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Arginine 313 of the putative 8th helix mediates $G\alpha_{q/14}$ coupling of human CC chemokine receptors CCR2a and CCR2b

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ABSTRACT

In man, two CC chemokine receptor isoforms, CCR2a and CCR2b, are present that belong to the rhodopsin-like G protein-coupled receptor family, and couple to G_i and G_q family members. The CCR2 receptors are known to regulate canonical functions of chemokines such as directed migration of leukocytes, and to potentially control non-canonical functions such as differentiation, proliferation, and gene transcription of immune and non-immune cells. We recently reported on the activation of phospholipase C isoenzymes and RhoA GTPases by coupling of the two CCR2 receptors to members of the G_q family, in particular $G\alpha_q$ and $G\alpha_{14}$. So far little is known about the structural requirements for the CCR2/$G_{q/14}$ interaction. Interestingly, the CCR2 receptor isoforms are identical up to arginine 313 (R^{313}) that is part of the putative 8th helix in CCR2 receptors, and the 8th helix has been implicated in the interaction of rhodopsin-like G protein-coupled receptors with $G\alpha_q$. In the present work we describe that the 8th helix of both CCR2a and CCR2b is critically involved in selectively activating $G\alpha_{q/14}$-regulated signaling. Refined analysis using various CCR2a and CCR2b mutants and analyzing their cellular signaling, e.g. ligand-dependent (i) activation of phospholipase C isoenzymes, (ii) stimulation of serum response factor-mediated gene transcription, (iii) activation of mitogen-activated protein kinases, (iv) internalization, and (v) changes in intracellular calcium concentrations, identified arginine 313 within the amino terminal portion of helix 8 to play a role for the agonist-mediated conformational changes and the formation of a $G\alpha_{q/14}$ binding surface. We show that R^{313} determines $G\alpha_{q/14}$ protein-dependent but not G_i protein-dependent cellular signaling, and plays no role in G_q/G_i-independent receptor internalization, indicating a role of R^{313} in biased signaling of CCR2 receptors.

1. Introduction

In man two CC chemokine receptors, CCR2a and CCR2b have been identified that differ only in their intracellular carboxyl-terminal-most portion, and are activated by the CC chemokine CCL2 [1–3]. The two CCR2 receptors are expressed particularly on monocytes, on endothelial cells, immature dendritic cells, and certain T cell subsets, and their ligand-induced signaling has been implicated in numerous inflammatory and neurodegenerative diseases such as atherosclerosis and asthma, and multiple sclerosis and neuropathic pain [3–7]. Upon ligand binding, the CCR2 receptors regulate the activity of various effector proteins, including adenylyl cyclases, phospholipase C isoenzymes, phosphoinositide (PI) 3-kinase (PI3-kinase), and mitogen-activated protein kinases (MAPK), resulting in a decrease of cAMP production, increase in inositol phosphate formation and changes in intracellular Ca^{2+} concentrations, phosphorylation of MAP kinases, and activation of transcription factors [8–15].

Chemokine receptors belong to the class A G protein-coupled receptors with rhodopsin as the best-characterized prototype [16–18]. Analysis of the crystal structure of rhodopsin confirmed the proposed seven transmembrane topology, and identified an intracellular located fourth loop, also referred to as 8th helix [16]. The 8th helix is located in near proximity to the conserved canonical NPXXY motif at the end of the 7th transmembrane domain, and has been predicted to form an amphipathic helix that is located at the inner leaflet of the plasma membrane and display a positively charged face [16,19]. In rhodopsin the 8th helix plays an important role for the interaction of the receptor with the Gα-subunit and probably Gγ-subunit of the transducing G_t protein [20,21]. Interestingly, in G_q-coupled class A GPCRs in particular the amino terminal portion of the 8th helix has been implicated in the interaction of the receptors with $G\alpha_q$ [22–24]. We recently reported that both CCR2a and CCR2b similarly couple to members of the $G\alpha_q$ family, in particular $G\alpha_q$ and $G\alpha_{14}$, to stimulate inositol phosphate formation via activation of phospholipase C isoenzymes, and to regulate

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RhoA GTPase-dependent activity of the transcription factor serum response factor [15]. Both human CCR2 receptors are identical in their primary amino acid sequence up to arginine 313 in the carboxyl terminal portion of the protein thereby sharing the predicted amino terminal portion of the putative 8th helix [1,2]. Interestingly, only one murine CCR2 receptor exist that resemble the human CCR2b. The amino acid sequence at the end of 7th transmembrane domain and beginning of the putative 8th helix of the mouse CCR2 differ in only one amino acid from two human receptors [25]. In the present work, we studied the role of the amino terminal portion of the 8th helix of human CCR2a and CCR2b in selectively activating $G\alpha_{q/14}$-regulated signaling, and report that arginine 313 plays a critical role in coupling of the receptors to G_q proteins.

2. Material and methods

2.1. Materials

Cell culture media and supplements were obtained from Invitrogen (Darmstadt, Germany), Biochrom (Berlin, Germany), PAA Laboratories (Cölbe, Germany), and Sigma Aldrich (Munich, Germany). Lipofectamine™ 2000 and OptiMEM® I were obtained from Invitrogen, and JetPrime® was obtained from PolyplusTransfection (Illkirch, France). For FACS analysis, monoclonal mouse anti-human CCR2 antibody and monoclonal mouse IgG_{2B} isotype control were purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany), and eBioscience (San Diego, USA). The secondary RPE-conjugated anti-mouse-IgG was purchased from DakoCytomation (Glostrup, Denmark). The antibody anti-$G\alpha_q$ was obtained from Santa Cruz (Dallas, USA). Anti-HA epitope tag, and anti-Flag epitope tag, anti-ERK1/2, and anti-P-ERK1/2 antibodies were purchased from Sigma-Aldrich (Munich, Germany) and Cell Signaling (Danvers, USA), respectively. The anti-β-actin-antibody (clone AC-15) was obtained from Sigma (Munich, Germany). For calcium mobilization studies, Fura-2 acetoxy-methyl-ester (Fura-2-AM) was obtained from Invitrogen (Thermo Fischer Scientific, Darmstadt, Germany). CCL2 (MCP-1) was purchased from PeproTech (Hamburg, Germany), and oleoyl-L-α-lysophosphatidic acid (LPA) was obtained from Sigma Aldrich (Munich, Germany). Pertussis toxin (PTX) was obtained from Alexis Biochemicals (San Diego, USA), and d-*myo*-[$2-^3$H]inositol (NET-114A or NET1156005MC) was purchased from Perkin-Elmer Life Sciences (Boston, USA). The $G\alpha_{q/11}$ inhibitor was purchased from WAKO (Osaka, Japan). CCL2-mCherry was produced in baculovirus-infected insect cells and purified from the supernatants as described in Moepps and Thelen [26].

2.2. Plasmids

The cDNAs encoding human HA epitope tagged CCR2a, Flag epitope-tagged CC2Rb, and human $G\alpha_q$ and $G\alpha_{14}$ were generated or obtained, respectively, as described [15]. The cDNAs encoding CCR2a and CCR2b receptor mutants were generated using the site directed mutagenesis kit from Invitrogen (Thermo Fischer Scientific, Darmstadt, Germany). Detailed information of the oligonucleotides used for site directed mutagenesis can be provided upon request.

2.3. Cell culture and transfection

COS-7 and HEK293 cells were maintained, grown, seeded, and transfected as described [15]. For cultivating of HEK293 cells Dulbecco's modified Eagle's medium (DMEM) (10% (*v*/v) fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) was additionally supplemented with HEPES and sodium pyruvate (both Sigma Aldrich, Munich, Germany). For transfection with JetPrime® the plasmid DNA was mixed with 2 μl of the transfection reagent per μg of DNA in transfection buffer. When necessary empty vector DNA was added to apply equal amounts of DNA to the cells.

2.4. Luciferase reporter assay

To analyze ligand-stimulated, receptor and G protein-mediated activation of transcription factor serum response factor (SRF, [27–29]) luciferase reporter assays were performed using the Dual-Luciferase® Reporter Assay System (Promega, Mannheim, Germany) following the instructions of the manufacturer and as described [15].

2.5. Analysis of inositol phosphate formation

Labelling of cells with 3 μCi/ml D-*myo*-[2-^{3}H]inositol and analyzing phospholipase C isoenzyme-mediated inositol phosphate formation was performed as described [29].

2.6. Membrane preparation and Western blot analysis

Membrane preparations of CCR2 receptor expressing cells were performed as described [15]. Proteins contained in equal aliquots of the lysates were subjected to Western blot analysis and immunoblotting using anti-HA, anti-Flag, and anti-β-actin antibodies. Alternatively, lysates obtained for the luciferase assays were analyzed for protein expression by SDS-PAGE and immunoblotting using anti-$G\alpha_q$ and anti-β-actin antibodies.

2.7. Flow cytometry

Flow cytometry was applied to analyze surface expression of human CCR2-receptor-expressing HEK293 cells. To this end 3×10^5 cells were resuspended in 100 μl PBS and incubated for 30 min at 4 °C with 1 μg/ml of monoclonal mouse anti-human CCR2 IgG_{2b} antibody or monoclonal mouse IgG_{2B} isotype control. The cells were washed once with PBS, were incubated for another 30 min at 4 °C in the dark with Alexa-488-conjugated secondary antibody at a dilution of 1:1000, washed again with PBS, fixed with 1% paraformaldehyde in PBS, and analyzed by flow cytometry (FACScalibur Becton-Dickinson Biosciences, Heidelberg, Germany).

2.8. Extracellular-signal regulated kinase phosphorylation and Western blotting

HEK293 cells were transfected in 12-well plates (3.2×10^5 cells/well) either with 1.0 μg empty vector pcDNA3.1+ or vector pcDNA3.1+ encoding human Flag-epitope-tagged CCR2b or Flag-epitope-tagged CCR2b mutants (each 1.0 μg per well). After 24 h HEK293 the CCR2 expressing cells were serum starved for 2 h. Cells were treated for 0 min or 5 min with 50 nM CCL2 or the indicated amounts of CCL2 (0.3 nM, 3 nM, 10 nM, and 100 nM) or equivalent amounts of solvent. Total cellular protein was obtained by lysing the cells with 200 μl of lysis buffer (10 mM Tris-HCl pH 7.5; 150 mM NaCl, 2 mM EDTA pH 7.5; 1% Triton-X 100, 10% glycerol, 1 mM dithiothreitol, 1× phosphor stop [Roche, Mannheim, Germany], 1× protease inhibitor cocktail [200× inhibitor cocktail, Sigma Aldrich, Deisendorf, Germany]). Ligand-induced phosphorylation of ERK1/2 was determined by Western blot analysis using either phospho-p44/42-MAPK (anti-P-ERK1/2) (# 4370, overnight at 4 °C, Cell Signaling, Frankfurt am Main, Germany) and goat anti-rabbit IgG (# A6154, room temperature for 1 h, Sigma-Aldrich), or total ERK1/2 antibody (# M5670, overnight at 4 °C, Sigma-Aldrich) and anti-mouse IgG antibody (A5278, room temperature for 1 h, Sigma-Aldrich). Proteins were visualized with ECL Western Blotting Detection Reagent (GE Healthcare Bio-Sciences, Munich, Germany) by exposing Agfa Cronex 5 film (Agfa, Bonn, Germany).

2.9. Internalization assay

HEK293 cells were transfected in 24-well plates (8×10^4 cells/well) either with 0.5 μg empty vector pcDNA3.1+ or vector pcDNA3.1+

encoding human Flag-epitope-tagged CCR2b or Flag-epitope-tagged CCR2b mutants (each 0.5 μg per well). HEK293 cells transiently expressing CCR2 wild type or mutant receptors grown on cover slips were incubated for 30 min with fluorescent CCL2-mCherry fusion protein (50 nM, [26,30]). Cells were washed twice with PBS, and were fixed for 10 min with 2% paraformaldehyde in PBS at room temperature. Nuclei were stained with 2.5 μg/ml Hoechst 33342 (Invitrogen, Karlsruhe) in PBS for 20 min. Cells on cover slips were washed once in buffer A, twice in in water and mounted on object slides using mowiol 4–88 solution (Roth, Karlsruhe, Germany). Fluorescence of cells was analyzed using an Olympus IX70 fluorescence microscope (Olympus, Hamburg, Germany).

2.10. Calcium mobilization assay

HEK293 cells in 10 cm dishes (4×10^6 cells/dish) were transfected either with 8 μg empty vector pcDNA3.1 + or vector pcDNA3.1 + encoding human Flag-epitope-tagged CCR2b or Flag-epitope-tagged CCR2b mutants (each 8.0 μg per dish). After 24 h cells were rinsed from the dish, washed once in 5 ml assay buffer [20 mM HEPES, 136 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, and 0.1% (*w/v*) glucose], and collected by centrifugation. Cells (1×10^6) were resuspended in 1 ml of assay buffer, and were incubated with Fura-2 acetoxy-methyl-ester (Fura-2, AM, 1 μM, Invitrogen, Thermo Fischer Scientific, Darmstadt, Germany) for 20 min at 37 °C in assay buffer. Cells were washed and resuspended in 1 ml of assay buffer and calcium mobilization was measured upon addition of indicated amounts of either CCL2 (50 nM, PeproTech, Hamburg, Germany) or LPA (20 μM, Sigma-Aldrich, Munich, Germany) to the incubation medium in a mechanically stirred cuvette using a luminescence spectrometer (LS50B, Perkin Elmer, Rodgau, Germany) at alternating excitation wavelength (340 nm and 380 nm).

2.11. Miscellaneous

SDS-PAGE and immunoblotting were performed according to standard protocols. Immunoreactive proteins were visualized using the ECL Western Blotting Detection System (GE Healthcare). All experiments were performed at least three times, and similar results and identical trends were obtained each time. Data from representative experiments are shown as means ± standard deviations of triplicate determinations performed on independently transfected cells.

3. Results

We recently showed that stimulation of CCR2 receptors, CCR2a and/or CCR2b induces activation of serum response factor (SRF)-dependent gene transcription, by coupling to G proteins of the $G\alpha_q$ family, in particular $G\alpha_q$ and $G\alpha_{14}$, and activating RhoA GTPases [15]. Interested in the structural requirements of the two receptors mediating this specific activation we set out to analyze the function of the putative 8th helix predicted to mediate $G\alpha_q$-interaction in other GPCRs such as PAR-1 or oxytocin receptor [22,23]. To this end we generated various CCR2 receptor mutants by replacing one or more amino acids within the 8th helix of the human CCR2 receptors by alanine and analyzed the capacity of these mutants to induce SRF-dependent transcriptional activation of a luciferase reporter gene [15] and to stimulate phospholipase C isoenzymes to produce inositol phosphates [15,29]. Since, the two receptors only share the first eight amino acids of the putative 8th helix that follows the NPXXY-motif, we first generated three mutants by exchanging either eight, five or three amino acids within the last part of the common eight amino acid motif (A^{306}FVGEKFR^{313}) in CCR2b (c.f. schema Fig. 1A). As shown in Fig. 1B, expression and stimulation of the wild type CCR2b proteins in COS-7 cells (50 nM CCL2) resulted in 5-fold induction of SRF-dependent gene transcription (Fig. 1B, left panel) and approximately 6-fold inositol phosphate formation (Fig. 1 B, right panel). In marked contrast, no CCL2-stimulated (50 nM) induction of SRF activity or inositol phosphate formation was observed in COS-7 cells expressing the CCR2b mutants CCR2b-8A (A^{306}-A^{313}), CCR2b-5A (A^{309}-A^{313}) or CCR2b-3A (F^{308}A, E^{308}A, R^{308}A) although receptor protein was found expressed in membranes of cells expressing the CCR2b mutants (Western blot Fig. 1C). Of note, receptor expression was controlled in additional experiments by immunoblotting using anti-Flag antibodies (Fig. 1C, *Anti-Flag upper panel*, for details see Material and Methods). Equal loading of proteins was determined by re-probing the blot using an anti-β-actin-antibody (*Anti-Actin, lower panel*). As shown, with the exception of CCR2b-8A (A^{306}-A^{313}), wild type and mutants were found expressed in similar amounts. The reason for the higher level of expression of CCR2b-8A (A^{306}–A^{313}) is unclear. As indicated by asterisks (*), monomeric and multimeric forms of the receptors were identified (Fig. 1C). Similar results in SRF activation and protein expression were obtained in HEK293 cells expressing CCR2b wild type and mutant proteins (data not shown). Next, we comparatively analyzed receptor function of wild type and mutants CCR2b-8A and -3A (c.f. schema Fig. 2A) in the presence of $G\alpha_q$. As shown in Fig. 2B co-expression of $G\alpha_q$ had little if any effect on either ligand-stimulated (50 nM CCL2) SRF activation or inositol phosphate formation induced by the CCR2b receptor mutant CCR2b-8A and -3A, but resulted in a marked synergistically further increase in SRF activation (approximately 2.5-fold), and inositol phosphate formation (approximately 2-fold) induced by the wild type protein (Fig. 2B). $G\alpha_q$ expression alone induced an increase in inositol phosphate formation, probably by coupling to endogenously expressed GPCRs. $G\alpha_q$ expression levels were found similar when coexpressed with CCR2b wild type or CCR2b mutant proteins (see Western blot analysis Fig. 2C, equal loading of proteins was determined as described). In addition, also coexpression of $G\alpha_{14}$ did not increase ligand-stimulated (50 nM CCL2) CCR2b-8A and CCR2b-3A induced SRF activity (data not shown).

Since replacement of valine V^{308}, glutamate E^{310} and arginine R^{313} as represented by the CCR2b-3A mutant almost completely abolished receptor-induced SRF activation, we next generated mutants carrying single alanine amino acid substitutions in position 308, 310, and 313 resulting in the receptor mutants: CCR2bV^{308}A, CCR2bE^{310}A, and CCR2bR^{313}A (schematically depicted in Fig. 3A). As shown in Fig. 3B replacement of E^{310} by alanine had little if any effect on ligand-stimulated SRF-induced activity or inositol phosphate formation (Fig. 3B, left and right panel, respectively). Replacement of V^{308} by alanine slightly reduced both receptor-induced activities. In marked contrast, replacement of R^{313} by alanine almost completely abolished both, receptor induced activation of SRF and inositol phosphate formation; indicating that this amino acid plays an important role in receptor-mediated activation of cellular signaling (Fig. 3B). Receptor protein of the three CCR2b mutants was found expressed in membranes of the cells similar to that of the CCR2b wild type, as deduced by immunoblotting using anti-Flag antibodies (Western blot Fig. 3C, equal loading of proteins was determined as described).

Next the question arose whether the positive charge and/or side chain projection at position 313 determine loss of receptor function. To address these questions three additional mutants were generated carrying either reduced side chain projections and positive charge or even negative charge at position 313 (schematically depicted in Fig. 4A). As shown in Fig. 4B replacement of R^{313} by lysine (R^{313}K) or histidine (R^{313}H) gradually reduced, and replacement by alanine (R^{313}A) and glutamate (R^{313}E) abolished ligand-stimulated receptor-induced SRF activation (Fig. 4B, left panel) and phospholipase C mediated inositol phosphate formation (Fig. 4B, right panel) in COS-7 cells. Expression of receptor proteins was again determined by Western blotting (Fig. 4C, equal loading of proteins was determined as described). Since both, CCR2b and CCR2a, carry R^{313} we next analyzed CCR2a mutants with the same substitutions at position 313 (schematically depicted in Fig. 5A). As shown in Fig. 5B similar to the observations made for CCR2b, also replacement of R^{313} by lysine, histidine, alanine and glutamate in CCR2a gradually reduced or abolished ligand-stimulated

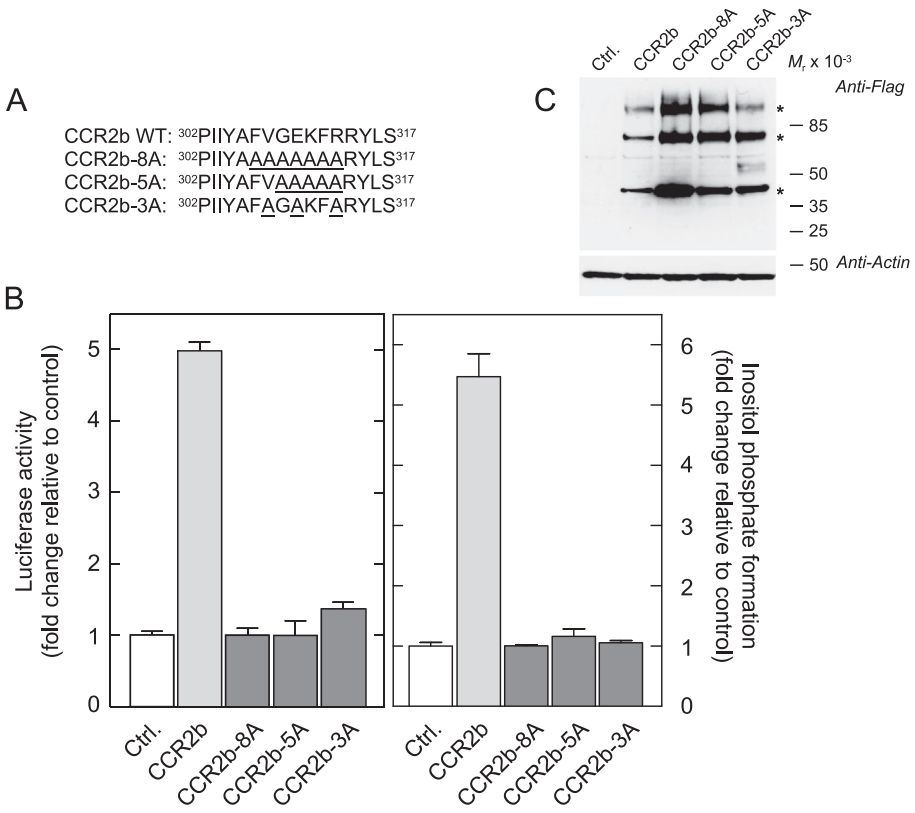

Fig. 1. Induction of cellular signaling by CCR2b and CCR2b-mutants of the 8th helix. A, Amino acid sequence changes introduced into the CCR2b protein. The amino acids exchanged by alanines are underlined. **B**, *Left panel* COS-7 cells were co-transfected in 48-well plates with reporter plasmids (pSRE.L and pRL-TK, each 30 ng/well), together with either empty vector pcDNA3.1 + (Ctrl.) or vector pcDNA3.1 + encoding human Flag-epitope-tagged CCR2b, CCR2b-8A, CCR2b-5A, and CCR2b-3A (*CCR2b*; *CCR2b-8A, CCR2b-5A,* and *CCR2b-3A*, each 100 ng per well) as indicated. Twenty-four hours after transfection, the cells were stimulated with CCL2 (each 50 nM) for 6 h. The cells were lyzed (40 µl/well, passive lysis buffer) and luciferase activity of the cell lysates was assayed as described under "Experimental Procedures". To correct differences in transfection efficiencies Firefly luciferase activities were normalized to the *Renilla* luciferase activities. Fold activation is given as luciferase activity relative to the activities determined for cells transfected with the empty vector control. *Right panel*, COS-7 cells were transfected in 48-well plates with empty vector (Ctrl.) vector pcDNA3.1 + or vector pcDNA3.1 + encoding human Flag-epitope-tagged CCR2b, CCR2b-8A, CCR2b-5A, and CCR2b-3A (*CCR2b*; *CCR2b-8A, CCR2b-5A,* and *CCR2b-3A*, each 100 ng per well). Twenty four hours after transfection, the cells were incubated for 17 h in the presence of D-*myo*-[2-^{3}H]inositol (3 µCi/ml) and 10 mM LiCl, and stimulated with CCL2 (50 nM) for a further 4 h in the presence of 10 mM LiCl. The levels of inositol phosphates were then determined as described under "Material and Methods". **C**, Western blot analysis of wild type and mutant CCR2b proteins. COS-7 cells were transfected in 6-well plates either with 1.6 µg empty vector pcDNA3.1 + (*Ctrl.*) or vector pcDNA3.1 + encoding human Flag-epitope-tagged wild type CCR2b (*CCR2b*) or CCR2b mutants CCR2b-8A, CCR2b-5A, and CCR2b-3A. Twenty-four hours after transfection, the cells were harvested and particulate fractions were prepared as described in "Material and Methods". Aliquots of the particulate fractions were subjected to SDS-PAGE and immunoblotting using antibodies reactive against Flag-epitope (*Anti-Flag*), and β-actin (*Anti-actin*) as indicated. Monomeric and multimeric receptor proteins are marked by asterisks (*).

receptor-induced SRF activation (Fig. 5B, left panel) and inositol phosphate formation (Fig. 5B, right panel) underlining the importance of R^{313} for CCR2 receptor signaling. Receptor protein of the CCR2a mutants and CCR2a wild type was found similarly expressed in membranes of the cells (Western blot, Fig. 5C, equal loading of proteins was determined as described). Next, we studied activation of the wild type receptors CCR2a and CCR2b and their $R^{313}A$ and $R^{313}E$ mutants in the presence of $G\alpha_q$ and $G\alpha_{14}$. As shown in Fig. 6A (both lower panels) in contrast to ligand-stimulated wild type CCR2a and CCR2b both mutants $CCR2aR^{313}A$ and $CCR2aR^{313}E$ (Fig. 6A, left panel) and $CCR2bR^{313}A$ and $CCR2bR^{313}E$ (Fig. 6A, right panel) failed to activate SRF in the presence of $G\alpha_q$ proteins in COS-7 cells. Similar observations were made when coexpressing $G\alpha_{14}$ (Fig. 6B, left and right panel). These findings emphasize the role of R^{313} for coupling of CCR2 receptors to $G\alpha_q$ and $G\alpha_{14}$. $G\alpha_q$ expression levels were found similar when coexpressed with CCR2b wild type or CCR2b mutant proteins (Fig. 6A, Western blot analysis, equal loading of proteins was determined as described). Immunodetection of coexpression of $G\alpha_{14}$ is not shown due to low sensitivity of commercially available antibodies and expression levels of exogenous $G\alpha_{14}$ when transfecting cells with 50 ng $G\alpha_{14}$-encoding cDNA [15]. We next analyzed and compared the activity of ligand-stimulated (50 nM CCL2) CCR2b and the $CCR2bR^{313}E$ and $CCR2bR^{313}A$ mutants in the presence of increasing amounts of $G\alpha_q$ in COS-7 and HEK293 cells (Fig. 7A and B). As shown CCR2b-induced SRF-activity was massively enhanced by cotransfection of increasing amounts of $G\alpha_q$ DNA (20 ng–200 ng/well) in COS-7 and HEK293 cells (Fig. 7A and B). In marked contrast, $CCR2bR^{313}A$ and $CCR2bR^{313}E$ failed to induce an increase in SRF-activity (Fig. 7A, COS-7), or marginally increased SRF-activity in the presence of increasing amounts of $G\alpha_q$ (Fig. 7B, HEK293). The latter finding indicates a cell-specific small residual coupling of the two mutants to $G\alpha_q$ probably due to the higher expression levels of the exogenously introduced receptors in HEK293 cells. $G\alpha_q$ expression levels were controlled by Western blotting (Fig. 7A and B, and equal loading of proteins was determined as described). In addition, we confirmed that CCR2 receptor induced SRF activity was G_q-dependent by using a now commercially available $G\alpha_{q/11}$ inhibitor YM-254890 [31]. As shown in Supplementary Fig. 1, ligand-induced SRF-activity in HEK293 cells expressing CCR2b alone or together with $G\alpha_q$ was massively reduced in the presence of the inhibitor (10 µM). Of note, also residual activity of the two mutants $CCR2bR^{313}E$ and $CCR2bR^{313}A$ was inhibited by YM-254890. Again $G\alpha_q$ expression levels were controlled by Western blotting, and equal loading of proteins was determined as described (Supplementary Fig. 1).

To exclude that lack of ligand-stimulated receptor-induced activation of SRF of $CCR2bR^{313}A$ and $CCR2bR^{313}E$ is due to changes in surface expression and/or lower affinity for CCL2 we analyzed CCR2 receptor expression by FACs analysis (Fig. 8A) and determined SRF activity in the presence of increasing concentrations of CCL2 (3 nM, 10 nM, 30 nM, 100 nM, 300 nM) in HEK 293 cells (Fig. 8B). As depicted in Fig. 8A both $CCR2bR^{313}A$ and $CCR2bR^{313}E$ were found expressed on the cell surface to a similar extend as the wild type CCR2b. The small differences in surface expression of the two mutants in comparison to the wild type receptor extend to 10%. However, in marked contrast to CCR2b-wild type expressing cells, little if any activation of SRF-activity was observed in cells expressing the two mutants ($CCR2bR^{313}A$ and $CCR2bR^{313}E$) in the presence of increasing concentrations of CCL2 (Fig. 8B), while stimulation of CCR2b-expressing cells resulted in an approximately 7.5-fold increase in SRF activation, with half-maximal effects at EC_{50}: 18 nM (Fig. 8B). The latter finding correspond to the

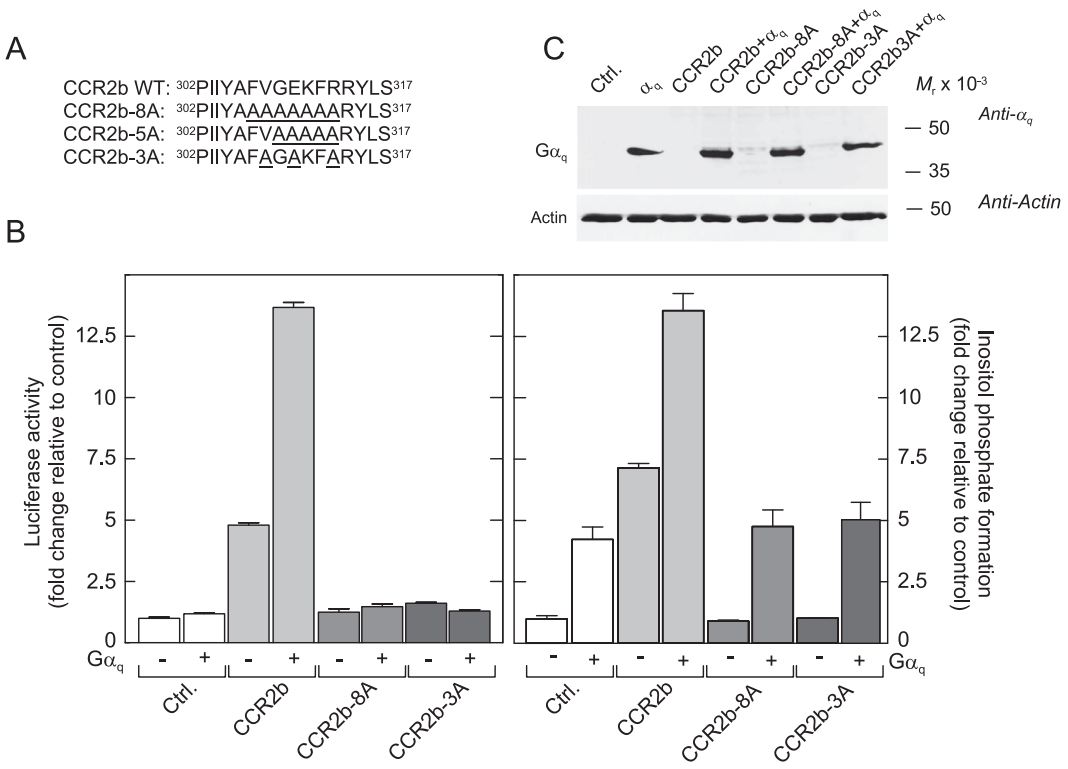

Fig. 2. Induction of cellular signaling by CCR2b and CCR2b-mutants of the 8th helix in the presence of exogenous $G\alpha_q$. A, Amino acid sequence changes introduced into the CCR2b protein. The amino acids exchanged by alanines are underlined. **B**, *Left panel* COS-7 cells were cotransfected in 48-well plates with reporter plasmids (pSRE.L and pRL-TK, each 30 ng/well), together with either empty vector pcDNA3.1 + (Ctrl.) or vector pcDNA3.1 + encoding human Flag-epitope-tagged CCR2b, CCR2b-8A, and CCR2b-3A and Gαq (*CCR2b*; *CCR2b-8A, CCR2b-5A,* and *CCR2b-3A*, each 100 ng per well, and *Gαq*, 50 ng per well) as indicated. Twenty four hours after transfection, cells were stimulated (50 nM CCL2, 6 h), cell lysates were assayed for luciferase activity, and luciferase activities and fold activation was determined as described in Fig. 1. *Right panel*, COS-7 cells were transfected in 48 -well plates with empty vector with either empty vector pcDNA3.1 + (Ctrl.) or vector pcDNA3.1 + encoding human Flag-epitope-tagged CCR2b, CCR2b-8A, and CCR2b-3A and $G\alpha_q$ (*CCR2b*; *CCR2b-8A, CCR2b-5A,* and *CCR2b-3A*, each 100 ng per well, and *Gαq*, 50 ng per well) as indicated. Twenty four hours after transfection, cells were labeled with D-*myo*-[2-^{3}H]inositol (17 h), stimulated with 50 nM CCL2 (4 h), and the levels of inositol phosphates were then determined as described in Fig. 1. **C**, Western blot analysis of $G\alpha_q$ expression. Aliquots (15 µl) of the soluble fractions of the cell lysates described in **(B)** were subjected to SDS-PAGE and immunoblotting using antibodies reactive against $G\alpha_q$ (*Anti-Gα$_q$*), and β-actin (*Anti-Actin*) as indicated.

observation made in COS-7 cells showing a though somewhat higher EC_{50} of 87 nM for the activation of SRF by CCR2b [15].

Interested in whether changes at amino acid position 313 of the carboxyl terminal portion of human CCR2 receptors influences other ligand-stimulated receptor-induced signaling we next comparatively analyzed stimulation of MAPKs ERK1/2 by wild type CCR2 receptors and the CCR2bR^{313}A and CCR2bR^{313}E mutants in HEK293 cells (Fig. 9A, upper panel). As shown the replacement of R^{313} by alanine or glutamate had little if any influence on CCL2-induced phosphorylation of ERK1/2 proteins (Fig. 9A, upper panel). Accordingly, a similar increase in ERK1/2 phosphorylation was observed for all three receptor proteins in the presence of increasing concentrations of CCL2 (Fig. 9B, a, b, c, each upper panels). Equal loading of protein was verified by reprobing of the blot membranes with anti-ERK1/2 antibodies (Fig. 9A, lower panel; Fig. 9B, a, b, c, each lower panels). These findings indicate that $G\alpha_q$ but not $G\alpha_i$ coupling is impaired in the two CCR2b mutants. Since activation of ERK1/2 kinases by CCR2 receptor has been attributed at least partially to coupling to pertussis toxin-sensitive G_i proteins [14], we next analyzed ligand-stimulated ERK1/2 activation by CCR2b wild type and CCR2bR^{313}A and CCR2bR^{313}E mutants in the presence of pertussis toxin (PTX; 30 ng/ml). As shown in Fig. 9C (upper panel), ERK1/2 activation by CCR2b wild type and CCR2b mutants was considerably reduced in the presence of PTX. This finding adds further support to the assumption that changes at position R^{313} impair $G\alpha_q$ but not $G\alpha_i$ coupling.

CCR2 receptors have been shown to functionally signal and to act as scavenger receptors with high capacity for the internalization of CCL2 ligand [30]. Signaling, scavenging, and internalization of chemokine receptors have been reported to occur in a G protein-dependent and/or G protein-independent but β-arrestin-dependent manner [32]. To analyze and compare the internalization and scavenging capacity of wild type CCR2b protein and the CCR2bR^{313}A and CCR2bR^{313}E mutants, we incubated CCR2 receptor expressing cells with a CCL2-mCherry fusion protein (50 nM [26,30]), and analyzed the internalization of the receptor/ligand complex by fluorescence microscopy. To address the role of G_i-proteins in internalization, the cells were pretreated with pertussis toxin (30 ng/ml overnight). As depicted in Fig. 10 wild type and mutant CCR2 receptors similarly internalized CCL2-mCherry fusion protein; both in the absence and presence of pertussis toxin (PTX), as visible by the accumulation of CCL2-mCherry in probably endosomal compartments near the nucleus. The nature of the compartment remains to be addressed. Corresponding to the somewhat smaller surface expression of the two CCR2b mutants internalization of CCL2-mCherry was slightly less prominent and the fluorescence slightly less intensive. Control cells transiently transfected with the empty expression plasmid showed no internalization of the chemokine (data not shown). Two major conclusions are drawn from these findings. First, the results show that internalization and scavenging of CCL2-mCherry occurs almost independently of G_i and G_q proteins, since neither treatment with PTX nor the usage of the CCR2b mutants CCR2bR^{313}A and CCR2bR^{313}E impaired the internalization of CCL2-mCherry. Second, the results indicate that R^{313} of the 8th helix is of minor importance for the

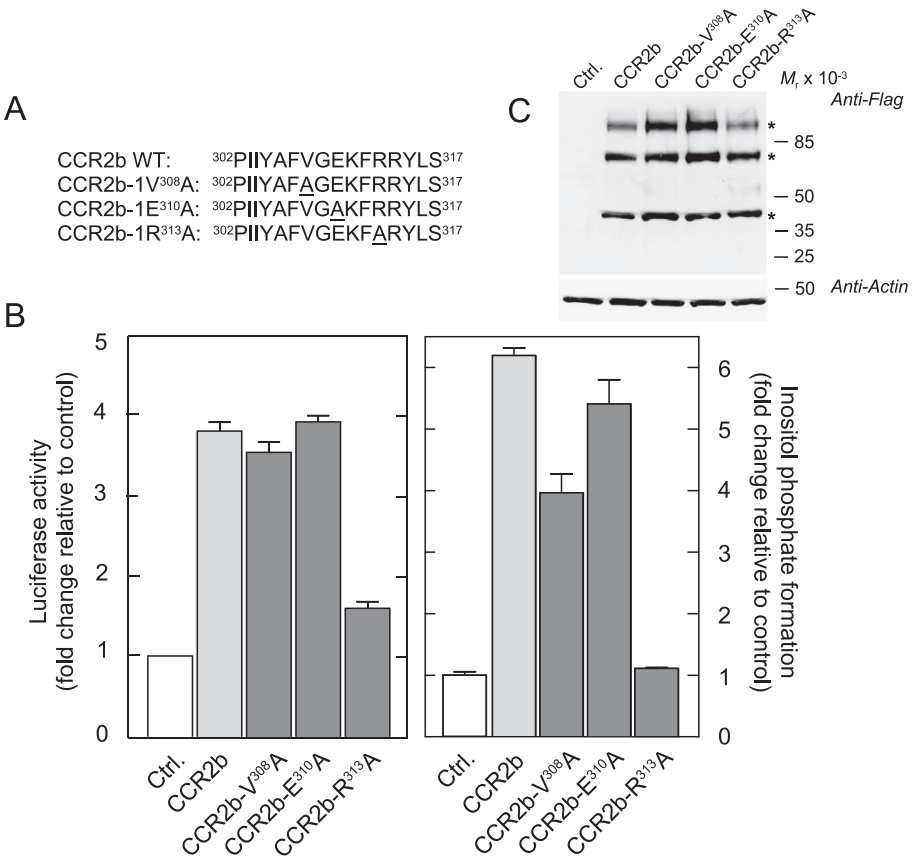

Fig. 3. Induction of cellular signaling by CCR2b and CCR2b-point mutants of the 8th helix. A, Amino acid sequence changes introduced into the CCR2b protein. The amino acids exchanged by alanines are underlined. **B**, *Left panel* COS-7 cells were cotransfected in 48-well plates with reporter plasmids (pSRE.L and pRL-TK, each 30 ng/well), together with either empty vector pcDNA3.1 + (Ctrl.) or vector pcDNA3.1 + encoding human Flag-epitope-tagged CCR2b, CCR2b-V^{308}A, and CCR2b-E^{310}A, and CCR2bR^{313}A (*CCR2b*; *CCR2b-V^{308}A, CCR2b-E^{310}A,* and *CCR2b-R^{313}A*, each 100 ng per well) as indicated. The cells were stimulated (50 nM CCL2, 6 h), cell lysates were assayed for luciferase activity, and luciferase activities and fold activation was determined as described in Fig. 1. *Right panel*, COS-7 cells were transfected in 48-well plates with either empty vector pcDNA3.1 + (Ctrl.) or vector pcDNA3.1 + encoding human Flag-epitope-tagged CCR2b, CCR2b-V^{308}A, and CCR2b-E^{310}A, and CCR2bR^{313}A (*CCR2b*; *CCR2b-V^{308}A, CCR2b-E^{310}A,* and *CCR2b-R^{313}A*, each 100 ng per well) as indicated. Twenty four hours after transfection, cells were labeled with D-*myo*-[2-^{3}H]inositol (17 h), stimulated with 50 nM CCL2 (4 h), and the levels of inositol phosphates were then determined as described in Fig. 1. **C**, Western blot analysis and immunodetection of wild type and mutant CCR2b proteins using *Anti-Flag* and *Anti-β-Actin* antibodies were performed as described in Fig. 1. Monomeric and multimeric receptor proteins are marked by asterisks (*).

internalization of CCR2 receptors.

Last, we analyzed the influence of the substitutions at position R^{313} on CCL2-induced changes in intracellular Ca^{2+} concentrations in HEK293 cells. As a control pcDNA3.1(+)-transfectd cells were used, and lysophosphatidic acid (LPA)-induced changes in Ca^{2+} concentrations due to endogenously expressed LPA receptors were recorded. Six LPA receptors have been described to induce changes in intracellular Ca^{2+} concentrations by coupling to G_q and/or to G_i [33,34]. In

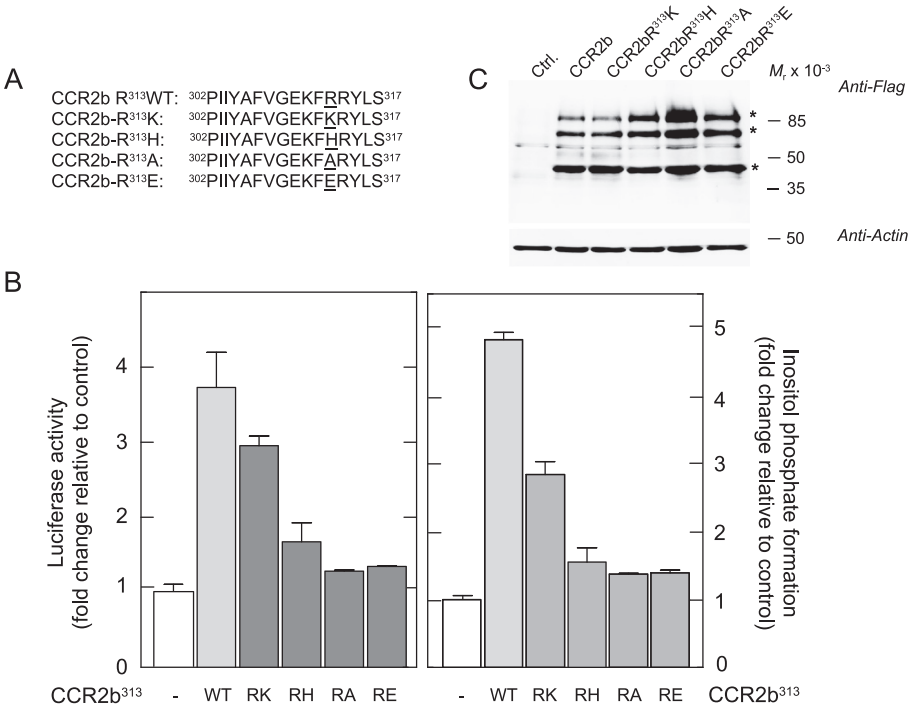

Fig. 4. Amino acid arginine 313 determines serum response factor activation and inositol phosphate formation by ligand-stimulated CCR2b. A, Amino acid sequence changes introduced into the CCR2b protein. The amino acids exchanged by alanines are underlined. **B**, *Left panel* COS-7 cells were cotransfected in 48-well plates with reporter plasmids (pSRE.L and pRL-TK, each 30 ng/well), together with either empty vector pcDNA3.1 + (Ctrl.) or vector pcDNA3.1 + encoding human Flag-epitope-tagged CCR2b, CCR2b-R^{313}K, and CCR2b-R^{313}H, and CCR2b-R^{313}A, and CCR2b-R^{313}E (*CCR2b*; *CCR2b-R^{313}K, CCR2b-R^{313}H, CCR2b-R^{313}A, and CCR2b-R^{313}E*, each 200 ng per well) as indicated. The cells were stimulated (50 nM CCL2, 6 h), cell lysates were assayed for luciferase activity, and luciferase activities and fold activation was determined as described in Fig. 1. *Right panel*, COS-7 cells were transfected in 48-well plates with either empty vector pcDNA3.1 + (Ctrl.) or vector encoding Flag-epitope-tagged CCR2b, CCR2b-R^{313}K, and CCR2b-R^{313}H, and CCR2b-R^{313}A, and CCR2b-R^{313}E (*CCR2b*; *CCR2b-R^{313}K, CCR2b-R^{313}H, CCR2b-R^{313}A, and CCR2b-R^{313}E*, each 200 ng per well) as indicated. Twenty four hours after transfection, cells were labeled with D-*myo*-[2-^{3}H]inositol (17 h), stimulated with 50 nM CCL2 (4 h), and the levels of inositol phosphates were then determined as described in Fig. 1. **C**, Western blot analysis and immunodetection of wild type and mutant CCR2b proteins using *Anti-Flag* and *Anti-β-Actin* antibodies were performed as described in Fig. 1. Monomeric and multimeric receptor proteins are marked by asterisks (*).

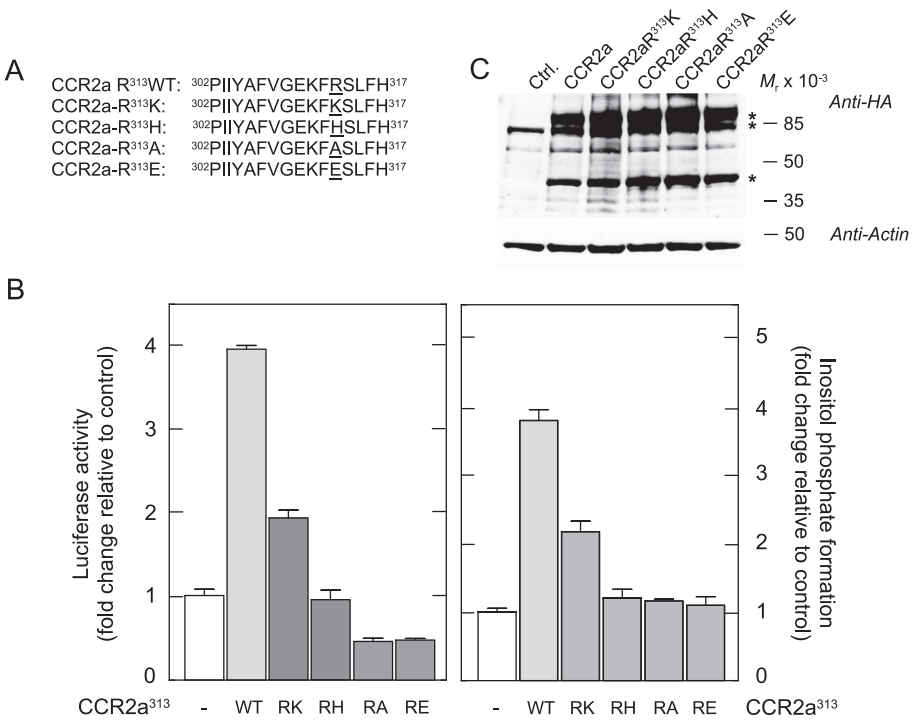

Fig. 5. Amino acid arginine 313 determines serum response factor activation and inositol phosphate formation by ligand-stimulated CCR2a. A, Amino acid sequence changes introduced into the CCR2a protein. The amino acids exchanged by alanines are underlined. **B**, *Left panel* COS-7 cells were cotransfected in 48-well plates with reporter plasmids (30 ng each per well of pSRE.L and pRL-TK), together with either empty vector pcDNA3.1 + (Ctrl.) or vector pcDNA3.1 + encoding human HA-epitope-tagged CCR2a, CCR2a-R^{313}K, and CCR2a-R^{313}H, and CCR2a-R^{313}A, and CCR2a-R^{313}E (*CCR2a*; *CCR2a-R^{313}K, CCR2a-R^{313}H, CCR2a-R^{313}A, and CCR2a-R^{313}E*, each 200 ng per well) as indicated. The cells were stimulated (50 nM CCL2, 6 h), cell lysates were assayed for luciferase activity, and luciferase activities and fold activation was determined as described in Fig. 1. *Right panel*, COS-7 cells were transfected in 48-well plates with either empty vector pcDNA3.1 + (Ctrl.) or vector encoding HA-epitope-tagged CCR2a, CCR2a-R^{313}K, and CCR2a-R^{313}H, and CCR2a-R^{313}A, and CCR2a-R^{313}E (*CCR2a*; *CCR2a-R^{313}K, CCR2a-R^{313}H, CCR2a-R^{313}A, and CCR2a-R^{313}E*, each 200 ng per well) as indicated. Twenty four hours after transfection, cells were labeled with D-*myo*-[2-^{3}H] inositol (17 h), stimulated with 50 nM CCL2 (4 h), and the levels of inositol phosphates were then determined as described in Fig. 1. **C**, Western blot analysis and immunodetection of wild type and mutant CCR2a proteins using *Anti-HA* and *Anti-β-Actin* antibodies were performed as described in Fig. 1. Monomeric and multimeric receptor proteins are marked by asterisks (*).

particular LPA2 and LPA3 receptors have been shown to mediate a strong increase in Ca^{2+} mobilization from intracellular stores [35,36]. mRNA expression of at least LPA1–3 receptors in HEK293 cells has been documented [37]. As shown in Fig. 11A CCR2b mutant R^{313}K-, R^{313}H-, R^{313}A-, and R^{313}E-expressing cells showed a reduced CCL2-induced (50 nM) Ca^{2+} release when compared to wild type CCR2b-expressing cells. However, in contrast to the observations that CCR2b R^{313}A and CCR2bR^{313}E are almost completely reduced in their capacity to regulate SRF-dependent activity, both mutants display significant CCL2-induced changes in intracellular Ca^{2+} concentrations (Fig. 11A). To delineate whether the latter activity was due to coupling of the CCR2b mutants to $G\alpha_i$ proteins, ligand induced Ca^{2+} release of CCR2bR^{313}A-expressing cells was monitored in the presence and absence of PTX. As shown in Fig. 11B, overnight pre-treatment of CCR2bR^{313}A-expressing cells with PTX (30 nM) resulted in complete loss of CCL2-induced changes in Ca^{2+} concentrations (Fig. 11 B, *right lower panel*). In marked contrast, cells expressing wild type CCR2b displayed only a reduction in intracellular Ca^{2+} concentrations (Fig. 11 B, *right upper panel*). These findings indicate (i) that CCL2-induced changes in intracellular Ca^{2+} concentration in CCR2b-expressing HEK293 cells is mediated by both $G\alpha_i$ and $G\alpha_q$ proteins, and (ii) that changes in position R^{313} impair the $G\alpha_q$-induced but not the $G\alpha_i$-induced Ca^{2+}-release. Also of note, CCL2-induced changes of intracellular Ca^{2+} concentration in wild type CCR2b-expressing cells probably results in heterologous desensitization (up to 65%) of endogenously expressed lysophosphatidic acid (LPA) receptors. This desensitization was gradually lost when analyzing the CCR2b mutant R^{313}K, R^{313}H-, R^{313}A-, and R^{313}E-expressing cells (Fig. 11A) reaching LPA-stimulated changes in intracellular Ca^{2+} concentration that resemble those observed in pcDNA3.1(+)-transfected control cells (*Ctrl.*). Furthermore, Ca^{2+} release induced by CCR2b in the presence of PTX continue to result in partially desensitization of LPA-induced changes in intracellular Ca^{2+} concentrations. These findings imply that the observed heterologous desensitization of endogenously expressed LPA receptors by CCR2b receptors involve coupling of the CCR2b receptor to G_q proteins.

4. Discussion

We recently reported on the activation of RhoA GTPases by coupling of the two receptors to members of the G_q family, particular $G\alpha_q$ and $G\alpha_{14}$, and proposed a role in regulating non-canonical functions such as differentiation, proliferation, and gene transcription of immune and non-immune cells [15]. In the current study, we determined the role of the amino terminal portion of the 8th helix of CCR2a and CCR2b for coupling to $G\alpha_q$ and $G\alpha_{14}$. Various studies showed that the 8th helix plays an important role in G_q protein coupling of class a GPCRs such as protease-activated receptor 1 (PAR-1), oxytocin receptor, cannabinoid receptor 1, and the virally encoded chemokine receptor homolog ORF 74 [22–24,38–40]. Also of interest, several findings indicated that in particular the hydrophilic interface at the amino terminal portion of the 8th helix is of importance for receptor function [22,23,39–44]. We identified amino acids within the putative 8th helix contributing to the coupling of the two CCR2 receptors to $G\alpha_q/G\alpha_{14}$ by using various CCR2a and CCR2b receptor mutants generated by replacing several (A^{306}-R^{313} or G^{309}-R^{313}), a combination (CCR2bV^{306}A/E^{310}A/R^{313}A), or single amino acids by alanine. Of note, the replacements seemed not to considerably interfere with protein expression of the receptor. We observed that receptor induced SRF activation and inositol phosphate formation was reduced or even lost by the substitution of distinct amino acids within the amino terminal portion of the 8th helix. In the PAR-1 receptor the acidic E^{377} corresponding to E^{310} in the CCR2 receptors were shown to mediate the interaction of the 8th helix with the first intracellular loop of the receptor and to be of importance for the interaction with $G\alpha_q$ [22]. The authors proposed a 7-8-1 receptor activation mechanism for PAR-1-mediated $G\alpha_q$ activation, whereby the TM7 interacts with the 8th helix, which in turns interacts with the i1-loop [22]. However, as shown in the present work for CCR2b, neither replacement of the acidic E^{310} by alanine, nor the hydrophobic V^{311} by alanine had little if any effect, or only mildly impaired, respectively, receptor-induced SRF activity and inositol phosphate formation. In marked contrast, replacement of the basic arginine at position 313 by alanine in CCR2b and CCR2a tremendously reduced receptor activity

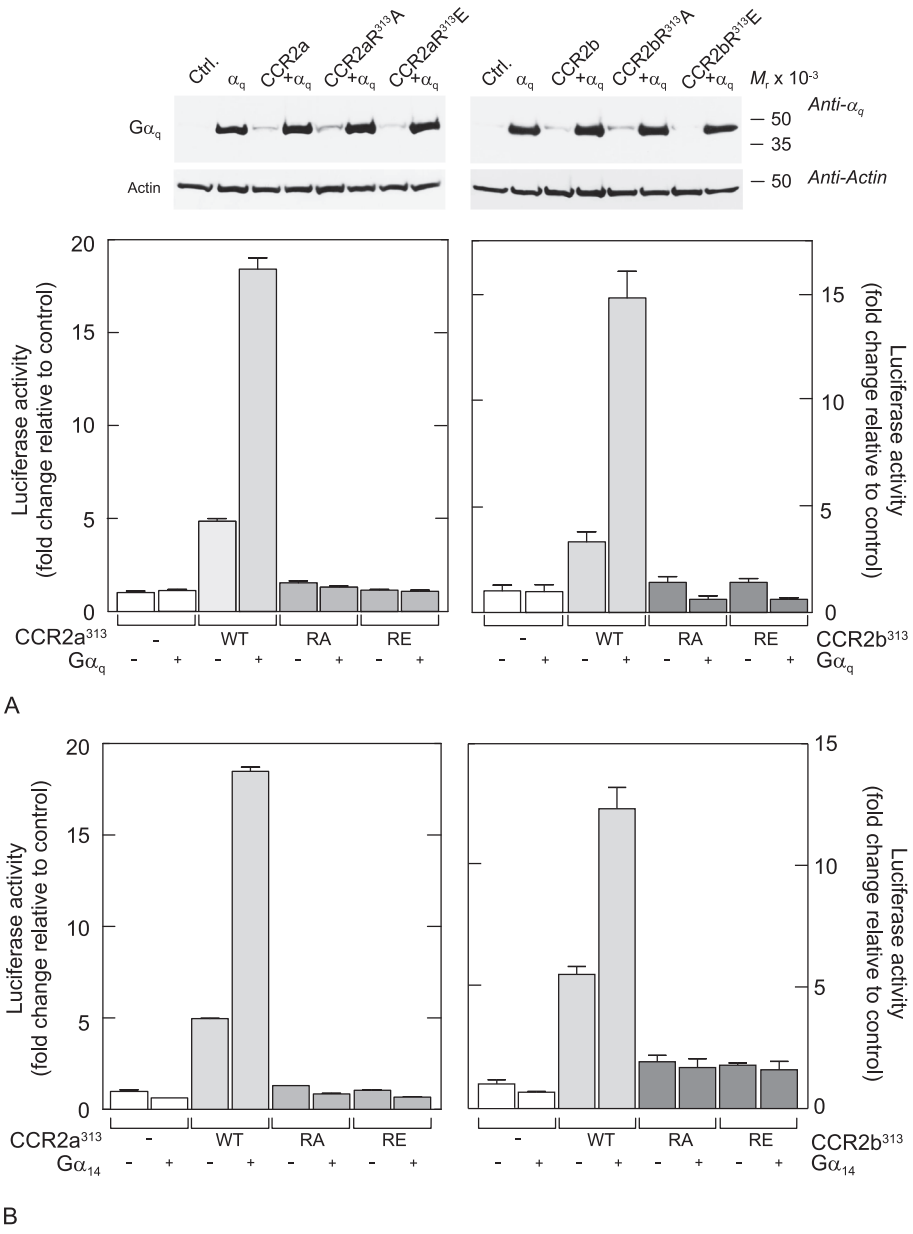

Fig. 6. Induction of cellular signaling by CCR2a, CCR2b, CCR2aArg^{313}- and CCR2b-Arg^{313} mutants in the presence of exogenous Gα_q. COS-7 cells cells were cotransfected in 48-well plates with reporter plasmids (30 ng each per well of pSRE.L and pRL-TK), together with pcDNA3.1 + (Ctrl.) or pcDNA3.1 + encoding (*left panel*) human HA-epitope-tagged CCR2a, CCR2aR^{313}A, and CCR2aR^{313}E, or encoding (*right panel*) human Flag-epitope-tagged CCR2b, CCR2bR^{313}A, an CCR2bR^{313}E (each 100 ng per well), and Gα_q (50 ng per well) (Fig. 6A, *lower panel*) or Gα_{14} (50 ng per well) (Fig. 6B, *lower panel*). The cells were stimulated (50 nM CCL2, 6 h), cell lysates were assayed for luciferase activity, and luciferase activities and fold activation was determined as described in Fig. 1. Fig. A. (*upper panel*), Western blot analysis of Gα_q expression. Aliquots (15 µl) of the soluble fractions of the cell lysates described in (Fig. A) were subjected to SDS-PAGE and immunoblotting using antibodies reactive against Gα_q (*Anti-Gα_q*), and β-actin (*Anti-Actin*) as indicated.

and coupling of the receptors to Gα_q and Gα_{14}. Similar findings have been described for the oxytocin receptor showing the importance of amino acids 343–355 within the 8th helix for the binding of G_q but not G_i proteins [40]. Interestingly, a role of hydrophilic residues located at the amino terminal portion of the 8th helix in PAR-1 receptor, and in the oxytocin receptor (E^{339} and R^{343}) for coupling to Gα_q has been reported [22,23]. Also in this line exchange of R^{332} (e.g. by tryptophan) within the 8th-helix of virally encoded oncogene ORF74 (also known as vGPCR), resulted in the loss of functional coupling to Gα_q, e.g. loss of activation of phospholipase Cβ isoenzymes [41]. Of note, despite the differences of CCR2 a and CCR2b in their amino acid sequences following the arginine at position 313 (R^{313}) changes in this amino acid resulted in severe impairment of coupling of CCR2a and CCR2b to both Gα_q and Gα_{14}. The results obtained with mutants in which R^{313} was replaced by lysine, histidine, alanine or glutamate showed that the side chain projection of R^{313} in the proposed hydrophilic interface of the 8th-helix (*c.f.* model Fig. 12) plays a major role in CCR2 receptor/G protein interaction. The structural model given in Fig. 12 that has been developed based on the known three-dimensional structure of the recently published human CCR2 receptor (RCSB-PDB: 5T1A, [45]) reveals the impact of replacing R^{313} by, e.g. lysine (K) to the functional integrity of helix 8. As shown in the model, the basic side chain projection of R^{313} are in close contact and hence likely interact with the main chain carbonyl groups of Y^{305}, which is present at the end of the terminal NPXXY-motif of the 7th helix, and with V^{308} present at the amino-terminal-most portion of the 8th helix. The distances between the guanidine group of R^{313} and the carbonyl oxygens are well within the hydrogen bonding distance of 2.6–4.2 Å (*c.f.* model Fig. 12). The replacement of R^{313} by lysine (K) is likely to at least reduce, and replacements by histidine (H), alanine (A), or glutamate (E) are even predicted to interfere with formation of these bonds. These observations indicate that changes in the basic amino acid at position 313 of CCR2 receptors potentially reduce the sterically constraining interaction between the end of 7th helix and the beginning of the 8th helix necessary for agonist-mediated conformational changes and the formation of the $G_{q/14}$ binding surface. The observations agree with the earlier mentioned mechanism described for PAR-1-mediated Gα_q activation by Swift et al. [22], proposing a direct interaction between 7th and 8th helix to facilitate a sequential coordinated movement of the two helices. For the virally encoded oncogene ORF74 it was shown that

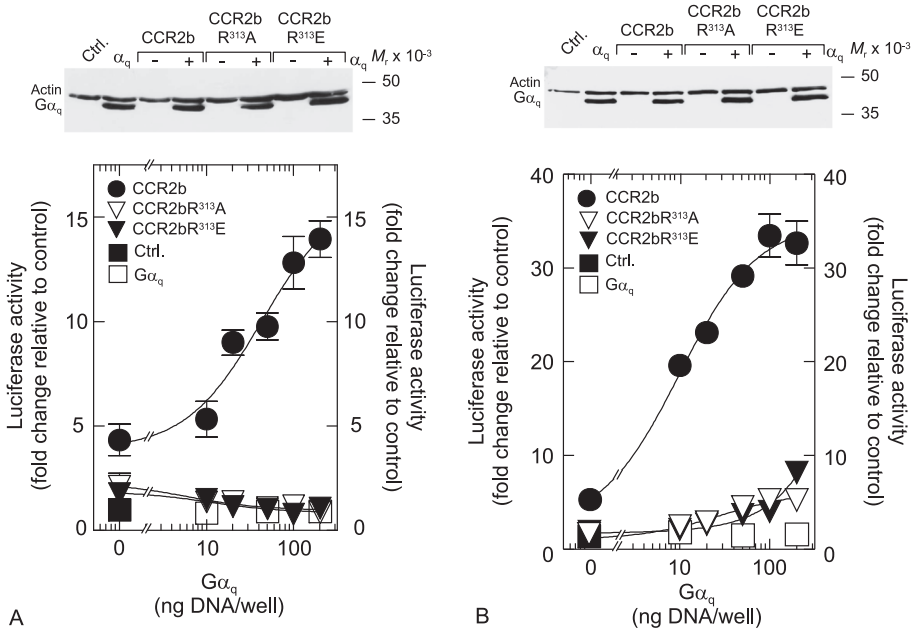

Fig. 7. Induction of cellular signaling by CCR2b, CCR2bR^{313}A, and CCR2bR^{313}E in the presence of increasing amounts of exogenous Gα$_q$. COS-7 cells (Fig. 7A) or HEK293 cells (Fig. 7B) were co-transfected in 48-well plates with reporter plasmids (30 ng each per well of pSRE.L and pRL-TK), together with pcDNA3.1+ (Ctrl., black square), Gα$_q$ (white square), or pcDNA3.1+ encoding human Flag-epitope-tagged CCR2b (black circle), CCR2bR^{313}A (white triangle), and CCR2bR^{313}E (black triangle) (each 200 ng per well), and increasing amounts of Gα$_q$ (20 ng–200 ng/well) as indicated. The cells were stimulated (50 nM CCL2, 6 h), cell lysates were assayed for luciferase activity, and luciferase activities and fold activation was determined as described (Fig. 7A and B, *lower panels*). Aliquots (15 µl) of cell lysates obtained from cells without cotransfected Gα$_q$-DNA (−) and cells cotransfected with 200 ng of Gα$_q$-DNA (+) were subjected to SDS-PAGE and immunoblotting using antibodies reactive against Gα$_q$, and β-actin. The detected proteins are indicated (Fig. 7A and B, *upper panels*).

R^{322} the arginine that correspond to R^{313} in CCR2 receptors has a specific role in stabilizing the end of transmembrane domain 7 by interacting with S^{315} and/or C^{316}. Disruption of these interactions by mutation of ORF74R^{322} distorts an H-bonding between conserved amino acids in TM2, TM7, and the 8th helix [24]. The latter is thought to be crucial for the positioning of the TM domain, and the coupling to Gα$_q$ [24,41]. Very recently, Carpenter et al. [46] solved the structure of the adenosine 2A receptor in complex with the α5 helix of an engineered Gs protein, mini Gs, and described extensive interactions between the end of 7th helix and the beginning of the 8th helix [46,47]. However, no interactions were observed between the H7-H8 boundary and Gs in the structure of the ß2 adrenergic receptor [48], indicating that the structural requirement for the interaction of receptors with signaling molecules such as G proteins display strong receptor specificity. In this line, although the primary amino acid sequences of the 8th helix of CCR2b and CCR5 receptors are almost identical, CCR5

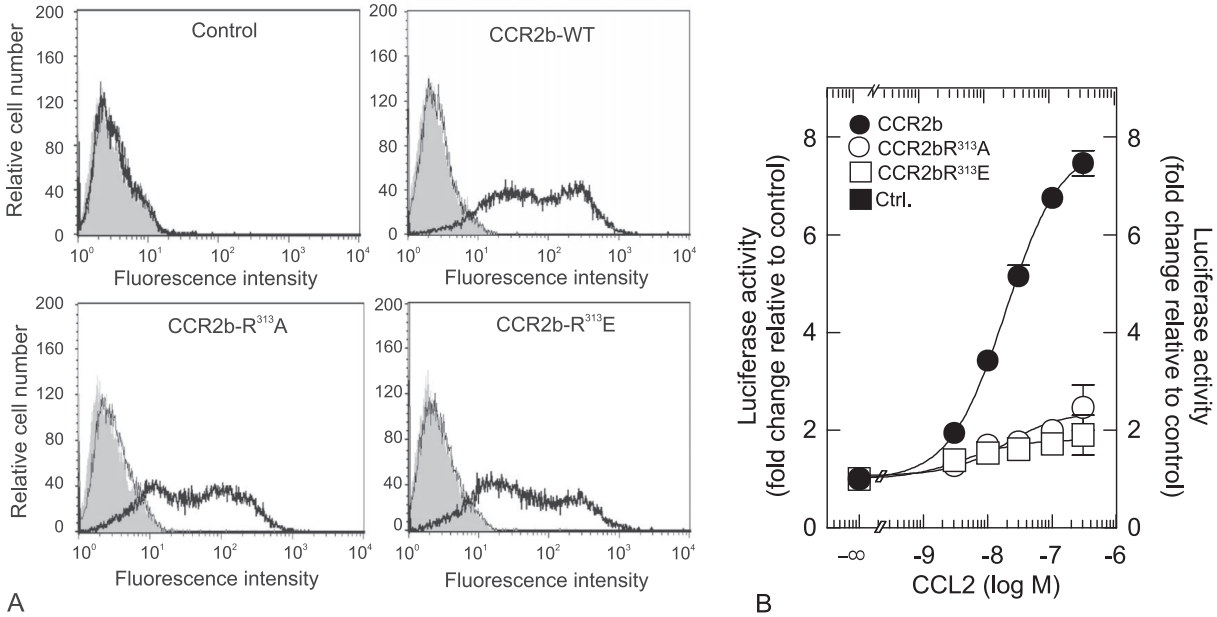

Fig. 8. (A) FACS analysis of CCR2 receptor expression of transiently transfected HEK293 cells. HEK293 cells were transfected in 12-well plates with either empty vector pcDNA3.1+ (Ctrl.) or vector pcDNA3.1+ encoding Flag-epitope-tagged CCR2b, CCR2b-R^{313}A, and CCR2b-R^{313}E (each 1 µg per well) as indicated. Surface expression of CCR2 receptors was analyzed by fluorescence-activated cell sorting (FACS) for transiently transfected HEK293 cells. Expression of CCR2b in control cells (Ctrl.), and cells transiently expressing CCR2b wild type protein (upper panels), and cells transiently expressing CCR2b-R^{313}A, and CCR2b-R^{313}E (lower panels) are shown. **(B) CCL2-stimulated SRF-dependent transcriptional activation by CCR2b and CCR2b mutants.** HEK293 cells were cotransfected in 48-well plates with reporter plasmids (30 ng each per well of pSRE.L and pRL-TK), with pcDNA3.1+ (black square) and 250 ng/well Flag-epitope-tagged wild type CCR2b-(*CCR2b,* black circle) or CCR2b-R^{313}A (*CCR2b-R^{313}A,* white circle)-, and CCR2b-R^{313}E-cDNA (*CCR2b-R^{313}E,* white square). Twenty-four hours after transfection, the cells were either incubated with solvent or with increasing amounts of CCL2 as indicated (*CCL2*; 3 nM, 10 nM, 30 nM, 100 nM, 300 nM) Cell lysates were assayed for luciferase activity, and luciferase activities and fold activation was determined as described in Fig. 1.

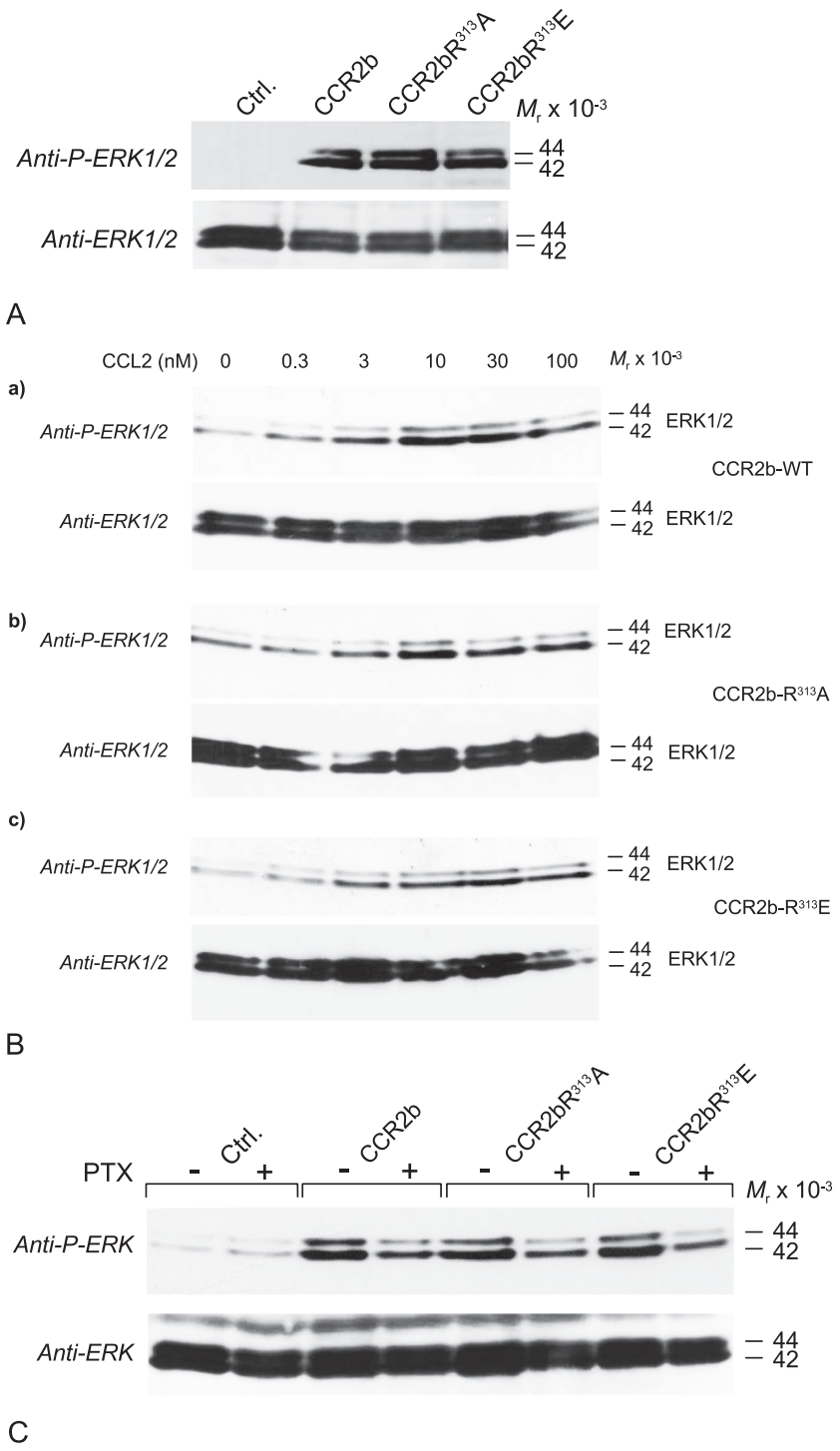

Fig. 9. (A) Effect of CCL2 on phosphorylation of extracellular signal-activated kinases in cells expressing CCR2b and CCR2b mutants. HEK293 cells were transfected in 12-well plates with either empty vector pcDNA3.1 + (Ctrl.) or vector pcDNA3.1 + encoding Flag-epitope-tagged CCR2b, CCR2bR^{313}A, and CCR2bR^{313}E (each 1 μg/per well) as indicated. HEK293 cells were serum-starved for 2 h and then incubated for 5 min with CCL2 (50 nM). Phosphorylated ERK1 and ERK2 kinases (ERK1/2) were detected in whole cell lysates by Western blotting using an anti-phospho-ERK1/2 antibody (*upper panel*). Equal protein loading was confirmed by probing the same blot with an anti-ERK1/2 antibody (*lower panel*). **(B) Phosphorylation of extracellular signal-activated kinases in cells expressing CCR2b and CCR2b mutants stimulated with increasing concentrations of CCL2.** HEK293 cells were transfected in 12-well plates with vector pcDNA3.1 + encoding Flag-epitope-tagged CCR2b (**a**), CCR2bR^{313}A (**b**), and CCR2bR^{313}E (**c**) (each 1 μg/per well) as indicated. HEK293 cells were serum-starved for 2 h and then incubated for 5 min with increasing concentrations of CCL2 as indicated (0.3 nM, 3 nM, 10 nM, 30 nM, 100 nM). Phosphorylated ERK1 and ERK2 kinases (ERK1/2) were detected in whole cell lysates by Western blotting using an anti-phospho-ERK1/2 antibody (*upper panels of **a**, **b**, and **c***). Equal protein loading was confirmed by probing the same blot with an anti-ERK1/2 antibody (*lower panels of **a**, **b**, and **c***). **(C) Influence of pertussis toxin pretreatment on CCL2-induced phosphorylation of extracellular signal-activated kinases in cells expressing CCR2b and CCR2b mutants.** HEK293 cells were transfected in 12-well plates with either empty vector pcDNA3.1 + (Ctrl.) or with vector pcDNA3.1 + encoding Flag-epitope-tagged CCR2b, CCR2b-R^{313}A, and CCR2b-R^{313}E (each 1 μg/well) as indicated. Nine hours after transfection the cells were further incubated in the absence (−) or presence (+) of pertussis toxin (30 ng/ml) for 16 h. Cells were serum-starved for 2 h and then incubated for 5 min with CCL2 (50 nM). Phosphorylated ERK1 and ERK2 kinases (ERK1/2) were detected in whole cell lysates by Western blotting using an anti-phospho-ERK1/2 antibody (*upper panel*). Equal protein loading was confirmed by probing the same blot with an anti-ERK1/2 antibody (*lower panel*).

receptors do not couple to Gα_q to activate SRF-dependent gene transcription [15]. Similarly, expression of mouse CCR2 that carry the conserved R^{313} together with Gα_q did not synergistically increase SRF activity in HEK293 cells (own unpublished data).

Importantly, as shown in the present work while R^{313} determines coupling of the two human CCR2 receptors to Gα_q/Gα_{14} thereby regulating SRF activity and inositol phosphate formation, other CCR2 receptor-induced cellular signaling, e.g. G$_i$-mediated MAPK ERK1/2 activation, internalization, and changes in intracellular Ca^{2+} concentrations were found only mildly or partially impaired, respectively. GPCRs, including chemokine receptors initiate a ligand induced rapid activation of extracellular signal-regulated kinases ERK1 and ERK2, involving coupling to G proteins and/or non-G proteins such as β-arrestins [14,49–54]. CCR2b-induced ERK1/2 activation has been reported to depend on coupling of the receptor to G$_i$ proteins, and the activation of protein kinase C, phosphoinositide-3 kinases (PI3-Ks) and Ras [14,55,56]. In contrast, β-arrestin scaffold function, and internalization of CCR2b receptors seem to play a minor role in the activation of the ERK1/2 pathway by CCR2 receptors [14]. The findings presented in the current work affirmed that CCR2 receptor-induced ERK1/2 phosphorylation is G$_i$ dependent, and that signaling via G$_i$ proteins is not affected by the changes in Arg^{313} of the 8th helix. Furthermore, these findings indicate that different structural determinants within the 8th helix influence the recognition of specific G proteins.

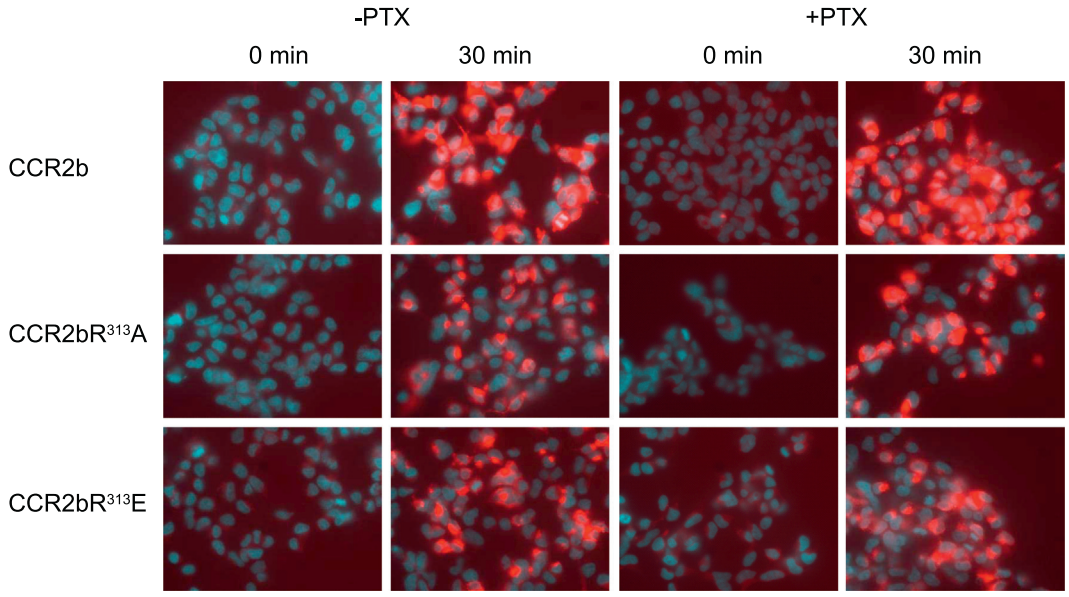

Fig. 10. CCL2-mCherry-induced receptor internalization of cells expressing CCR2b and CCR2b mutants. HEK293 cells were transfected in 24-well plates with vector pcDNA3.1 + encoding Flag-epitope-tagged CCR2b, CCR2b-R^{313}A, and CCR2b-R^{313}E (each 500 ng/per well). Nine hours after transfection the cells were further incubated in the absence (−) or presence (+) of pertussis toxin (PTX; 30 ng/ml) for 16 h. The cells were incubated with CCL2-mCherry (50 nM) in serum-free medium and incubated for 30 min at 37C. Cells were fixed, and the nuclei counterstained with Hoechst solution as described in Material and Methods. Fluorescence of cells was analyzed and pictures were taken using an Olympus IX70 fluorescence microscope (Olympus, Hamburg, Germany).

Amino acids of the 8th helix have been described to be of importance for folding and trafficking of several class A receptors [57]. For example, changes in a di-leucin motif ($L^{304}L^{305}$) within the 8th helix of the leukotriene B4 receptor results in enhanced ligand-induced internalization [58]. Interestingly, changes in K^{315} of the bradykinin receptor B2 two amino acids upstream of the corresponding R^{313} in CCR2 receptors not only affect G protein coupling but also impair receptor internalization [59]. However, loss of the positive charge at position 313 of the 8th helix of the CCR2b receptor had only little effect (up to 10%) on the cell surface expression and only mildly impaired internalization as deduced by FACS analysis and by using fluorescent CCL2-mCherry fusion proteins. These findings exclude an important role of R^{313} in these processes, and indicate that coupling of the receptors to $G\alpha_q$ proteins is not a major prerequisite for internalization. Also of note, pretreatment of receptor-expressing cells with PTX did not interfere with receptor-mediated internalization of fluorescent CCL2-mCherry fusion proteins. This observation points to a G_i protein-independent process, thereby confirming afore made observations of Garcia et al. [49] showing a clathrin-dependent and -independent internalization of CCR2b that is scarcely blocked by pretreatment with PTX [49].

In a few cases, changes in amino acids of the 8th helix have been correlated with constitutive activity of GPCRs [43,60]. Exchange of L^{304} and L^{305} in the 8th helix of the BLT-1 receptor resulted in a moderate constitutive receptor activation [60], and charge reversal of R^{384} to glutamate (E) in the β1-adrenergic receptor resulted in a receptor with increased basal activity and enhanced phosphorylation by G protein-coupled receptor kinases [43]. However, the CCR2 receptor mutants including the charge reversed R^{313}E showed little if any increase in basal activity in the signaling pathways analyzed. Interestingly, replacement of R^{322} by tryptophan (W) within the 8th helix of the virally encoded oncogene ORF74 was reported to influence chemokine binding and/or inhibition of constitutive receptor activity by an inverse agonist [24]. However, changes in basic arginine at position 313 of the CCR2 receptor only mildly impaired binding and internalization of CCL2-mCherry. Whether changes in amino acid 313 influences binding of one of the other possible ligands to CCR2 receptors, e.g. CCL7, CCL8, CCL11, or CCL13 [5] awaits further experimentation. All these findings indicate that the interface generated by the characteristics of specific amino acids within the 8th helix and the resulting structural interactions are unique for each receptor and determine its G protein coupling, internalization, and/or receptor trafficking.

Another functional response stimulated by CCL2 on CCR2b is the induction of changes in intracellular Ca^{2+} concentration [1,8,61]. The results of the present work show that ligand-induced CCR2b-receptor mediated changes in intracellular Ca^{2+} concentrations depend on both G_i- and G_q-coupling at least in HEK293 cells expressing exogenous CCR2 receptors. In accordance, the mutant CCR2bR^{313}A that are massively reduced in coupling to $G\alpha_{q/14}$ proteins displayed severely reduced ligand-stimulated changes of intracellular Ca^{2+} concentrations. Furthermore, pretreatment of CCR2bR^{313}A expressing cells with pertussis toxin completely abolished the residual ligand-induced Ca^{2+} response, indicating that ligand-induced Ca^{2+} response of this mutant comprises G_i-coupling. In addition, we observed that stimulation of CCR2b receptors and coupling to G_q but not G_i proteins resulted in a reduction of Ca^{2+} release induced by endogenously expressed lysophosphatic acid (LPA) receptors, potentially by cross desensitizing of the latter receptors. Like chemokines, LPA regulate a variety of cellular functions including directed migration natural killer cells [62], and monocytes/macrophages [63], and LPA receptors have been described in numerous cell types, and are known to play a role in physiological and pathophysiological conditions [33,34]. Crosstalk of receptors controlling migration of neutrophils, e.g. receptors stimulated by formulated peptides (fMLP) and chemokine receptors has been reported [64]; a process referred to as heterologous or cross desensitization [65]. As shown, pretreatement of murine neutrophils with fMLP render the cells unresponsive to other chemoattractants, e.g. the chemokine KC [64]. Also of interest, chemokine receptors and opioid receptors were shown to counter control their activity via heterologous desensitization [66,67]. The potential physiological role of CCR2 chemokine receptor-induced cross talk with LPA receptors remains to be elucidated.

Very recently, a structure of an engineered CCR2b (truncation of the carboxyl terminal residues 329–360, and addition of T4 lysozyme into the intracellular domain 3, and exchange of the amino acids L226-R240 by the corresponding stretch of the muscarinic receptor S226-K240) in a ternary complex with the allosteric antagonist CCR2-RA[R] has been solved [45]. The authors identified a binding pocket including the

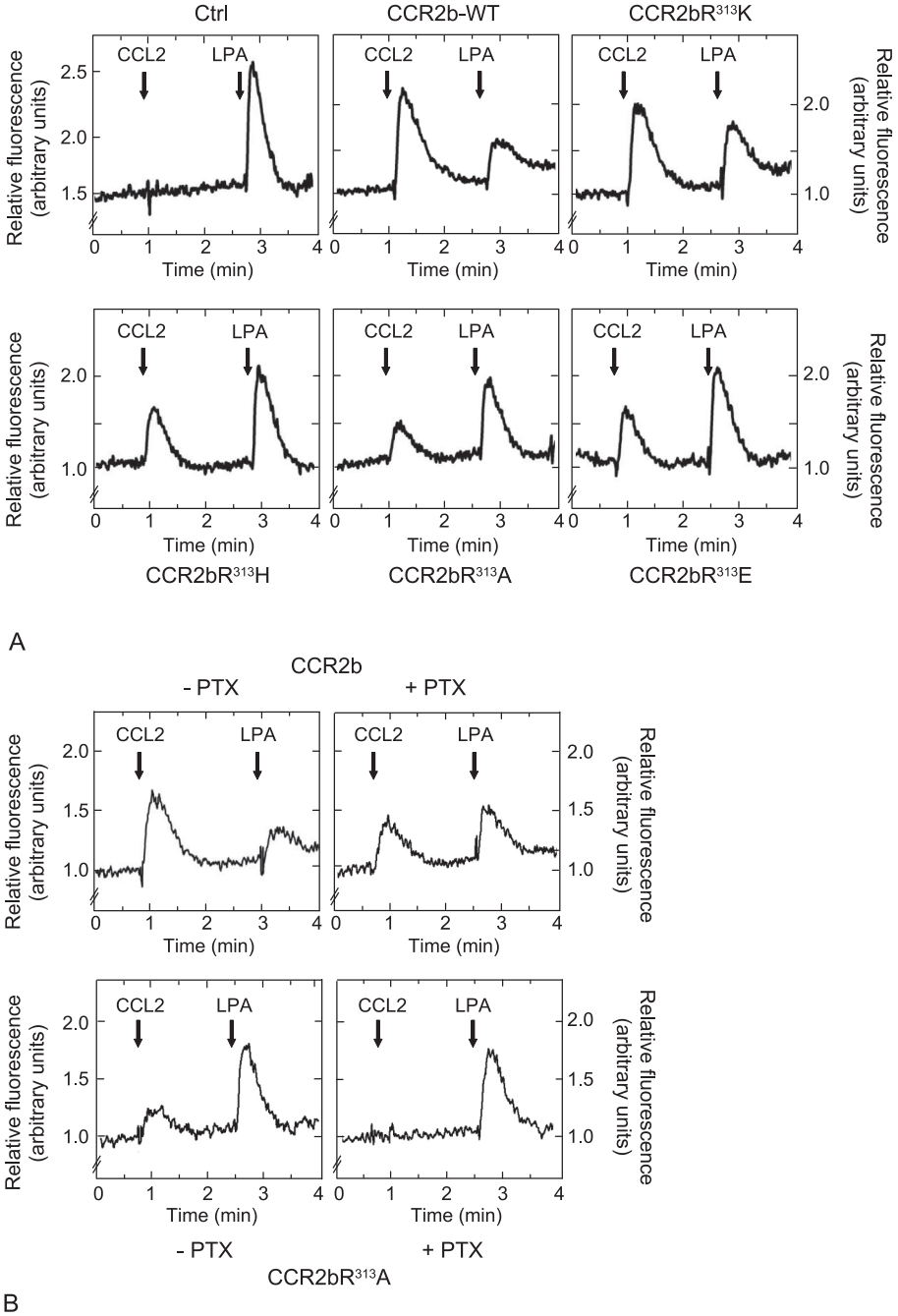

Fig. 11. (A) CCL2-stimulated changes in concentration of cytosolic free Ca^{2+} in cells expressing CCR2b and CCR2b mutants. HEK293 cells were transfected in 10 cm dishes with either empty vector pcDNA3.1 + (Ctrl.) or with vector pcDNA3.1 + encoding Flag-epitope-tagged CCR2b, CCR2b-R^{313}A, and CCR2b-R^{313}E (each 8 μg/well). Twenty four hours after transfection 2×10^6 cells were loaded with Fura 2-AM (1 μM). At the times indicated by arrows, CCL2 (50 nM) or LPA (20 μM) were added to the cells. The fluorescence due to changes in intracellular Ca^{2+} concentrations was recorded. **(B) CCL2-stimulated changes in concentration of cytosolic free Ca^{2+} in cells expressing CCR2b and CCR2b mutant R^{313}A in the absence and presence of PTX.** HEK293 cells were transfected in 10 cm dishes with either empty vector pcDNA3.1 + (Ctrl.) or with vector pcDNA3.1 + encoding Flag-epitope-tagged CCR2b, CCR2bR^{313}A, and CCR2bR^{313}E (each 8 μg/per well). Nine hours after transfection the cells were further incubated in the absence (−) or presence (+) of pertussis toxin (30 ng/ml) for additional 16 h. HEK293 cells (2×10^6) were loaded with Fura 2-AM at the times indicated by arrows, CCL2 (50 nM) or LPA (20 μM) were added to the cells. The fluorescence due to changes in intracellular Ca^{2+} concentrations was recorded.

hydrophobic amino acids V^{244}, Y^{305}, K^{311} and F^{312} to be of importance for the binding of the allosteric antagonist CCR2-RA[R]. By comparing the structures of the rhodopsin/$G\alpha_t$ and β_2-adrenergic/Gs receptor/G protein interfaces with the solved CCR2 structure they identified the residues G^{309}, E^{310}, and K^{311} in CCR2 potentially involved in G protein coupling. Similar, Zweemer et al. reported in 2014 that exchange of L^{311} or F^{312} by alanine severely reduced ligand-induced GTPγS binding [68]. These findings indicate that L^{311} and F^{312} are involved in coupling of the CCR2b receptor to G_i proteins. However, as reported herein, coupling of CCR2 receptor to $G\alpha_q$ proteins is preferentially mediated by R^{313}. Whether changes in L^{311} and F^{312} directly impairs coupling to $G\alpha_{q/14}$ and/or influences interaction of R^{313} with $G\alpha_{q/14}$ awaits further experimentation. Based on the identification of receptor domains that participate in effector binding and in the signaling conformations of GPCRs, positive allosteric modulators (PAM) or negative allosteric modulators (NAM) have been described for various class A receptors [69,70]. The latter include the CXC chemokine receptor CXCR4, CXCR1 and CXCR2, and as mentioned above CCR2 [68–70]. Interestingly, the pepducin ATI2341 acting on CXCR4 has the ability to bias G protein signaling, in particular G_i, from β arrestin signaling [71]. Also of interest, for the prostaglandine F2α receptor a compound was identified acting as PAM on agonist-induced $G\alpha_q$ signaling and NAM on $G\alpha_{12}$ signaling [72]. However, the finding that arginine 313 plays a critical role for $G_{q/14}$ protein-dependent but not for G_i protein-dependent cellular signaling or for G_q/G_i-independent receptor internalization, may promote the development of allosteric modulators of non-canonical signaling of CCR2 receptors.

5. Conclusions

Taken together, using various CCR2a and CCR2b mutants we showed that R^{313} within the amino terminal portion of the putative 8th

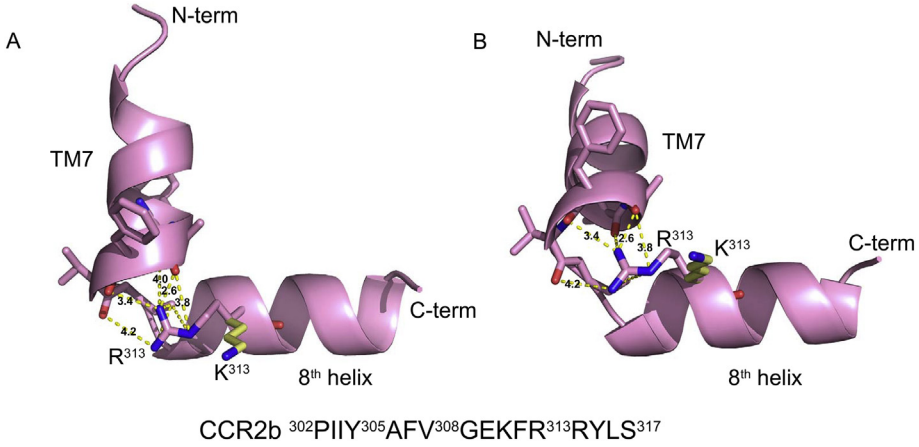

Fig. 12. Modeling CCR2b-arginine 313 side chain interactions. The known structure of the human CCR2b receptor (RCSB-PDB: 5T1A; [45] was modeled (Swiss-Model and PyMOL Molecular Graphics System) to visualize hydrogen bond interactions of R^{313} in the CCR2b wild type protein compared to the CCR2b $R^{313}K$ replacement mutant. The primary sequence of the amino acids at the end of the transmembrane domain 7 (7TM), and part of the 8th helix is given below. The amino terminus (N-term) end carboxyl terminus (C-term) of the receptor protein fragment is accordingly labeled. **Figs. A** and **B**, The positions of the 7TM and 8th helix (pink) are indicated, and the side chains or main chains of R^{313} (pink and blue), Y^{305} (red) and V^{308} (red), respectively, in a stick model are given. The location of the side chain of the modeled K^{313} is shown in green and blue. The potential hydrogen bonds of R^{313} with the major chains of Y^{305} (red) and V^{308} (red) at the end of the 7TM or at the amino-terminal most portion of the 8th helix, respectively, are displayed in yellow. The distances of predicted hydrogen bonds of R^{313} range from 2.6–4.2 Å in this model. The replacement of R^{313} by K^{313} is likely to at least reduce formation of hydrogen bonding of the side chain. Given is a side view (**Fig. A**) and a view of the 8th helix after the camera was rotated by 40° about the x axis of the presentation. (**Fig. B**). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

helix of CCR2 receptors is of major importance for $G\alpha_{q/14}$-mediated biased signaling. Changes in the basic amino acid at position 313 of human CCR2 receptors potentially interfere with the hydrogen bonding that may control coordinated movements of TM7 and helix 8 necessary for the generation of a $G\alpha_{q/14}$ interacting surface. Of note, the observation that selectivity of coupling to Gα proteins can be attributed to a single amino acid within the putative 8th helix of human CCR2 receptors may allow to develop specific allosteric modulators to address biased G_q-mediated CCR2 receptor signaling.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2018.10.007.

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