brave et al., 2010).

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, and not in ECL-3. This conserved bridge can be formed in all endogenous receptors except for CXCR6, which lack the N-terminal Cys. The impacts of these two disulfide bridges have been studied in a handful of chemokine receptors, where disruption of either bridge was found to reduce receptor surface expression (CXCR4 and CCR5) (Chabot et al., 1999;Blanpain et al., 1999) and/or abolish chemokine binding (CXCR2, CCR5 and CCR6) (Blanpain et al., 1999;Limatola et al., 2005;Ai and Liao, 2002). 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., 2007b), 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 -5. 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 non-peptide agonists and antagonists. Thus, the two chemokine agonists CCL3 and CCL5 were included together with the CCR1-selective non-peptide antagonist BX471 (Horuk, 2005) as well as two chemically different series of small-molecule agonists: a novel carbamide-based series (Petersen et al., 2013;Jensen et al., 2011) and metal-ion chelators (Jensen et al., 2008;Thiele et al., 2012). As CCR5 is the

[1] The nomenclature for the positions of transmembrane-located residues within the 7TM receptors are provided according to the nomenclature suggested by Schwartz/Baldwin (Baldwin, 1993;Schwartz, 1994)followed by the one suggested by Ballesteros (Ballesteros and Weinstein, 1995).

closest homolog to CCR1, and overlap in ligand-binding profiles with CCR1 (Thiele et al., 2011;Jensen et al., 2008;Thiele et al., 2012;Murphy et al., 2000), 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.

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MATERIALS AND METHODS

Materials – CCL3 were purchased from R&D. The cDNA for WT human CCR1 was kindly provided by Tim Wells (Serono Pharmaceutical Research Institute, Geneva, Switzerland), whereas WT CCR5 was cloned in house from a spleen cDNA library. ^{3}H-*myo*-Inositol (PT6-271) and ^{125}I-CCL3 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The promiscuous chimeric G-protein GαΔ6qi4myr, was kindly provided by Evi Kostenis (University of Bonn, Germany).

Site-directed Mutagenesis – Point mutations were introduced in the receptor, using the polymerase chain reaction overlap extension technique with human chemokine receptors as template. All reactions were carried out using the Pfu polymerase (Stratagene). The mutant receptors were cloned into the eukaryotic expression vector pcDNA3.1+, and expressed in E. coli cells. The mutations were verified by DNA sequencing by WMG.

Transfections and tissue culture – COS-7 cells were grown at 10% CO_2 and 37°C in Dulbecco's modified Eagle's medium with glutamax (Gibco, Cat. No 21885-025) adjusted with 10% fetal bovine serum, 180 u/ml penicillin and 45 ug/ml streptomycin (PenStrep). Transfection of COS-7 cells was performed by the calcium phosphate precipitation method. For 75 cm^2 flasks, 10 μg receptor DNA and 15 μg GΔ6αqi4myr were mixed with 30 μl 2 mM $CaCl_2$ and TE buffer to a total volume of 240 μl. 240 μl 2xHBS was added and the mixture was left in 45 min for precipitation before added to the cells together with cloroquine 2 mg/ml. The flasks were incubated for 5 h at 37°, before replacing the media with 10 ml fresh COS-7 media.

Inositol-Phosphate assay (IP-turnover) – COS-7 cells were transfected as described above. The co-transfection with GαΔ6qi4myr, turns the Gi signal into a Gq coupled signal, making it possible to measure the PLC activation as IP-turnover. One day after transfection, the cells were seeded in 24 well plates (1,5 x 10^5 cells/well) and incubated with 2 μCi of ^{3}H-*myo*-Inositol in 0.3 ml growth medium for 24 hours. Cells were washed twice with HBSS supplemented with $CaCl_2$ and $MgCl_2$ (GIBCO14025) and afterwards incubated for 15 min in 0.2 ml buffer supplemented with 10 mM LiCl prior to ligand addition followed by 90 min incubation. Cells were extracted by addition of 1 ml 10mM formic acid followed by incubation on ice for 30 min. The generated [3H]inositol phosphate were purified on AG 1X8 anion exchange resin. Determinations were made in duplicates

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Binding experiments – COS-7 cells were transiently transfected, and transferred to culture plates and left over night. The number of cells seeded per well was determined by the apparent expression efficiency of the receptor and was aimed to obtain 5 – 10% specific binding of the added radioactive ligand. The competition binding was carried out for three hours incubation at 4°C using 10-15 pM ^{125}I-CCL3 along with the unlabeled ligand in 0.3 ml 50mM Hepes buffer pH 7.4 supplemented with 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 0,5 % BSA. After incubation, the cells were washed twice in 4°C binding buffer supplemented with 0.5 M NaCl. Nonspecific binding was determined as the binding in the presence of 0.1 µM unlabeled CCL1, -3 or -5 respectively. Determinations were made in duplicates. B_{max} values were calculated based on the homologous competition binding experiments by the following fomula: ((Top of curve in CPM – bottom of curve in CPM)*IC50 (M)*assay volume (L)*10^{15})/(total radioactivity (in CPM)*10^5cells) = fmol/10^5 cells

Surface Enzyme-Linked Immunosorbent Assay (ELISA) – 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 TBS (50 mM Tris-base, 150 mM NaCl, pH 7.6), and subsequently fixed in 150 µl 4% formaldehyde for 15 min. After three washes in TBS, the cells were blocked in TBS containing 2% BSA for 30 min. Subsequently, the cells were incubated with mouse M1 anti-FLAG antibody 2 µg/ml in TBS containing 1% BSA and 1 mM $CaCl_2$ for 2 hours. Following three washes in TBS with 1 mM CaCl, the cells were incubated with goat anti-mouse horseradish peroxidise-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 peroxidise substrate according to the manufacturer's instructions.

β-arrestin recruitment - Recruitment of β-arrestin was measured using the PathHunter™ β-arrestin assay (DiscoverX). 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 PCR. Assays were performed in a CHO-K1 EA-arrestin cell line stably expressing β-$arrestin_1$ or β-$arrestin_2$ 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 reagent (0.15 µl/well). 24 hours after transfection the medium is removed and 100 ul Opti-MEM® I (Gibco®) is added. The next day,

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cells are stimulated with varying concentrations of CCL3 or CCL5 for 90 minutes at 37 °C. The Detection Reagent Solution® is added prior to 60 minutes incubation at room temperature and the β-arrestin recruitment is measured as chemiluminescence using Perkin Elmer EnVision 2104 Multilable Reader.

Bioinformatics - The nucleotide sequences of the chemokine receptors were acquired from GenBank of NCBI. The alignment was done in Geneious 6.0.5 using MAFFT v6.814b. 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 web-based program weblogo.berkeley.edu.

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RESULTS

Highly conserved localization of the disulfide bridges and length of extracellular regions in chemokine receptors. When comparing 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 (Mirzadegan 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., 2007a) (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 is exactly 25 amino acids to the ProVII:17/7.50 (Fig. 1F, black symbols) and when counted from ProVI:15/6.50 there is 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, give 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 lengths of the extracellular loops (and of the region between the N-terminal Cys

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and TM-1) are very similar within the chemokine family. When assessing the published structures of CXCR1 and CXCR4 the same tendencies are observed (Park et al., 2012;Wu et al., 2010). We therefore looked at the primary structures in these regions of all endogenous chemokine receptors. As shown in Supplemental Figure 1 there is generally low amino acid conservation in the extracellular receptor parts compared to the transmembrane regions, reflecting that chemokine-recognition relies on extracellular receptor regions (Allen et al., 2007a). However, there is a high degree of similarity in certain smaller extracellular areas, like for instance the WxFG 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 (Park et al., 2012;Wu et al., 2010). 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. In order 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 Gαi-subunit, but transduces a Gαq signal. Consequently, G protein-mediated activation was measured by IP3-accumulation. As seen in Fig. 2A-B and Table 1, CCL3- and CCL5-mediated activation was highly impaired and for some mutations completely absent, whereas they acted as previously described on CCR1 WT (Jensen et al., 2008;Neote et al., 1993). ELISA-based determination of surface expression uncovered WT-like expression of [C24A]- and [C273A]-CCR1 whereas that of [C106A]- and [C183A]-CCR1 (with disrupted 7TM-conserved bridge) was reduced up to 6-fold compared to CCR1 WT (Table 1).

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Given the preserved receptor expression, the impaired activation (Fig. 2A and B) could either be due to impaired ligand *binding* or that the mutations interfered 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. 2C 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, whereas these act independently of the chemokine-conserved bridge. Given the small size and thus more restricted binding mode compared to the endogenous ligands (Jensen et al., 2008;Handel and Lau, 2004), 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 due 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, whereas these ligands acted with WT-like potencies and efficacies in the absence of the chemokine-conserved bridge (Fig. 4A and B, Table 2). The same pattern was observed for the more potent carbamide-based CCR1 agonist TUG-584 (Supplemental Figure 2A and B), and thus demonstrated that the chemokine-conserved bridge – in contrast to the 7TM-conserved bridge - is not essential for activation as such, but only for activation mediated by the chemokines.

Positive allosteric modulators shift towards 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 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

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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 ^{125}I-CCL3 binding upon disruption of the 7TM-conserved disulfide bridge, and in fact displayed an entirely different binding pattern, as they shifted towards 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 (Fig. 4A 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:06/7.39 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 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, heterologous competition binding experiments were performed 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 across the whole binding pocket interacting with TM-1, -2, -3 and -7 (Vaidehi et al., 2006). As seen in Supplemental Figure 2C and D, BX471 displaced ^{125}I-CCL3 with WT-like affinities in all four Cys-substituted receptors, indicating that the main binding crevice is maintained in the absence of the disulfide bridges.

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Different roles of the extracellular areas in CCR5 and CCR1. As CCR5 ligand-binding profile overlaps with that of CCR1 (Murphy et al., 2000;Thiele et al., 2011;Thiele et al., 2012;Jensen et al., 2008) 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 (Table 3 and Fig. 5A and B). This was not due to 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 (data not shown). 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. 5C 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 (Fig. 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, Supplemental Figure 2E and F and Table 3), however compared to 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 and Table 3), as was the case in CCR1 (Fig. 4C and D). Thus, there was no difference between CCR1 and -5 with regard to the impact of the chemokine-conserved bridge for small-molecule agonist action, whereas in CCR5, small-molecule activation was maintained despite disruption of the 7TM-conserved bridge.

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DISCUSSION

Here we assess the importance of the conserved disulfide bridges for CCR1 function, and compare it with CCR5. The strong positional conservation for the implicated cysteine residues and thereby also 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 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 rigidity, as well as to provide general important mechanisms for receptor regulation (Peeters et al., 2011). The disulfide bridge between TM-3 and ECL-2 is present in 92% of all class A receptors (Storjohann et al., 2008), and early (pre-crystal) structure studies showed an essential role of this bridge for rhodopsin stability (Davidson F.F et al., 1994). Similar experiments in the gonadotropin- and thyrotropin-releasing hormone receptors, μ-opioid, bradykinin, muscarinic, vasopressin, and NK1 receptors have shown reduced ligand activation caused by impaired ligand binding upon disruption of this bridge (Cook and Eidne, 1997;Perlman et al., 1995;Zhang P. et al., 1999;Herzig et al., 1996;Zeng et al., 1999;Storjohann et al., 2008;Conner et al., 2007;Elling et al., 2000;Davidson F.F et al., 1994). Experiments in chemokine receptors include CXCR4, where the 7TM-conserved bridge was found to be important for maintained receptor structure, as a series of structure-dependent antibodies was not able to recognize CXCR4 with single or double Ala substitutions of the corresponding Cys residues (Chabot et al., 1999). Moreover, disruption of the disulfide bridge retained at least 90% of the receptor population intracellularly (Chabot et al., 1999). Similar findings were made in CCR5, where Ala substitution of these Cys residues reduced surface expression by 40-70% and structure-dependent antibodies and CCL4 failed to recognize any of the mutant receptors (Blanpain et al., 1999). The differences in surface expression between these results, and what was observed in present study, are likely explained by differences in cell-lines and transfection-procedures. In CXCR2, Ala

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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, e.g. the MC receptors (Benned-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 conserved (Fig. 1C).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 (Rasmussen et al., 2007;Warne et al., 2008;Jaakola VP et al., 2008;Hanson et al., 2008;Palczewski et al., 2000;Scheerer 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, firstly allowing the ligand to enter the binding crevice and secondly to stabilize the ligand-induced receptor conformations (Peeters et al., 2011;Avlani et al., 2007).

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, and thereby provide important mechanisms of regulating receptor activity and ligand binding (Jaakola VP 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 (Fig. 2 and 5), as also suggested by previous studies in CCR5, CXCR1, -2 and -4 (Blanpain et al., 1999;Leong et al., 1994;Limatola et al., 2005;Chabot et

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al., 1999). Yet, this may not hold true in all chemokine receptors, as a study in CCR6 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 (Dragic et al., 1998;Samson et al., 1997). Furthermore, consistent with our findings in CCR5, Blanpain and coworkers have shown that extracellular conformation-dependent antibodies failed to recognize CCR5 when one or the other disulfide bridge was destroyed (Blanpain et al., 1999). Taken together with the overall extracellular binding mode of chemokines (Pease et al., 1998;Xanthou et al., 2003), this explains why CCL3 and -5 were not able to bind to CCR5. However, the maintained small molecule activation in CCR5 (Fig. 6 and Supplemental Figure 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 and colleagues (Pease et al., 1998;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, CX_3CL1 depends on residues in the N-terminus as well as ECL-3 of CX_3CR1 (Chen Y. et al., 2006) and CXCL16 depends on all extracellular regions of CXCR6 (Petit S.J. et al., 2008). Likewise, we and others have reported that the chemokine-induced activation is critically dependent upon the receptor N-terminus in CCR1, -2, and -8 (Jensen et al., 2008;Jensen et al., 2011;Samson et al., 1997).

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 (Jensen et al., 2007;Jensen et al., 2008;Vaidehi et al., 2006;Berkhout et al., 2003;de Mendonca et al., 2005;Maeda et al., 2006;Rosenkilde et al., 2007;Thiele

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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, and 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 (Jaakola VP et al., 2008;Palczewski et al., 2000;Wu et al., 2010;Cherezov et al., 2007;Warne et al., 2008). Likewise, mutational studies have revealed participation of ECL-2 in small-molecule ligand binding in CCR1, -5 and -8 (Jensen et al., 2011;Thiele et al., 2011;Maeda et al., 2006).

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 of for possible ligand interaction to otherwise non-accessible conformations. These considerations could explain alterations in efficacy and B_{max} values of both small-molecule and chemokine agonists. Thus, a shift in receptor population towards 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_{max} values) however at a concentration to low to observe activation.

Concerning the 7TM-conserved disulfide bridge we find that proper constrainment of ECL-2 is important for the *action*, but not *binding* of chemokine and small-molecule agonists in CCR1 (Fig. 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 7TM-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.

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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 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.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Rummel, Rosenkilde

Conducted experiments: Rummel, Thiele, Hansen

Contributed reagents: Petersen, Ulven

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

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FIGURE LEGENDS

Figure 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.

Figure 2. Disruption of conserved disulfide bridges impairs chemokine action, but maintain binding. IP-accumulation experiments in transiently transfected COS-7 cells, given in CPM values (A and B), and binding experiments curves, normalized against own CCL3 binding (C and D), of CCR1 WT (□), [C24A]-CCR1 (▼), [C106A]-CCR1 (▲), [C183A]-CCR1 (■) and [C272A]-CCR1 (●). (n=3-78).

Figure 3. β-arrestin recruitment depend on maintained disulfide bridges. CCL3 induced β-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 CCL-3 induced β-arrestin recruitment at CCR1 WT. (n=3).

Figure 4. Small molecule activation in CCR1 is impaired and shift towards competitive antagonism when the 7TM-conserved bridge is disrupted. A and B, IP-accumulation experiments of 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).

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Figure 5. Endogenous ligand activation and binding is not retained in CCR5, when disrupting either of the two disulfide bridges. IP-accumulation experiments in transiently transfected COS-7 cells, given as CPM values (A and B), and binding experiments curves, normalized against CCL3 binding on CCR5 WT (C and D), of CCR5 WT (□), [C20A]-CCR1 (▼), [C101A]-CCR1 (▲), [C178A]-CCR1 (■) and [C269A]-CCR1 (●). (n=3-78).

Figure 6. Small molecule ligand activation is retained when disrupting either of the disulfide bridges in CCR5. IP-accumulation experiments of CuBip (circles) and CuPhe (squares) on CCR5 WT (open symbols) and mutant receptors (closed symbols). A, [C178A]-CCR5 represents results from disrupted 7TM-conserved bridge. B, [C269A]-CCR5 represents results from disrupted chemokine-conserved bridge. All results are normalized against CCL3 induced activation of CCR1 WT, and are the mean of at least 4 experiments.

Figure 7. Summary of the dependencies of CCL3 and metal-ion chelators (MC) for extracellular disulfide bridges in CCR1 (A) and CCR5 (B). CCR1 and CCR5 WT are shown in the upper panel. Receptors with a disrupted 7TM-conserved bridge are shown in the middle panel and receptors with a disrupted chemokine-conserved bridge in the lower panel. A, left column: In the presence of CCL3, CCR1 WT is activated (green color), whereas the mutant receptors binds CCL3, but remains inactive (grey color). The chemokine is suggested to bind in an altered manner when the 7TM-conserved bridge is disrupted. MC's (middle column) activate both CCR1 WT and mutant receptors with a disrupted chemokine-conserved bridge. A, right column: upon co-administration of MC and CCL3, MC binding increases the affinity of CCL3 in both WT and upon disruption of the chemokine-conserved bridge. In constructs with a disrupted 7TM-conserved bridge, CCL3 is suggested to bind at a site that overlaps with the MC's, so that the two ligands compete. B, left column: In the presence of CCL3, CCR5 WT is activated (green color), whereas CCL3 does not bind to the mutant receptors. Contrary, MC's activate both CCR5 WT and mutant receptors (middle column). B, right column: As previously described, MC's work as positive allosteric modulators on

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CCL3 binding to CCR5 (Thiele et al., 2012). However, as CCL3 binding was absent in the mutant receptors, heterologous binding experiments were not possible to perform.

TABLES

Table 1 Molecular interaction of endogenous ligands with CCR1.

Mutant receptors were screened for surface expression, IP3-accumulation and homologous binding experiments. Surface expression is given as a % of CCR1 wt expression, and the number of experiments is given in "n". The IP-accumulation data displays the potency as logEC_{50} and EC_{50} (nM). The differences in potency of a given ligand compared to the WT receptor is given as "fold". NA means "no activation" and ND means "not determined". Binding experiments are given as B_{max} (in fmol/10^5 cells) and affinity as logIC_{50} and IC_{50} (nM). The difference in affinity of a given ligand compared to the WT receptor is given as “fold”. The numbers of experiments are given in “n”. NB means “no binding”.

	Surface expression		**IP-accumulation – CCL3**				**IP-accumulation – CCL5**				**^{125}I-CCL3/CCL3 binding**				
			Potency				Potency					Affinity			
	% of CCR1 WT	(n)	EC_{50} SEM	EC_{50}	Fold	(n)	EC_{50} SEM	EC_{50}	Fold	(n)	B_{max} SEM	IC_{50} SEM	IC_{50}	Fold	(n)
			(log)	*(nM)*			*(log)*	*(nM)*			*(fmol/10^5 cells)*	*(log)*	*(nM)*		
CCR1 WT	100	(3)	-8,43 ± 0,04	3,7	1,0	(78)	-8,78 ± 0,09	1,6	1,0	(42)	1711 ± 300	-8,04 ± 0,06	9,2	1,0	(19)
C24A	132 ± 4,5	(3)	<-6	<1000	>272	(3)	<-6,5	<716	>436	(3)	48 ± 25	-8,40 ± 0,35	4,0	0,44	(3)
C106A	19 ± 1,3	(3)	NA		NA	(4)	NA		NA	(3)	38 ± 10	-8,11 ± 0,10	7,8	0,85	(4)
C183A	17 ± 2,3	(3)	<-6	<1000	>272	(8)	<-6	<1000	>609	(5)	114 ± 58	-8,57 ± 0,23	2,7	0,29	(5)
C273A	123 ± 21	(3)	<-6	<1000	>272	(4)	<-6	<1000	>609	(4)	110 ± 47	-8,28 ± 0,04	5,2	0,57	(4)
C106A-E286A	22 ± 0,9	(3)	NA		NA	(2)	NA		NA	(2)	NB				(3)
C183A-E286A	14 ± 1,2	(3)	NA		NA	(2)	NA		NA	(2)	NB				(3)

Table 2. Molecular interaction of small-molecule ligands with CCR1.

Ala substitutions of extracellular Cys residues were screened in IP3-accumulation and heterologous binding experiments. The IP-accumulation data displays the potency as logEC_{50} and EC_{50} (nM). The difference in potency of a given ligand compared to the WT receptor is given as "fold". Binding affinity is given as logIC_{50} and IC_{50} (nM). The difference in affinity of a given ligand compared to the WT receptor is given as "fold". Results highlighted in bold display allosteric displacement (in contrast to allosteric enhancement). The numbers of experiments is given in ”n”

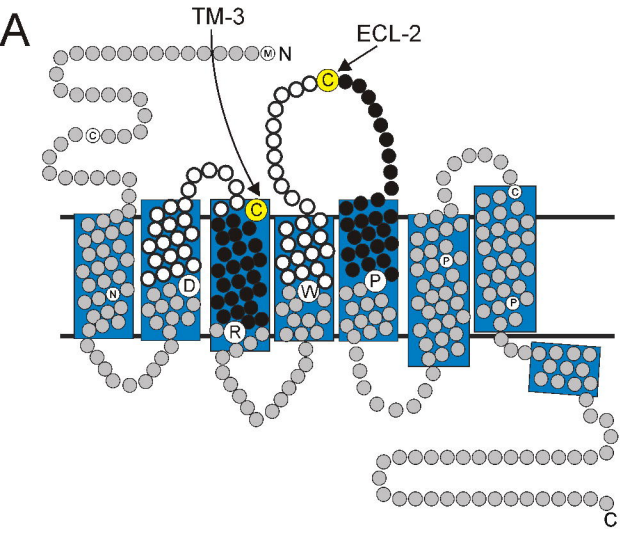
	IP-accumulation - CuBip				**IP-accumulation - CuPhe**				**^{125}I-CCL3/CuBip binding**			**^{125}I-CCL3/CuBip binding**			**IP-accumulation – TUG-584**			
	Potency				Potency				Affinity			Affinity			Potency			
	EC_{50} SEM	EC_{50}	Fold	(n)	EC_{50} SEM	EC_{50}	Fold	(n)	IC_{50} SEM	IC50	(n)	IC_{50} SEM	IC_{50}	(n)	EC_{50} SEM	EC_{50}	Fold	(n)
	(log)	*(μM)*			*(log)*	*(μM)*			*(log)*	*(μM)*		*(log)*	*(μM)*		*(log)*	*(μM)*		
CCR1 WT	-4,68 ± 0,11	21	1,0	(14)	-4,97 ± 0,09	11	1,0	(14)	-4,32 ± 0,03	48	(9)	-4,57 ± 0,06	27	(10)	-6,43 ± 0,09	0,37	1,0	(9)
C24A	-4,40 ± 0,10	40	1,9	(3)	-5,19 ± 0,07	6,4	0,6	(3)							-6,09 ± 0,10	0,82	2,2	(3)
C106A	<-3	<1000	>48	(3)	<-3	<1000	>91	(3)							<-4	<100	>270	(3)
C183A	<-3	<1000	>48	(4)	<-3	<1000	>91	(4)	**-5,68 ± 0,10**	**2,1**	(5)	**-5,88 ± 0,20**	**1,3**	(6)	<-4	<100	>270	(3)
C273A	-4,41 ± 0,11	39	1,8	(6)	-4,92 ± 0,17	12	1,1	(5)	-4,42 ± 0,03	38	(4)	-4,67 ± 0,07	22	(4)	-6,22 ± 0,24	0,61	1,6	(3)

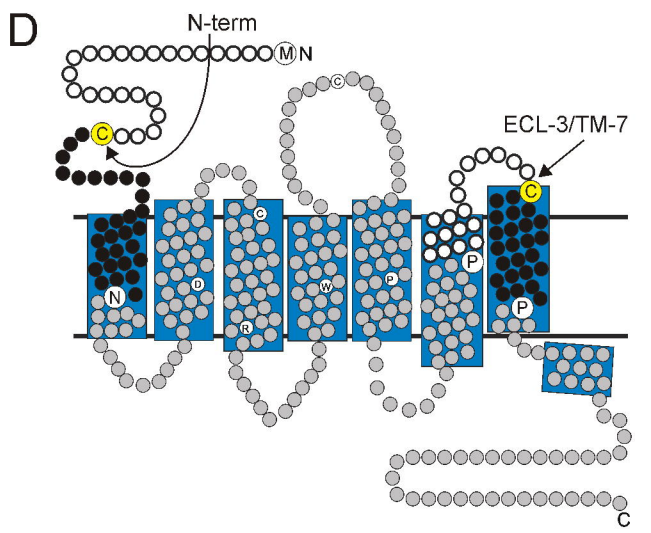
Table 3. Molecular interaction of endogenous and small-molecule ligands with CCR5.

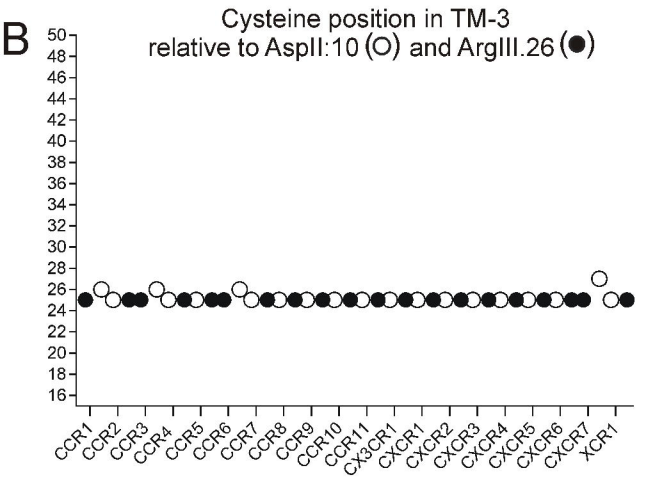
Ala substitutions of extracellular Cys residues were screened for surface expression, IP3-accumulation and homologous binding experiments. Surface expression is given as a % of CCR5 WT expression, and the number of experiments is given in "n". The IP-accumulation data displays the potency as logEC_{50} and EC_{50} (nM). The differences in potency of a given ligand compared to the WT receptor is given as "fold". NA means "no activation".

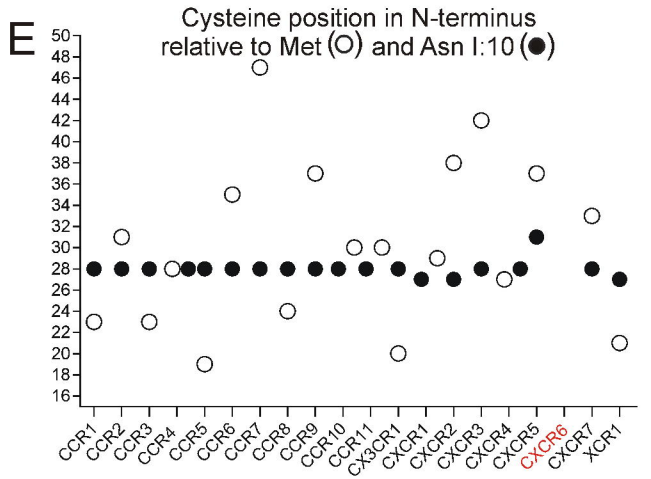
	Surface expression		**IP-accumulation – CCL3**				**IP-accumulation – CuBip**				**IP-accumulation – CuPhe**				**IP-accumulation – TUG-588**			
			Potency				Potency				Potency				Potency			
	% of CCR5 WT	(n)	EC_{50} SEM *(log)*	EC_{50} *(nM)*	Fold	(n)	EC_{50} SEM *(log)*	EC_{50} *(μM)*	Fold	(n)	EC_{50} SEM *(log)*	EC_{50} *(μM)*	Fold	(n)	EC_{50} SEM *(log)*	EC_{50} *(μM)*	Fold	(n)
CCR5 WT	100 ± 0,0	(3)	-8,00 ± 0,17	10	1,0	(8)	-4,70 ± 0,05	20	1,0	(6)	-5,01 ± 0,22	9,8	1,0	(5)	-5,75 ± 0,18	1,8	1,0	(3)
C20A	81 ± 11	(3)	<-6	<1000	>100	(3)	-4,67 ± 0,09	21	1,1	(3)	-5,46 ± 0,26	3,5	0,36	(3)	-5,27 ± 0,21	5,4	3,0	(3)
C101A	52 ± 16	(3)	NA		NA	(3)	-4,78 ± 0,12	16	0,82	(3)	-4,56 ± 0,32	28	2,8	(4)	-5,44 ± 0,19	3,6	2,0	(3)
C178A	61 ± 16	(3)	NA		NA	(4)	-4,99 ± 0,20	10	0,5	(3)	-5,13 ± 0,36	7,4	0,75	(4)	-5,32 ± 0,09	4,7	2,6	(3)
C269A	64 ± 14	(3)	<-6	<1000	>100	(4)	-4,73 ± 0,12	19	0,9	(3)	-5,37 ± 0,32	4,2	0,43	(3)	-5,79 ± 0,11	1,6	0,91	(3)

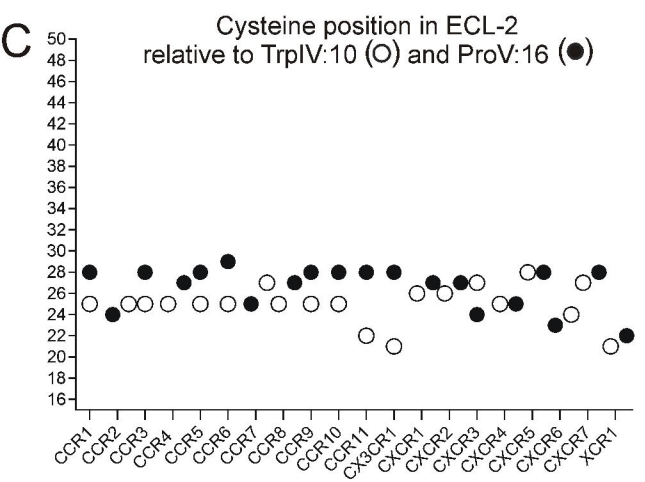
Figure 1


A
TM-3
ECL-2
N
C
D
R
W
P


D
N-term
ECL-3/TM-7
N
C
D
R
W
P


B
Cysteine position in TM-3
relative to AspII:10 (O) and ArgIII.26 (●)
CCR1 CCR2 CCR3 CCR4 CCR5 CCR6 CCR7 CCR8 CCR9 CCR10 CCR11 CX3CR1 CXCR1 CXCR2 CXCR3 CXCR4 CXCR5 CXCR6 CXCR7 XCR1


E
Cysteine position in N-terminus
relative to Met (O) and Asn I:10 (●)
CCR1 CCR2 CCR3 CCR4 CCR5 CCR6 CCR7 CCR8 CCR9 CCR10 CCR11 CX3CR1 CXCR1 CXCR2 CXCR3 CXCR4 CXCR5 CXCR6 CXCR7 XCR1


C
Cysteine position in ECL-2
relative to TrpIV:10 (O) and ProV:16 (●)
CCR1 CCR2 CCR3 CCR4 CCR5 CCR6 CCR7 CCR8 CCR9 CCR10 CCR11 CX3CR1 CXCR1 CXCR2 CXCR3 CXCR4 CXCR5 CXCR6 CXCR7 XCR1

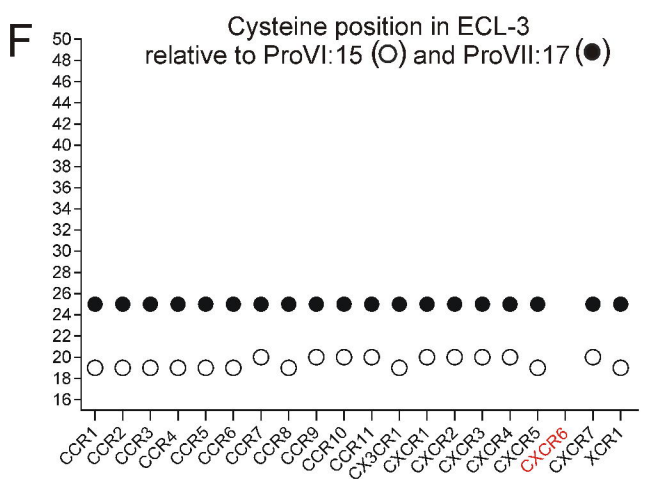

F
Cysteine position in ECL-3
relative to ProVI:15 (O) and ProVII:17 (●)
CCR1 CCR2 CCR3 CCR4 CCR5 CCR6 CCR7 CCR8 CCR9 CCR10 CCR11 CX3CR1 CXCR1 CXCR2 CXCR3 CXCR4 CXCR5 CXCR6 CXCR7 XCR1

Figure 2

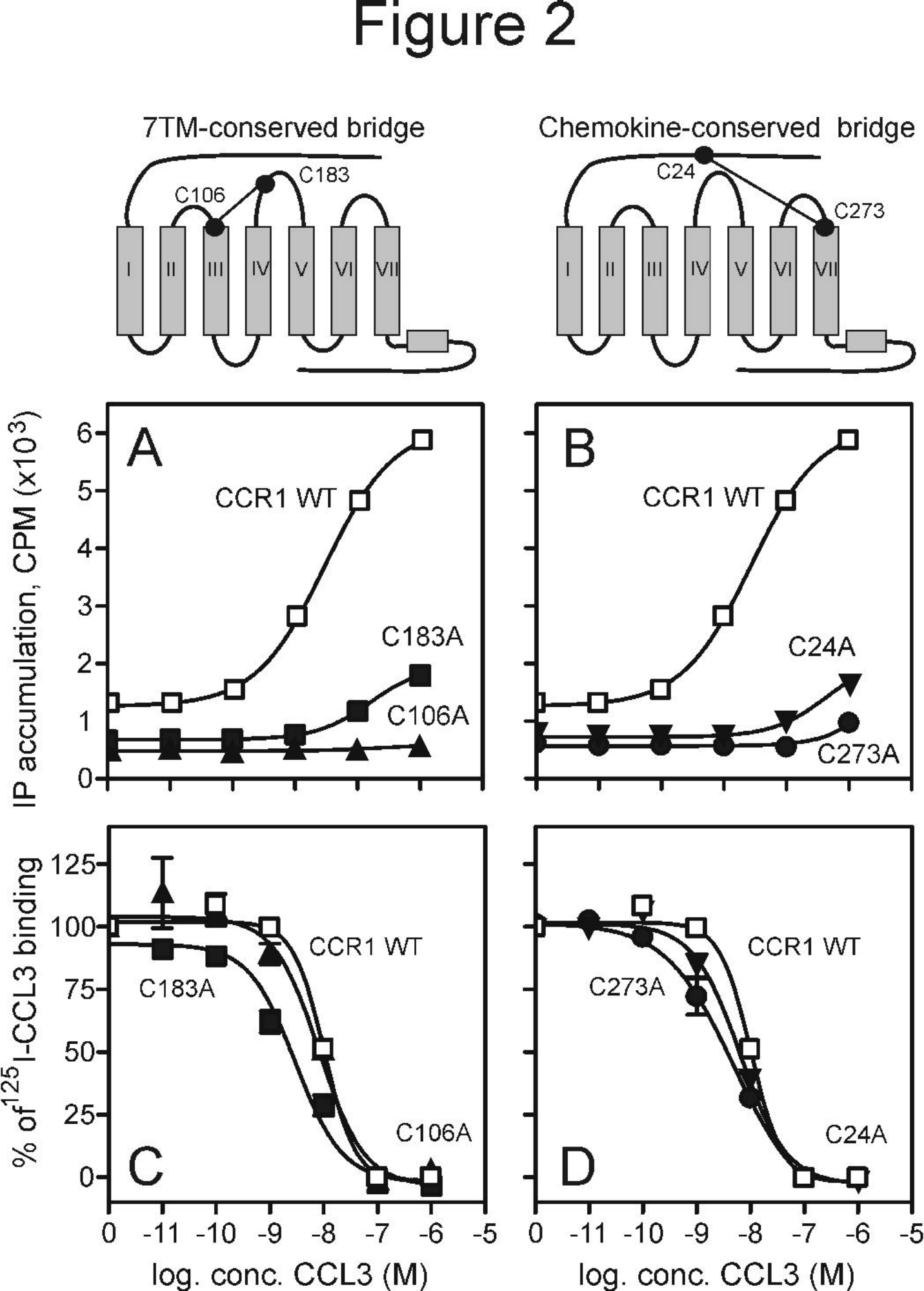

7TM-conserved bridge
C106
C183
I
II
III
IV
V
VI
VII
Chemokine-conserved bridge
C24
C273
A
CCR1 WT
C183A
C106A
B
CCR1 WT
C24A
C273A
IP accumulation, CPM (x10^3)
0
1
2
3
4
5
6
% of ^{125}I-CCL3 binding
0
25
50
75
100
125
C
CCR1 WT
C183A
C106A
D
CCR1 WT
C273A
C24A
0 -11 -10 -9 -8 -7 -6 -5
log. conc. CCL3 (M)
0 -11 -10 -9 -8 -7 -6 -5
log. conc. CCL3 (M)

Figure 3

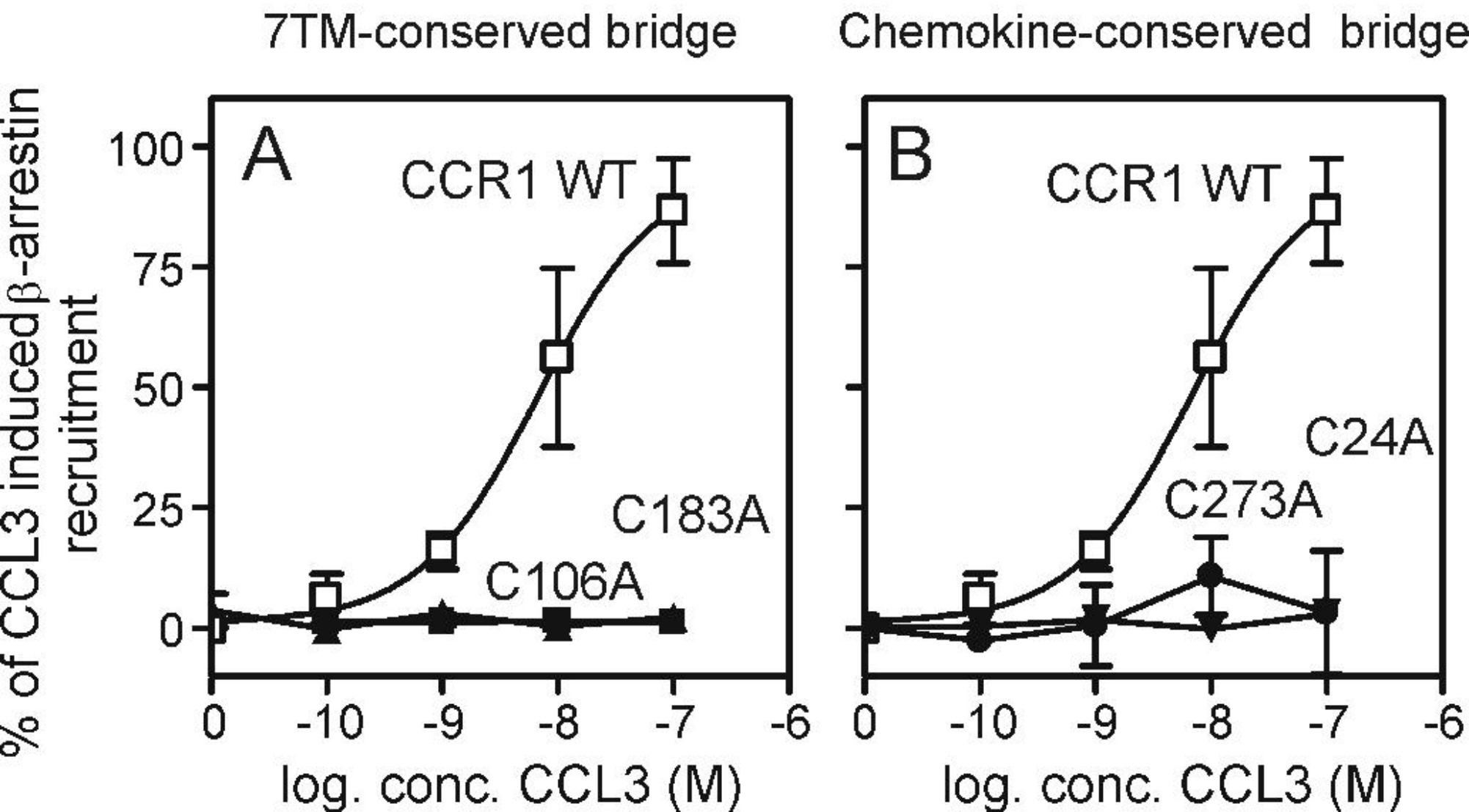

7TM-conserved bridge
Chemokine-conserved bridge
A
B
CCR1 WT
CCR1 WT
C183A
C106A
C24A
C273A
100
75
50
25
0
0
-10
-9
-8
-7
-6
% of CCL3 induced β-arrestin recruitment
log. conc. CCL3 (M)
log. conc. CCL3 (M)

Figure 4

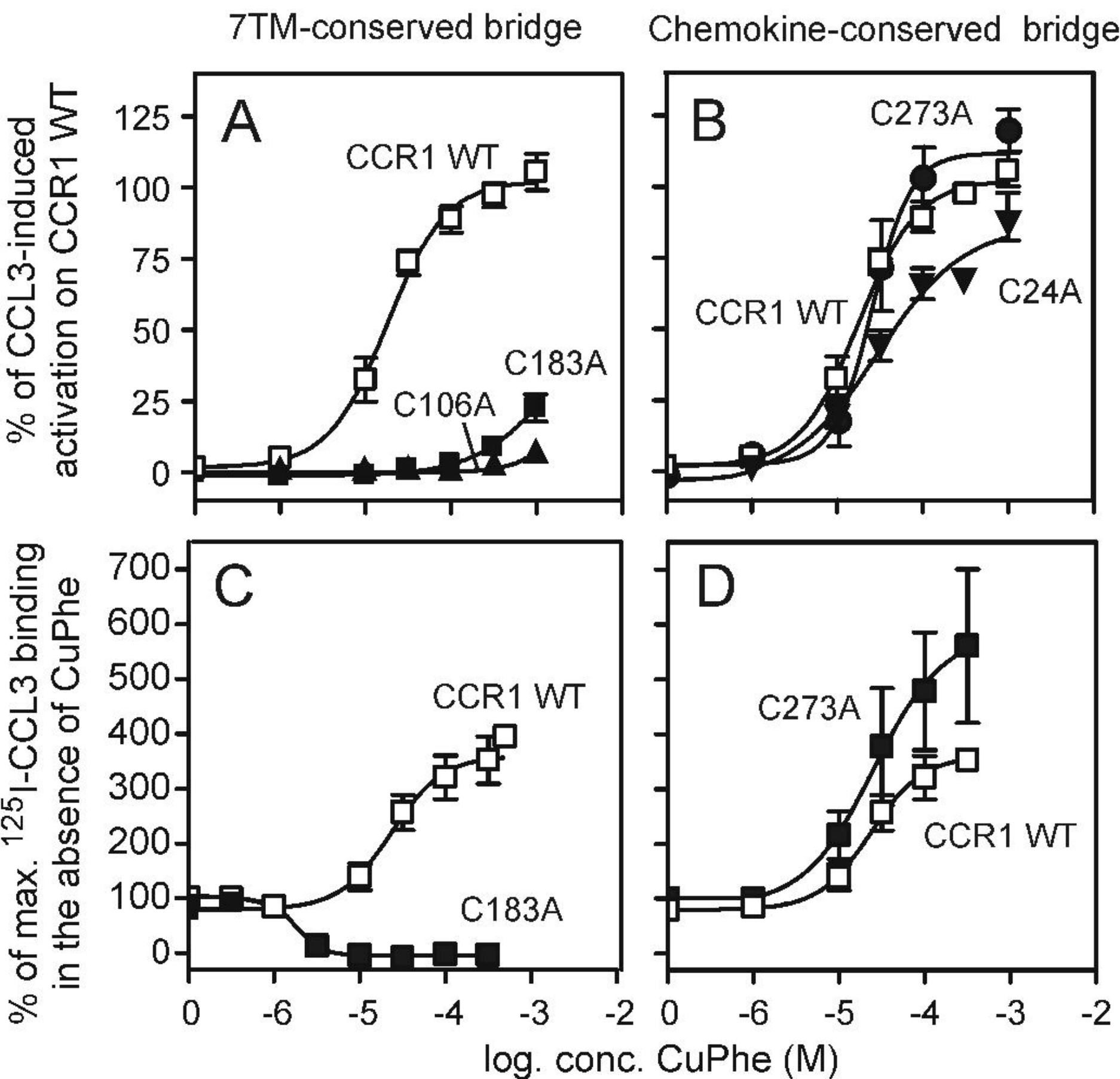

7TM-conserved bridge
Chemokine-conserved bridge
A
CCR1 WT
C183A
C106A
B
C273A
CCR1 WT
C24A
C
CCR1 WT
C183A
D
C273A
CCR1 WT
% of CCL3-induced activation on CCR1 WT
% of max. 125I-CCL3 binding in the absence of CuPhe
log. conc. CuPhe (M)
125
100
75
50
25
0
700
600
500
400
300
200
100
0
-6
-5
-4
-3
-2

Figure 5

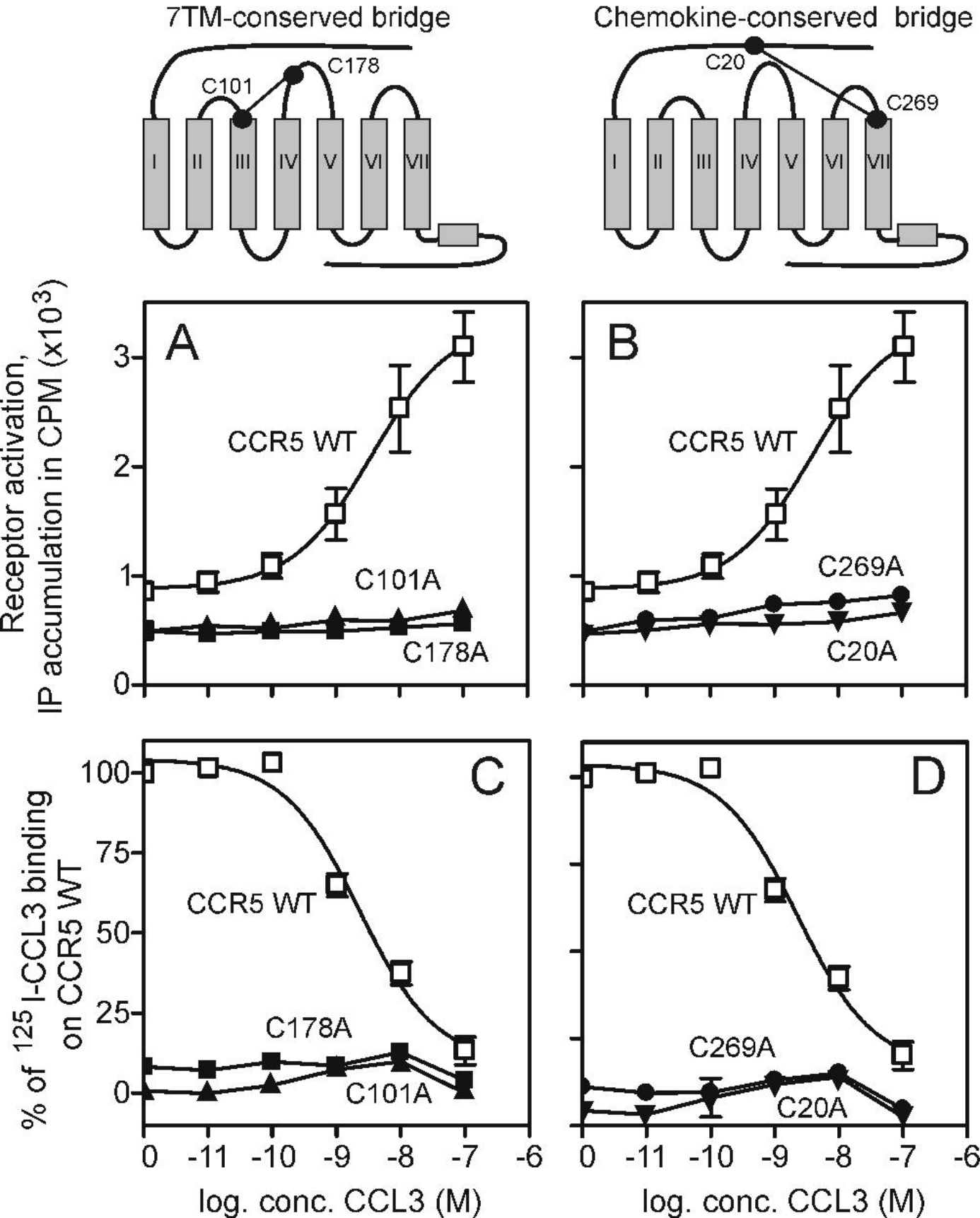

7TM-conserved bridge
C101
C178
I
II
III
IV
V
VI
VII
Chemokine-conserved bridge
C20
C269
Receptor activation,
IP accumulation in CPM (x10^3)
A
CCR5 WT
C101A
C178A
B
CCR5 WT
C269A
C20A
% of ^{125}I-CCL3 binding
on CCR5 WT
C
CCR5 WT
C178A
C101A
D
CCR5 WT
C269A
C20A
log. conc. CCL3 (M)
log. conc. CCL3 (M)

Figure 6

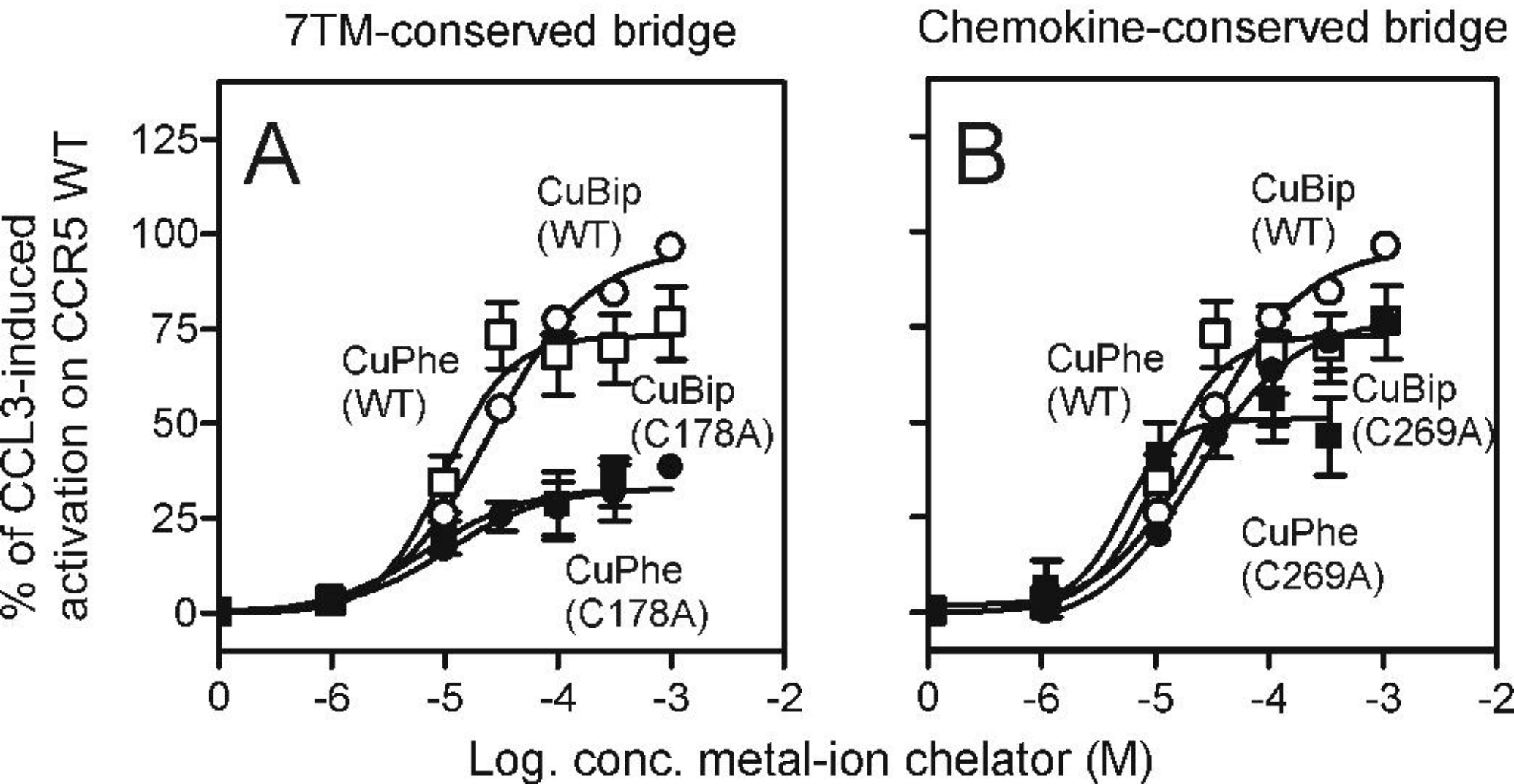
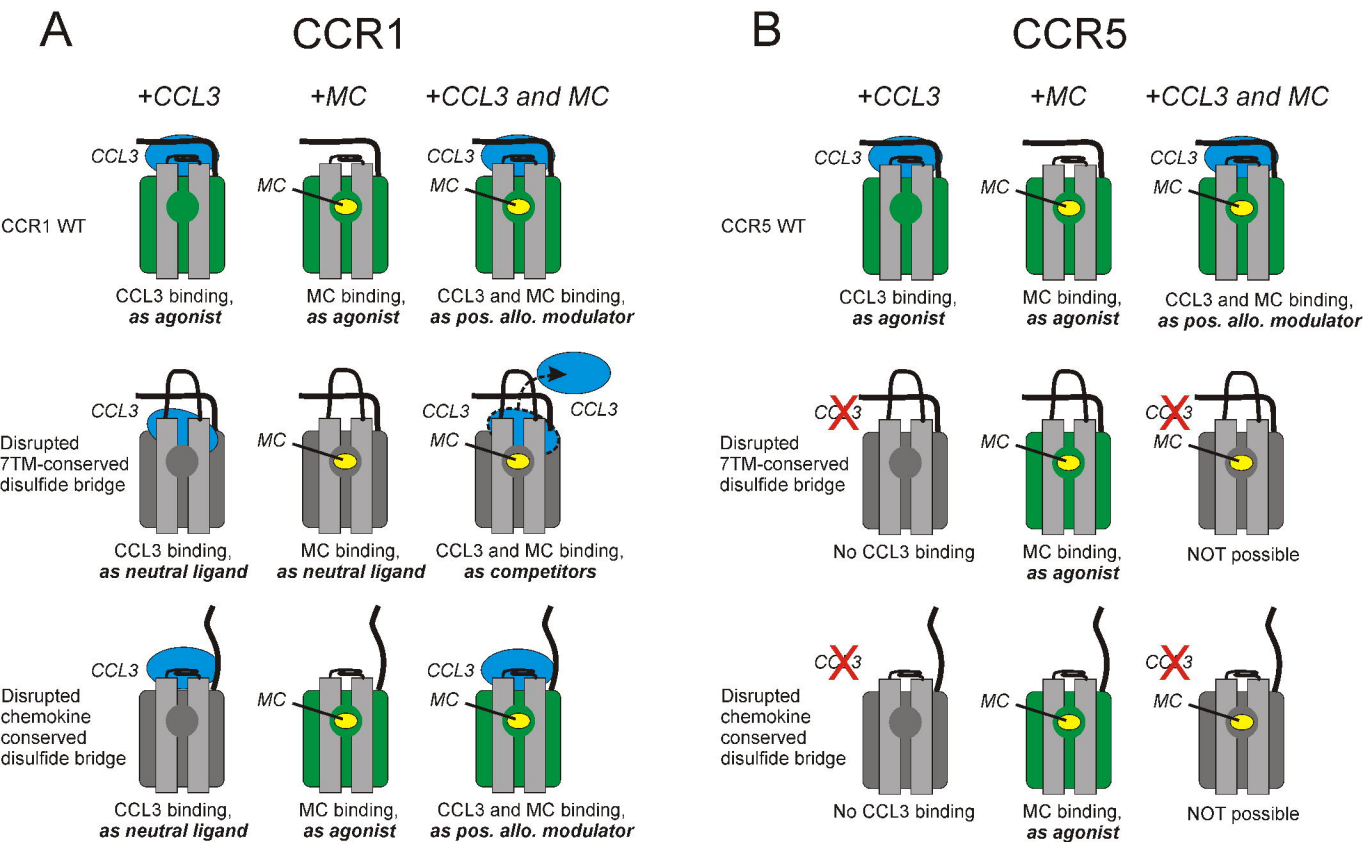

7TM-conserved bridge
Chemokine-conserved bridge
A
B
CuBip (WT)
CuPhe (WT)
CuBip (C178A)
CuPhe (C178A)
CuBip (C269A)
CuPhe (C269A)
% of CCL3-induced activation on CCR5 WT
125
100
75
50
25
0
0
-6
-5
-4
-3
-2
Log. conc. metal-ion chelator (M)

Figure 7


A
CCR1
+CCL3
+MC
+CCL3 and MC
CCR1 WT
CCL3
MC
CCL3 binding, as agonist
MC binding, as agonist
CCL3 and MC binding, as pos. allo. modulator
Disrupted 7TM-conserved disulfide bridge
CCL3 binding, as neutral ligand
MC binding, as neutral ligand
CCL3 and MC binding, as competitors
Disrupted chemokine conserved disulfide bridge
CCL3 binding, as neutral ligand
MC binding, as agonist
CCL3 and MC binding, as pos. allo. modulator
B
CCR5
+CCL3
+MC
+CCL3 and MC
CCR5 WT
CCL3 binding, as agonist
MC binding, as agonist
CCL3 and MC binding, as pos. allo. modulator
Disrupted 7TM-conserved disulfide bridge
No CCL3 binding
MC binding, as agonist
NOT possible
Disrupted chemokine conserved disulfide bridge
No CCL3 binding
MC binding, as agonist
NOT possible