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Mutational analysis of the extracellular disulphide bridges of the atypical chemokine receptor ACKR3/CXCR7 uncovers multiple binding and activation modes for its chemokine and endogenous non-chemokine agonists

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ABSTRACT
The atypical chemokine receptor ACKR3/CXCR7 plays crucial roles in numerous physiological processes but also in viral infection and cancer. ACKR3 shows strong propensity for activation and, unlike classical chemokine receptors, can respond to chemokines from both the CXC and CC families as well as to the endogenous peptides BAM22 and adrenomedullin. Moreover, despite belonging to the G protein coupled receptor family, its function appears to be mainly dependent on β-arrestin. ACKR3 has also been shown to continuously cycle between the plasma membrane and the endosomal compartments, suggesting a possible role as a scavenging receptor. So far, the molecular basis accounting for these atypical binding and signalling properties remains elusive. Noteworthy, ACKR3 extracellular domains bear three disulphide bridges. Two of them lie on top of the two main binding subpockets and are conserved among chemokine receptors, and one, specific to ACKR3, forms an intra-N terminal four-residue-loop of so far unknown function. Here, by mutational and functional studies, we examined the impact of the different disulphide bridges for ACKR3 folding, ligand binding and activation. We showed that, in contrast to most classical chemokine receptors, none of the extracellular disulphide bridges was essential for ACKR3 function. However, the disruption of the unique ACKR3 N-terminal loop drastically reduced the binding of CC chemokines whereas it only had a mild impact on CXC chemokine binding. Mutagenesis also uncovered that chemokine and endogenous non-chemokine ligands interact and activate ACKR3 according to distinct binding modes characterized by different transmembrane domain subpocket occupancy and N-terminal loop contribution, with BAM22 mimicking the binding mode of CC chemokine N terminus.

1. Introduction
Chemokine receptors are class A G protein-coupled receptors (GPCRs) present at the surface of various cell types. By interacting with their cognate chemokines, they regulate vital cellular mechanisms, including cell trafficking, development, immune-modulation and adhesion as well as growth and survival [1]. They are also involved in pathological processes such as inflammation, cancer and HIV-1 infection [2,3].

ACKR3, the atypical chemokine receptor 3, formerly known as CXCR7, is one of the most recently deorphanized chemokine receptors [4]. It is expressed in various cells such as B and T lymphocytes, neurons and endothelial cells and plays a crucial role in many processes including cardiovascular and neuronal development as well as in migration and homing of hematopoietic stem/progenitor cells [5–11]. ACKR3 is also present in many cancer cell types and on tumour-associated vasculature and accumulating evidence demonstrates its involvement in metastasis development [12–15]. ACKR3 binds to two endogenous chemokines, CXCL12 and CXCL11, which are also the ligands for CXCR4 and CXCR3, respectively [10,16–19]. In addition, ACKR3 and CXCR4 can interact with vCCL2, the human herpesvirus 8 (HIV-8)-encoded chemokine, which is an antagonist for a broad

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spectrum of chemokine receptors, including CXCR4, but acts as agonist towards ACKR3 [19–22]. Unlike CXCR4 and CXCR3, which signal via G protein pathways, ACKR3 activity relies mainly on β-arrestin recruitment, although its ability to trigger signalling may be cell context-dependent [22–25]. In addition ACKR3 is proposed to act as a scavenger or “sink” receptor for CXCL12, CXCL11 and vCCL2, regulating their availability for other chemokine receptors [19,26–29]. Recently, it has also been shown that ACKR3 is a high-affinity receptor for BAM22, a non-chemokine peptide derived from the proenkephalin A family, which plays a role in the modulation of circadian glucocorticoid oscillation [30]. Similarly, based on phenotypic analysis of knock-out mice, ACKR3 was also proposed as a scavenger receptor for adrenomedullin (ADM), a pro-angiogenic peptide involved in the regulation of vascularisation [31].

Over the last few years, the tridimensional structures of several chemokine receptors, including CXCR4, have been resolved in complexes with small molecules or chemokines [32–38]. These structures revealed the typical receptor architecture comprising a flexible extracellular N terminus followed by a bundle of seven hydrophobic membrane-spanning α-helices (TM1–TM7) connected by three extracellular (ECL1–3) and three intracellular (ICL1–3) hydrophilic loops. In addition to the conserved disulphide bond linking the top of TM3 (end of ECL1) to the middle of ECL2, present in a large majority of class A GPCRs, all currently available tridimensional structures of chemokine receptors reveal a second disulphide bridge between the N terminus and the top of TM7 (end of ECL3) [39,40]. As a consequence, the last residues of the receptor N terminus form an additional extracellular loop (“ECL4”) connecting TM1 and TM7 and closing the receptor into a ring [39,40] (Fig. 1A and B). These structures together with recent mutational and functional studies were also key to refining our current understanding of chemokine-receptor interactions, moving from a simple two-step mechanism to a more continuous one characterized by extensive contacts between the two partners and 1:1 stoichiometry [33,41–44]. These interactions involve the core of the chemokine, including the N-loop region, with the flexible N terminus of the receptor (chemokine recognition site 1, CRS1), a key determinant for selectivity [41,43,45] ensuring optimal chemokine orientation with respect to the top of the ligand-binding pocket and ECL4 (CRS1.5) [33,39]. This enables the insertion of the flexible chemokine N terminus into the receptor transmembrane cavity (CRS2), made of the transmembrane segments and the extracellular loops, stabilizing an active state of the receptor and leading to intracellular signalling [33,43,46–48]. These tridimensional structures also showed that the orientation relative to the receptor varies for the different classes of chemokines and confirmed the presence of two distinguishable binding pockets – the major ligand binding pocket formed by the TM3, 4, 5, 6 proposed to be mainly occupied by the N terminus of CXC chemokines, and the minor pocket delineated by TM1, 2, 3, 7 suggested to accommodate the N terminus of CC chemokines (Fig. 1D) [33,34,48–50].

We have recently shown that ligand binding and activation of ACKR3 is in some measure different compared with CXCR4 and CXCR3 [51]. Indeed, ACKR3 has a strong propensity to activation, possibly
linked to its scavenging role, and as opposed to CXCR4 and CXCR3, the most N-terminal residues of the chemokine ligands as well as the N-loop have a minor importance for ACKR3 binding and activation. Moreover, vCCL2 seems to stabilize a different conformation of ACKR3 than do its endogenous CXC chemokine ligands, whereas very little is known on the binding mode of BAM22 and adrenomedullin to ACKR3 [19,30].

An intriguing particularity of ACKR3, which may contribute to its atypical nature, is the presence of two additional cysteine residues at positions 21 and 26, which have recently been shown to be linked by a disulphide bridge, giving rise to an intra-N terminus four-residue loop (Fig. 1) [32,39,52]. With the exception of CXCR3 that bears two additional successive cysteines at positions 37 and 38, other chemokine receptors do not have analogous cysteine residues. Given the crucial role of the flexible receptor N terminus in chemokine recognition, the intra-N terminus loop in ACKR3 could account for the existence of either a unique receptor structure or an unusual mode of interaction with its ligands [39,41].

Here, by targeted mutagenesis, we examined the role of the extracellular disulphide bridges in chemokine receptors CXCR4 and ACKR3. Using various binding and functional assays, we investigated the role of the unique ACKR3 intra-N terminus disulphide bridge for receptor-ligand interactions and compared the importance of the ECL4-forming disulphide bridge as well as the one linking TM3 and ECL2 for chemokine binding, receptor integrity and activation. We demonstrated that, in contrast to classical chemokine receptors, the other conserved extracellular disulphide bridges are not essential for ACKR3 surface expression, ligand binding and activation. In addition, we showed that the unique N terminal loop of ACKR3 is important for activation by CC but not CXC chemokines and that BAM22 mimics the binding mode of CC chemokine N terminus.

2. Material and methods

2.1. Peptides and chemokines

Chemokines CXCL12, CXCL11, vCCL2, CXCL10 and CXCL9 were purchased from PeproTech. Alexa Fluor 647-labelled CXCL12 (CXCL12-AF647) was purchased from Almac. BAM22 and adrenomedullin (ADM) were purchased from Bachem. Peptides derived from the N terminus of chemokines and peptide TPA (21-CNSSDC-26, with C21-C26 cyclization) were purchased from JPT. Peptides CCS (encompassing residues 1-MDLHLFDYSEPGNFSDISWPCNSSDCIVVDTVMSPNMPNKS-40, with a disulphide bridge between cysteines 21 and 26 and a cysteine-to-serine mutation at position 34), SSS (encompassing residues 1-MDLHLFDYSEPGNFSDISWPSNSSDSIVVDTVMSPNMPNKS-40, in which the three cysteine residues were mutated to serines) and SSSscrbld (1-PPYDVISSMLKDSINENFVSLPNGPSDTVHMWDNFMDS-40, in which all residues were randomly permuted) were purchased from ChinaPeptides. All peptides contain a free amine at the N terminus and an amide group at the C terminus to avoid additional negative charge.

2.2. Generation of U87 cell lines expressing wild-type and mutant ACKR3 and CXCR4

pBABE.puromycin vectors (Addgene) encoding wild type ACKR3, variants bearing double cysteine-to-serine mutations (C21S-C26S, C34S-C287S and C117S-C186S) or glycine-substituted variant (C21-G4-C26), and wild-type CXCR4 or C285-C274S or C109S-C186S mutants were transfected into U87 cells [51]. This cellular background was chosen for its absence of endogenous CXCR4, ACKR3 and CXCR3 as previously demonstrated [19]. Cells stably expressing the modified receptors were obtained following puromycin selection and subsequent single-cell sorting using BD FACSAria II cell sorter (BD Biosciences). The presence of the mutations and the surface expression level of the mutated receptors were verified by genomic DNA sequencing and flow cytometry using antibodies recognising the proximal N terminal part of

Table 1

<table>
<thead>
<tr>
<th>Binding</th>
<th>ACKR3</th>
<th>CXCL12-AF647</th>
<th>CXCL11</th>
<th>vCCL2</th>
<th>BAM22</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEC50 ± SEM</td>
<td>8.60 ± 0.04</td>
<td>8.79 ± 0.07</td>
<td>8.58 ± 0.07</td>
<td>8.84 ± 0.06</td>
<td>8.61 ± 0.06</td>
</tr>
<tr>
<td>IC50 (nM)</td>
<td>2.5</td>
<td>100</td>
<td>40</td>
<td>7</td>
<td>100</td>
</tr>
<tr>
<td>Fold WT</td>
<td>1.0</td>
<td>2.5</td>
<td>4.1</td>
<td>2.7</td>
<td>3.9</td>
</tr>
<tr>
<td>pIC50 ± SEM</td>
<td>8.32 ± 0.05</td>
<td>6.21 ± 0.15</td>
<td>8.41 ± 0.06</td>
<td>8.41 ± 0.06</td>
<td>8.41 ± 0.06</td>
</tr>
<tr>
<td>EC50 (nM)</td>
<td>3.0</td>
<td>622.5</td>
<td>14.3</td>
<td>3.9</td>
<td>11.2</td>
</tr>
<tr>
<td>Fold WT</td>
<td>1.0</td>
<td>43.4</td>
<td>106 ± 1</td>
<td>39.4</td>
<td>34.4</td>
</tr>
</tbody>
</table>

* CXCL12-AF647 binding to ACKR3 mutants was normalized to receptor cell surface expression based on staining with mAb 8F11-M16 considering WT receptor as 100%.
CXCR4 (clone 4G10, Santa Cruz Biotechnology) or ACKR3 (clones 11G8, R&D Systems and 9C4, MBL Life Science). CXCR3 in transiently transfected U87 cells was detected using the mAb 1C6 (BD Pharmingen).

2.3. Chemokine and peptide binding

U87 cells expressing wild-type or mutant ACKR3 or CXCR4 were distributed into 96-well plates (15 × 10⁴ cells per well) and incubated with Alexa Fluor 647-labelled CXCL12 at concentrations ranging from 35 pM to 115 nM for 90 min on ice or for 45 min at 37 °C, respectively. Non-specific binding of CXCL12-AF647 was evaluated on ACKR3- and CXCR4-negative U87 cells and subtracted. For binding competition studies, U87 cells expressing wild-type or mutant ACKR3 or CXCR4 were incubated with CXCL12-AF647 at concentrations equivalent to twice the EC₅₀ values determined for each ACKR3 variant in saturation binding experiments (Table 1) or 11.5 nM for CXCR4, mixed with unlabelled CXCL12, CXCL11, vCCL2, CXCL10, BAM22 or ADM at concentrations ranging from 6 pM to 1 μM. All binding experiments were performed in PBS containing 1% BSA and 0.1% NaN₃ (FACS buffer). Non-specific chemokine binding was evaluated by the addition of 250-fold excess of unlabelled CXCL12 and the signal obtained was used to define 0% specific binding. The signal obtained for CXCL12-AF647 in the absence of unlabelled chemokines was used to define 100% binding. Chemokine binding was quantified by mean fluorescence intensity on a BD FACS Fortessa cytometer (BD Biosciences).

2.4. β-arrestin recruitment

β-arrestin-2 recruitment to wild-type and mutant ACKR3, CXCR4 or CXCR3 induced by chemokines, chemokine N-terminus-derived peptides or BAM22 was monitored by NanoLuc complementation assay (NanoBit, Promega) [51,53,54]. 1.2 × 10⁶ U87 cells were plated in 10 cm-culture dishes and 48 h later transfected with pNBe vectors containing human β-arrestin-2 N-terminally fused to LgBiT and receptors C-terminally fused to SmBiT. 48 h post-transfection cells were harvested, incubated 40 min at 37 °C with 200-fold diluted Nano-Glo Live Cell substrate and distributed into white 96-well plates (5 × 10⁴ cells per well). β-arrestin-2 recruitment in response to chemokines, N-terminus derived peptides or BAM22 was evaluated after 10-minute incubation with a Mithras LB940 luminometer (Berthold Technologies). For each receptor and each experiment the maximum signal recorded with a saturating concentration (200 nM) of full agonist (i.e CXCL12 for ACKR3 and CXCR4) was set as 100%.

2.5. Neutralisation of mAb 9C4 by ACKR3 N-terminal peptides

The mAb 9C4 (3.3 μg/ml) was first incubated for 30 min at room temperature with peptides CCS, SSS (100 nM) or TPA (100 μM) derived from the N terminus of ACKR3. Peptide SSS scrambled (100 nM) was used as negative control. The antibody-peptide mix was then incubated for 60 min at 4 °C with U87 cells expressing wild-type ACKR3 (15 × 10⁴ cells/well in a 96-well plate). The binding of 9C4 to ACKR3...
was revealed with an allophtocyanin-conjugated goat anti-mouse IgG F(ab')2 (Jackson ImmunoResearch) and quantified by mean fluorescence intensity on a BD FACS Fortessa cytometer (BD Biosciences).

2.6. Immunocytochemistry and fluorescent imaging

For microscopic analysis of CXCR4 and ACKR3 distribution, 5 × 10^4 U87 cells were plated on sterile coverslips in a 24-well plate and cultured overnight. Cells were transiently transfected with equal amounts of pBabe plasmid encoding wild type or mutated CXCR4 or ACKR3 using X-tremeGENE 9. 48 h later, cells were washed with PBS and fixed for 20 min on ice with 4% (w/v) paraformaldehyde. After one washing step with PBS supplemented with 50 mM NH4Cl and two washes with PBS, cells were permeabilised with 0.1% Triton X-100 for 20 min at room temperature. Subsequently, cells were blocked with 10% (w/v) normal goat serum for 1 h and incubated overnight at 4 °C with the mAb 4G10 (Santa Cruz Biotechnology) diluted 1:75 for CXCR4 or the mAb 11G8 (R&D Systems) diluted 1:100 for ACKR3 staining in 1% BSA PBS, respectively. After three washing steps, cells were incubated for 1 h at room temperature with a goat anti-mouse Alexa Fluor 647-conjugated secondary antibody (abcam) diluted 1:1200 in PBS complemented with 5% normal goat serum. Cells were washed twice, stained with Hoechst 33,342 dye (Sigma) for 10 min at 4 °C, washed twice and mounted on glass slides with 5 μl ProLong Diamond anti-fade mounting medium (Molecular Probes). Images were acquired using a 63 × oil-immersion Apotome.2 and a Colibri LED illumination system. Representative images of two independent experiments are shown.

2.7. Data and statistical analysis

Concentration-response curves were fitted to the four-parameter Hill equation using an iterative, least-squares method (GraphPad Prism version 7.02) to provide pEC50, pIC50, EC50 or IC50 values, standard errors of the mean (SEM) and the Hill coefficient. Fitting was performed on data from at least three independent experiments (n = 3). Unpaired t tests were used to analyse the differences in pEC50/pIC50 for each ligand using the wild-type receptor as reference. p value of < 0.05 was considered as statistically significant.

3. Results

3.1. Extracellular disulphide bridges are dispensable for ACKR3 surface expression and folding

3.1.1. The conserved disulphide bridges

First, the importance for ACKR3 conformational fidelity of the disulphide bridge between the N terminus and the top of TM7 (C34-C287) as well as the one between TM3 and ECL2 (C117-C196) was evaluated and compared to CXCR4 (Fig. 1A and B). The impact of cysteine pair mutations on the receptor cellular distribution was monitored by flow cytometry and fluorescence microscopy using antibodies detecting linear epitopes located in the receptor N terminus (11G8 for ACKR3 and 4G10 for CXCR4) or recognising more complex epitope spread over different extracellular domains (8F11-M16 for ACKR3 and 12G5 for CXCR4).

In line with previous reports, a higher proportion of the wild-type ACKR3 was present intracellularly compared to the cell surface, while CXCR4 was localised at the plasma membrane (Fig. 2E). Mutation of the cysteine pair engaged in the disulphide bridge between the N terminus and top of TM7 (the ECL4-forming bridge), typical of chemokine receptors, had little effect on ACKR3 (C34S-C287S) and CXCR4 (C28S-C274S) surface expression but strongly impacted CXCR4 architecture, as shown by the inability of the conformational mAb 12G5 to recognise the receptor (Fig. 2A and B). In contrast, recognition of the ACKR3-C34S-C287S variant by the conformational mAb 8F11-M16 was equivalent to that of wild-type receptor. The disruption of the TM3-ECL2 bridge (C117S-C196S) only moderately affected the ACKR3 distribution (Fig. 2E), in stark contrast to CXCR4, for which the equivalent mutation (C109S-C186S) abolished the receptor surface expression (Fig. 2B) and resulted in a strong intracellular retention (Fig. 2E). Nonetheless, a 75% reduction of ACKR3 surface expression was observed with the conformational mAb 8F11-M16 compared to the wild-type receptor (Fig. 2A), indicating that the TM3-ECL2 bridge does play a role in ACKR3 structural integrity, although it is not critical for receptor export.

Overall, these results show that the receptor surface expression and architecture in the absence of disulphide bridges was much less affected for ACKR3 than for CXCR4.

3.1.2. The atypical disulphide bridge of ACKR3

Next, the potential role played by the unique four-residue intra-N terminal loop of ACKR3 formed by the disulphide bridge between residues C21 and C26 (TPA for TetraPeptidyl Arch) in receptor expression, localisation and conformation was assessed. No difference in ACKR3 surface expression or folding was observed when each of the four residues within the loop (NSSD) was substituted with a glycine (C21-G4-C26) (Fig. 2A and B). The disruption of the loop-forming cysteine bridge (C21S-C26S) did however have an effect on the receptor cellular distribution, resulting in an apparent 40% increase in surface expression compared with wild-type ACKR3 (Fig. 2A and E). Similar tendency was observed for CXCR3 variant in which the two N-terminal cysteines were mutated to serines (C37S-C38S) (Fig. 2C).

Surprisingly, although the mutants C21S-C26S and C21-G4-C26 were detected at the cell surface using the mAb 11G8, they were not recognised by the mAb 9C4, also raised against the N terminal domain of ACKR3 (Fig. 2D) [55]. In line with this observation, 9C4 binding to the wild-type ACKR3 was reduced by 86% in the presence of the cyclic peptide OC5, derived from the N terminus of ACKR3 and bearing the TPA, whereas the equivalent linear SSS peptide caused only a 25% reduction (Fig. 2F). Peptide SSS_cyclic had no effect on 9C4 binding to the wild-type receptor. These results show that the four-residue intra-N terminal loop of ACKR3 (TPA) is a crucial contributor of the 9C4 binding, although it is not the sole determinant of its epitope as demonstrated by the inability of the shorter cyclic peptide comprising only the TPA (C21-SSS-C26) to neutralise the antibody binding to the wild-type receptor, even at a concentration as high as 100μM. In addition, these data reveal that the TPA is well exposed and accessible to the extracellular ligands and makes mAb 9C4 a useful probe to evaluate its presence on the receptor in different cellular contexts.

3.2. CXC and CC chemokines have different binding and activation modes on ACKR3

The binding of Alexa Fluor 647-labelled CXCL12 to cysteine-mutated ACKR3 and CXCR4 was first evaluated and compared in U87 cell lines stably expressing wild-type or mutated receptor.

In contrast to CXCR4, all cysteine mutants of ACKR3 retained their ability to bind CXCL12-AF647, although the maximal binding and potency were affected to different extents. The mutation of the TM3-ECL2 cysteine bridge (C117S-C196S) had the most pronounced effect, resulting in a marked reduction of potency because of which no EC50 nor maximum binding could be determined (Fig. 3B). The mutation of the ECL4-forming cysteine bridge linking the N terminus and TM7 of ACKR3 had less effect, but still led to a 60%-decrease of the maximum CXCL12-AF647 binding and a significant 4-fold increase in EC50 as compared to the wild-type receptor (Fig. 3B, Table 1). Interestingly, the analogous mutant of CXCR4 (C28S-C274S) was no longer capable of binding CXCL12 (Fig. 3A and Table 1), further confirming the stronger tolerance of ACKR3 to alterations in TM-linking disulphide bridges. The C21-G4-C26 mutant showed an EC50 value equivalent to that of wild-type ACKR3, while the C21S-C26S mutation led to an approximately
two-fold increase in EC$_{50}$ (Fig. 3B, Table 1) indicating that it is the constraint brought by the intra-N terminus loop rather than its decorating residues that plays a role in CXCL12 binding.

The effect of cysteine mutations on the ACKR3 interactions with its other chemokine ligands was then evaluated in binding competition studies with CXCL12-AF647. CXCL11 binding to mutant ACKR3.C34S-C287S was reduced by approximately two-fold compared to the wild-type receptor, while vCCL2 binding was improved by nearly 4-fold (Fig. 3C and D, Table 1). Both CXCL11 and vCCL2 were less potent in displacing CXCL12-AF647 from ACKR3.C21S-C26S where the binding of the viral chemokine was the most impacted (2.4- and 14.3-fold increase in IC$_{50}$ values respectively). Binding of CXCL11 and vCCL2 to the mutant C21-G4-C26 was affected to a similar extent (3-fold increase in IC$_{50}$ values), despite the absence of effect of the mutation on CXCL12-AF647 binding. These results suggest that the presence of the TPA has an impact on the binding of all chemokines with a significantly higher effect on vCCL2 binding while the residues of the TPA themselves appear to be involved in interactions with vCCL2 and CXCL11 but not CXCL12.

The effect of disulphide bridge disruption on ACKR3 activation was then monitored in a β-arrestin-2 recruitment assay. Overall two clearly contrasting trends could be observed in how disrupting the disulphide bridges affected the ability of chemokines to activate ACKR3, one - for the two CXC chemokines, CXCL12 and CXCL11, second - for the CC chemokine vCCL2. Firstly, β-arrestin-2 recruitment induced by CXCL12 and CXCL11 was only negatively impacted by the mutation disrupting the TM3-ECL2 disulphide bridge located on top of the major ligand-binding pocket (6.5- and 6.5-fold increase in EC$_{50}$ values, respectively) (Fig. 3F and G, Table 2). This mutation however had no effect on vCCL2 potency to induce β-arrestin-2 recruitment (Fig. 3H, Table 2). Interestingly, whereas the TM3-ECL2 mutation resulted in a non-functional CXCR4, in case of ACKR3 it allowed CXCL11 and vCCL2 to achieve efficacies equivalent to CXCL12, as opposed to their partial response (75%) observed with the wild-type receptor and all the other cysteine mutants. The mutation of ECL4 (C34S-C287S) did not affect β-arrestin-2 recruitment to the TPA-modified ACKR3 variants in response to CXCL12 and CXCL11 was comparable to the wild-type receptor, whereas it was considerably impaired in response to vCCL2 (4.6- and 6.6- fold reduction for the C21S-C26S and C21-G4-C26 mutants, respectively), showing the importance of the TPA in the vCCL2-induced ACKR3 activation. By comparison, mutagenesis of cysteine residues at position 37 and 38 of the CXCR3 N terminus had no significant impact on the receptor activation by CXCL11, CXCL10 but decreased the potency of CXCL9 by more than 2 fold (Table 4).

Overall, these data reveal that whereas all cysteine-mutated ACKR3 variants were able to bind and efficiently respond to chemokine ligands, the disruption of the disulphide bridges in CXCR4, without exception,
led to severe impairment of the receptor functionality. Furthermore, the differences in the impact of cysteine pair mutations for the CXC chemokines on one side and vCCL2 on the other clearly point to distinct binding and activation modes for ACKR3, either largely relying on the TM3-ECL2 disulphide bridge or on the N-terminal TPA (Fig. 5).

To gain further insight into the determinants involved in the recognition and activation of ACKR3, peptides derived from the N-terminal region of CXCL12, CXCL11 and vCCL2 were tested for their ability to induce β-arrestin-2 recruitment to the wild-type and mutated receptors. Similarly to what has previously been shown for the wild-type ACKR3 [51], CXCL12-, CXCL11- and vCCL2-derived peptides covering the flexible N terminus, the cysteine motif and the N loop (CXCL12, CXCL11 and vCCL2) were able to trigger β-arrestin-2 recruitment to the cysteine-mutated receptors, although differences were observed between the peptides. The mutation of the cysteine bridge between TM3 and ECL2, covering the major binding pocket, strongly reduced the activity of CXCL12 and vCCL2 (EC50 increase by > 20 and 6.5 fold, respectively) and to a lesser extent that of CXCL11 and vCCL2 (1.8 fold). Surprisingly however, this mutation had little impact on vCCL2, in contrast to what was observed for full-length vCCL2, indicating that the core of the viral chemokine is the main contributor in the interactions with TPA.

3.3. The endogenous peptide BAM22 reveals a vCCL2-like binding and activation mode towards ACKR3

Among the different non-chemokine ligands described to bind to ACKR3, only BAM22, a 22-amino acid peptide showing sequence similarities with chemokine N termini (Fig. 1E), was able to compete with the binding of labelled CXCL12 to wild-type ACKR3 (IC50 = 32.2 nM, pIC50 = 7.49 ± 0.03). No displacement was observed with adrenomedullin (ADM) in the concentration range tested (Fig. 4A, Table 1).

BAM22 was then tested towards the cysteine mutants to provide the first information about its binding and activation modes. Reminiscent of what was observed for vCCL2, the mutation C37S-C38S disrupting ECL4, reduced the IC50 of BAM22 binding by nearly two-fold compared to the wild-type receptor (Fig. 4B). However, in contrast to vCCL2, mutations of the TPA had no effect on BAM22 binding to ACKR3. BAM22 was also able to induce β-arrestin-2 recruitment towards all ACKR3 mutants (Fig. 4C). The strongest impairment was observed with the C117-C196 mutant, just like for the CXC chemokines, all small chemokine N-terminal peptides but not for vCCL2. While the mutation in ECL4 had a modest effect on ACKR3 functionality, the two mutations affecting the TPA significantly impacted the potency of BAM22 to induce β-arrestin-2 recruitment (2.5 fold for C21S-C26S and 2.9 fold for C21-G4-C26, respectively), comparable to what was observed for vCCL2 and its N-terminal peptides. These data suggest that the endogenous peptide BAM22 has a vCCL2-like ACKR3 binding and activation modes, consistent with its sequence homology with the N-terminal region of vCCL2 (Fig. 1E).

4. Discussion

ACKR3 is involved in different vital physiological but also pathological processes and has emerged as a highly relevant drug target, especially for cancer therapy. Although many efforts have been undertaken over the last years, no small molecule antagonist of ACKR3 has been identified so far. ACKR3 shows several distinctive functional characteristics some of which have been suggested to be linked to its chemokine scavenging function. The most remarkable ones are its lack of G protein coupling, its continuous cycling, predominantly intracellular localization, responsiveness to both CXC and CC chemokines as well as to endogenous non-chemokine peptides, and its high propensity for activation. In addition to these functional particularities, an uncommon structural feature of ACKR3 is the disulphide bridge within its N terminus, which creates a small loop whose function and impact on the receptor biology remain unknown [32,41,52]. In this study, by disrupting this additional disulphide bridge, as well as the two conserved disulphide bridges, we uncovered new functional properties of ACKR3 and demonstrated that chemokine and endogenous non-chemokine ligands interact and activate ACKR3 following different binding modes characterized by distinct transmembrane domain subpocket occupancy and intra-N terminus loop contribution.

The roles of the conserved disulphide bridges for chemokine receptor functions have previously been shown to reflect unique traits of a single receptor rather than being shared between receptors, even within the same subfamily [40]. Their impact was also shown to vary depending on the ligand, thus offering an interesting means to explore different binding and/or activation modes for a specific receptor. Indeed, the two conserved disulphide bridges are positioned at opposing critical regions of the receptor extracellular surface and shape the entrance of the ligand-binding subpockets (Fig. 1D).

Our data showed that ACKR3 folding and export is largely independent of the presence of its extracellular disulphide bridges. Only the disruption of the TM3-ECL2 disulphide reduced the presence of the receptor at the plasma membrane and moderately impacted the structure of the receptor. These results markedly contrast with the observations for TM3-ECL2 disulphide mutants in this and previous studies for CXCR4 [56] or CCR6 [57], which were both fully retained within the cell. However, maintenance of folding and export were reported for CXC2 and CCR1 [40,58].

The substitution of the two N-terminal cysteine residues engaged in the formation of the intra-N terminus loop (TPA) resulted in a slight increase of surface expression levels compared to the wild-type receptor. Similar results were observed for the CXC3 variant bearing the C37S-C38S mutation, suggesting that this apparent higher surface expression in N-terminal cysteine mutants may either reflect a facilitated transfer through the cell secretory pathway or simply a better accessibility of the epitope to the probe. A recent mutagenesis study of ACKR3 focusing on its N-terminal features reported corroborating results and found that the disruption of the N terminus-TM7 or the TPA-forming disulphide bridges did not significantly affect ACKR3 surface expression [52].

The maintenance of cell surface expression for all ACKR3 mutants allowed us to further probe the role of the disulphide bridges in ligand binding and receptor activation, providing new insights into the importance of the different extracellular regions and subpockets of ACKR3. The absence of the disulphide bridge linking TM3 to ECL2, lying on top of the major subpocket, reduced the ability of ACKR3 to bind CXCL12 and respond to CXC chemokines, but not the CC chemokine. Noteworthy, the disruption of this disulphide turned CXCL11 and vCCL2 from partial to full agonists, suggesting that this structural constraint regulates the maximal responsiveness of the receptor to certain chemokines. Conversely, the disruption of the disulphide bridge linking the N-terminal domain to TM7, surrounding the minor ligand binding pocket, enhanced the apparent binding of vCCL2, which was likely linked to the weaker interaction of the fluorescent tracer and may point to differential ACKR3 binding pocket occupancy by CXC and CC chemokines. Strikingly, the disruption of or substitution within the N-terminal loop (TPA) markedly impaired the recognition and activation by CC and to a lesser extent by CXC chemokines. Altogether these results provide strong evidence for a different binding modes for CXC versus CC chemokines to ACKR3 characterised by different roles of the N-terminal arch and different ligand subpocket occupancies. Our data
### Table 2
β-arrestin-2 recruitment induced by full-length chemokines and BAM22 to ACKR3.

<table>
<thead>
<tr>
<th>ACKR3</th>
<th>CXCL12</th>
<th>CXCL11</th>
<th>vCCL2</th>
<th>BAM22</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC_{50} ± SEM</td>
<td>EC_{50} (nM)</td>
<td>Fold WT</td>
<td>Max (%)</td>
</tr>
<tr>
<td>WT</td>
<td>8.89 ± 0.07</td>
<td>1.27</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>C34S-C287S</td>
<td>8.83 ± 0.07</td>
<td>1.44</td>
<td>1.1</td>
<td>100</td>
</tr>
<tr>
<td>C117S-C196S</td>
<td>8.07 ± 0.13</td>
<td>8.45</td>
<td>6.5</td>
<td>100</td>
</tr>
<tr>
<td>C21S-C26S</td>
<td>8.93 ± 0.09</td>
<td>1.17</td>
<td>0.9</td>
<td>100</td>
</tr>
<tr>
<td>C21-G4C26</td>
<td>8.90 ± 0.16</td>
<td>1.25</td>
<td>1.0</td>
<td>100</td>
</tr>
</tbody>
</table>

β-arrestin-2 recruitment was monitored in U87 cells using split Nanoluciferase complementation assay (n = 3).

### Table 3
β-arrestin-2 recruitment induced by peptides derived from chemokine N-terminal regions to ACKR3.

<table>
<thead>
<tr>
<th>ACKR3</th>
<th>CXCL12</th>
<th>CXCL11</th>
<th>vCCL2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC_{50} ± SEM</td>
<td>EC_{50} (nM)</td>
<td>Fold WT</td>
</tr>
<tr>
<td>WT</td>
<td>6.22 ± 0.04</td>
<td>610</td>
<td>1.0</td>
</tr>
<tr>
<td>C34S-C287S</td>
<td>6.23 ± 0.7</td>
<td>600</td>
<td>1.0</td>
</tr>
<tr>
<td>C117S-C196S</td>
<td>4.85 ± 0.18</td>
<td>14270</td>
<td>23.4</td>
</tr>
<tr>
<td>C21S-C26S</td>
<td>6.18 ± 0.09</td>
<td>660</td>
<td>1.1</td>
</tr>
<tr>
<td>C21-G4C26</td>
<td>6.20 ± 0.12</td>
<td>630</td>
<td>1.0</td>
</tr>
</tbody>
</table>

β-arrestin-2 recruitment was monitored in U87 cells using split Nanoluciferase complementation assay (n = 3).

### Table 4
β-arrestin-2 recruitment induced by full-length chemokines to CXCR3.

<table>
<thead>
<tr>
<th>CXCR3</th>
<th>CXCL11</th>
<th>CXCL10</th>
<th>CXCL9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC_{50} ± SEM</td>
<td>EC_{50} (nM)</td>
<td>Fold WT</td>
</tr>
<tr>
<td>WT</td>
<td>8.03 ± 0.19</td>
<td>9.4</td>
<td>1.0</td>
</tr>
<tr>
<td>C38S-C38S</td>
<td>8.68 ± 0.10</td>
<td>8.7</td>
<td>0.9</td>
</tr>
</tbody>
</table>

β-arrestin-2 recruitment was monitored in U87 cells using split Nanoluciferase complementation assay (n = 3).
suggest that CXC chemokines mainly occupy the major subpocket, whereas vCCL2 interacts with structural determinants located in the vicinity of the minor subpocket, including the TPA. These observations are supported by structural data and comparative models of the binding mode of CXCL12 and vCCL2 to CXCR4, which shares 29% identity with ACKR3, that indicate that although the tip of the N-termini of the two chemokines reach similar depths in the transmembrane region, they occupy the extracellular surface and the receptor subpockets differently. While vCCL2 lies mainly above TM1 and TM7 with its N terminus spanning the minor binding pocket, CXCL12 shows a rotation of approximately 80° relative to vCCL2, consequently positioning it more on top of the major binding pocket (TM5-TM6) [33,59].

In this study, no interaction between adrenomedullin and ACKR3 was detected in the concentration range for which all other ligands were highly potent. This observation may be due the requirement of additional partners for adrenomedullin binding to occur, which are absent in the U87 cells. The binding and activation by BAM22, showed an apparent mixed CXC/CC profile for the effect of disulphide bridge disruptions. BAM22 interactions was negatively impacted by the disruption of the disulphide bridge TM3-ECL2 similarly to what was observed for CXC chemokines, suggesting that it also activates the receptor by occupying mainly the major binding pocket (Fig. 5). However, its binding and activity were also significantly affected by the disruption or the substitution of the TPA indicating that its C terminus is most likely positioned more on top of the minor ligand binding pocket reminiscent of vCCL2 binding mode. These binding similarities between vCCL2 and BAM22 may be partially explained by the sequence identity between the N-loop of vCCL2 and the C terminus of BAM22, both regions bearing the YQKR motif. We recently demonstrated that the N-loop of vCCL2, but not that of CXCL12 and CXCL11, is important for ACKR3 binding and activation further supporting the observation that this stretch could interact with the TPA by for instance forming electrostatic interactions with negatively charged residues (D25) located within the TPA [51]. Sequence alignment revealed that D25 occupies a position similar to that of the sulfotyrosine sY21 in the CXCR4 N terminus, a residue known to be crucial for CXCR4-chemokine interactions [42]. Furthermore, the shortening of CR51 resulting from the formation of the TPA would also bring the potential sY8 of ACKR3 at a position equivalent to that of sY7 in CXCR4. Moreover, although it is highly speculative, the oxidation state of the two TPA-forming cysteines, by influencing the strength of ACKR3 interactions with internalised ligands, may regulate their handling or release during the trafficking through the different intracellular compartments. Finally, it cannot be excluded that this arch could also support the binding of other yet unknown ligands or interacting partners.

**Acknowledgments**

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**Conflict of interest**

The authors declare no conflict of interest.


