

Ligand-independent CXCR2 dimerization

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Running title: CXCR2 receptor functions as a dimer.

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Summary

Homo- and hetero-oligomerization have been reported for several G protein coupled receptors (GPCRs). The CXCR2 is a GPCR that is activated, amongst the others, by the chemokines CXCL8 (IL-8) and CXCL2 (GRO β) to induce cell chemotaxis. We have investigated the oligomerization of CXCR2 receptors expressed in HEK cells and generated a series of truncated mutants to determine if they could negatively regulate the wild-type (wt) receptor functions. CXCR2 receptor oligomerization was also studied by co-immunoprecipitation of GFP- and V5-tagged CXCR2. Truncated CXCR2 receptors retained their ability to form oligomers only if the region between the aminoacids A106 and K163 was present. In contrast, all the deletion mutants analysed were able to form heterodimers with the wt CXCR2 receptor, albeit with different efficiency, competing for wt/wt dimer formation. The truncated CXCR2 mutants were not functional, and when co-expressed with wt CXCR2 interfered with receptor functions, impairing cell signalling and chemotaxis. When CXCR2 was expressed with the AMPA-type glutamate receptor GluR1, CXCR2 dimerization was again impaired in a dose-dependent way, and receptor functions were prejudiced. In contrast CXCR1, a chemokine receptor that shares many similarities with CXCR2, did not dimerize alone or with CXCR2 and when co-expressed with CXCR2 did not impair receptor signalling and chemotaxis. The formation of CXCR2 dimers was also confirmed in cerebellar neuron cells. Taken together, we conclude from these studies that CXCR2 functions as dimer, and that truncated receptors negatively modulate receptor activities competing for the formation of wt/wt dimers.

Introduction

Homo- or hetero-dimerization of G-protein coupled receptors (GPCRs) has recently emerged as a constitutive or ligand-induced property of several receptor types. Receptor oligomerization has functional implications in terms of cell surface expression, ligand binding, signalling, and receptor trafficking (1). Many different GPCR have been proved to undergo homo- or heterodimer formation; these include the receptors for dopamine that can form both homo (2-4) and heterodimers with somatostatin receptors (5), angiotensin A1 and A2 (6), adrenergic (7,8), GABAB (9-11), δ - and κ -opioid (12-15), rhodopsin (16), mGlu (17), vasopressin V2 (18), vasopressin/ossitocin (19), muscarinic (20), glucagone (21), Ca^{2+} (22) sphingosine-1-phosphate (23), and the chemokine CXCR4, CCR2 and CCR5 receptors (24-28). The molecular mechanism of receptor oligomerization varies between different receptors and involves transmembrane (TM) domains as well as extra-membrane regions. Cysteine residues in the fourth TM segment are responsible for D2 dopamine receptors dimerization (4) and disulfide bonds are also involved in the oligomerization of Ca^{2+} (22), κ opioid (13), sphingosine 1-phosphate (23), and V2 vasopressin receptors (18); sequences in the N-terminal extracellular domains are involved in mGluR (17) and CCR5 (24-27) homodimer formation; while the C-terminal region is essential for δ -opioid receptor dimerization (12). The seventh TM domain controls non-covalent hydrophobic interactions for adrenergic receptors, which also require receptor glycosilation (7,8). Contrasting consequences are also reported for receptor functional properties: in some cases receptor dimerization is essential for receptor function, as demonstrated for GABAB (9-11) and for the taste receptors (29): for other GPCRs, hetero-dimerization impairs or changes the features of their activation, as shown for the angiotensin II (6), the opioid and dopamine (30,31), and for the β adrenergic receptors (32). Furthermore, both agonist-dependent and agonist-independent receptor dimerization have been reported. From all these studies it clearly appears that there are not unique molecular mechanisms of dimerization for GPCRs, and that the functional effects of receptor oligomerization are likely far to be completely understood.

The oligomerization of chemokine receptors has been shown to be relevant for receptor biology (24,26,27): the homo-dimerization of the chemokine receptor CCR5 with a natural genetic mutation found in some ethnic groups (*ccr5Δ32*) confers some resistance to HIV-1 infection due to a sort of loss of function of the hetero-dimers (24). Both CCR5 and CXCR4 receptors have been reported to homo-dimerize but the role of agonist stimulation on receptor oligomerization is debated (25,27,28). CCR5 receptor oligomerization occurs in the endoplasmic reticulum, consistent with a constitutive receptor assembly under resting conditions (27). CCR2 and CCR5 receptors heterodimerize upon treatment with a combination of chemokines, and heteromeric receptors may induce different functional responses (26). Since chemokine receptor dimerization may add further complexity to the biology of this promiscuous receptor family (33), we decided to investigate the oligomerization of the chemokine receptor CXCR2. We have recently demonstrated that the CXCR2 receptors, expressed by cerebellar neurons or transfected on HEK cells, physically and functionally interact with the α -amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) type glutamate receptors (34,35). Here, we investigated whether CXCR2 receptors could also form oligomers, and what consequences oligomerization might produce on CXCR2 functions.

In this paper we describe that CXCR2 forms oligomers when expressed in HEK cells and in native neuronal systems and that oligomer formation is independent of receptor activation by agonist. Attempting to define the molecular regions responsible for receptor oligomerization, we have generated several drastic deletion mutants of CXCR2. We demonstrated that most of these mutants act as dominant negative inhibitors of receptor function, in terms of cell signalling and chemotaxis. Furthermore, CXCR2 receptors mutated in different molecular domains and expressed in HEK cells do not complement the original function. From all these data, we suggest that the active form of the CXCR2 receptor is a dimer and that each individual subunit is activated with an intra-molecular mechanism (*cis*-activation). To our knowledge, this is the first demonstration that CXCR2 receptors function in oligomerized state.

EXPERIMENTAL PROCEDURES

Materials-Monoclonal and polyclonal antibodies against human CXCR2 (E2, C19), anti ERK2, polyclonal antibody against rat CXCR2 (K19) and against GFP (FL-1) were purchased from Santa Cruz Biotechnology (Santa Cruz CA); polyclonal antibody against human GluR1 (Ab1504) was purchased from Chemicon Int. (Temecula CA); anti-phospho Akt (Ser473), anti Akt, and anti-phospho-p44/p42 MAP kinase (Thr202/Tyr204) mAb were from New England Biolabs Inc. (Hertfordshire, UK); recombinant rat CXCL2 and human CXCL8 were from Peprotech (London UK); transwell cell culture inserts were from Becton Dickinson Labware (Franklin Lakes, NJ); BCA protein assay was from Pierce (IL, USA). All culture media and monoclonal V5 Ab were purchased from Life Technology Italia (Milan, Italy).

Generation of CXCR2 mutants-cDNAs encoding for CXCR2 and CXCR1, cloned in pCEP4 expression vector, were kindly provided by Dr. Massimo Locati (University of Brescia, Italy). cDNA for human GluR1 (flip) was from ATCC (Manassas, VA). In order to obtain CXCR2-tagged proteins, cDNA encoding for CXCR2 (Swiss-Prot # P25025) was subcloned in pEGFP-C3 (Clontech, Palo Alto, CA) expression vector using the primers 5'-ATAACTCGAGATGGAAGATTTTAAC-3' and 5'-GTCAGAATTCTTAGAGAGTAGTG-3'; and in pcDNA3.1D/V5-His-TOPO (Invitrogen, Groningen, The Netherlands) using the primers 5'-AGACAAGCTTATGGAAGATTTTAAC-3' and 5'-ATAACTCGAGTGGAGAGTAGTG-3'. C-(A315, F183, K163, V142) and N-terminal (Y49, A106, D143) CXCR2 deletion mutants were generated by PCR and cloned into the expression vector pCEP4 (Invitrogen, Groningen, The Netherlands). The following couples of primers were used for the PCR reactions: 5'-GATCAAGCTTATGGAAGATTTTAAC-3' and 5'-GATCCTCGAGTTAGGCGTAGATGAG-3' for CXCR2-A315; 5'-GATCAAGCTTATGGAAGATTTTAAC-3' and 5'-GATCCTCGAGTTAGAAAAGTAAGACAG-3' for CXCR2-F183; 5'-GATCAAGCTTATGGAAGATTTTAAC-3' and 5'-GATCCTCGAGTTATTTGACCAAGTA-3' for CXCR2-K163; 5'-GATCAAGCTTATGGAAGATTTTAAC-3' and 5'-

GATCCTCGAGTTACACACTGATGCAG-3' for CXCR2-V142; 5'-GATCAAGCTTATGTATTTTGTGGTC-3' and 5'-TATCCTCGAGTTAGAGAGTAGTGGA-3' for Y49-CXCR2; 5'-GATCAAGCTTATGGCCTCCAAGGTG-3' and 5'-TATCCTCGAGTTAGAGAGTAGTGGA-3' for A106-CXCR2; 5'-GATCAAGCTTATGGACCGTTACCTG-3' and 5'-TATCCTCGAGTTAGAGAGTAGTGGA-3' for D143-CXCR2. The fidelity of all the CXCR2 constructs was checked by DNA sequencing and their expression in HEK cells was confirmed by western blot and immunofluorescence analysis. GluR1-GFP fusion proteins were obtained as previously described (35).

Cell transfection and signalling studies-HEK 293 (HEK) and human CXCR2-stably transfected HEK 293 (HEK-CXCR2) cells (kindly provided by Dr. Massimo Locati, University of Brescia, Italy and by Dr. Adit Ben-Baruch, Tel-Aviv University, Israel), were transfected with Lipofectamine 2000 plus reagent (Invitrogen, Groningen, The Netherlands). Routinely, cells were used for experiments 48 h after transfection. When used for cellular signalling, 18 h after transfection, cells from different transfections were trypsinized and re-seeded on 12 well plates. After additional 6 h cells were serum-starved for 16 h and further incubated for additional two h in Locke's buffer (35). When necessary, cells were pre-incubated with PTX (100 ng/ml, 16 h) or LY294002 (50 μ M, 1 h) and stimulated with chemokine (120 nM for CXCL8 and 30 nM for CXCL2) in this same buffer. After 10 min, the chemokine-containing medium was removed and cells were washed with ice-cold PBS, lysed in Triton X-100 buffer (described below) and analysed for protein content with a commercial kit. Same amounts of cellular proteins (10-20 μ g) were analysed by SDS-PAGE and western blot analysis with Abs specific for phospho-ERK1/2, phospho-Akt, ERK2 and Akt. For experiments with cerebellar neurons, cerebellar granule neurons (CGNs) were obtained from 3 or 7-day-old Wistar rats, as described (35), stimulated with CXCL2e and analyzed as above.

Immunoprecipitation-Transiently transfected HEK 293 cells were washed with phosphate-buffered saline (PBS), and lysed for 15 min on ice in buffer containing 50 mM Tris-HCl pH 8, 20 mM

EDTA, 1% Nonidet P-40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 15,000x g for 20 min at 4°C, cell lysates were precleared with normal mouse serum or with pre-immune rabbit IgG and then incubated at 4°C for 16 h with mAb anti-CXCR2 (8 µg/ml), mAb V5 (10 µg/ml), or polyclonal anti-GFP (2 µg/ml). The resulting immunocomplexes were separated on SDS polyacrylamide gel by electrophoresis and analysed by Western blotting with anti-GluR1 antibody (Ab 1504), anti-V5 antibody (V5), or anti-CXCR2 antibody (C19).

Expression of wt and mutant CXCR2 proteins-HEK cells were transiently transfected with different combinations of CXCR2 constructs and lysed by addition of hot SDS Laemmli buffer 2X or by using a Triton X-100 lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% TritonX-100, 20 mM sodium pyrophosphate, 20 mM NaF, 1 mM sodium orthovanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM CaCl₂). Expression of wt and mutant CXCR2 was analysed by western blotting of SDS polyacrilamide electrophoresis gel. MAb E2, recognising a N-terminal epitope, was used to analyse wt, A315, F183, K163 and V142 proteins; polyclonal Ab C19, recognising a C-terminal epitope was used to analyse wt, Y49, A106 and D143 proteins.

Chemotaxis assay-CXCL8-induced chemotaxis was investigated in HEK cells transfected with CXCR2 alone or with different combinations of truncated mutants. 48 h after transfection, cells were trypsinized, washed twice in chemotaxis medium (FBS-free DMEM plus BSA 0,1% and 25 mM Hepes pH 7,4) and plated (500,000 cells/well) on collagen-pre-coated 12 mm transwells (12 µm pore size filters) in the same medium. The lower chambers of the transwell system contained vehicle (water) or CXCL8 (6 nM) in chemotaxis medium. After 2 h of incubation at 37° C, cells were washed with PBS and treated with trichloroacetic acid on ice for 10 minutes. Cells adhering to the upper side of the filter were scraped off, while cells on the lower side were stained with a solution containing 50 % isopropanol, 1 % formic acid and 0,5 % (w/v) brilliant blue R250. Stained

cells were visually counted in at least 20 fields using a 20 x objective. Chemotactic index is obtained by the ratio between the number of migrating cells in chemokine-treated vs. untreated cells for each type of transfection.

Results

CXCR2 forms dimers in HEK cells and in cerebellar neurons.

To examine whether CXCR2 forms oligomers, as already demonstrated for other chemokine receptors, we generated CXCR2 constructs with either a V5 (CXCR2-V5) or a GFP (GFP-CXCR2) tail, at the C- and N-terminal domains, respectively. These constructs were transfected in HEK cells and the cell lysates, obtained 48 h later, were immunoprecipitated with GFP Ab and analysed by western blot for V5 immunoreactivity. Results in Fig. 1A show that the complex CXCR2-V5/GFP-CXCR2 is immunoprecipitated by the GFP Ab only when the two constructs are simultaneously expressed. Similar results were obtained when the immunoprecipitation was performed with V5 Ab and the corresponding western blots were analysed for GFP immunoreactivity (not shown). Co-immunoprecipitation was not detected in a mixture of lysates from cells individually expressing the tagged receptors, excluding the possibility of non-specific receptor aggregation (not shown). Fig. 1B shows that when HEK 293 cells, transfected with plasmids containing CXCR2 (wt), were lysed with SDS Laemmli buffer and analysed by western blot, both monomeric and oligomeric forms of the receptor become evident. A mutated form of the CXCR2 receptor (A315), obtained by the complete truncation of the intracellular C-terminal tail, has a similar shifted pattern. This supports the view that the high molecular weight bands are constituted by aggregated receptors. When CXCR2-transfected cells were treated with tunicamycin and analysed for receptor oligomerization, we observed the disappearance of glycosylated monomeric CXCR2, and a shift in the putative CXCR2 dimer band (Fig. 1C). This indicates that CXCR2 receptors dimerize before they appear on the plasma membrane, during the biosynthetic pathway. To investigate whether receptor oligomer formation could be modulated by receptor activation, transfected cells were treated with the specific agonist CXCL8 for 5 min, and analysed as above. Fig. 1D indicates that the formation of CXCR2

oligomers (wt or A315) was not affected by receptor activation, showing that CXCR2 oligomer formation was ligand-independent. CXCR2 dimers were resistant to membrane solubilization with detergent, being present both in cell lysates obtained with Triton X-100 buffer and with hot SDS buffer, and were also resistant to reducing conditions, since the Laemmli buffer we used always contained 50 mM DTT (see methods).

We have previously described the functional expression of CXCR2 in rat cerebellar granule neurons (36). Lysates obtained from 8 day-cultured neurons, analysed by western blot with a polyclonal CXCR2 Ab (K19), revealed a main band at 40 kDa and an additional protein at about 78 kDa, which likely represents CXCR2 dimers (Fig. 1E).

To establish the receptor domain(s) directly involved in CXCR2 receptor oligomerization, deletion mutants were generated at the C- or N- terminal regions (see Fig. 2) and tested for dimer formation by western blot analysis. For each kind of transfection, protein expression was also confirmed by immunofluorescence (not shown). Fig. 3A (right part, and Fig. 1B) shows that when the whole intracellular C-terminal (CXCR2-A315) region was deleted, the truncated CXCR2 receptors retained their ability to form homodimers, as evidenced by the immunological detection of both the monomeric deleted receptors and additional proteins with molecular weights compatible with truncated dimers. Progressive deletions at the C- terminal region lead to the generation of receptors truncated at the aminoacids F183, K163 and V142, as detailed in Fig. 2. Dimeric forms of CXCR2-F183 were detectable (Fig. 3A, left side), while CXCR2-V142 was only present as monomer (shown in Fig. 3B). Interestingly, the C-terminally deleted mutants A315, F183 and V142, when co-expressed together with the wild type CXCR2, retained the ability to bind the wild type (wt) receptors, as evidenced by the bands with molecular weight corresponding to the sum of the wt and deleted receptor types (here called “heterodimers” wt/A315 and wt/F183, Fig. 3A and wt/V142, Fig. 3B). Interestingly, the C-terminal mutant CXCR2-K163 also retained the ability to homodimerize, as demonstrated in Fig. 3C.

When the whole extracellular N-terminal (Y49-CXCR2) region was deleted, both homo- and heterodimers (wt/Y49) could be formed (Fig. 3D, left side). Further truncation of the CXCR2 receptor from the N-terminal region, in particular at the aminoacid D143 (described in Fig. 2) results in receptors with different properties: D143-CXCR2 mutants did not dimerize, but formed heterodimers with wt CXCR2 (Fig. 3D, right side). Intermediate N-terminal mutants obtained truncating the CXCR2 at the aminoacid A106 (A106-CXCR2) retained the ability to form both homo- and heterodimers (Fig. 3E).

CXCR2 dimer formation is impaired by GluR1 co-expression in HEK cells.

We have previously reported that CXCR2 physically interacts with various subunits of the AMPA-type glutamate receptors (GluR) both in HEK cells and cerebellar granule neurons, and that this interaction negatively modulates CXCR2-mediated cell chemotaxis (35). To investigate whether CXCR2/GluR1 co-expression interferes with CXCR2 receptor oligomerization, HEK cells were transfected with raising amounts of plasmid containing the GluR1 subunit cDNA. Fig. 4A shows that increasing the amount of GluR1 cDNA co-transfected with a fixed quantity of CXCR2 resulted in a dose-dependent reduction of CXCR2 dimers. This effect was GluR1-specific, as demonstrated when CXCR2 was co-transfected (in a cDNA ratio 1:1) together with CXCR1: in this case the levels of CXCR2 dimers are comparable with those obtained from CXCR2-transfected cells (Fig. 4B). We then investigated whether GluR1 could interact with the CXCR2 mutants. In a previous work, we have already demonstrated that GluR1 interacts with CXCR2-A315, which lacks the whole intracellular C-terminal region (35). Fig. 5A shows that also the other C-terminal deletion mutants CXCR2-F183, CXCR2-K163 and CXCR2-V142 interact with GFP-GluR1. On the other hand, Fig. 5B shows that the N-terminal mutant Y49-CXCR2 interacts with GluR1 but after a more severe N-terminal truncation, at the aminoacid D143, this interaction is lost.

Deletion mutants of CXCR2 behave as dominant negative receptors, impairing CXCR2-mediated cellular signalling.

It is well established that CXCR2 is a GPCR that regulates the intracellular signalling through the activation of many different phospholipases and protein kinases (reviewed in 37). The CXCR2-mediated activation of both the ERKs and the PI3-K/Akt pathways has been described in several cellular systems, where these pathways can be either coupled (as in neutrophils, see 38) or independent each other (as in neurons, see 39). We investigated the activation of the phosphorylation of ERK1/2 and Akt following CXCL8 treatment of HEK cells transfected with CXCR2 alone, in combination with the different CXCR2 deletion mutants or together with GluR1. Results obtained indicate that CXCR2-transfected HEK cells respond to CXCL8 with the rapid and sustained phosphorylation of ERK1/2 and Akt, which is already detectable after 1 min and lasts up to 30 min (only shown for the time point of 10 min in Fig. 6A and B). These phosphorylations are inhibited by cell pre-treatment with pertussis toxin (PTX, 100 ng/ml, 16 h), indicating receptor coupling to PTX-sensitive G proteins (Fig. 6 A and B). In the presence of LY294002 (50 μ M, 1 h), a specific PI3-K inhibitor, Akt phosphorylation was completely inhibited (Fig. 6A), while some CXCL8-induced ERK1/2 phosphorylation was still detectable (Fig. 6B), indicating the presence of both PI3-K-dependent and PI3-K-independent ERK1/2 activation in HEK cells. When CXCR2 was co-expressed, in HEK cells, together with different CXCR2 deletion mutants, CXCL8-induced signalling was greatly impaired. In particular, there was a reduction of ERKs (Fig. 7A) and Akt (Fig. 7B) phosphorylation, which was more evident with Y49-CXCR2, but that was clearly detectable with all the mutants analysed. When the deleted CXCR2 receptors were expressed alone, in HEK cells, no obvious CXCL8-induced signalling was evident (shown in Fig. 7A and B). These data indicate that the CXCR2 deletion mutants may act as dominant negative inhibitors of CXCR2 function, reducing its signalling activities, likely due to their interaction with the wt receptor. This interaction in its turn would compete for wt receptor dimer formation.

When CXCR2 was co-expressed with GluR1, CXCL8 induced ERK1/2 phosphorylation was similar to CXCR2 expressing cells, while Akt phosphorylation was almost undetectable (Fig. 7A-B). The co-expression of two CXCR2 mutants, deleted in different regions, A315 and Y49 did not restore CXCL8-induced ERK1/2 and Akt phosphorylation (Fig. 7A-B), indicating that the truncated receptors do not complement their mutations. As a control, CXCR2 was co-expressed in HEK cells together with CXCR1; under these experimental conditions, CXCR2 dimer formation was not impaired (see above, Fig. 4B). To avoid a direct CXCR1 activation by CXCL8, CXCL2 at low dose (30 nM) was used as more specific agonist of CXCR2. Results obtained demonstrate that CXCR1 was only faintly activated by CXCL2 (analysed as ERK1/2 and Akt phosphorylation, Fig. 7C), and that CXCR2 activity was not impaired by its co-expression with CXCR1, in line with a specific effect of both GluR1 and the CXCR2 truncated receptors on CXCR2 oligomerization.

We have previously reported that in CGNs, obtained from newborn rats (p3), the CXCR2 receptor was not associated with GluR subunits, and that these neurons migrated in response to CXCL2 (35). In contrast, neurons from older animals (p7-p11), where the CXCR2/GluR complex was present, failed to migrate upon CXCL2 treatment, indicating an inhibitory effect of GluRs on CXCR2 function (36). In order to determine whether these differences resulted in coherent variations in cellular signalling, experiments were performed on neurons obtained from the cerebella of p3 or p7 rats. When cell lysates obtained from cerebellar neurons of p3 and p7 rats were analysed for CXCR2 oligomers, no significant differences were observed in the amount of CXCR2 oligomers (not shown). Nevertheless, when the same cells were treated with CXCL2 and analysed for ERK1/2 and Akt phosphorylation, neurons from p3 animals respond with a significantly higher frequency (Fig. 8).

The co-expression of deletion mutants of CXCR2 with wt CXCR2 impairs CXCL8-mediated chemotaxis

Since chemokine main function is to regulate cell chemotaxis, we decided to investigate whether the inhibitory behaviour of the truncated CXCR2 on signalling had any effect on the chemotactic activity of the wt receptor. With this aim, HEK cells were transfected with CXCR2 constructs alone or mixed in different combinations. Results, shown in Fig. 9, indicate that the chemotaxis induced by CXCL8 in CXCR2-expressing HEK cells was significantly reduced by the co-expression of the deletion mutants. In general, the effects of the co-expression on chemotaxis were comparable with those obtained on cellular signalling, with inhibitory effects more pronounced for the less drastic mutants Y49-CXCR2 and CXCR2-A315; while the inhibitory effect was almost undetectable for the shorter mutant analysed, CXCR2-V142. For the C-terminally truncated mutants, it is interesting to note that the progressive truncation reduces consistently the rate of chemotaxis inhibition (Fig. 9). As already shown for cellular signalling, the expression of the CXCR2 mutants A315, Y49 and D143, alone, was not sufficient to make cells responsive to CXCL8 (Fig. 9). Similarly, the co-expression of Y49-CXCR2 and CXCR2-A315 could not produce assembled functional receptors (Fig. 9). These results indicate that the truncated receptors behave as dominant negative inhibitors of CXCR2-mediated chemotaxis.

Discussion

In spite of a general initial scepticism, due to the deeply rooted classic view of GPCR as single working unit in the lipid bilayer, the evidence for GPCR oligomers that signal in physical conjunction with other receptors and signalling partners has been substantially accepted in recent years (40). GPCR homo- and hetero-dimerization may have many functional implications: the possibility to modify receptor properties following its interaction with other receptors (GPCRs or others) enormously increases the signalling properties of the single members of this receptor superfamily. This may have many interests for the chemokine receptor family, which comprises a number of proteins (about 20) far below the number of the known putative specific agonists (about 40). This study was undertaken to investigate the oligomerization of the chemokine receptor CXCR2, which is the target of seven known different chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8). The main results obtained in this paper can be summarized as follows: i) CXCR2 exists in unstimulated HEK cells and cerebellar neurons both as monomer and oligomers, with predominant dimers; ii) CXCR2 receptor oligomerization occurs during biosynthesis, and requires the presence of a region comprised between aminoacids A106 and K163, which contains the first extracellular loop, the TM3, and the second intracellular loop; iii) deletion mutants of CXCR2 receptors, which are not functional when expressed alone in HEK cells, behave as dominant negative inhibitors of receptor function when co-expressed with wt CXCR2 receptors; iv) CXCR2 interaction with GluR1 involves a domain spanning aminoacids Y49 and V142.

We presented evidence that CXCR2 receptors oligomerize in HEK cells and in cerebellar granule neurons independently of agonist stimulation. The description of CXCR2 dimers in tunicamycin-treated HEK cells indicates that receptor oligomerization takes place during receptor biosynthesis and could be an essential step for its proper expression in the plasma membrane. This is in line with other reports on GPCR oligomerization, which converge toward the endoplasmic reticulum as the site of receptor homo- and hetero-dimerization. GABAB receptors 1 and 2

heterodimerization, in fact, occurs during receptor biosynthesis, and is essential to produce functional receptors and to localize them properly at the plasma membrane (10). Similarly, oxytocin and vasopressin receptors form homo- and hetero-dimers during the biosynthetic pathway (19); the constitutive oligomerization of CCR5 receptors explains the inhibitory effect of the *ccr5*Δ32 mutants on plasma membrane expression of CCR5 dimers (24,27); D2 dopaminergic receptors (4), β adrenergic receptors (41), and CXCR4 are all already found in the homo-dimeric form before any agonist treatment, although CXCL12 treatment increases CXCR4 dimer formation (28, but see also 25). On the other hand, for others GPCRs, oligomerization occurs only after agonist binding, as reported for dopamine and somatostatin complex (5), and for CCR2 and CCR5 receptors (26).

The molecular analysis of the receptor region(s) involved in CXCR2 oligomerization lead us to identify a putative “dimerization motif” in the sequence between the aminoacids A106 and K163; this sequence includes the first extracellular loop, the TM3, and the second intracellular loop. We describe that the CXCR2 deletion mutants that contain only a portion of this region, i.e. the CXCR2-V142 and the D143-CXCR2, did not form dimers when expressed in HEK cells, identifying in this region the presumed dimerization interface. However, we cannot exclude that the region responsible for the physical coupling could be only a part of the whole sequence comprised between A106 and K163. In particular, we observe that the C-terminally deleted CXCR2-V142 mutant does not dimerize, while CXCR2-K163, which only differs for the additional presence of the second intracellular loop, makes dimers. Nevertheless, the N-terminally deleted mutant D143-CXCR2, whose sequence begins with the second intracellular loop, does not dimerize, suggesting a stabilizing role, more that a direct one, for the second intracellular loop in CXCR2 oligomerization. In addition, the SDS resistance of the physical interaction present in CXCR2 oligomers, would point to the TM3 as the main region involved in receptor assembly. On the other hand, the observation that the CXCR2-V142 and the D143-CXCR2 mutants can both form “heteromers” with wt CXCR2, even if much less efficiently in comparison with the other mutants, indicates that the presence of wt CXCR2 compensates for structural deficits of the mutated CXCR2 proteins.

We also report that the co-expression of the CXCR2 deletion mutants with wt CXCR2 impairs receptor function in term of cell signalling and chemotaxis. The rate of functional inhibition for most of the different mutants is inversely correlated with their physical interaction with wt CXCR2: the Y49-CXCR2, which abundantly interacts with wt CXCR2, has the strongest inhibitory effects on ERK1/2 and Akt phosphorylation, and on cell chemotaxis. On the other hand CXCR2-V142, which interacts with wt CXCR2 with the lowest efficiency, does not impair CXCL8-mediated cell chemotaxis and Akt phosphorylation, and only partially affects ERK1/2 phosphorylation. The other tested mutants have intermediate behaviour both on cell signalling and chemotaxis. We hypothesize that the inhibitory effects exerted by truncated CXCR2 proteins on wt CXCR2 might reflect an abnormal trafficking of the wt/mutant complex at the plasma membrane, as already reported for other GPCRs (9-11,18,24).

We describe that, in HEK cells, the activation of the ERK1/2 and Akt pathways by CXCR2 are not completely coupled, as already shown in other cellular systems (39), but in contrast with others (38). This is proved by our observation that cell pre-treatment with LY294002, which drastically eliminated Akt phosphorylation, did not abolish CXCL8-induced ERK1/2 phosphorylation. In contrast, both pathways are coupled to PTX-sensitive G proteins as already described in other systems (42,43), but differently from others (39). It is interesting to note that the co-expression of two different CXCR2 mutations, one lacking the N-terminal (involved in chemokine binding) domain, Y49-CXCR2, and one lacking the whole C-terminal domain (involved in signalling), CXCR2-A315, did not restore cell signalling and chemotaxis, indicating that receptor activation upon agonist binding implicates an intra-molecular mechanism. This is different from what has been reported for other GPCRs, like the LH receptors (44), and indicates a mechanism of *cis*-activation for CXCR2 composing the dimers.

We have previously reported that the interaction of CXCR2 with GluR1, in HEK and CGNs, resulted in a drastic inhibition of cell chemotaxis upon CXCL2 treatment (35). In this paper we describe that GluR1 co-expression with CXCR2 results in a dose-dependent inhibition of CXCR2

dimer formation, further supporting the hypothesis of CXCR2 working as functional dimer. We mapped the CXCR2 domain involved in GluR1 interaction between the aminoacids Y49 and V142. The partial overlapping of this domain with the “dimerization motif” might explain the competition produced by GluR1 for CXCR2 dimer formation.

We observe that GluR1/CXCR2 co-expression also reduced CXCL8-mediated signalling, with a specific effect on Akt phosphorylation. This would explain the inhibition of cell chemotaxis observed in CXCR2/GluR1 co-expressing cells (35), since CXCL8-mediated chemotaxis is reported to be dependent on the PI3-K/Akt pathway (38). On the other hand, given the lack of inhibition of ERK1/2 phosphorylation in CXCR2/GluR1 transfected cells, we hypothesize that the CXCR2/GluR1 complex acquires new properties in terms of signalling and function (45). All together these results strongly indicate that the physical interaction of CXCR2 with GluR1 or with the truncated CXCR2 proteins competes for CXCR2 dimer formation and this results in a general impairment of CXCR2 functions. This interpretation is consistent with the data we obtained with rat CGNs: neurons from p7 rats were poorly or not responsive to CXCR2 agonist treatment in term of cell signalling, while CGN from p3 rats were responsive. We had previously reported that CGNs obtained from p3 rats responded to CXCR2 stimulation with cell chemotaxis, while CGNs from p7 rats, where CXCR2 co-immunoprecipitated with GluR1/2/3 subunits, were unresponsive (35). Furthermore, the AMPA receptor antagonist CNQX impairs the neurotrophic activity of CXCR2 (45) and reverts the inhibition of cell chemotaxis in CXCR2/GluR1 expressing cells without hampering receptor co-immunoprecipitation (45). All together these data point to the existence of CXCR2/GluR1 complexes, activated by CXCR2 agonists, whose functions can be blocked with CNQX.

In conclusion, we provide evidence that CXCR2 forms dimers, and that receptors assembly occurs early in biosynthesis, before receptor glycosilation and independently of receptor activation. The region involved in receptor assembly comprises the TM3 and the adjacent extra- and intracellular regions. It is interesting to remember that CXCL8, a well-established CXCR2 agonist, is present in

solution both as monomer and dimer, in equilibrium at physiological, nanomolar, concentrations (46), and that these molecular forms can be equally active (47,48). The existence of CXCL8 dimers could represent a physiological stimulus for dimerized CXCR2 receptors. In addition, the formation of CXCR2 dimers is obstructed by the presence, in the same cells, of the AMPA-type glutamate receptor GluR1, which competes for CXCR2 dimer formation. GluR1/CXCR2 heteromers lose the signalling properties and functions of CXCR2 homodimer receptors to acquire new properties. This may have functional implications regulating cell responsiveness to chemokines in cells that co-express chemokine and glutamate receptors, like neurons, during development and adulthood.

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Abbreviations: GPCR: G-protein coupled receptor; AMPA: α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CGNs: cerebellar granule neurons; ERK: extracellular-signal regulated kinase; PI3-K: phosphatidylinositol 3 kinase; PTX: pertussis toxin; TM: transmembrane.

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Figure legends

FIG.1. CXCR2 receptor forms ligand-independent dimers in HEK cells and in cerebellar granule neurons. (A) HEK cells were transiently co-transfected with 1 μ g of GFP-and 1 μ g of V5-tagged CXCR2 receptors (+). For single transfections, 1 μ g of empty vector cDNA (pCEP) was added to reach the same amount of total cDNA in each transfection. 48 h after transfection cells were immunoprecipitated with anti GFP Ab and western blot analysed for V5 immunoreactivity. Total cell lysates (lysates) of co-transfected cells indicates the position of CXCR2-V5 (indicated by an arrow on the left). (B) HEK cells were transfected with wt CXCR2 (wt) or with a C-terminally deleted CXCR2 (A315). Lysates obtained from these cells were analysed by western blot for receptor oligomerization with E2 Ab. Arrows indicate the position of monomers, dimers and oligomers for both kinds of transfected receptors. Molecular weight markers are indicated on the right. (C) HEK cells were transfected with CXCR2 and 24 h later were treated with (+ tun) or without (- tun) tunicamycin (5 mg/ml) for additional 24 h; cell lysates were analysed as above. (D) Wt or A315 CXCR2 were transfected in HEK cells; 48 h after transfection, cells were starved as described in the experimental section and stimulated with CXCL8 (120 nM) for 10 min. The corresponding cell lysates were analysed as in B. (E) Lysates of cerebellar granule neurons, cultured for 8 days in vitro, were analysed for CXCR2 receptor expression and oligomerization, with anti CXCR2 (K19) or pre-immune IgG. Arrows indicate the position of putative CXCR2 monomer and dimer. Molecular weight markers are indicated.

FIG. 2. Schematic structure of the CXCR2 deletion mutants generated. Deletion mutants of CXCR2 receptors were obtained by PCR as described in the experimental procedures. The numbers indicate the position of the last aminoacid present for the C-terminally deleted receptors A315, F183, K 163 and V142; for the N-terminally deleted receptors Y49, A106 and D143, the numbers indicate the first aminoacid present in the sequence. Regions in red indicated the putative “dimerization motif”.

FIG. 3. Analysis of the homo- and hetero-oligomerization of the CXCR2 receptor deletion mutants. HEK cells were transfected with the indicated combinations of CXCR2 receptors and the corresponding cell lysates analysed by western blotting analysis. The mutated forms of CXCR2 receptors (1 μ g of plasmid cDNA) were expressed alone (with 1 μ g of the empty vector pCEP) or together with the wt receptors (1 μ g), as indicated. The arrows indicate the different forms (monomer, homo-dimer or hetero-dimer) of receptor detected for the corresponding transfection for C-terminally-deleted CXCR2 (*A*, *B* and *C*, E2 Ab) and for N-terminally deleted CXCR2 (*D* and *E*, C19 Ab).

FIG. 4. The AMPA-type GluR1 receptor subunit competes for CXCR2 dimer formation in a dose-dependent way. HEK cells were transfected with cDNAs encoding for CXCR2 in the presence of increasing amounts of GluR1 (*A*) or together with CXCR1 (*B*). Variable (indicated in *A*) or fixed (1 μ g in *B*) amounts of pCEP were also transfected to keep constant the total amount of cDNA in each transfection. Numbers on top of the figures indicate the amount (μ g) of cDNAs used for each vector. Lysates obtained from transfected cells were analysed by western blot for CXCR2 oligomer formation (top *A* and *B*) and GluR1 (*A* bottom) or CXCR1 (*B* bottom) protein expression. Arrows indicate the position of CXCR2 monomers and dimers, and of CXCR1 protein.

FIG. 5. The AMPA-type GluR1 receptor subunit co-immunoprecipitates with CXCR2 wt and with the CXCR2 deletion mutants. (*A*) HEK cells, co-transfected with GluR1 and with the indicated CXCR2 constructs: pCEP (mock), wt, A315 or V142, were immunoprecipitated with anti CXCR2 Ab (E2) and analysed by western blotting with anti GluR1 Ab (Ab1504). Arrows indicate the position of GluR1 and IgG. (*B*) HEK cells, co-transfected with GluR1-GFP and with the indicated CXCR2 constructs: pCEP (mock), wt, Y49 or D143, were immunoprecipitated with anti

GFP Ab (FL-1) and analysed by western blotting with anti CXCR2 Ab (C19). Arrows indicate the position of wt CXCR2, Y49-CXCR2 and IgG.

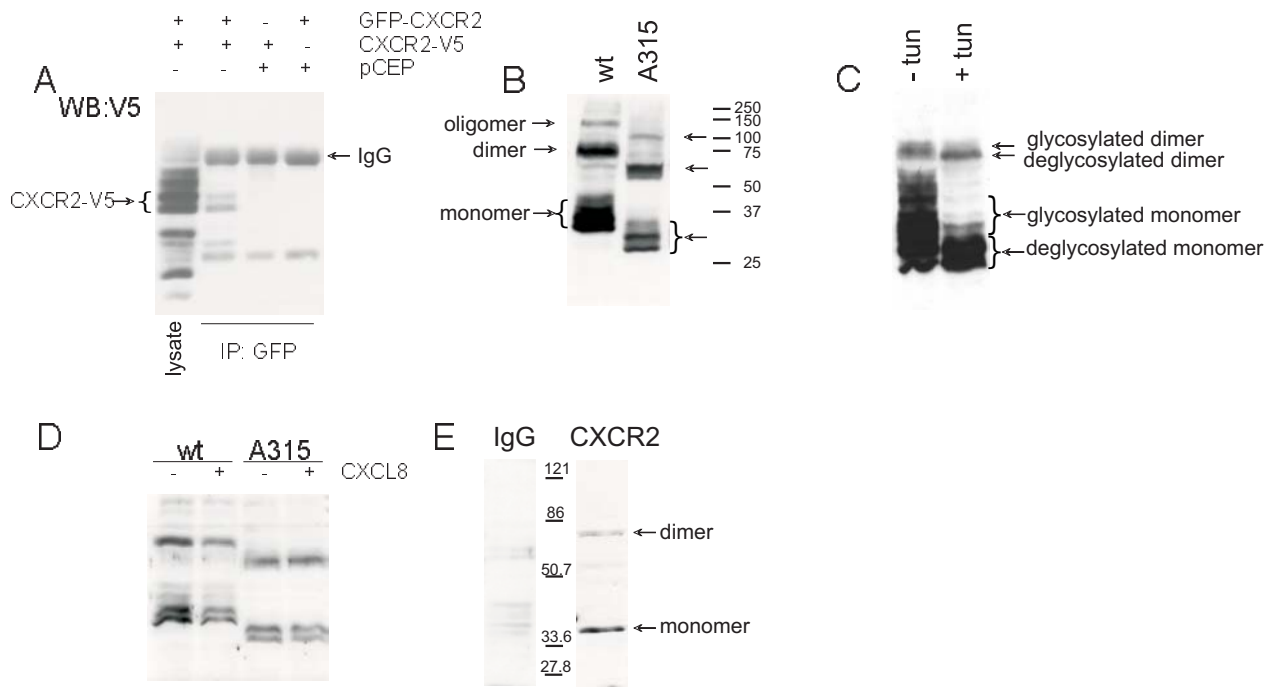
FIG. 6. CXCL8-induces ERK1/2 and Akt phosphorylation. CXCR2 transfected HEK cells were serum starved for 16 h, pre-treated with PTX (100 ng/ml, 16 h), LY294002 (50 μ M, 1 h), or vehicle (untreated) and stimulated (+) or not (-) with CXCL8 for 10 min, as indicated. Cell lysates were analysed by western blot for Akt (*A*, top) and ERK1/2 (*B*, top) phosphorylation; total Akt (*A*, bottom) and ERK2 (*B*, bottom) were determined as control of equal protein loading, as indicated. Similar results were obtained in three independent experiments.

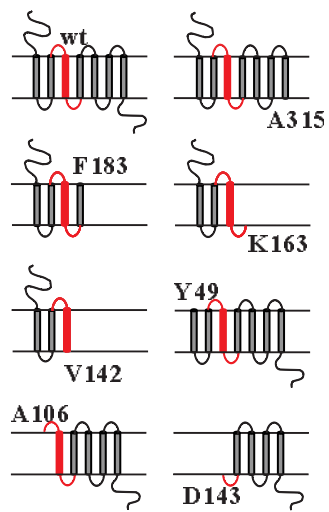
FIG. 7. The co-expression of CXCR2 deletion mutants with wt impairs CXCL8-mediated cell signalling. HEK cells were transfected with cDNAs indicated and stimulated with CXCL8 (120 nM, *A* and *B*) or CXCL2 (30 nM, *C*) for 10 min. The same amounts (20 μ g of total protein) of the corresponding cell lysates were then analysed by western blot for ERK1/2 (*A* and *C*) or Akt (*B* and *C*) phosphorylation. Data reported represent the means \pm S.E. of at least 4 different experiments.

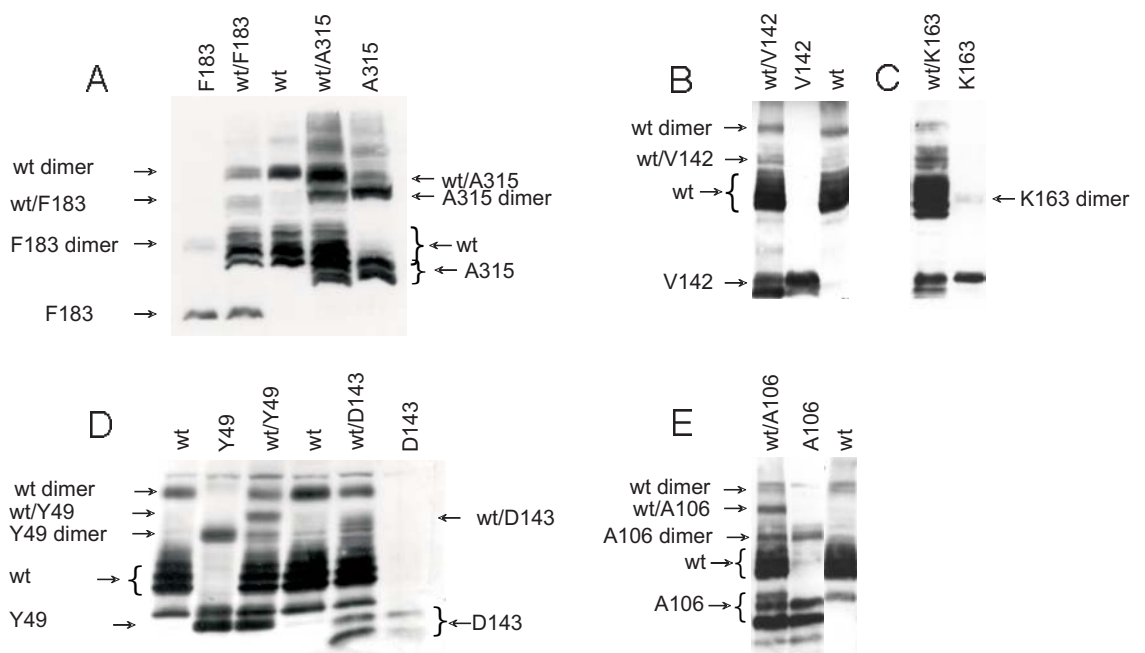
FIG. 8. CXCR2 receptor stimulation induces ERK1/2 and Akt phosphorylation in cultured cerebellar granule neurons obtained from p3 animals. Cerebellar granule neurons, obtained from rats of p3 and p7, were stimulated for 60 s with rat CXCL2 and analysed for ERK1/2 and Akt phosphorylation. Band intensity was determined by densitometric analysis and the results from 4 independent experiments are reported \pm SD. * Student's t test analysis, p values < 0,02.

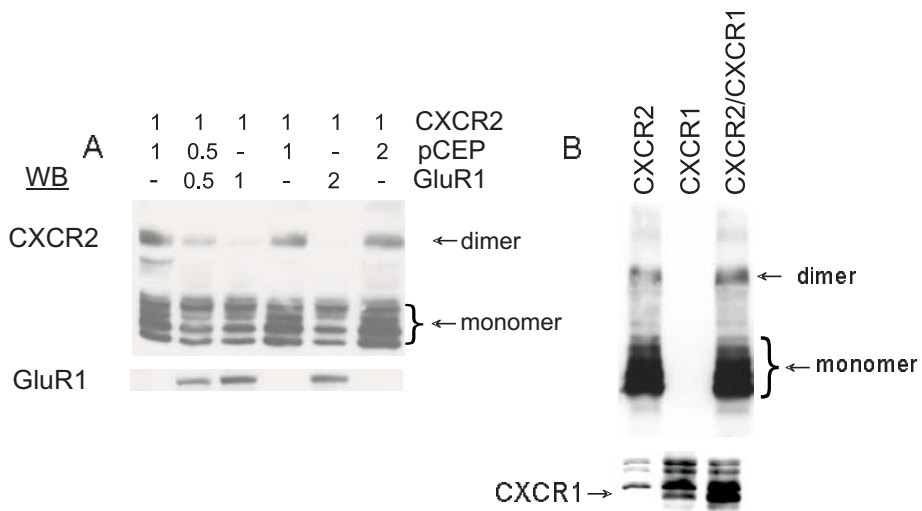
FIG. 9. Chemotactic assay. HEK cells were transfected with the indicated CXCR2 constructs, and analysed for chemotactic responses to CXCL8 treatment. 48 h after transfection, cells were plated on top of collagen pre-coated polycarbonate filters (12 μ m pores) in transwell inserts; the lower chambers contained CXCL8 (6 nM) or vehicle (water). After two h, cells adhering to the lower side

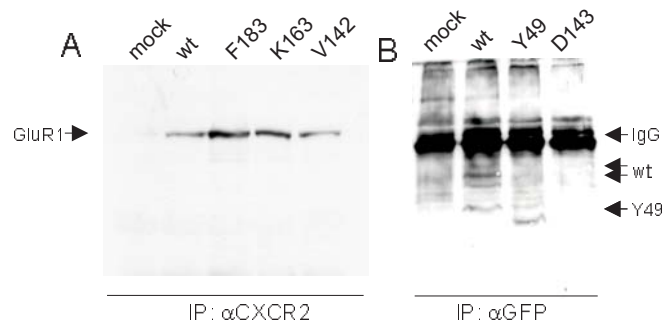
of the filters (migrated cells) were counted as described in the text; the ratio between the numbers of cells migrated in vehicle-treated samples (control) vs CXCL8-treated samples (stimulated) represents the chemotactic index. This is calculated for each type of transfection: for wt, the chemotactic index is $3,0 \pm 0,4$. Results are reported as the % of wt chemotactic index for each separate experiment (at least 4 for each transfection).

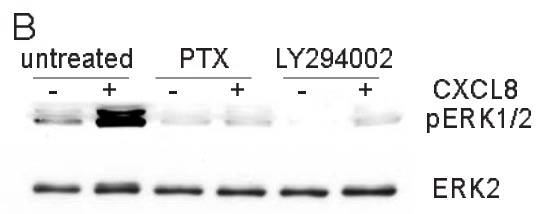
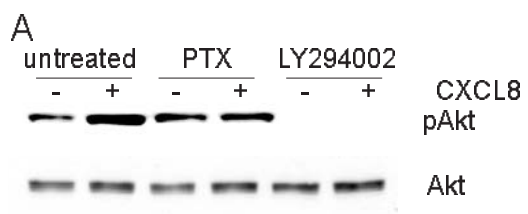


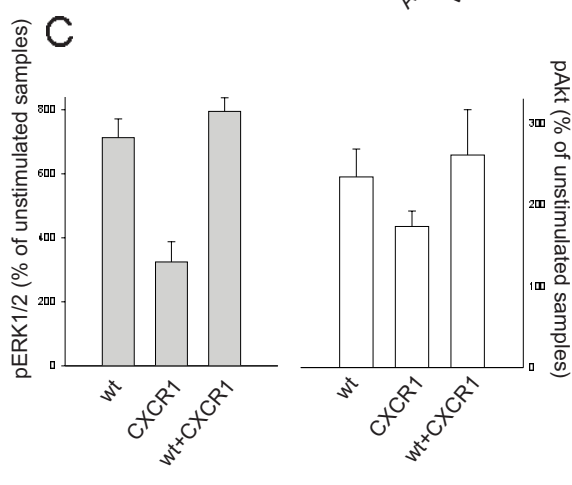
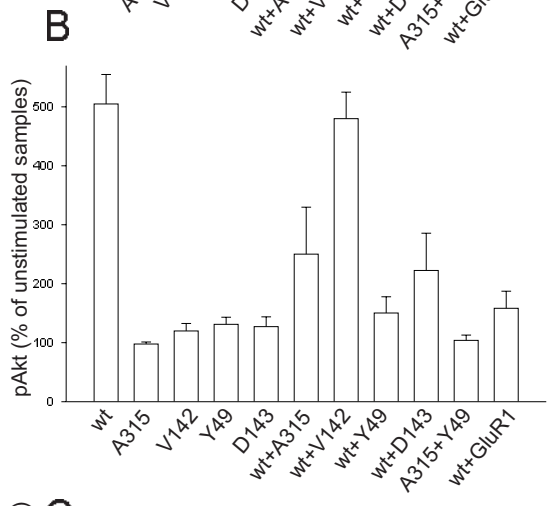
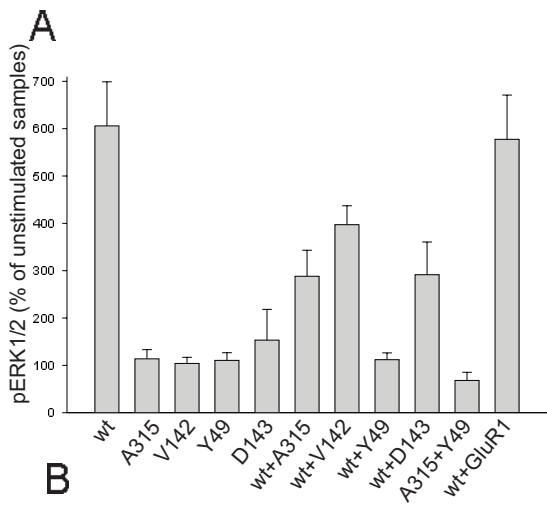


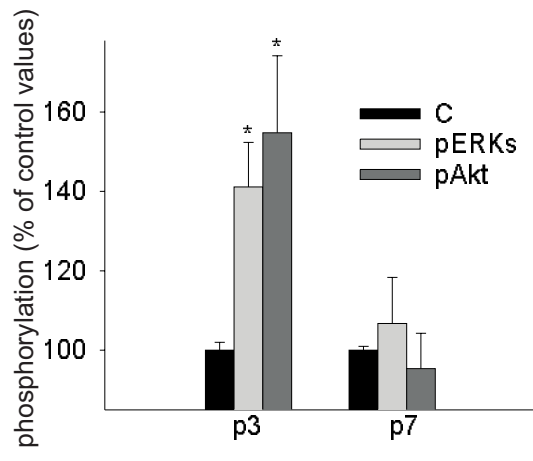


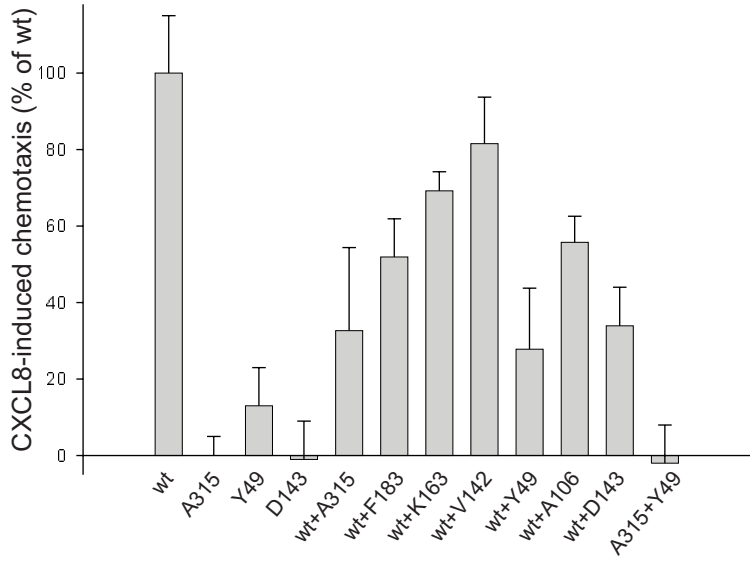












Ligand-independent CXCR2 dimerization

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