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Functional and structural consequences of chemokine (C-X-C motif) receptor 4 activation with cognate and non-cognate agonists

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Abstract

Chemokine (C-X-C motif) receptor 4 (CXCR4) regulates cell trafficking and plays important roles in the immune system. Ubiquitin has recently been identified as an endogenous non-cognate agonist of CXCR4, which activates CXCR4 via interaction sites that are distinct from those of the cognate agonist C-X-C motif chemokine ligand 12 (CXCL12). As compared with CXCL12, chemotactic activities of ubiquitin in primary human cells are poorly characterized. Furthermore, evidence for functional selectivity of CXCR4 agonists is lacking and structural consequences of ubiquitin binding to CXCR4 are unknown. Here we show that ubiquitin and CXCL12 have comparable chemotactic activities in normal human peripheral blood mononuclear cells, monocytes, vascular smooth muscle and endothelial cells. Chemotactic activities of the CXCR4 ligands could be inhibited with the selective CXCR4 antagonist AMD3100 and with a peptide analogue of the 2nd transmembrane domain of CXCR4. In human monocytes, ubiquitin- and CXCL12-induced chemotaxis could be inhibited with pertussis toxin and with inhibitors of phospholipase C, phosphatidylinositol 3 kinase and extracellular signal-regulated kinase 1/2. Both agonists induced inositol trisphosphate production in vascular smooth muscle cells, which could be inhibited with AMD3100. In β -arrestin recruitment assays, ubiquitin did not sufficiently recruit β -arrestin2 to CXCR4 ($EC_{50} > 10 \mu M$), whereas the EC_{50} for CXCL12 was 4.6 nM (95% confidence interval: 3.1–6.1 nM). Both agonists induced similar chemical shift changes in the ^{13}C - 1H -heteronuclear single quantum correlation (HSQC) spectrum of CXCR4 in membranes,

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Conflict of Interest

The authors declare no conflict of interest.

whereas CXCL11 did not significantly alter the ^{13}C - ^1H -HSQC spectrum of CXCR4. Our findings point towards ubiquitin as a biased agonist of CXCR4.

Keywords

CXCL12; stromal cell-derived factor-1 α ; ubiquitin; chemotaxis; nuclear magnetic resonance spectroscopy; biased agonist

Introduction

The G protein coupled receptor (GPCR) chemokine (C-X-C motif) receptor (CXCR) 4 is abundantly expressed on immune cells and functions as a key regulator of leukocyte trafficking, stem cell mobilization and homing [1]. CXCR4 is involved in various disease processes and AMD3100 (plerixafor), a selective antagonist of CXCR4, is already approved by the Federal Drug Administration to mobilize hematopoietic stem cells to the peripheral blood in lymphoma patients [1]. Upon binding to the cognate agonist C-X-C motif chemokine ligand 12 (CXCL12), CXCR4 couples to guanine nucleotide-binding protein α_i ($\text{G}\alpha_i$) and recruits β -arrestin-1/2 to the receptor, leading to termination of G protein-mediated signaling, receptor internalization and activation of G protein-independent signaling [2, 3]. Recently, ubiquitin has been identified as a non-cognate non-chemokine agonist of CXCR4 [4]. While previous studies suggest that ubiquitin and CXCL12 bind to distinct contact sites on CXCR4 [5], multiple lines of evidence indicate that both agonists induce $\text{G}\alpha_i$ -mediated signaling, receptor internalization and regulate cell movements via CXCR4 in various cell types [4–14]. Chemotactic activities of ubiquitin in primary human cells, however, are poorly characterized. Furthermore, it remains unclear whether both agonists show functional selectivity and information on ligand-induced structural rearrangements of CXCR4 upon ubiquitin binding is not available. Thus, in the present study we compared chemotactic activities of CXCL12 and ubiquitin in various primary human cells and further evaluated structural and functional consequences upon agonist binding to CXCR4.

Materials and Methods

Proteins and reagents

Ubiquitin was purchased from R&D systems and C-X-C motif chemokine ligand 11 (CXCL11) and CXCL12 from Protein Foundry. Bovine serum albumin, AMD3100, pertussis toxin and U73122 were purchased from Sigma, LY94002 and U0126 from Cell Signaling and 6bK from Tocris. The peptide analogue of transmembrane helix (TM) 2 of CXCR4 (LLFVITLPFWAVDAVANWYFGNDD-NH₂) was as described [15].

Cells

All procedures were approved by the Loyola Institutional Review Board; informed consent was obtained from all blood donors. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood from healthy volunteers by standard density gradient centrifugation. Monocytes were then positively selected using the EasySep Human CD14

positive selection kit (Stemcell Technologies) and suspended in Roswell Park Memorial Institute medium (RPMI), 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. Human primary aortic smooth muscle cells (hVSMCs PCS-100-012) and human primary pulmonary artery endothelial cells (PPAEs PCS-100-022) were obtained from ATCC. Cells were cultured using vascular basal cell media (PCS-100-030, ATCC) with the addition of supplemental growth factors (hVSMCs PCS-100-042, PPAE PCS-100-041 (ATCC)) and 100 U/mL penicillin, 100 µg/mL streptomycin. Cells were cultured in a humidified environment at 37°C, 5% CO₂ and utilized within passages 2–5. The HTLA cell line, a HEK293 cell line stably expressing a tetracycline transactivator (tTA)-dependent luciferase reporter and a β-arrestin2-Tobacco Etch Virus (TEV) fusion gene [16], was generously provided by the laboratory of Dr. Bryan Roth and maintained in high glucose Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated FBS, 1x non-essential amino acids (ThermoScientific), 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL hygromycin B, and 2 µg/mL puromycin at 37°C, 5% CO₂ in a humidified environment.

Chemotaxis assay

Cell migration was assessed using the ChemoTx 96-well cell migration system, as described [5, 15]. The chemotactic index (CI) was calculated as the ratio of cells that transmigrated through the filter in the presence versus the absence (= PBS/control) of the test solutions.

Inositol trisphosphate (IP₃) enzyme-linked immunosorbent assay

IP₃ enzyme-linked immunosorbent assays were purchased from LS Bio and performed according to the manufacturer's protocol (LS BIO F10644). In brief, confluent hVSMCs were pre-incubated with 10 µM AMD3100 (Sigma) or vehicle (30 min, 37°C) and then stimulated with vehicle or 1 µM of ubiquitin or CXCL12 (15 min, 37°C). Cells were washed with cold phosphate buffered saline (PBS), 225 µL of PBS were added to each well and cells were lysed by ultrasonication. The hVSMC lysate was centrifuged (10 min, 1500 g, 4°C) and total protein concentrations in the supernatant determined with the Bio-RAD DC Protein Assay according to manufacturer's protocol (Bio-Rad 500-0116). Equivalent amounts of protein were added to the ELISA strips diluted in the provided sample diluent. The assay was then completed as per manufacturer's protocol. Optical densities were read on a Biotek Synergy II microplate reader (absorbance at 450 nm) and IP₃ concentrations were extrapolated from the standard curve.

PRESTO-Tango β-arrestin recruitment assay

The PRESTO-Tango assay was performed as recently described [16]. The CXCR4-Tango plasmid was a gift from Dr. Bryan Roth (Addgene plasmid # 66262). HTLA cells (2.5×10⁵/well) were seeded in a 6-well plate and transfected with 1500 ng of the CXCR4-Tango plasmid using Lipofectamine 3000 (ThermoScientific). The following day, transfected HTLA cells (1×10⁵ cells/well) were plated onto Poly-D-Lysine pre-coated (Sigma-Aldrich) 96-well microplates (Corning) and allowed to attach to the plate surface for at least 4 hours prior to treatment. Proteins used for treatment were prepared at twice the final concentration in culture media, added at a 1:1 volume/volume ratio and incubated overnight at 37°C, 5% CO₂ in a humidified environment. The following morning, media was removed from cell

culture plates and replaced with a 100 μ L 1:1 mixture of Bright-Glo (Promega) and 1x HBSS (Gibco), 20mM HEPES (ThermoScientific) solution. Plates were then incubated at room temperature before measuring luminescence on a Biotek Synergy 2 plate reader.

Reductive Methylation of Membrane Preparations

ChemiSCREEN Chem-1 membrane preparations for recombinant human CXCR4 (HTS004M) were purchased from EMD Millipore. Reductive methylation of the membrane preparations was performed as described previously [17, 18]

Heteronuclear Single Quantum Coherence (HSQC) NMR

Final samples (200 μ L) contained 50% of membrane preparations, 10% D₂O, 2% DMSO-d₆. Ubiquitin, CXCL12, and CXCL11 were added at a final concentration of 1 μ M. ¹H-¹³C HSQC NMR experiments were carried out on a 900-MHz Bruker Avance Spectrometer equipped with a cryogenic probe. ¹³C and ¹H chemical shifts in the HSQC spectra reveal the chemical and magnetic environments of the ¹³CH₃ groups as well as their dynamic properties. Data were processed and analyzed using the NMRPipe/NMRDraw software [19]. To determine the mean chemical shift difference (δ_{CH}) or chemical shift perturbations (CSPs) [20–22], we used the following equation:

$$\Delta \delta_{CH} = \sqrt{\frac{(\Delta \delta H)^2 + (\Delta \delta C)^2 / 33}{2}}$$

where δ_{CH} is the weighted average chemical shift difference for the ¹H and ¹³C nuclei in ppm; δH and δC are the chemical shift differences between the ¹³C labeled CXCR4 membranes in the presence and absence of ligands (CXCL12, ubiquitin, and CXCL11); the value 33 is a scaling factor α for ¹³C nuclei. CSPs (δ_{CH}) greater than the average plus one standard deviation were considered significant.

Data analyses

Data are expressed as mean $\pm$ SE. Data were analyzed with Student's t-test, one-way analysis of variance with Dunnett's post-test or with non-linear regression analysis using the GraphPad-Prism-6 software. A 2-tailed p<0.05 was considered significant.

Results and Discussion

CXCR4 activation with ubiquitin and CXCL12 induces chemotaxis and activates similar signaling pathways in primary human cells of hematopoietic origin

We first compared chemotactic activities of ubiquitin and CXCL12 in human PBMNCs and monocytes. Both proteins induced chemotaxis with a bell-shaped average dose-response profile (Fig. 1A/B); maximal chemotactic activities of CXCL12 and ubiquitin were comparable. It should be noted that we observed two different types of dose-response profiles in monocyte preparations from individual donors within the tested range of concentrations: a bell-shaped dose response (Fig. 1C) and a saturable dose-response (Fig. 1D). While the reason for these different dose-responses is unknown, both types have

previously been reported in chemotaxis experiments with CXCL12 in human monocytes [23–25]. We then tested whether the chemotactic activities can be inhibited with the selective CXCR4 antagonist AMD3100 and with a TM2 peptide analogue of CXCR4, which has previously been shown to inhibit CXCR4 in the human glioblastoma cell line U87 and in primary human monocytes and vascular smooth muscle cells [10, 15, 18, 26]. As shown in Fig. 2A–D, AMD3100 and the TM2 peptide inhibited chemotaxis of human PBMNCs and monocytes towards CXCL12 and ubiquitin, suggesting that both agonists induce chemotaxis via CXCR4. Thus, the observation that the chemotactic dose-responses to ubiquitin and CXCL12 are different among monocyte preparations from individual donors suggests inter-individual variations in CXCR4 expression levels or CXCR4 reactivity.

Next, we tested a panel of inhibitors of intracellular signaling pathways that are known to be involved in CXCR4 signaling (Fig. 2E). Consistent with coupling of CXCR4 to $G\alpha_i$, CXCL12 and ubiquitin-induced chemotaxis of monocytes could be inhibited with pertussis toxin. Furthermore, the phospholipase C inhibitor U73122, the phosphoinositide 3-kinase inhibitor LY294002 and the mitogen activated protein (MAP) kinase kinase (MEK1/2) inhibitor U0126 inhibited monocyte movements towards both agonists to a similar degree. In agreement with previous reports on ubiquitin-mediated chemotaxis in the monocytic cell line THP-1 and in primary human T-regulatory cells [5, 10, 13], our observations demonstrate that ubiquitin exerts chemotactic activity via CXCR4 in primary human PBMNC and monocytes and suggest that both agonists activate similar signaling pathways.

CXCR4 activation with ubiquitin and CXCL12 induces chemotaxis and activates similar signaling pathways in primary human cells of non-hematopoietic origin

We then compared chemotactic activities of CXCL12 and ubiquitin in primary human aortic vascular smooth muscle cells (Fig. 3A–C). Both CXCR4 agonists showed comparable chemotactic activities and dose-response profiles when tested in parallel experiments (Fig. 3A); CXCL12 and ubiquitin-induced chemotaxis could be inhibited with AMD3100 (Fig. 3B) and the TM2 peptide analogue (Fig. 3C). To confirm that both agonists activate similar signaling pathways in human vascular smooth muscle cells, we then measured inositol trisphosphate (IP_3) production as a read-out for $G\alpha_i$ -coupled signaling. As shown in Fig. 3D, ubiquitin and CXCL12 stimulation of human vascular smooth muscle cells increased IP_3 concentrations, which could be inhibited with AMD3100. Next, we tested whether both agonists also induce chemotaxis via CXCR4 in human primary pulmonary artery endothelial cells (Fig. 3E–G). As observed for human smooth muscle cells, both CXCR4 agonists showed comparable chemotactic activities and dose-response profiles in human pulmonary artery endothelial cells when tested in parallel experiments (Fig. 3E); the chemotactic activities of ubiquitin and CXCL12 could be inhibited with AMD3100 (Fig. 3F) and the TM2 peptide analogue (Fig. 3G). It has been previously reported that ubiquitin induces chemotaxis in B16-F10 (skin melanoma), 4T1 (breast cancer), RM-9 (prostate cancer) cells and regulates movements of isolated rat cardiac microvascular endothelial cells via CXCR4 [7, 9]. Thus, our findings confirm previous observations and suggest that ubiquitin and CXCL12 activate CXCR4, which then couples to $G\alpha_i$ to induce chemotaxis in primary human cells of hematopoietic and non-hematopoietic origin.

Ubiquitin does not sufficiently recruit β -arrestin 2 to CXCR4

While CXCR4 activation with CXCL12 leads to $G\alpha_i$ protein-mediated signaling and β -arrestin1/2 recruitment [4, 7, 10], direct evidence for β -arrestin recruitment to CXCR4 upon ubiquitin binding has not been provided. Thus, we utilized the PRESTO-Tango (parallel receptorome expression and screening via transcriptional output, with transcriptional activation following arrestin translocation) assay to compare β -arrestin2 recruitment to CXCR4 [16]. In this assay, the EC_{50} for CXCL12 mediated β -arrestin2 recruitment was 4.6 nM (95% confidence interval: 3.1–6.1 nM). Ubiquitin, however, induced β -arrestin2 recruitment to CXCR4 only at very high concentrations ($EC_{50} > 10 \mu M$) (Fig. 4A). Albumin and CXCL11, a CXCR3 and ACKR3 agonist that does not bind to CXCR4 [1], did not induce β -arrestin2 recruitment. This suggests that the positive signals at high concentrations of ubiquitin in this assay are specific (Fig 4A). Insulin-degrading enzyme (IDE), which is expressed on the cell surface of many cells, has been shown to cleave the C-terminal signaling site of ubiquitin, leading to C-terminal truncated ubiquitin, which binds to CXCR4 but lacks agonist activity [6]. The selective IDE inhibitor 6bK, however, did not affect ubiquitin signaling in the PRESTO-Tango assay (Fig. 4A). This suggests that C-terminal truncation of ubiquitin is unlikely to account for the lack of β -arrestin2 recruitment to CXCR4 at lower concentrations and could point towards agonist selective signaling of CXCR4 upon activation with CXCL12 and ubiquitin. Furthermore, we observed that ubiquitin did not antagonize CXCL12 induced β -arrestin2 recruitment to CXCR4 (Fig. 4B). As we previously provided evidence that ubiquitin and CXCL12 bind to CXCR4 through different contact sites on the receptor [5], it appears possible that differences in receptor folding or post-translational receptor modifications between endogenous CXCR4 and recombinant CXCR4 in expression systems may alter the ubiquitin binding site, but may not affect the CXCL12 receptor binding site. Thus, our observations could also be consistent with the inability of ubiquitin to bind to and activate recombinant CXCR4.

Ubiquitin and CXCL12 induce similar chemical shift changes in the ^{13}C - 1H -heteronuclear single quantum correlation (HSQC) spectrum of CXCR4 in membranes

We then evaluated whether ubiquitin and CXCL12 interact with CXCR4 expressed in mammalian cells employing nuclear magnetic resonance (NMR) spectroscopy. We used ^{13}C -labeled methylated membranes prepared from cells overexpressing CXCR4 to closely mimic native conditions for receptor folding and interactions with the plasma membrane. We have utilized this strategy previously to assess ligand binding to CXCR4 and α_{1a} -adrenergic receptors [18]. The overlaid ^{13}C - 1H heteronuclear single quantum coherence (HSQC) spectra of CXCR4 with and without 1 μM of CXCL12, ubiquitin or CXCL11 are shown in Fig. 4C–E. Addition of CXCL12 and ubiquitin induced similar line-broadening effects in the NMR spectra of CXCR4-containing membranes (Fig. 4D/E, signals 2 and 3), which are indicative of a global structural rearrangement of the receptor induced by ligand binding. These effects could not be detected upon addition of CXCL11, a chemokine that does not bind to CXCR4 (Fig. 4F). The most upfield signal (Fig. 4C, signal 1) likely representing the ^{13}C -methylated N-terminal amino group [17] was significantly perturbed by the addition of CXCL12 (Fig. 4D). This signal was not significantly affected ($CSP < 0.0058$) by ubiquitin and CXCL11 (Fig. 4D/E). Consistent with our previous observations [5], this result suggests that CXCL12 interacts with the unstructured N-terminus of CXCR4 (Fig.

4C/D, signal 1), whereas ubiquitin lacks this interaction but shares with CXCL12 the ability to bind the extracellular loops of the receptor (Fig. 4D/E, signals 2 and 3). These findings provide initial biophysical evidence for specific ubiquitin binding to CXCR4 in membranes.

The observation that ubiquitin did not antagonize CXCL12 in the β -arrestin 2 recruitment assays may therefore reflect that the affinity of CXCL12 is much higher than the affinity of ubiquitin for recombinant CXCR4 [4]. This assumption would be consistent with the finding that ubiquitin recruits β -arrestin 2 to CXCR4 only at very high concentrations, in the presence and absence of a submaximal concentration of CXCL12. As both CXCR4 agonists appear to interact with CXCR4 via distinct binding sites and CXCR4 is believed to exist as a homodimer [5, 27], alternative explanations could be that ubiquitin binding does not prevent CXCL12 binding to CXCR4 or that both agonists can bind to CXCR4 in parallel. In such a ligand binding model, binding of one ligand to the receptor may affect the signaling properties of the other ligand, e.g. via allosteric interactions, which could explain partially synergistic effects on CXCR4 signaling that we have previously observed upon co-activation of CXCR4 with both agonists [10].

In conclusion, the present study extends previous observations that ubiquitin functions as a CXCR4 agonist in cell lines to primary human cells of hematopoietic and non-hematopoietic origin. Furthermore, we provide direct evidence for structural rearrangements of CXCR4 upon CXCL12 and ubiquitin binding. While CXCL12 binding to CXCR4 leads to G protein-mediated signaling and β -arrestin2 recruitment, ubiquitin binding leads to G protein-mediated signaling but fails to efficiently recruit β -arrestin2. β -arrestin 1 and 2 have been described to be involved in CXCL12 mediated chemotaxis and in CXCR4 internalization upon CXCL12 binding [28–30]. As several lines of evidence suggest that ubiquitin binding to CXCR4 expressing cells also leads to co-internalization of the ligand-receptor complex [4, 10–12, 14], the mechanisms underlying CXCR4 internalization upon ubiquitin and CXCL12 binding may be different. Nevertheless, the findings of the present study, in combination with our previous observation that ERK1/2 phosphorylation upon ubiquitin activation of CXCR4 was rapid and transient, whereas ERK1/2 phosphorylation upon CXCL12 activation was persistent, suggest that ubiquitin functions as a biased CXCR4 agonist that preferentially signals via G protein-mediated pathways [5, 31]. Thus, it is likely that activation of CXCR4 with CXCL12 and ubiquitin fulfills distinct roles in biology.

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Figure 1. Ubiquitin and CXCL12 induce chemotaxis in primary human cells of hematopoietic origin

Migration of human peripheral blood mononuclear cells (PBMNC, $n = 3$) (A) and monocytes ($n = 7$) (B) towards CXCL12 (circles) and ubiquitin (Ub, squares). C/D: Typical examples for bell-shaped (C) and saturable (D) chemotactic dose-responses for CXCL12 and Ub in individual monocyte preparations. CI: chemotactic index.

Figure 2. CXCR4 activation with ubiquitin and CXCL12 activates similar signaling pathways in primary human monocytes

Pharmacological inhibition of CXCL12 and ubiquitin (Ub) induced chemotaxis in human monocytes. Cells were incubated with vehicle or AMD3100 (10 μ M, PBMNCs: n = 7, **(A)**; monocytes: n = 4 **(C)**) or with vehicle and the TM2 peptide (100 μ M, PBMNCs: n = 3 **(B)**; monocytes: n = 4, **(D)**) and migration towards 10 nM of CXCL12 or Ub tested. CI (% ctrl.): chemotactic index in % of vehicle treated cells (= 100%). *: p<0.05 vs. vehicle. **E**: Monocytes (n = 3) were pre-incubated at 37°C with vehicle, pertussis toxin (PTX)-100 ng/mL, 2 h; U73122-5 μ M, 30 min; LY294002-50 μ M, 1 h; U0126-10 μ M, 30 min. Cells were then washed and migration towards 10 nM of CXCL12 or Ub tested. CI (% ctrl.): chemotactic index in % of vehicle treated cells (= 100%). *: p<0.05 vs. vehicle.

Figure 3. CXCR4 activation with ubiquitin and CXCL12 induces chemotaxis and activates similar signaling pathways in primary human cells of non-hematopoietic origin

A: Migration of human primary aortic smooth muscle cells (hVSMC, $n = 6$) towards CXCL12 (circles) and ubiquitin (Ub, squares). **B/C:** hVSMC were incubated with vehicle or AMD3100 (10 μM , $n = 4$, **(B)**) or with vehicle and the TM2 peptide (100 μM , $n = 4$, **(C)**) and migration towards 10 nM of CXCL12 or Ub tested. CI (% ctrl.): chemotactic index in % of vehicle treated cells (= 100%). *: $p < 0.05$ vs. vehicle. **D:** Inositol trisphosphate (IP_3) production of hVSMC upon stimulation with ubiquitin or CXCL12. Cells were pre-incubated with 10 μM AMD3100 or vehicle (30 min, 37°C) and then stimulated with vehicle or 1 μM of ubiquitin or CXCL12 (15 min, 37°C). $N = 3$. **E:** Migration of human primary pulmonary artery endothelial cells (hPPAE, $n = 3$) towards CXCL12 (circles) and ubiquitin (Ub, squares). **F/G:** hVSMC were incubated with vehicle or AMD3100 (10 μM , $n = 3$, **(F)**) or with vehicle and the TM2 peptide (100 μM , $n = 3$, **(G)**) and migration towards 10 nM of CXCL12 or Ub tested. CI (% ctrl.): chemotactic index in % of vehicle treated cells (= 100%). *: $p < 0.05$ vs. vehicle.

Figure 4. Ubiquitin induces chemical shift changes in the NMR spectrum of CXCR4 in membranes but fails to recruit β -arrestin 2 to CXCR4

A. β -arrestin 2 recruitment assay (PRESTO-Tango) for CXCR4. Ub: ubiquitin. Ub + 6bK: Ub + 1 μM 6bK. Alb: albumin. RLU (%): relative luminescence units in % of the RLU after treatment with 1 μM CXCL12 (= 100%). N = 3. **B.** β -arrestin 2 recruitment assay (PRESTO-Tango) for CXCR4. Ub was tested in the presence of 10 nM CXCL12. RLU (%): relative luminescence units in % of the RLU after treatment with 1 μM CXCL12 (= 100%). N = 3. **C–F:** ^1H - ^{13}C HSQC spectra of reductively methylated CXCR4 membrane preparations were recorded without (blue, (C)) and with (red) 1 μM CXCL12 (D), ubiquitin (E) or CXCL11 (F). Signal 1 represents the N-terminus of CXCR4, signal 2 and signal 3 represent lysine residues that have been successfully modified with two ^{13}C labeled methyl groups. Black arrows indicate the difference in chemical shifts or broadening (loss) of the signal. Chemical shift perturbations (CSPs) of the N-terminus of reductively methylated CXCR4 were 0.0064, 0.0014 and 0.0024 in the presence of CXCL12, ubiquitin and CXCL11, respectively; CSPs greater 0.0058 were considered significant.