Mutational Analysis of Atypical Chemokine Receptor 3 (ACKR3/CXCR7) Interaction with Its Chemokine Ligands CXCL11 and CXCL12*

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Atypical chemokine receptors do not mediate chemotaxis or G protein signaling, but they recruit arrestin. They also efficiently scavenge their chemokine ligands, thereby contributing to gradient maintenance and termination. ACKR3, also known as CXCR7, binds and degrades the constitutive chemokine CXCL12, which also binds the canonical receptor CXCR4, and CXCL11, which also binds CXCR3. Here we report comprehensive mutational analysis of the ACKR3 interaction with its chemokine ligands, using 30 substitution mutants. Readouts are radioligand binding competition, arrestin recruitment, and chemokine scavenging. Our results suggest different binding modes for both chemokines. CXCL11 depends on the ACKR3 N terminus and some extracellular loop (ECL) positions for primary binding, ECL residues mediate secondary binding and arrestin recruitment potency. CXCL12 binding required key residues Asp-1794.60 and Asp-2756.58 (residue numbering follows the Ballesteros-Weinstein scheme), with no evident involvement of N-terminal residues, suggesting an uncommon mode of receptor engagement. Mutation of residues corresponding to CR52 in CXCR4 (positions Ser-1032.63 and Gln-3017.39) increased CXCL11 binding, but reduced CXCL12 affinity. Mutant Q301E7.39 did not recruit arrestin. Mutant K118A3.26 in ECL1 showed moderate baseline arrestin recruitment with ablation of ligand-induced responses. Substitutions that affected CXCL11 binding also diminished scavenging. However, detection of reduced CXCL12 scavenging by mutants with impaired CXCL12 affinity required drastically reduced receptor expression levels, suggesting that scavenging pathways can be saturated and that CXCL12 binding exceeds scavenging at higher receptor expression levels. Arrestin recruitment did not correlate with scavenging; although Q301E7.39 degraded chemokines in the absence of arrestin, S103D2.63 had reduced CXCL11 scavenging despite intact arrestin responses.

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Atypical chemokine receptors (ACKRs) are chemokine receptors that do not activate G protein-dependent signaling cascades upon agonist binding; they also do not mediate chemotaxis (1). Rather, chemokine binding to ACKRs leads to rapid chemokine internalization and degradation. Therefore, ACKRs act as chemokine scavengers and play a role in chemokine gradient reshaping, maintenance, and resolution.

ACKR3, also known as CXCR7, is an atypical chemokine receptor for the constitutive chemokine CXCL12 (SDF-1), which also binds CXCR4 as a cognate canonical chemokine receptor (2). The second ACKR3 ligand is the highly regulated inflammatory chemokine CXCL11 (I-TAC), which also binds the canonical receptor CXCR3. Being unable to raise G protein responses in most cell types, agonist binding to ACKR3 nevertheless induces arrestin recruitment as a signaling response (3) and rapid degradation of both CXCL12 and CXCL11 (4). ACKR3 has been identified as a potentially promising drug target (2). Given the highly divergent role and regulation of its two ligands, selective interference with their ACKR3 interaction and scavenging may be of interest, warranting better understanding of ligand engagement and scavenging.

The structures of chemokine receptors are beginning to be unraveled by crystallography, and the results are in line with those reported earlier from receptor mutagenesis-based approaches (5–7). The reported chemokine receptor crystals required co-crystallization in complex with receptor antagonists. CXCR4 was the first receptor crystallized in complex with an antagonistic chemokine ligand, the virally encoded vMIP-II, which has also permitted refined modeling of the putative CXCR4-CXCL12 complex (8). Overall, these structures refine an early two-step binding model that provides a conceptual framework for the interactions of chemokines with canonical chemokine receptors (for a recent critical review of this model see Kleist et al.; Ref. 9). Following this model, initial chemokine interaction with the receptor critically depends on the receptor N terminus, and some extracellular loop residues. This is then followed by secondary interaction mainly involving the chemokine N terminus with receptor binding pocket residues; this second interaction is required for receptor activation. Of note, for CXCR4 the two-step interaction model is supported by

1 The abbreviations used are: ACKR, atypical chemokine receptor; BRET, boluminolucence resonance energy transfer; CR51, chemokine recognition site 1; ANOVA, analysis of variance; TM, transmembrane.
experimental data (10). Both CXCL11 and CXCL12 interactions with their canonical receptors conform this model, with key roles for the chemokine N-loop in receptor recognition, and deletion of the chemokine N terminus reportedly affecting signaling (11–13). Besides divergent sequences of the chemokine N terminus (KPVSLSYRC in CXCL12 compared with FPMFKRGRC in CXCL11), one difference between the two chemokines may be the relative inability of CXCL11 to dimerize, due to particularities in the β1 strand (14, 15).

At present, it remains largely unknown how chemokines interact with ACKRs. Of note, all known synthetic ACKR3 ligands, including those that act as antagonist on CXCR4, turned out to trigger activation of arrestin recruitment (3, 16, 17); this circumstance may complicate crystallization of an ACKR3-ligand complex. To date, no extensive mutagenesis study on ACKR-chemokine interactions has been reported. To gain insight into ACKR3 interactions with its chemokine ligands, we here report investigation of ACKR3 substitution mutants of candidate residues in the extracellular parts of the receptor (for a schematic overview see Fig. 1). We investigate chemokine binding using radioligand competition, arrestin recruitment using bioluminescence resonance energy transfer (BRET)-based assays, and for a subset of mutants, chemokine degradation as a functional readout.

**Results**

**Identification of the ECL4-forming Disulfide Bridge**—In G protein-coupled receptors, a conserved disulfide bridge links extracellular loops 1 and 2 (ECL1 and ECL2); in chemokine receptors, an additional conserved disulfide bridge links the receptor N-terminal to the base of ECL3 to form the so-called ECL4 pseudoloop (18). This ECL4-forming disulfide bridge is essential for CXCR4 ligand binding and activation (19, 20). ACKR3 has three cysteine residues in its N terminus, Cys-21, Cys-26, and Cys-34; based on sequence alignments, either Cys-21 or Cys-34 has been predicted to form ECL4 with Cys-287.25 in ECL3 (18, 21) (residue numbering follows the Ballesteros-Weinstein scheme; Ref. 22); it is unknown whether the potential additional disulfide bridge between the remaining two N-terminal cysteines is of functional relevance. To clarify these issues, we tested three cysteine double mutants, C21S/C26S, C26S/C34S, and C34S/C287S fused to the yellow fluorescent protein YFP.

Mutant surface expression was assessed by flow cytometry. As shown in Fig. 2, A–C, cells transfected with C21S/C26S and C34S/C287S were well stained, whereas transfection with C26S/C34S resulted in a ~50% reduction of staining intensity. YFP expression remained, however, unaffected (Fig. 2B), suggesting that the mutant was not unstable, as would be expected for grossly misfolded proteins. However, there was a complete absence of specific binding of 125I-CXCL12 at tracer concentrations (50 pM) to C26S/C34S- or C34S/C287S-expressing cells (Fig. 2D), whereas C21S/C26S bound the tracer to similar extent as wild type ACKR3. Specific binding of 125I-CXCL11 could not be detected at tracer concentration, even to wild type ACKR3, probably because of too weak affinity. Therefore, binding competitions throughout this study were conducted using 125I-CXCL12 as a tracer, thus assessing CXCL11 binding by heterologous competition. Trace amounts of endogenous
ACKR3 induced by transfection with empty plasmid or endogenous CXCR4 did not lead to significant specific $^{125}$I-CXCL12 binding (Fig. 2 E). Unlabeled CXCL12 competed with the tracer on mutant C21S/C26S with the same IC$_{50}$ as on wild type ACKR3 (Table 1). Accordingly, arrestin recruitment of C21S/C26S in response to either ligand was identical to wild type ACKR3 (Fig. 2, F and G).

These results suggest that Cys-34 is engaged in a disulfide bridge with Cys-287 and in formation of the ECL4 pseudo-loop, and not Cys-21, as was previously speculated on the basis of a preceding conserved proline (21). Moreover, we observed no role in binding or arrestin recruitment for a potential disulfide bond formed between Cys-21 and Cys-26.

**Point Mutations in the ACKR3 N Terminus**—For canonical chemokine receptors, the N terminus is an essential part of chemokine recognition site 1 (CRS1) (21). CRS1 interactions often involve acidic receptor residues and can involve post-translational receptor modifications. Specifically, CXCR4 is modified by sulfate at tyrosine Tyr-21 and to a lesser degree at Tyr-7 and Tyr-12 (23) but also by N-linked or O-linked glycosylations at asparagine 11 and serine 18 (23–25). We addressed the potential role of ACKR3 N-terminal acidic residues by creating substitution mutants D2N, D7N, E10Q, D16N, D25N, and D30N. The sulfation candidate tyrosine 8 was mutated as well as Tyr-45 (mutants Y8F and Y45F), and the potential N-glycosylation (N13A, N22A, and N39A) and O-glycosylation (S23A/S24A) sites (Fig. 1).

As shown in Fig. 3A, cells transfected with most mutants were stained to a similar extent as cells transfected with wild type ACKR3. To account for possible involvement of mutated residues in the conformational epitope of the monoclonal antibody 358426, two different antibodies were used (358426 and 11G8). Indeed, mutants D7N and Y8F did not stain with antibody 358426, and mutant D16N did not stain with antibody 11G8. Because they were fully recognized by the respective other antibody, we concluded that the substitutions affected the respective antibody epitopes rather than mutant receptor surface expression.

Trace radioligand $^{125}$I-CXCL12 bound to mutant receptors to similar extent as to wild type ACKR3, suggesting no major impact of any of these mutants on CXCL12 binding (Fig. 3B). This was confirmed by dose-response competition experi-
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Table 1: Binding and activation data of N-terminal CXCR7/ACKR3 mutants

| Receptor/mutant | Residue | \(^{125}\text{I-CXCL12 tracer binding (\% of ACKR3)}\) |
|-----------------|---------|--------------------------------------------------|
|                 | IC\(_{50}\) | log IC\(_{50}\) ± S.D. | EC\(_{50}\) | log EC\(_{50}\) ± S.D. |
| ACKR3           | 100     | -8.9 ± 0.2 | 4.5 | -8.3 ± 0.2 |
| C21S/C26S       | 83 ± 16 | -8.4 ± 0.2 | 5.5 | -8.3 ± 0.2 |
| D2N             | 90 ± 12 | -8.9 ± 0.2 | 4.0 | -8.4 ± 0.2 |
| D7N             | 97 ± 13 | -9.0 ± 0.2 | 3.3 | -8.5 ± 0.2 |
| E10Q            | 94 ± 18 | -8.8 ± 0.2 | 3.3 | -8.5 ± 0.2 |
| D16N            | 101 ± 22 | -8.7 ± 0.3 | 4.7 | -8.3 ± 0.1 |
| D25N            | 87 ± 19 | -9.0 ± 0.1 | 3.1 | -8.5 ± 0.2 |
| D30N            | 83 ± 24 | -9.0 ± 0.2 | 3.5 | -8.5 ± 0.3 |
| N13A            | 85 ± 21 | -9.2 ± 0.2 | 3.8 | -8.4 ± 0.2 |
| N22A            | 87 ± 23 | -9.8 ± 0.3 | 3.3 | -8.5 ± 0.1 |
| N39A            | 109 ± 8  | -8.6 ± 0.4 | 7.4 | -8.1 ± 0.1 |
| S23A/S24A       | 103 ± 13 | -9.0 ± 0.2 | 2.7 | -8.6 ± 0.3 |
| Y8F             | 100 ± 22 | -8.9 ± 0.2 | 3.2 | -8.5 ± 0.2 |
| Y45F            | 104 ± 30 | -8.9 ± 0.3 | 2.0 | -8.7 ± 0.2 |

\( \alpha \) p < 0.01, \( \beta \) p < 0.0001, \( \gamma \) p < 0.001, \( \delta \) p < 0.05.

As shown in Fig. 5A, most mutants were well detected at the cell surface by at least one of the monoclonal antibodies, with the exception of mutant K118A, which stained less with both antibodies. However, mutant E114Q (ECL1) was relatively less detected by antibody 11G8, whereas E207Q (ECL2b) was inversely less detected with 358426, again suggesting impairment of the respective conformational antibody epitopes. 125I-CXCL12 tracer specifically bound to most mutants at similar levels as wild type CXCR7. Notable exceptions were mutants D179N and D275N but also for mutants E114Q in ECL1 (for both chemokines), D275N and E207Q in ECL2b, which are thus crucial for CXCL12 binding (Fig. 5B).

Tracer binding to K118A was also reduced but remained proportional to the reduced antibody staining, suggesting reduced cell surface expression of this mutant. Binding competition dose-response experiments revealed no major effects of any of the other substitutions on CXCL12 IC\(_{50}\) (Fig. 5C, Table 2). Minor, but significant, effects on CXCL11 IC\(_{50}\) were found for the ECL2 mutants K184A and E202Q (Fig. 5C). Of note, we could not test potential contributions of D179N, K206D, and D275N to CXCL11 binding because of the lack of tracer binding. Finally, the mutation K118A significantly increased competition of CXCL11 with the tracer.

We then tested BRET arrestin recruitment to the mutants. Although none of the substitutions led to a significant decrease of the maximal arrestin recruitment signal observed with either chemokine (with the notable exception of K118A, see below), several substitutions increased the EC\(_{50}\) of the recruitment responses, reaching significance in several instances (Fig. 5D). This was the case, as expected, for mutants D179N and K206D, and D275N but also for mutants E114Q in ECL1 (for both chemokines) and ECL2 mutant R197A (significant only for CXCL12). As shown above, substitution of K118A, a basic residue in ECL1 just above TM3 and highly conserved among chemokine receptors, has reduced surface expression and binds a correspondingly reduced amount of tracer. Indeed, although the CXCL12 competition IC\(_{50}\) is unaltered, CXCL11 competition is increased with decreased IC\(_{50}\) on K118A (Fig. 6). Arrestin recruitment assays revealed that K118A yielded moderately...
increased baseline BRET, suggesting constitutive arrestin recruitment. However, the mutant remained largely unresponsive to ligand challenge (Fig. 6).

When chemokine scavenging by selected mutants was tested, we found a striking difference between CXCL12 and CXCL11. Although none of the substitutions affected scavenging of CXCL12, mutants D179N, K206D, and D275N strongly reduced CXCL11 scavenging (Fig. 7A and C). The lack of effect of reduced receptor affinity of CXCL12 to some mutants was unexpected. We reasoned that this might relate to receptor expression levels, although the impact of CXCL11 affinity on degradation was readily detected at standard experimental conditions (1 μg of plasmid DNA per well). Indeed, a 100-fold reduction of the amount of transfected DNA (10 ng/well) was required to observe reduced CXCL12 degradation by mutants D179N and D275N (but not K206D) (Fig. 7B), whereas 10-fold reduction was without effect. This illustrates the high efficiency of CXCL12 degradation by minute quantities of ACKR3 compared with CXCL11, which required higher expression levels, probably due to differences in affinity. Of note, this difference may be biologically relevant.

Residues Ser^2.63 and Gln^7.39—Sequence comparison of ACKR3 with CXCR4 revealed that residues Asp^2.63 and Glu^7.39 of CXCR4, which directly contact the CXCL12 N-terminal lysine CRS2 (8), are not conserved in ACKR3, which has Ser^2.63 and Gln^7.39 instead. To test their implication in ACKR3 binding, activation, and scavenging, we created and tested substitutions S103D^2.63 and Q301E^7.39, restoring the charges found in CXCR4. Although S103D^2.63 was well expressed at the cell surface, it bound significantly less radiola-
Some of the residues identified correspond to those identified by reports on the CXCL11 interaction with its canonical receptor CXCR3. CXCR3 D186N4.60 was found important for CXCL11 binding (30), as were Asp-2786.58 and ECL2(C+9) (29), although not by all studies (30, 31). Moreover, CXCR3 S304A7.39 was reported important, as well as D116N2.63 (Ref. 29 but not Refs. 30 and 31). However, other CXCL11-CXCR3 key residues played no role in ACKR3 (CXCR3 Glu-2937.28, Ref. (29)) or are not conserved in ACKR3 (CXCR3 Arg-216 in ECL2, Asp-2826.62, Refs. 29 and 30). Overall, despite some discrepancies among the CXCR3 studies, CXCL11-ACKR3 and CXCL11-CXCR3 interactions appear similar, with some subtle differences.

Comparison of the CXCL12-ACKR3 Interaction with CXCL12-CXCR4—The surprising apparent absence of significant effects of single N-terminal substitutions in the CXCL12-ACKR3 interaction is in stark contrast to the CXCL12-CXCR4 interaction, where the N terminus clearly contributes to primary binding (12, 20, 23, 27, 28). It appears unlikely that our results simply reflect methodological issues, as we readily identified the effects of the same substitutions on CXCL11-ACKR3 binding. Rather, the most straightforward explanation for these observations is that initial CXCL12 binding does not rely on the ACKR3 N-terminal but that initial CXCL12 binding (CRS1) mainly relies on ECL residues (Asp-1794.60, Asp-2756.58, and Lys-2067.28), and proteins are not conserved in ACKR3 (CXCR3 Arg-216 in ECL2, Asp-2826.62, Refs. 29 and 30). Overall, despite some discrepancies among the CXCR3 studies, CXCL11-ACKR3 and CXCL11-CXCR3 interactions appear similar, with some subtle differences.

Discussion

This work set out to delineate the modes of interactions between the scavenger receptor ACKR3, also known as CXCR7, and its chemokine ligands CXCL11 and CXCL12 using a large collection of receptor substitution mutants in the first comprehensive mutational analysis of chemokine interaction with an atypical chemokine receptor. Comparison of ACKR3 binding and arrestin recruitment revealed a number of differences between CXCL11 and CXCL12. Although no singular contribution of the ACKR3 N-terminal residues to CXCL12 binding was identified, N-terminal acidic residues clearly contribute to CXCL11 binding of ACKR3. Key residues for CXCL12 binding are in the extracellular loops (Asp-1794.60 and Asp-2756.58 and to a lesser degree Lys-2067.28) (position C+10). The same residues probably also contribute to CXCL11 binding, although this could not be directly tested because of the lack of tracer binding. Other residues specifically contributing to CXCL11 binding were Lys-184 and Glu-202 in ECL2. The key binding residues Asp-179/Lys-206/Asp-275 also affected arrestin recruitment potency of both chemokines. Of note, residues E114Q and R197A were specifically involved in arrestin recruitment potency but had no effect on binding. Finally, CRS2 residues also contributed to binding, although with opposite effect on the two chemokines; mutations S103D or Q301E increased CXCL11 affinity, whereas S103D decreased CXCL12 binding, probably differently affecting the placement of the respective chemokine N termini.
with the atypical receptor ACKR2 (32). Nevertheless, the role of ECL4 for CXCL12 binding appears similar between CXCR4 (8, 19, 20) and ACKR3. Of note, ECL4 is also required for ligand-independent CXCR4 activation (19); whether this is also the case for ACKR3 remains to be studied.

As has been noted earlier (33), the binding pockets of CXCR4 and ACKR3 are quite divergent, and none of the residues corresponding to CXCR4 CRS2, which engage the CXCL12 N-terminal lysine (Asp^{2.63}, Asp-187^{ECL2}, and Glu^{7.39} (8)) is conserved in ACKR3. This implies different accommodation of the CXCL12 N-terminal by ACKR3 compared with CXCR4, with S103D^{2.63} nevertheless affecting CXCL12 binding. The inability of ACKRs to raise G protein-dependent responses has been hypothesized to be caused by variation in the ACKR3 intracellular DRY motif (34). However, insertion of the corresponding region from CXCR4 into ACKR3 did not restore G protein signaling (4, 35). It may be speculated that differences in the CRS2-CXCL12 interaction between CXCR4 and ACKR3 also contribute to the absence of G protein signaling in ACKR3.

**ACKR3 Chemokine Scavenging**—ACKR3 chemokine scavenging was highly efficient and reached 25–40% that of the...
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**TABLE 2**

Binding and activation data of CXCR7/ACKR3 mutants

One-way ANOVA with Dunnett’s post test is shown in the footnotes. ND, not determined. Residues are given using Ballesteros-Weinstein numbering (22). Values in boldface type are significantly different from the wild type.

| Receptor/ mutant Domain Residue | 125I-CXCL12 tracer binding (% of ACKR3) | IC50 SD | log IC50 ± S.D. | EC50 SD | log EC50 ± S.D. | IC50 SD | log IC50 ± S.D. | EC50 SD | log EC50 ± S.D. |
|---------------------------------|------------------------------------------|---------|-----------------|---------|-----------------|---------|-----------------|---------|-----------------|
| ACKR3                           | 100                                      | 3       | −8.5 ± 0.3      | 4.6     | −8.3 ± 0.2      | 16      | −7.8 ± 0.3      | 8       | −8.1 ± 0.3      |
| K40A ECL4                        | 111 ± 34                                 | 4       | −8.4 ± 0.3      | 4.2     | −8.3 ± 0.2      | 23      | −7.7 ± 0.5      | 6       | −8.2 ± 0.2      |
| N14Q ECL1                        | 89 ± 21                                  | 2.5     | −8.6 ± 0.3      | 35*     | −7.5 ± 0.4*     | 50      | −7.3 ± 0.3      | 59*     | −7.2 ± 0.2*     |
| K118A ECL1                       | 3.26                                     | 4       | −8.4 ± 0.4      | ND      | ND              | 2.1*    | −8.7 ± 0.3*     | ND      | ND              |
| D179N ECL2a                      | 15 ± 12 a                                | ND      | ND              | ND      | ND              | ND      | ND              | ND      | ND              |
| K184A ECL2a                      | 86 ± 3                                   | 4       | −8.4 ± 0.1      | 6       | −8.2 ± 0.5      | 63*     | −7.2 ± 0.2*     | 7       | −8.1 ± 0.5      |
| E193Q ECL2a                      | 99 ± 19                                  | 4       | −8.4 ± 0.1      | 13      | −7.9 ± 0.9      | 50      | −7.3 ± 0.1      | 9       | −8.0 ± 0.4      |
| R197 A ECL2b                     | 105 ± 28                                 | 4.1     | −8.4 ± 0.2      | 18*     | −7.8 ± 0.1*     | 10      | −8.0 ± 0.3      | 21      | −7.7 ± 0.2      |
| E202Q ECL2b                      | 94 ± 23                                  | 5       | −8.3 ± 0.3      | 18      | −7.8 ± 0.4      | 80*     | −7.2 ± 0.2*     | 11      | −8.0 ± 0.4      |
| K206D ECL2b                      | 46 ± 17                                  | ND      | ND              | 26*     | −7.6 ± 0.2*     | ND      | ND              | 40*     | −7.4 ± 0.2*     |
| E207Q ECL2b                      | 123 ± 42                                 | 6       | −8.2 ± 0.2      | 8       | −8.1 ± 0.2      | 50      | −7.3 ± 0.3      | 16      | −7.8 ± 0.1      |
| E213Q ECL2b                      | 5.39                                     | 146 ± 77| 6       | −8.2 ± 0.3      | 4.1     | −8.4 ± 0.1      | 10      | −8.0 ± 0.4      | 6       | −8.2 ± 0.1      |
| D275N ECL3                       | 6.58                                     | 18 ± 10b| ND              | ND      | 34*            | ND      | ND              | 75*     | −7.1 ± 0.4*     |
| R288A ECL3                       | 67 ± 10                                  | 3       | −8.6 ± 0.3      | 8       | −8.1 ± 0.1      | 16      | −7.8 ± 0.4      | 8       | −8.1 ± 0.3      |
| E290Q ECL3                       | 7.28                                     | 108 ± 48| 8       | −8.1 ± 0.3      | 2.5     | −8.6 ± 0.4      | 10      | −8.0 ± 0.3      | 5       | −8.3 ± 0.1      |
| S103D Minor pocket               | 2.63                                     | 44 ± 3  | 9*             | −8.0 ± 0.2b | 5.8     | −8.2 ± 0.1      | 1.4*    | −8.8 ± 0.3*     | 13      | −7.9 ± 0.1      |
| Q301E Minor pocket               | 7.39                                     | 54 ± 2  | 2.5            | −8.6 ± 0.2 | ND      | ND              | 3.3*    | −8.5 ± 0.2b     | ND      | ND              |

* p < 0.0001.

**FIGURE 6.** Binding competition and arrestin recruitment to mutant K118A3-26. Binding competition curves (left panels) of 125I-CXCL12 by unlabeled CXCL12 (upper left panel; red) or CXCL11 (lower left panel; blue) to wild type ACKR3 (circles) or mutant K118A (colored symbols). Dose-response curves of arrestin recruitment (right panels) to wild type ACKR3 (circles) or mutant K118A (colored symbols) by CXCL12 (upper right panel; red) or CXCL11 (lower right panel; blue).

input at 50 pm within 3 h. Of note scavenging occurred at concentrations that did not permit detection of binding of a significant fraction of radiolabeled tracer at equilibrium in some combinations (125I-CXCL11 with wild type ACKR3 or 125I-CXCL12 with mutants D179N and D275N), illustrating its cumulative nature. Expression of very low receptor levels was required to observe reduced scavenging resulting from reduced CXCL12 affinity. This suggests that CXCL12 degradation pathways are saturable and that CXCL12 binding largely exceeds scavenging at higher ACKR3 expression levels. The high efficiency of CXCL12 scavenging thus appears to be driven by the rapid rate of degradation of a relatively small fraction of the bound chemokine at high receptor expression levels. For CXCL11, our data are in line with the conclusion that reduced scavenging reflected reduced CXCL11 affinity. These observations highlight the potential biological relevance of the different CXCL12 and CXCR11 affinities for ACKR3: cells expressing low endogenous receptor levels would efficiently scavenge CXCL12 but not CXCL11.

Intriguingly, scavenging data obtained with CRS2 mutants suggested independence between scavenging and arrestin recruitment. Indeed, efficient scavenging by Q301E occurred in the absence of significant arrestin recruitment. Complementing this observation, the reduced scavenging of CXCL11 by S103D occurred despite increased binding and intact arrestin recruitment. This suggests that arrestin recruitment may be
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Cell Culture and Transfection—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium, 1% penicillin-streptomycin (Invitrogen), and 10% fetal bovine serum (Invitrogen). Transient transfections were performed in six-well plates using the polyethyleneimine method; if not stated otherwise, 1 µg of plasmid DNA was used per well. The total amount of transfected DNA was kept constant at 2 µg/well for all transfections by adding empty vector.

Flow Cytometry—Receptor mutant surface expression was assessed by flow cytometry. Cells were stained for 30 min on ice followed by washing and analysis using a FACSCalibur (BD Biosciences) cytometer. Overall, mutant protein expression was quantified by the C-terminal YFP moiety and did not differ significantly between mutants. Data analysis was performed using FlowJo software.

Radioligand Binding Assays—HEK293 cells were transfected with ACKR3-YFP or the indicated mutants in six-well plates. After overnight incubation, cells were washed with PBS and incubated for 5 min at 37 °C with 100 µM phenylarsine oxide followed by washing. Cells were collected, washed, and incubated with 50 pm 125I-CXCL12 tracer in binding buffer (50 mM HEPES, pH 7.4, 5 mM MgCl2, 1 mM CaCl2, 0.2% BSA) in the presence or absence of the indicated concentrations of unlabeled CXCL12 or CXCL11 for 30 min at 37 °C. After removal of unbound tracer, bound radiolabel was quantified in a gamma counter. Mutant bindings were run with wild type ACKR3 controls within the same experiment.

Arrestin Recruitment—β-Arrestin-2 recruitment to ACKR3 mutants was monitored as previously described using a BRET-based assay (45). Briefly, HEK293E cells were transiently transfected with receptor-YFP fusion and β-arrestin-2-RLuc constructs. Transfected cells were seeded onto poly-D-lysine treated 96-well plates. At 48 h post-transfection culture medium was replaced with PBS supplemented with 0.5 mM MgCl2 and 0.1% BSA. Cells expressing -YFP and -RLuc fusions were mixed in a ratio resulting in BRETMAX were stimulated with chemokine ligands for 5 min at 37 °C followed by the addition of coelenterazine h to 5 µM (Nanolight Technology) for 10 min. Fluorescence and luminescence readings were collected using a Mithras LB940 plate reader (Berthold Technologies, Bad Wildbad, Germany). Experiments were performed with cells that remained attached to the plastic surface of assay plates. BRETnet is calculated by subtracting

neither sufficient nor required for scavenging and will deserve further study. Indeed, although arrestin recruitment by ACKR2 and ACKR4 is described, its contribution to ACKR2 chemokine scavenging is a matter of debate (for review, see Ref. 36). It is possible that arrestin influences ACKR2 scavenging indirectly via modulation of receptor trafficking (37–40). For ACKR3, a report from the zebrafish model that arrestin regulates intracellular receptor transport rather than endocytosis points into the same direction (41). Finally, arrestin double-knock-out murine embryonic fibroblasts accumulated significantly less chemokine than their wild type counterparts, but the accumulation was still above background levels (42); this study, however, did not directly assess degradation. Thus, the exact arrestin contribution to ACKR3 scavenging remains to be clarified as well as the mechanism by which S103D affects scavenging.

ACKR3 K118A Constitutively Recruits Arrestin—Unexpectedly, we found that the K118A substitution, at the top of TM3, remained largely unresponsive to stimulation despite the increased affinity for CXCL11 while showing increased baseline association with arrestin. Although being beyond the scope of the present study, this may be suggestive of a conserved determinant for arrestin recruitment in chemokine receptors. Indeed, Lys-3.26 is highly conserved in chemokine receptors, and corresponding mutants in CXCR2 and CCR7 were found unable to signal via G proteins (43, 44). It is possible, although not reported, that these mutants constitutively recruit arrestin, i.e. are constitutively desensitized. Along these lines, the involvement of Glu-114 adjacent to Lys-118 in the potency of arrestin recruitment, but not in chemokine binding, further supports a role of TM3 in ACKR3 activation.

Experimental Procedures

Materials—Recombinant chemokines were kind gifts from Amanda Nevis and Brian Volkman or from PeproTech Inc. (Rocky Hill, NJ); radiolabeled chemokines were from (PerkinElmer Life Sciences). Coelenterazine h (for BRET1 experiments) was from NanoLight Technology (Pinetop, AZ). The anti-CXCR7 monoclonal antibodies (clones 358462 and 11G8) directly coupled to phycoerythrin or allophycocyanin were from R&D Systems (Minneapolis, MN).

Generation of ACKR3 Mutants—Receptor mutants were generated by PCR in ACKR3-YFP, which has been described and analyzed before (3); the sequences of the resulting mutants were checked by sequencing.

![FIGURE7: Chemokine scavenging by mutants of charged residues in the receptor extracellular loops. Shown is relative degradation of 125I-CXCL12 (A and B) or of 125I-CXCL11 (C) by cells expressing wild type or mutant ACKR3. Cells have been transfected with 1 µg DNA/well (A and C) or 10 ng/well (B) (6-well plates).](image)
FIGURE 8. Expression, chemokine binding, arrestin recruitment, and scavenging to mutants S103D$^{2.63}$ and Q301E$^{7.39}$. A, relative surface expression of mutants compared with wild type ACKR3, detected by flow cytometry using monoclonal antibodies 11G8 (gray bars) or 358426 (black bars). B, specific binding of $^{125}$I-CXCL12 to cells expressing wild type ACKR3 or mutants. C, radioligand competition (left panels) and arrestin recruitment (right panels) to mutant S103D; upper row (red), CXCL12; lower row (blue), CXCL11. D, radioligand competition (left panels) and arrestin recruitment (right panels) to mutant Q301E; upper row (red), CXCL12; lower row (blue), CXCL11. The wild type ACKR3 BRET control is the same as in Fig. 6 (mutant K118A). E, chemokine degradation of CXCL12 (upper panel) or CXCL11 (lower panel). Values are from at least three independent experiments conducted in duplicate; ANOVA with Dunnett’s post test: ***, $p < 0.001$; ****, $p < 0.0001$. 

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the background BRET ratio observed in cells expressing β-arrestin-2-Rluc alone.

**Chemokine Scavenging**—To determine receptor-mediated chemokine degradation, we essentially followed a protocol described in Hoffmann et al. (35). Cells were transfected in 6-well plates with 1 μg of coding plasmid. In a subset of experiments, only 10 ng/well were transfected. The total amount of transfected DNA was complemented with empty vector to 2 μg/well. 24 h post transfection, cells were transferred to 24-well plates. For degradation, medium was replaced with 200 μl of 125I-labeled chemokine in HEPES-buffered serum-free DMEM containing 1% BSA for 3 h at 37 °C. Supernatants were collected, and cells were washed in glycine buffer, pH 2.8, to remove surface-attached chemokine; washing buffer was pooled with supernatant and subjected to 12.5% TCA precipitation. Precipitate represents degraded chemokine, whereas the supernate contains chemokine degradation products; radioactivity that remained associated with the cells was also counted. Typically, 20–40% of the chemokine input were degraded by CXCR7-transfected cells under these conditions.

**Data Analysis**—Data analysis was performed using GraphPad Prism6 software (GraphPad Software Inc., San Diego, CA).

**Author Contributions**—B. B., M. G., and N. H. designed the study. B. B. and M. G. created the ACKR3 mutants. B. B., M. G., D. R., and G. St.-O. performed the experiments. B. B., M. G., D. R., G. St.-O., and N. H. analyzed the data. N. H. wrote the manuscript with input from the other authors. All authors approved the final version of the manuscript.

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**Note Added in Proof**—In the version of this article that was published as a Paper in Press on November 14, 2016, the bars in Fig. 7, A and C, were mislabeled. This error has now been corrected and does not affect the results or conclusions of this work.

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