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Review

Function, diversity and therapeutic potential of the N-terminal domain of human chemokine receptors

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ABSTRACT

Chemokines and their receptors play fundamental roles in many physiological and pathological processes such as leukocyte trafficking, inflammation, cancer and HIV-1 infection. Chemokine-receptor interactions are particularly intricate and therefore require precise orchestration. The flexible N-terminal domain of human chemokine receptors has regularly been demonstrated to hold a crucial role in the initial recognition and selective binding of the receptor ligands. The length and the amino acid sequences of the N-termini vary considerably among different receptors but they all show a high content of negatively charged residues and are subject to post-translational modifications such as O-sulfation and N- or O-glycosylation. In addition, a conserved cysteine that is most likely engaged in a receptor-stabilizing disulfide bond delimits two functionally distinct parts in the N-terminus, characterized by specific molecular signatures. Structural analyses have shown that the N-terminus of chemokine receptors recognizes a groove on the chemokine surface and that this interaction is stabilized by high-affinity binding to a conserved sulfotyrosine-binding pocket. Altogether, these data provide new insights on the chemokine-receptor molecular interplay and identify the receptor N-terminus-binding site as a new target for the development of therapeutic molecules. This review presents and discusses the diversity and function of human chemokine receptor N-terminal domains and provides a comprehensive annotated inventory of their sequences, laying special emphasis on the presence of post-translational modifications and functional features. Finally, it identifies new molecular signatures and proposes a computational model for the positioning and the conformation of the CXCR4 N-terminus grafted on the first chemokine receptor X-ray structure.

Contents

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1. Introduction

Chemokine receptors are rhodopsin-like G protein-coupled receptors (GPCRs) displaying a structure typical of this family that consists of seven hydrophobic membrane-spanning α-helices separated by alternating hydrophilic extracellular (ECL) and intracellular (ICL) loops. The N-terminus of the receptor is situated on the outside of the cell and participates in ligand binding whereas the C-terminal tail is located on the intracellular side. Upon ligand binding, chemokine receptors activate intracellular heterotrimeric G proteins triggering downstream signaling pathways that result in a variety of cellular responses. Additionally, non-signaling receptors such as decoy receptors were shown to control the cellular response to chemokines by sequestration and modulation of their local concentration [1,2].

Chemokines and their receptors regulate vital cellular mechanisms including migration, adhesion as well as growth and survival [3,4]. Chemokines control processes such as embryonic development, angiogenesis and hematopoiesis but can be also released under stress. These inducible chemokines play crucial roles in adaptive and innate immune response, wound healing and organ repair by attracting effector cells to the site of infection or injury [5–7]. Many chemokines are also involved in pathological processes including inflammatory and autoimmune diseases as well as cancer [4,8–10]. In addition, some pathogens interfere with the host chemokine/chemokine receptor network to promote their own survival by either encoding chemokine receptors/chemokines or co-opting chemokine receptors for host cell entry.

Chemokines are generally believed to interact with their cognate receptors according to a two-step model [11,12]. The initial step corresponds to the anchoring of the chemokine to the N-terminus of the receptor and is followed by the binding of the flexible chemokine N-terminus to the extracellular loops and the transmembrane segments of the receptor. Numerous studies illustrate the great importance of the extracellular parts, and in particular the receptor N-terminus, in discriminating between the various chemokine ligands.

Despite their low level of sequence identity, all chemokines display a common monomeric structure consisting of a flexible N-terminus followed by an N-loop, three anti-parallel β-strands and a C-terminal α-helix [13,14]. The N-terminal domain contains one or two cysteines implicated in structure-stabilizing disulfide bonds. Based on the positioning of these cysteines, chemokines are divided into four groups: C, CC, CXC and CX_3C [15]. Accordingly, chemokine receptors are named CR, CCR, CXCR or CX_3CR. The chemokine-receptor network is very complex and a given chemokine may bind to several receptors, while a chemokine receptor usually has multiple ligands. To date, 50 chemokines and 20 receptors have been identified in humans.

The N-terminal domains vary considerably in length between different chemokine receptors, also within subfamilies. They do however display a number of common features, including high content of negatively charged residues, tyrosine sulfation motifs and N-glycosylation sites. In addition, a highly conserved disulfide bond links the N-terminus and the third extracellular loop. Some of these characteristics of the chemokine receptor N-terminal domain have been shown to strongly influence ligand binding as well as the cellular responses.

Given the implication of the chemokine network in many pathologies, a better understanding of the mechanisms driving ligand binding to chemokine receptors is essential for the development of highly specific therapeutic molecules targeting either the receptors or more recently the chemokines. To accurately comprehend these interactions, three-dimensional structures of chemokine receptors would be needed. Yet, their resolution has proven particularly arduous mainly due to the difficulties in purifying and crystallizing these proteins. To date, CXCR4 is the only chemokine receptor for which the three-dimensional structure has been resolved [16]. The spatial arrangement of its N-terminus, however, could not be determined. Nevertheless, multiple alternative approaches have been used to investigate the interactions of the N-terminus of chemokine receptors with their ligands. Chimeric, mutated or truncated receptors have long been widely exploited [17–23]. In parallel, soluble synthetic peptides derived from the N-termini of chemokine receptors have been used as models for the binding of ligands to full-length receptors [24,25]. In particular, the NMR studies of interactions between the receptor N-terminus-derived peptides and chemokines have provided substantial functional and structural information in this regard [26–33]. Additionally, grafting of the N-terminus together with another extracellular loop on the B1 domain of protein G soluble scaffolds allowed examining ligand interactions in contexts that are more reminiscent of native receptors [34,35]. Other approaches aimed to investigate the N-terminus of chemokine receptors in more membrane-like environments such as micelles or phospholipid bilayers as well as in fusion with membrane proteins [24,36,37]. Thanks to this constantly growing arsenal of methods and increasingly powerful tools, remarkable progress has been made towards the elucidation of ligand interactions with chemokine receptors.

The present review gives an outline of the information currently available on the diversity and function of human chemokine receptor N-terminal domains. Additionally, it provides a comprehensive annotated inventory of the chemokine receptor N-terminal sequences, laying special emphasis on the presence of post-translational modifications, sequence signatures and functional features. In this review, chemokines and chemokine receptors will be referred to by their systematic nomenclature.

2. Sequence diversity of chemokine receptor N-terminal domains

2.1. Length and molecular signatures

Chemokine receptors present relatively short N-terminal domains ranging from 26 (CX3CR1) to 65 (DARC) amino acids compared to the N-terminal domains of up to 600 amino acids in other GPCRs. Notably, in all chemokine receptors except for CXCR6, the N-terminal domains bear a conserved cysteine residue in their second moiety. This cysteine is likely to be engaged in a disulfide bridge with the third extracellular loop of the receptor (ECL3) and delimits two functional parts characterized by different sequence features: the M–C part including residues from the N-terminal methionine (M) to the cysteine (C) and the C–TM part including the residues from the cysteine to the first transmembrane segment (TM). While the M–C parts are in general described as very flexible, the C–TM parts link the TM1 and TM7 through a disulfide bridge forming a pseudo-loop at the surface of the receptor (Fig. 1A and B) (see Section 2.2.1).

In all chemokine receptor families, the M–C parts show variable length, low sequence identity, overall negative charges and contain multiple tyrosine and asparagine residues that are post-translationally modified. The size of the M–C parts varies from 21 to 51 amino acids and is not fixed within a family. Moreover, there seems to be no correlation between their length and the selectivity of the receptor. Low identity observed in the M–C parts supports their implication in ligand selectivity. Except for their overall negative charges, the presence of sulfotyrosines (see Section 2.2.2) and of potential N-glycosylation sites (see Section 2.2.3), no specific signatures seem to be present and conserved in the M–C parts.

The C–TM parts are shorter (5–20 residues), display variable net charges within the CC and decoy receptor families and are neutral

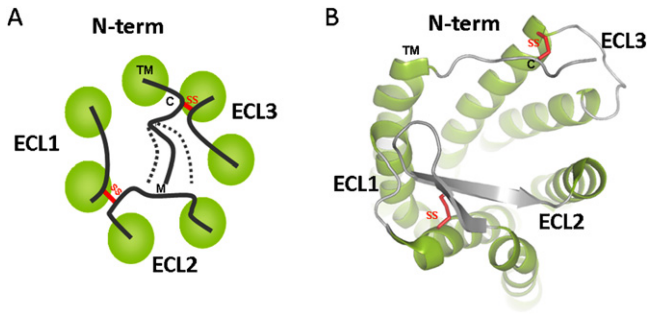

Fig. 1. Top-down representation of chemokine receptor surface (A) Schematic representation. The seven transmembrane (TM) segments are represented as green circles. The two disulfide bridges connecting the N-terminus to ECL3 and ECL1 to ECL2 are colored in red and indicated by SS. The N-terminal part of the N-terminus (M–C part) is flexible and unstructured in the absence of chemokine. The conserved cysteine (C) forms a disulfide bridge (red) with ECL3 linking TM1 and TM7. (B) X-ray structure (PDB ID: 3OEO), clear density was only observed for C–TM part, starting at residue P28.

or negative in CXC receptors, contain no sulfated tyrosines or glycosylation sites. The only exception is CXCR7 which bears a putative N-glycosylation site three residues before the predicted TM1. Despite the apparent low identity and size variation, we identified new signatures conserved within the C–TM parts of different chemokine receptor families. Receptors CCR1, CCR2, CCR3, CCR4, CCR5 and CCR9 present longer C–TM parts characterized by a length of 18 residues and the conservation of a scattered motif K-X_3-K/R-X_7-PPLYS/W separated from the cysteine by one residue. In contrast, CCR6, CCR7, CCR8, CCR10, all CXC receptors, D6 and CCX-CKR display shorter C–TM parts (10 or 11 residues) characterized by the conservation of a negative charge ($E/D_{+3/+4}$) 3 or 4 residues after the cysteine and a positive charge ($K/R_{+9/+10}$) preceding the TM1. In other receptors such as XCR1, CX3CR1 and DARC, no particularities or features allowing their classification in one of these two families were found.

2.2. Post-translational modifications

2.2.1. Disulfide bridge

Chemokine receptors typically bear one cysteine residue in each extracellular domain. While the two cysteines present in ECL1 and ECL2 are a characteristic of nearly all rhodopsin-like GPCRs and form a structurally and functionally critical disulfide bridge [38], the other two cysteines situated in the N-terminus and ECL3 are a particularity of chemokine receptors and their role is not as well-established. Indeed, although the conservation of these residues as a pair in all chemokine receptors except CXCR6 indicates their importance for receptor biology, most likely through disulfide bridge formation, somewhat diverging results have been reported in the literature.

In an early study, it was shown that CXCR1 treatment with diamide, a bifuctional sulfhydryl reagent that oxidizes thiol groups and leads to formation of disulfide bonds, resulted in a functionally inactive receptor and reduced CXCL8 (IL-8) binding [39]. Approaches using alkylating agents also pointed to the existence of free thiols in the extracellular domains of CXCR1 [39] and in the N-terminus and ECL3 of CCR6 [40].

The results from numerous other studies however strongly put forward the role of the N-terminus–ECL3 cysteine pair in receptor functions. Indeed, the mutation of one or both cysteines from the N-terminus and ECL3 decreased chemokine CCL2 (MCP1), CCL5 (RANTES), CXCL12 (SDF1), CXCL8 (IL-8) binding and chemokine-induced signaling in CCR2, CCR5, CXCR4, CXCR1 and CXCR2, respectively [37,41–44] as well as DARC interactions with chemokines [45]. However, it was shown for CCR2 that the cysteine present in the N-terminus is not directly involved in the interactions with CCL2 [37]. Similarly, a study with a constitutively active N119S-CXCR4 demonstrated that mutants carrying a salt bridge C28R/C274E or an aromatic pair C28F/C274F retained some of the activity of the receptor. It was further proposed that the N-terminus–ECL3 cysteine pair may stabilize the active state of CXCR4 [46]. Interestingly, the mutation of this cysteine pair in the two major HIV-1 co-receptors, CXCR4 and CCR5, seems to have little effect on the gp120 binding to the receptors [41,47].

The most compelling and direct evidence of the existence of a disulfide bridge between the N-terminus–ECL3 cysteines arises from one of the recently resolved CXCR4 X-ray structures (PDB ID: 3OEO), in which the N-terminus cysteine at position 28 is linked to ECL3 cysteine at position 274 (Fig. 1B). Interestingly, the helix VII of CXCR4 is two turns longer than in other GPCR structures, allowing the optimal positioning of C274 for this interaction [16]. Moreover, the proline residue directly preceding C28, also present in many other chemokine receptors, may play a crucial role in orienting the N-terminus regions in the vicinity of this cysteine to facilitate the disulfide bridge formation. Long-time molecular dynamics simulation confirmed the continuous presence of the disulfide bridge and suggested that its formation may be favored by the interactions between other residues from the M–C part and ECL3 (unpublished results, see Supplementary data [159,160]). The constraint imposed by the disulfide bond may fashion the chemokine binding pocket and/or be of importance in the correct positioning of the M–C part for chemokine binding or for its further interactions with the receptor (site II) (see Section 3.1). Indeed, in the case of CXCR4, the disulfide bond delocalizes the M–C part from TM1 to the top of TM7 and facing the second extracellular loop, which is proposed to participate in the second step of the binding mechanism. Additionally, by linking the TM1 and TM7 the disulfide bridge may stabilize the three-dimensional structure of chemokine receptors by locking the transmembrane segments in a circular arrangement (Fig. 1). Moreover, since ECL3 connects TM6 and TM7, which are proposed to participate in conformational changes that trigger receptor activation, the N-terminus–ECL3 disulfide bridge was suggested to have a role in the coupling of ligand binding to receptor activation [48].

However, it still remains to be determined whether the disulfide bridge observed in CXCR4 structure is also present in other chemokine receptors and whether it is permanent or dynamic (i.e. formed upon ligand binding). In addition, the impact of the C–TM length differences as well as the presence of signatures among the newly identified families (see Section 2.1) on disulfide bridge formation and on the distance between TM1–TM7 need to be addressed.

2.2.2. Tyrosine sulfation

In addition to their high glutamate and aspartate content, all M–C parts of chemokine receptor N-termini display at least one tyrosine residue that may potentially be post-translationally modified by the addition of a negatively charged sulfate to their hydroxyl groups. The reaction of tyrosine O-sulfation is catalyzed by the Golgi tyrosylprotein sulfotransferases (TPST-1 and TPST-2) and has been shown to play important roles in the regulation of protein–protein interactions of many secreted and transmembrane proteins [49]. Studies with sulfated chemokine receptors however have proven to be difficult mainly due to the lability of the sulfate group. To date, the presence of sulfated tyrosines has been demonstrated for only six human chemokine receptors: CCR2b, CCR5, CXCR3, CXCR4, CX_3CR1 and DARC (see Table 1) [22,50–55]. By means of various approaches including site-directed mutagenesis, treatment with sulfation inhibitors or sulfatases, using both whole receptors and N-terminus-derived peptides, it could be

shown that O-sulfation of their N-termini is critical for high-affinity binding to chemokines as well as for the recognition of the HIV-1 gp120 protein [22,50–55]. Notably, all these chemokine receptors bear a sulfated tyrosine located approximately nine residues before the conserved cysteine. Sequence analysis indicates that this potential sulfation site ($_{p}$sY) is present in almost all the receptors, arguing for the existence of a common sulfotyrosine-dependent ligand binding mode. Although the exact importance of sulfotyrosines within the chemokine receptor N-termini is not fully understood, the distribution of highly polarizable electrons on both the sulfate and the phenyl group make sulfotyrosines perfectly suitable to be accommodated by the positively charged pocket at the surface of the receptor ligands [32,33,56,57]. Indeed, recent structural modeling and NMR measurements suggest that all chemokines harbor a conserved sulfotyrosine-binding pocket, providing a molecular basis for sulfotyrosine conservation observed among chemokine receptors (Fig. 4). The presence of such sulfotyrosine-binding pocket was experimentally determined for four chemokines representative of the different families (XCL1 (Lymphotactin), CCL5 (RANTES), CXCL12 (SDF1) and CX3CL1 (Fractalkine)) [58]. In particular, for CXCL12, structural data demonstrated that the sulfotyrosine-binding pocket is defined by the residues $V18^{CXCL12}$, $R47^{CXCL12}$ and $V49^{CXCL12}$ located near the hydrophobic groove delimited by the N-loop and the third beta-strand (see Section 3.1, Fig. 4B).

However, besides the presence of the conserved potential sulfation site, many chemokine receptors bear multiple tyrosine residues whose post-translational modification is not equally important for ligand recognition [53,59,60]. These sulfotyrosines however also seem to contribute to the high-affinity chemokine binding as illustrated for the CXCR4/CXCL12 interactions, in which sulfation of the receptor tyrosine 7 and 12 in addition to the conserved sY21 increases the affinity for the chemokine over six-fold ($K_D sY_{21} \approx 1.3$ μM versus $K_D sY_{7/12/21} = 0.2$ μM) [61]. However, while the interacting partner of sY12, the $K27^{CXCL12}$, is well identified on the monomeric form of the chemokine, the interaction site of sY7 is not clearly defined and may involve a pocket formed upon chemokine dimerization or interaction with other receptor extracellular domains (Fig. 4B and C). Interestingly, the involvement of $K27^{CXCL12}$ in heparin binding may also suggest that the N-terminus negatively charged residues and in particular sulfotyrosines play a role in heparin displacement prior to receptor binding [29]. Sulfation of tyrosines may additionally favor an extended conformation of the M–C part of the N-terminus. Indeed, we performed long time molecular dynamics for CXCR4, with or without sulfate groups at position 7, 12 and 21 and demonstrated that repulsive interactions caused by the negative charges of the sulfate groups prevent the internal collapse of the N-terminal domain thereby maintaining it in an open conformation accessible for ligand binding (Fig. 2) (see Supplementary data).

The prediction of protein tyrosine sulfation sites remains problematic. Nevertheless, although a specific signature could not be clearly identified among the proteins that are O-sulfated, several consensus features seem to be required for TPSTs activity. (a) Acidic residues are generally found in the vicinity of sulfated tyrosines, whereas basic amino acids abolish the reaction [62,63]. Another possible determinant for TPST activity is (b) a certain degree of flexibility of the peptide chain, as small or turn-inducing residues are often present close to sulfation sites [62,63]. Moreover, (c) disulfide bridges and N-glycosylation sites have been proposed to interfere with tyrosine sulfation [63,64]. Similarly, in silico identification of modified tyrosines remains challenging as sulfation prediction algorithms are often very restrictive. The sulfation prediction tool Sulfinator [65] for instance fails to identify the sulfation of tyrosines 7 and 12 of CXCR4, which has been determined experimentally. Moreover, in vitro sulfation of N-terminus peptides derived from receptors bearing multiple sulfotyrosines was shown to be sequential but also incomplete, giving rise to products displaying a variety of sulfation patterns that differentially affect the binding to chemokines. These observations point to the existence of a mechanism for regulation of ligand affinity/specificity towards sulfated receptors [55]. Moreover, TPST-1 and 2 show different tissue expression patterns and play distinct but overlapping biological roles [64,66–68]. The two isoenzymes also display different kinetic properties and show differences in substrate specificities as well as pH optima, which strengthens their possible involvement in chemokine-receptor network regulation [69,70].

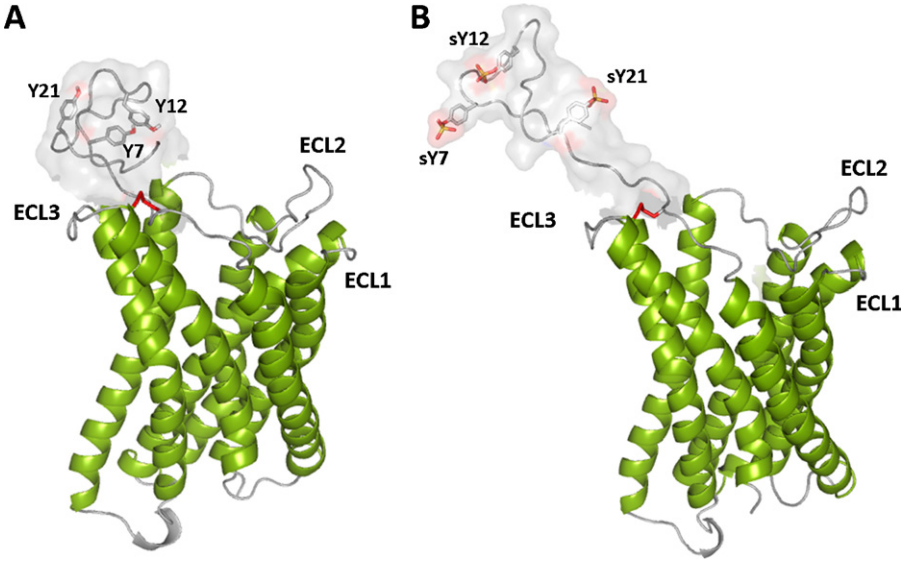

Fig. 2. Impact of tyrosine sulfation on CXCR4 N-terminus conformation. CXCR4 N-terminus with non-sulfated tyrosines (A) and CXCR4 N-terminus with sulfotyrosines (B) derived from the last snapshot (20 ns) of MD simulation carried out with the whole receptor. Receptor helical structures are shown in green; ECLs, ICLs and N-terminus are represented in gray; tyrosine and sulfotyrosine residues are displayed as sticks and the disulfide bond between the N-terminus and ECL3 is colored in red. Guided MD simulations suggest that in absence of sulfate groups the N-terminus tends to collapse forming a condensed structure, whereas tyrosine sulfation creates repulsive interactions promoting the adoption of a an extended structure largely accessible for chemokine binding (see Supplementary data).

Table 1
Sequence, length, charge and post-translational modifications of C chemokine receptor N-terminus.

Receptor	Chemokine[a]	Pathology[b]	Sequence	N-term	M-C	C-TM	$_{p}$sY	N-Glyco
XCR1	XCL1 XCL2	RA	MESSGNPESTTFFYYDLQSQPC-ENQAWVFAT	31 (-4)	22 (-3)	9 (-1)	2	0

2.2.3. Glycosylation

Like other transmembrane receptors, chemokine receptors may also be post-translationally modified by the addition of sugar moieties either to the amide group of asparagine residues (N-glycosylation) or to hydroxyl groups of serine or threonine residues (O-glycosylation). N-glycosylation occurs at the consensus sequence N-X-S/T, where X is any amino acid except proline, while O-glycosylation sites are less well characterized and generally comprise serine/threonine-rich regions. These post-translational modifications occur in the Golgi and are catalyzed by a series of glycosyltransferases and glycosidases that shape the carbohydrate chains. Most chemokine receptors bear one or two putative N-glycosylation sites as well as serine/threonine doublets or triplets within their M–C part. While no specific position or molecular signature can be defined for N-glycosylation, clusters of serine or/and threonine residues are generally found about two to four amino acids on either side of the conserved sulfated tyrosine (see Section 2.2.2). Experimental data on human chemokine receptor glycosylation are however scarce and only five receptors have been shown to carry N-linked (CCR2B, CXCR2, CXCR4 and DARC [50,55,71–73] or O-linked (CCR5 [74]) carbohydrate moieties in their N-terminus (see Tables 1–5). The exact role of N-terminal domain glycosylation remains unclear. Similarly to other GPCRs, glycosylation of the extracellular domains of chemokine receptors has been proposed to increase their flexibility or to directly participate in ligand binding. Indeed, depending on the nature of the carbohydrate chains, glycosylation may provide additional negatively charged moieties for electrostatic interactions with the positively charged chemokines. While the presence of sialyted O-glycans in CCR5 N-terminus (S6 and S7) was shown to be important for high-affinity binding to CCL3 (MIP1α) and CCL4 (MIP1β) [74], N-glycosylation of CXCR2 (N17), CXCR4 (N11) and DARC (N16) appears to have no influence on CXCL7 (NAP2), CXCL12 and CXCL8 interactions, respectively [73,75,76]. CXCR2 glycosylation was however shown to be crucial for receptor maintenance on the cell surface, chiefly by protecting it against protease degradation. Furthermore, N-glycosylation patterns have been suggested to have an impact on the subcellular distribution of CXCR2 [73]. Additionally, although in the case of CCR5 it has been shown that O-glycosylation at S6 and S7 does not impair sulfation of Y10 [74], the vicinity of carbohydrate chains was proposed to negatively influence tyrosine sulfation [64]. It was also postulated that differential CXCR4 N-glycosylation may contribute to the presence of structurally and functionally distinct receptor isoforms [77]. Therefore, glycosylation of the receptor N-terminus is likely to be of greater importance than initially appreciated and in particular cell-dependent glycosylation patterns may represent an additional level in the finely tuned regulation of the chemokine network. In addition, glycosylation of the CXCR4 N-terminus was shown to influence HIV-1 co-receptor usage (see Section 3.2).

Table 2
Sequences, lengths, charges and post-translational modifications of CC chemokine receptors N-termini.

Receptor	Chemokine[a]	Pathology[b]	Sequence	N-term	M-C	C-TM	$_{p}$sY	N-Glyco	Ref
CCR1	CCL3 CCL5 CCL7 CCL8 CCL13 CCL14 **CCL15** CCL16 **CCL23**	AR AS AT CA COPD HIV MS PS RA	METPNTTEDYDTTTEFDYGDATPC-QKVNERAFGAQLLPPLYS	42 (-6)	24 (-7)	18 (+1)	2	1	
CCR2	**CCL2** CCL7 CCL8 CCL11 CCL13 CCL16	AS CA COPD HIV LP MS RA	MLSTSRSRFIRNTN*ESGEEVTTFFDY*DYGAPC-HKFDVKQIGAQLLPPLYS	50 (-0)	32 (-2)	18 (+2)	2	1	(50)
CCR3	CCL2 CCL5 CCL7 CCL8 **CCL11** CCL13 CCL15 **CCL24** **CCL26** CCL28	AS CA COPD HIV	MTTSLDTVETFGTTSYYDDVGLLC-EKADTRALMAQFVPPLYS	42 (-4)	24 (-4)	18 (0)	2	0	(59, 60)
CCR4	CCL17 CCL22	AD AS CA DI IBD PS	MNPTDIADTTLDESIYSNYYLYESIPKPC-TKEGIKAFGELFLPPLYS	47 (-4)	29 (-4)	18 (0)	4	0	
CCR5	CCL3 to CCL5 CCL8 CCL11 CCL14 CCL16	AR AS AT CA CH COPD HIV IBD MS PS RA	MDY*QVS*S*PIY*DINY*Y*TSEPC-QKINVKQIAARLLPPLYS	38 (0)	20 (-3)	18 (+3)	4	0	(51, 74, 163)
CCR6	CCL20	CA IBD PS	MSGESMNFSDVFDSSEDYFVSVNTSYYSVDSEMLLC-SLQEVRQFSRL	47 (-6)	36 (-7)	11 (+1)	3	2	
CCR7[w]	CCL19 CCL21	CA IBD MS	QDEVTDDYIGDNTTVDYTLFESLC-SKKDVRNFKA	34 (-4)	24 (-7)	10 (+3)	2	1	
CCR8	**CCL1** CCL4 CCL16 CCL17	AD AS	MDYTLDLSVTTVTDYYYPDIFSSPC-DAELIQTNGKL	36 (-5)	25 (-4)	11 (-1)	4	0	
CCR9	CCL25	CA IBD	MTPTDFTSPIPNMADDYGSESTSSMEDYVNFNFTDFYC-EKNNVRQFASHFLPPLYW	56 (-5)	38 (-7)	18 (+2)	3	1	
CCR10	CCL27 CCL28	AD CA PS	MGTEATEQVSWGHYSGDEEDAYSAEPLPELC-YKADVQAFSRA	42 (-6)	31 (-7)	11 (+1)	2	0	

Table 3
Sequences, lengths, charges and post-translational modifications of CXC chemokine receptors N-termini.

Receptor	Chemokine[a]	Pathology[b]	Sequence	N-term	M-C	C-TM	$_{p}$sY	N-Glyco	Ref
CXCR1	CXCL1 CXCL6 **CXCL8**	AS CA COPD IBD PPP RA	MSNITDPQMWDFDDLNFTGMPPADEDYSPC-ML*E*TETLN*K*Y	40 (-8)	30 (-7)	10 (-1)	1	2	(27)
CXCR2	CXCL1 to CXCL3 CXCL5 to CXCL8	AS AT CA COPD IBD PS RA	MEDFNMESDSFEDFWKGEDLSN*Y SYSSTLPPFLLDAAP C-EP*E*SLEIN*K*Y	49 (-10)	39 (-8)	10 (-2)	2	1	(50)
CXCR3	CXCL9 to CXCL11	AR AS AT CA CH COPD DI IBD LP MS PS RA	MVLEVSDHQVLNDAEVAALLENFSSSY*DY*GENESDSC CTSPPC-PQ*D*FSLNFD*R*A	54 (-9)	43 (-8)	11 (-1)	2	2	(22, 54)
CXCR4	CXCL12	AS AT CA HIV RA	MEGISIY*TSDN*Y*TEEMGSGDY*DSMKEPC-FREENANFN*K*I	39 (-6)	28 (-6)	11 (0)	3	1	(29, 52, 61)
CXCR5	CXCL13	CA LP	MNYPLTLEMDLENLEDLFWELDRLDNYNDTSLVENHL C-PAT*E*GPLMASF*K*A	51 (-8)	38 (-8)	13 (0)	2	1	
CXCR6[ψ]	CXCL16	CA MS	MAEHDYHEDYGFSSFNDSSQEEHQDFLQFS*K*V	32 (-4)	32 (-4)	-	2	1	
CXCR7	CXCL11 CXCL12	CA	MDLHLFDYSEPGNFSDISWPC-NSS*D*CIVVDTVMCPNMPN*K*S	41 (-4)	21 (-3)	20 (-1)	1	3	

Table 4
Sequence, length, charge and post-translational modifications of CX3C chemokine receptors N-terminus.

Receptor	Chemokine[a]	Pathology[b]	Sequence	N-term	M-C	C-TM	$_{p}$sY	N-Glyco	Ref
CX3CR1	CX3CL1	AT CA IBD PS	MDQFPESVTENFEY*DD LAEAC-YIGDI	26 (-8)	21 (-7)	5 (-1)	1	0	(53)

3. Ligand binding mode

3.1. Binding of chemokines to chemokine receptor N-terminus

Numerous studies conducted with whole receptors [17–23,36,37] or receptor-derived synthetic peptides [24–33,78] have demonstrated that the N-terminal domain of chemokine receptors holds an important role in ligand binding. Based on some of these results and the observation that chemokine binding and receptor activation are separable events driven by distinct molecular mechanisms and involving different structural determinants, a general two-site model was proposed by different authors to describe the interaction of chemokines with their cognate receptors [11,12] (Fig. 3A–C). In this model, the receptor

Table 5
Sequences, lengths, charges and post-translational modifications of decoy receptors N-termini.

Receptor	Chemokine[a]	Pathology[b]	Sequence	N-term	M-C	C-TM	$_{p}$sY	N-Glyco	Ref
D6	CCL2 to CCL8, CCL11 to CCL14 CCL17 CCL22	CA	MAATASPQPLATEDADAENSSFYYYDYLDEVAFMLC-RK*D*AVVSFG*K*V	47 (-6)	36 (-7)	11 (+1)	4	1	
DARC	CCL2 CCL5 CCL7 CCL11 CCL13 CCL14 CCL17 CXCL1 CXCL3 CXCL5 CXCL6 CXCL8 CXCL11	PL PS	MGNCLHRAELSPSTEN*SSQLDFEDVWN*SSYGVN*DSF PDGDYGANLEAAAPC-HSCNLLDDSALPFF	65 (-9)	51 (-8)	14 (-1)	2	2	(45, 55, 71, 72, 75)
CCX-CKR	CCL19 CCL21 CCL25 CXCL13	CA	MALEQNQSTDYYYEENEMNGTYDYSQYELIC-IKE*D*VREFA*K*V	42 (-7)	31 (-7)	11 (0)	6	2	

Tables present the length and (charge) for the complete N-terminal domains (N-term), M–C and C–TM parts. M–C encompasses residues starting from the amino terminal methionine (M) to the cysteine (C) involved in a disulfide bridge with the third extracellular loop (ECL3). C–TM corresponds to the sequence starting from the cysteine (C) to the first amino acid of the first transmembrane segment 1 (TM). – delimits the M–C part from the C–TM part. Negatively charged residues are represented in bold. Tyrosine residues present in the M–C part that are potentially sulfated ($_{p}$sY) are highlighted in gray. Potential N-glycosylation sites (NxS/T) are underlined. *Denotes post-translational modifications that were experimentally demonstrated. Double underlined italic residues highlight (1) the conserved K-K/R-PPLYS/W motif located in the C–TM parts at positions C+2, C+6 and C+13 respectively or (2) the negatively and positively charged residues conserved at positions +3/4 and +10/11. Charge corresponds to the sum of negatively (D, E) and positively (K, R, H) charged residues. [ψ]CCR7 N-terminal sequence presents a signal peptide of 24 residues. Processing prediction indicates Gln25 as the amino terminal residue of CCR7 N-terminus. [ψ]CXCR6 does not present a cysteine in its N-terminal domain. AD: atopic dermatitis, AR: allograft rejection, AS: asthma, AT: atherosclerosis, CA: cancers, CH: chronic hepatitis, COPD: chronic obstructive pulmonary disease, DI: type I diabetes, HIV: human immunodeficiency virus infection, IBD: inflammatory bowel disease, LP: lupus, MS: multiple sclerosis, PL: plasmodium infection, PPP: palmoplantar pustulosis, PS: psoriasis, RA: rheumatoid arthritis. [a]Based on [161] the principal endogenous agonists are represented in bold. [b]Adapted from [162].

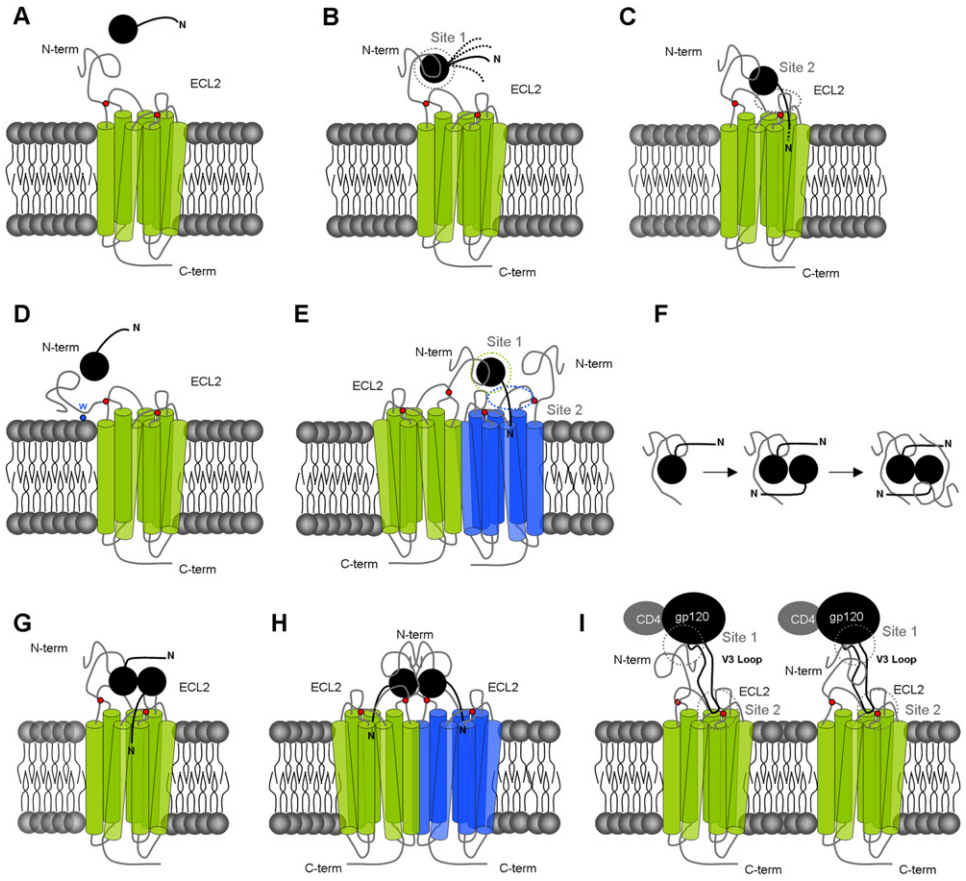

Fig. 3. Interaction models for chemokine receptors. (A–C) Putative two-site mechanism for the interaction between chemokines and chemokine receptors. (A and B) First step: interactions between the N-loop (site I) of the chemokine and the N-terminal domain of the receptor. (B and C) Step two: interactions between the flexible N-terminus (site II) of the chemokine and the extracellular loops as well as the transmembrane segments of the receptor. The disulfide bridges between N-term/ECL3 and ECL1/ECL2 are depicted as red dots. (D) Anchoring of the N-terminal domain of CXCR1 into the membrane through hydrophobic contacts mediated by an aromatic residue (W) (blue dot). (E) Receptor trans-activation. Chemokine binds the N-terminal domain (site I) of receptor 1 (green) and trans-activates receptor 2 (blue) through its binding at site II. (F) Induction of CXCL12 dimerization upon binding of a sulfated peptide corresponding to the N-terminus of CXCR4 (residues 1–38). A chemokine monomer binds to one N-terminal peptide which increases the interaction interface and facilitates the binding of the second chemokine monomer. The second N-terminal peptide binds to the second monomer leading to the formation of a symmetric 2:2 complex [29]. (G and H) Possible stoichiometries of CXCR4 interactions with CXCL12 dimer (G) Monomeric CXCR4 binds a dimer of CXCL12. (F) Dimeric CXCR4 binds a dimer of CXCL12. (I) Putative two-site binding mode describing the interactions between the gp120 protein and CCR5 [135]. Site I: the N-terminal domain of the receptor binds to the bridging sheet and the base of the V3 loop of the gp120 protein trough electrostatic interactions. Site II: the crown of the V3-loop interacts with the second extracellular loop of the receptor. Representation of the putative orientation of the N-terminus with respect to two different docking models based on NMR studies of gp120 associated to synthetic peptides derived from CCR5 N-terminus [136,137].

N-terminus plays a crucial role in the initial recognition of the chemokine through the binding of its N-loop (site I). This primary interaction is likely to contribute to correct chemokine orientation, promoting the binding of its flexible N-terminus (site II) to the extracellular loops and the transmembrane segments of the receptor, triggering its activation.

To date, little information about the structure of chemokine receptor N-termini is available. The N-terminal domains of chemokine receptors, especially their M–C part, are generally proposed to be highly flexible, showing an extended form when unbound and only adopting a fixed structure upon chemokine binding [27,48]. This hypothesis is in line with the lack of clear electron density for the M–C part in the CXCR4 X-ray structures [16].

The N-termini are the most variable extracellular domains of chemokine receptors in terms of sequence and length and this diversity is most probably an important determinant dictating the specificity of the receptor. The chemokine receptor N-termini display net negative charges and their binding to chemokines is proposed to be typically driven by electrostatic but also hydrophobic interactions. There exists a considerable amount of data on the importance of many individual residues within the N-terminus, obtained mainly from binding studies with mutated receptors [17,18,42,79,80]. These residues are however rarely conserved among the receptors with the exception of a tyrosine found approximately nine residues before the C–TM part (see Section 2.2.2). These observations suggest the existence of a common mechanism for N-terminus binding involving the conserved sulfotyrosine but also relying on non-conserved residues that may determine the selectivity of the receptors. In accordance with this hypothesis, NMR studies conducted with labeled chemokines in the presence of receptor N-terminus-derived peptides identified a groove delimited by the N-loop and the β-sheet as the receptor N-terminus binding site. Although not identical, this binding site seems highly conserved among different chemokines [32,33,56].

In particular, for CXCL8, Skelton et al. demonstrated that a small modified peptide covering residues 9–29 (M_9WDFDD$_{14}$-linker-M_{20}PPADEDYSP$_{29}$) of the CXCR1 N-terminus (K_i = 13 μM) occupies a cleft between the N-loop and the third β-strand in an extended fashion and with only a limited number of contact residues (in bold: **P$_{21}$PADEDYSP$_{29}$**) (Fig. 4A) [27]. In the complex, P21 and P22 formed hydrophobic interactions with L43^{CXCL8} and L49^{CXCL8} residues while P29 preceding the conserved cysteine wrapped

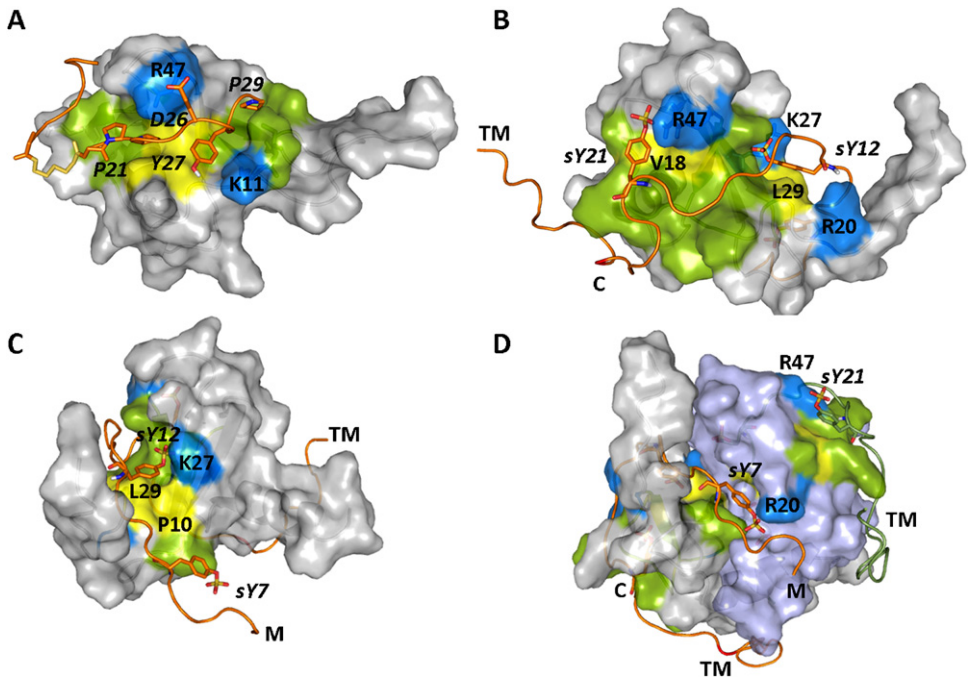

Fig. 4. Structures of chemokine/N-terminus derived peptide complexes. Chemokines are represented as surface and colored in gray. The hydrophobic N-terminus binding groove is colored in green and yellow. N-terminus-derived peptides are represented as cartoon, colored in orange and annotated in Italic. (A) NMR structure of the CXCL8–CXCR1 N-terminus complex [27]. Tyrosine 27-binding site includes residues I10, Y13, L49 (yellow) and K11 (blue). D46 of CXCR1 forms electrostatic interactions with R47 of CXCL8. N-terminus P21, P22 and P29 residues form hydrophobic interactions with the groove of the chemokine. (B–D) NMR structures of CXCL12 in complex with full-length CXCR4 N-terminus bearing sulfotyrosines at positions 7, 12 and 21 [29]. (B) Recognition sites for sulfotyrosines sY12 and sY21. Conserved sY21 binds a pocket defined by V18 and V49 (yellow) and overhung by residue R47 (blue) while sY12 interacts with a similar pocket formed by residues L29, P10 (yellow) and K27 (blue). (C) Binding of sulfotyrosine sY7 and sY12 to a CXCL12 monomer (60° rotation relative to B). sY12 occupies a defined binding pocket while sY7 points in the opposite direction making no clear interaction with the chemokine monomer. (D) Binding of sulfotyrosine sY7 to a dimer of CXCL12. sY7 occupies the cleft at the interface between two chemokine monomers and interacts with residues V24 and R20 of the second monomer. The second N-terminus peptide binding to the second monomer is represented as cartoon and colored in dark green.

around the chemokine β-sheet making hydrophobic contacts ($I10^{CXCL8}$ and $I40^{CXCL8}$). Y27, conserved in almost all chemokine receptors and most probably O-sulfated in the native CXCR1 receptor (see Section 2.2.2), interacts with a pocket delimited by $I10^{CXCL8}$, $K11^{CXCL8}$, $Y13^{CXCL8}$ and $L49^{CXCL8}$. The binding is stabilized by an additional electrostatic interaction between D26 and chemokine $K11^{CXCL8}$. The importance of these residues was confirmed by site-directed mutagenesis of the complete CXCR1, indicating that the binding mode deduced from the NMR study most likely reflects the interaction of the chemokine with the complete receptor [43].

More recently, Veldkamp et al. reported the NMR structure of a strictly dimeric form of CXCL12 in complex with a full-length CXCR4 N-terminal domain peptide [1–38] bearing sulfotyrosines at positions 7, 12 and 21 [29]. This study provided the first structural evidence of the existence of sulfotyrosine recognition sites and demonstrated that the CXCR4 N-terminal peptides adopt an extended conformation with sulfotyrosines 12 (sY12) and 21 (sY21) binding to one chemokine monomer and sulfotyrosine 7 (sY7) interacting with the second monomer (Fig. 4B–D). Interestingly, in the complex, sY21 is orientated in the opposite direction compared to the equivalent Y27 in CXCR1 and interacts with a hydrophobic pocket defined by $V18^{CXCL12}$ and $V49^{CXCL12}$ and with the overhanging basic residue $R47^{CXCL12}$, which in CXCR1 is occupied by P21 (Fig. 4A and B). It is noteworthy that a residue equivalent to $R47^{CXCL12}$ is also present in CXCL8 ($R47^{CXCL8}$) but is involved in stabilizing electrostatic interactions with E25 of CXCR1. Similarly, a positively charged residue equivalent to $K11^{CXCL8}$ is also present in CXCL12 ($R20^{CXCL12}$) but does not interact with any of the CXCR4 N-terminus residues. Furthermore, this study also provided structural data on the binding mode of the two other CXCR4 sulfotyrosines, sY7 and sY12, that are not strictly conserved in other receptor N-termini. In particular, sY12 was shown, just like sY21, to bind a hydrophobic pocket defined by $P10^{CXCL12}$, $L29^{CXCL12}$ and $K27^{CXCL12}$, whereas sY7 had no interacting partners on the first chemokine monomer and occupied a cleft delimited by the interface of the dimer forming an electrostatic interaction with $R20^{CXCL12}$ of the second monomer.

Altogether these data demonstrated that sulfotyrosine recognition, critical for high affinity interactions with chemokines, occurs at particular binding sites sharing a similar architecture and that a given chemokine can display several sulfotyrosine-binding sites. Moreover, other interactions supported by non-conserved residues scattered along the N-terminal domains most probably also play essential roles in sulfotyrosine recognition and in further stabilization of the chemokine-receptor complexes, possibly providing the molecular basis for the differences in affinity and selectivity observed among the different receptors.

However, while the N-terminus plays the predominant role in the initial chemokine binding, other extracellular parts have also been shown to participate in chemokine binding, in which case the combination of multiple low-affinity interactions provides high-affinity binding energy in chemokine-receptor interactions. Consistent with this assertion is the observation that by simultaneously grafting peptides corresponding to the CCR2 N-terminus and ECL3 on a stabilized variant of the protein G B1 domain, the affinity for CCL2 is 100 times as high as when only the N-terminus is present on the scaffold [35]. Similar results were obtained for CCR3 [34]. Other extracellular parts such as ECL2, which is involved in the formation of site II, could be of importance for the overall affinity of the receptor.

Moreover, accumulating data suggest that the mechanism underlying chemokine binding to their receptors is likely to be more complex than a simple two-site model. It has been proposed that site I and site II interactions may be far from independent. Indeed, conformational changes in both the chemokine and the receptor that follow the initial chemokine binding to the N-terminus of the receptor may energetically influence the subsequent interactions at site II [81]. This model may for instance explain why while CXCL8 binds CXCR1 with a significantly higher affinity than CXCL1, both chemokines bind the N-terminus of the receptor with similar affinities [24]. Therefore, in contrast to CXCL8, the changes resulting from the binding of CXCL1 to site I would negatively affect further interactions of the chemokine at site II. Such coupling between the two binding steps may thus have a major role in the regulation of chemokine affinity and selectivity for their receptors, providing yet another molecular basis for the complexity of chemokine–chemokine receptor network [82]. It is also conceivable that upon binding of the chemokine at site I, the area for further interactions with site II increases, either as a result of conformational changes in the chemokine/receptor [82] or by complementation of sites that are partly present on the receptor N-terminus and partly on the chemokine.

Recently, the possible role held by the vicinity of the cell membrane in the regulation of receptor N-terminus interactions with the ligand has also been put forward. Indeed, it has been demonstrated that the CXCR1 N-terminal peptides interact with membranes or membrane-mimicking micelles in extended but constrained conformation that may energetically facilitate the interactions with the chemokine [24,83]. NMR studies using a phospholipid bilayer-embedded CXCR1 receptor or an N-terminus–TM1 construct suggested that the CXCR1 N-terminal domain may be anchored to the membrane via a tryptophan residue at position 10 (Fig. 3D). The release of the N-terminus from the membrane upon strong interactions with the chemokine may thus be considered, at least for CXCR1, as the earliest step of the ligand binding mechanism [36]. Such hydrophobic association of the N-terminus with the cell membrane may have a great impact on its binding properties. Indeed, the affinity of the CXCR1 N-terminal fragment for CXCL8 was shown to be 20-fold higher in detergent micelles than in solution ($K_D \approx 1$ μM versus 20 μM). Moreover, membrane-like environment has been reported to influence the binding selectivity of the receptor N-terminal domains [24].

Another poorly understood aspect is the stoichiometry of chemokine-receptor interactions. Since many chemokine receptors are known to form homo- or heterodimers, the possibility of a cross-talk in which site I and site II interaction would take place on separate receptors should not be excluded (Fig. 3E). In accordance with this hypothesis, Monteclaro et al. demonstrated that the CCL2 binding to the CCR2 N-terminus fused to CD8 can activate in trans a chimeric CCR2 that carries an irrelevant N-terminus [37]. Besides the receptors, many chemokines as well have been shown to exist in different oligomeric states. Furthermore, in the case of CXCL12, it has been demonstrated that binding to the N-terminus of CXCR4 induces its dimerization in a symmetric 2:2 complex in which the dimerization interface is shared by both the residues from the N-terminal domain and the chemokine [30] (Figs. 4D and 3I). While CXCL12 dimerization has been suggested to be physiologically irrelevant [33], recent data recorded with strictly dimeric chemokine demonstrated distinct signaling pathways and differential chemotactic effect depending on the oligomeric state of CXCL12 [84]. Moreover, structural data showed that the CXCR4 N-terminus binds differentially to CXCL12 monomers and dimers [84]. In particular, while residues 4–9 of the CXCR4 N-terminal peptide make strong interactions with CXCL12 monomers, they are only weakly associated with the chemokine in its dimeric form. Similar 2:2 interactions were proposed for CXCL8 and CXCR1 N-terminus but remain controversial [26,81]. One cannot rule out the possibility that such dimerization may reflect the experimental setup, where in the absence of other possibilities of interactions with the receptor, chemokine dimerization is energetically favored. On the contrary, it has been proposed for CXCL8 that the binding of the N-terminal domain of CXCR1 to the chemokine dimer could promote its dissociation [81].

In an emerging concept, chemokine receptor response specificities underlie the differences in receptor trafficking. Particularly, it has been proposed that determinants of receptor internalization rates following ligand binding may be harbored by the N-terminus of chemokine receptors [23]. By swapping the N-termini of CXCR1 and CXCR2, two chemokine receptors that share 77% of sequence identity but show different binding and signaling profiles towards CXCL8, it was demonstrated that the trafficking profiles of the chimeric receptors were defined by the N-terminus and translated in temporal differences in activation of ERK1/2 signaling pathways, which are important for different signaling specificities. However, these determinants remain hitherto unidentified.

3.2. Binding of pathogen proteins to chemokine receptor N-terminus

To subvert the host immune system and promote their pathogenesis, viruses such as herpesviruses, poxviruses and retroviruses have evolved various strategies to interfere with the host chemokine network, for instance by expressing chemokine analogs (for review see [85,86]).

The Human herpes virus 8 (HHV-8), also named Kaposi's sarcoma-associated herpesvirus, expresses three viral macrophage inflammatory proteins (vCCL1, vCCL2 and vCCL3) that share homologies with CCL3 and CCL4 [87]. The characterization of these proteins revealed that vCCL2 has the unique ability to cross-bind to various CC and CXC chemokine receptors [88,89]. In particular, vCCL2 binds to CXCR4 and CCR5 and is capable of inhibiting the interaction with their cognate chemokine ligands as well as infection of host cells by HIV-1 [88,89]. Although vCCL2 displays a typical chemokine structure [90], very little information about its binding mode is currently available. Whether its ability to interact with chemokine receptors of both subfamilies involves the same or distinctive determinants remains to be elucidated.

Chemokine receptors can also be hijacked by pathogens to allow their entry into specific cell types. Two striking examples of such piracy are the malaria parasites (*Plasmodium vivax* and *Plasmodium knowlesi*) and the human immunodeficiency virus (HIV-1).

P. vivax and *P. knowlesi* belong to the five *Plasmodium* species responsible for human malaria, a mosquito-borne infectious disease causing fever and headache and progressing in the most severe cases to coma and death. *P. vivax* and P. *Knowlesi* infect human erythrocytes by using the decoy chemokine receptor DARC (Duffy blood group antigen) [91–93] that binds various CC and CXC chemokines [94]. *Plasmodium* interaction with DARC was shown to be mainly mediated by a conserved cysteine-rich domain present in the parasite Duffy binding proteins (PvDBP and PkDaBP) [95] and by a modified 35-amino acid fragment (residues 8–42) of the receptor N-terminus [96]. The tyrosine residues at position 30 and 41 of the N-terminus of DARC are sulfated although only the second one was reported as critical for PvDBP and PkDaBP binding [55]. Interestingly, erythrocytes interaction with PvDBP-expressing cells can be inhibited by a peptide derived from DARC N-terminus (IC_{50} = 1 μM) [96] while sulfation of tyrosine 30 and 41 in the peptide results in a more efficient inhibition (IC_{50} = 5 nM) [55]. Recent data point to the existence of a sulfotyrosine-binding pocket for DARC N-terminus on the interface of DBP dimer of *P. vivax* [97].

The human immunodeficiency virus (HIV-1), the causative agent of AIDS, uses chemokine receptors CCR5 and CXCR4 to specifically infect monocytes and macrophages (M-tropic) or T-cells (T-tropic), respectively [9,98–101]. This multi-step process is mainly mediated by envelope glycoproteins gp120 and gp41 organized in heterotrimer spikes on the outer surface of the viral membrane [102,103]. Gp120 is constituted of an alternation of five constant domains (C1–C5) and five variable loops (V1–V5). The domains C1, C2 and C4 form a four-stranded antiparallel β-sheet called the bridging sheet. Upon binding to CD4, its primary receptor, HIV-1 envelope glycoprotein gp120 undergoes conformational changes resulting in the spatial reorientation of the bridging sheet and the variable V1/V2 and V3 loops exposing specific binding sites for the co-receptors [104–107]. Binding of gp120 to CXCR4/CCR5 leads to a rearrangement of gp41, bringing together the cellular and the viral membranes and allowing their fusion [108].

Interactions between gp120 and chemokine receptors CCR5 and CXCR4 have been investigated using different approaches including chimeric receptors [109–115], site-directed mutagenesis [41,79,116–120] and other biochemical and immunological methods [9,121–127]. All these studies point to the importance of the co-receptor extracellular domains in gp120 binding, especially the receptor N-terminus and ECL2, although their relative contribution depends on the HIV-1 strain [128]. Discrimination between CCR5 and CXCR4 has been shown to mainly depend on the determinants present in the V3 loop (±35 aa) of gp120 such as positively charged amino acids at positions 11, 24 and 25, the overall charge and the distribution of the electrostatic potential [129,130]. However, co-receptor usage has also been shown to be affected by amino acid composition and glycosylation of the V1/V2 stem [131,132].

In particular, the interaction between the V3 loop and the N-terminus of CCR5 has been shown to depend on a cluster of negatively charged and tyrosine residues (D2, Y3, Y10, D11, Y14, Y15, E18) and suggested to be driven by electrostatic interactions [18,113,116,117,119]. Besides, the co-receptor function of CCR5 was also associated to other determinants such as S6, S7, I9, N13, Q21 and K22 [18,117,119]. Like for chemokines, sulfation of tyrosine residues, in particular Y10 and Y14, was identified to critically affect the binding of gp120 while O-glycosylation of serine residues had little effect [51,74,119,125].

Together with structural analyses of the V3 loop [106,107,133,134], these studies revealed the role of spatially distinct domains of gp120 in CCR5 interaction and led to the development of a two-site binding model, similar to that proposed for chemokines [135]. In this model, the conserved four-stranded bridging sheet (C4) and the base of the V3 loop bind to the CCR5 N-terminus (residues 2–15) (site I) through electrostatic interactions, while the crown of the V3 loop interacts with the co-receptor ECL2 (site II) (see Fig. 3I).

In the absence of high-resolution structures, new insight into the molecular details of gp120-coreceptor interactions arose from NMR studies of gp120 bound to synthetic peptides derived from specific co-receptor domains [136,137]. NMR study of a sulfated CCR5 N-terminus peptide (sY10–sY14 CCR5 2–15) in complex with gp120 revealed a well-defined structure for residues 7–15. The docking of this peptide into the crystal structure of gp120–CD4 suggested that CCR5 N-terminus binds to gp120 at the intersection of the bridging sheet and the V3 loop (Fig. 3I, left panel) [136]. Residues S7 and P8 bind to the V3 stem while sY10, N11, Y15 interact with $R327^{gp120}$, $R440^{gp120}$, $I439^{gp120}$, respectively. The pocket between the bridging sheet and V3 encircles sY14 and rigidifies the V3 stem into a β-hairpin structure. A more recent study performed with a longer sulfated peptide (sY10–sY14 CCR5 1–27) showed that residues 7–23 bind to gp120–CD4 with P8–S17 and A20–I23 forming helical structures [137]. This study also provided a clearer picture of the main CCR5 binding determinants, emphasizing the importance of the previously identified residues D2, Y3, sY10, D11, sY14, Y15, E18 while contradicting the results regarding V5, I9, I12 and T16. The integration of these data in a gp120 structural model suggested the interaction of residues 2–22 with the fourth constant domain as well as the stem of the V3 loop (site I). In contrast to the previous docking model, here, the peptide is flipped by 180° with sY14 fitting into a binding pocket and strongly interacting with $R440^{gp120}$ while sY10 binds to $R32^{gp120}$ (Fig. 3I, right panel).

The binding of gp120 to CXCR4 probably occurs via a similar mechanism, although the N-terminus seems less important for infection by certain isolates [114,138,139]. In contrast to CCR5, no precise cluster of residues critically affected virus entry. Mutagenesis studies however revealed the role of individual residues (Y7, N10, Y12, N20, Y21, N22, S23 and E26) for the co-receptor function of CXCR4, although the extent of their contribution was strain-dependent [47,79,140]. The sulfation of tyrosine residues, in particular Y21, only had a minor effect on the entry of X4-tropic HIV-1 [52], while controversial data were obtained regarding the impact of glycosylation. Mutation of the N-glycosylation site of CXCR4 N-terminus (N11) was initially shown to slightly facilitate R5 [141] or R5X4 [142] virus entry while having no effect on X4 viruses [79,118,142]. In another study however, the replacement of N11 with Q11 enhanced the binding and entry of X4 and R5 viruses [143].

4. Therapeutic discoveries targeting N-terminus interactions

Since their discovery twenty years ago, chemokines and their receptors have emerged as fundamental regulators of human physiology. The interest in chemokine biology also arises from their key roles in such pathologies as cancer, inflammatory and autoimmune diseases as well as HIV-1 infection (see Tables 1–5). Therefore much effort has been put into exploring ways to interfere with these processes, by either targeting the receptors or their ligands.

Therapeutic strategies directed against chemokine receptors have already proven efficacious in clinic. Two small molecules are currently on the market, namely the CXCR4 antagonist, AMD3100 (plerixafor, Mozobil™ developed by Genzyme) used for hematopoietic stem cell mobilization prior to autologous transplantation in patients with lymphoma and multiple myeloma and Maraviroc (Selzentry™, developed by Pfizer) for the treatment of CCR5-tropic HIV-1 infection [144,145]. These compounds, like the vast majority of chemokine receptor inhibitors, bind pockets in the transmembrane regions and do not interact with the N-terminal domain. However, because of their key roles in ligand recognition, the N-termini of chemokine receptors may also represent highly relevant targets for drug discovery. To the best of our knowledge, small molecules specific to the N-terminus have never been reported and this is certainly due to the unstructured, highly flexible nature of this domain. These characteristics however can also be regarded as advantageous for the generation of therapeutic antibodies able to block the initial site I-interaction of intact receptors. Immunization with synthetic N-terminus derivatives allows for instance to circumvent the need for receptor purification or avoid elicitating antibodies against irrelevant epitopes in whole-cell antigens but may overlook the post-translational modifications often present in the extracellular domains [146,147]. Antibodies recognizing linear or conformational epitopes exclusively or partly present in the N-terminus are commonly used in research and may also be exploited for therapeutic applications. Indeed, given that receptors which share ligands can at the same time have very distinct N-termini (see Tables 1–5) it is conceivable that highly specific, clinically relevant antibodies can be raised against these fragments.

To date, there are no anti-chemokine receptor antibodies approved for clinical use. However, clinical trials for at least two anti-N-terminus mAbs are in progress. This includes the anti-CCR5 mAb PRO140 currently evaluated against HIV infection as well as the CCR4-specific mAb KW-0761 for the treatment of adult T-cell leukemia–lymphoma and peripheral T-cell lymphoma [148,149].

Other rather encouraging results from studies with antibodies recognizing the chemokine receptor N-terminus have been published. Recently, a dromedary-derived VHH, CA52, directed against the N-terminus of DARC, efficiently inhibiting *P. vivax* invasion and able to displace CXCL8 from the receptor was described [150]. Similarly, sera from rabbits immunized with the first seven CCR5 N-terminus amino acids fused to T-helper cell epitope from tetanus toxoid were shown to inhibit HIV-1 infection of primary macrophages [147].

An attractive alternative to receptor inhibition consists of neutralizing the ligand, in particular by blocking the N-terminus-recognition site [151]. The report on the human mAb 10F8 whose epitope overlaps with the binding pocket of CXCR1 N-terminal domain (see Section 3.1, [27]), illustrates well the feasibility of such a strategy [152]. This antibody was shown to interact with CXCL8 with picomolar affinity and to inhibit its binding to neutrophils (IC_{50} 0.3 nM) as well as chemokine-induced neutrophil activation and chemotaxis. In addition, it proved relatively efficient in treating palmoplantar pustulosis, an inflammatory disease in which CXCL8 plays a predominant role.

Moreover, a considerable therapeutic potential can be expected from approaches targeting specifically sulfotyrosine-binding pockets, as sulfotyrosine-mediated interactions seem widely exploited not only in chemokine biology but also by pathogen proteins. In line with this assumption is the report of a sulfated peptide corresponding to the first 60 residues of DARC N-terminus and blocking at low nanomolar concentration the association of plasmodium PvDBP and PkDaBP with the receptor [55]. Attempts to neutralize chemokines or the HIV-1 envelope protein gp120 using N-terminus-derived peptides have so far proven unsuccessful mainly due to their low affinity and poor stability. It may however be possible to improve the affinity and pharmacokinetic properties of these peptides for example by incorporating in the sequence non-natural residues such as D-amino acids or chemical derivatives like the acid-stable sulfotyrosine mimic, (p-sulfomethyl)-phenylalanine [153,154]. Interestingly, recent high-throughput in silico screening of small molecules targeting the $sY21^{CXCR4}$ sulfotyrosine-binding pocket on CXCL12 identified several lead compounds of which one (ZINC 310454) bound CXCL12 with an affinity of 64 μM [155]. Extending the screening target to larger parts of the chemokine/N-terminus interaction surface may provide molecules of higher specificity and/or affinity.

5. Discussion

Chemokines are a family of small highly basic proteins that display a common fold but share little sequence similarities. By binding to chemokine receptors, they participate in many vital processes. The chemokine-receptor network is characterized by an apparent redundancy and many chemokines can bind to several receptors, while a chemokine receptor usually has multiple ligands. This overlapping selectivity reflects however sophisticated regulation mechanisms that are still not fully elucidated.

The N-terminus of chemokine receptors has a critical role in the initial step of chemokine binding as well as in determining the specificity and affinity of this interaction. At first sight, the N-termini vary remarkably between different receptors in terms of length and amino acid sequence. However, on closer examination several common characteristics and signatures can be discerned.

One such feature is the conserved cysteine residue involved in a disulfide bond that links the N-terminus and ECL3 and delimits two distinct regions within the N-terminus, the M–C and C–TM parts. While this disulfide bridge has been shown to be important for chemokine receptor biology, the exact way of how it exerts its function remains unclear [37,41–44]. It is likely that by linking the N-terminus to ECL3, this disulfide bridge participates in the positioning of the M–C part above TM7 in an arrangement favoring the presentation of the chemokine to site II. Moreover, as a large part of chemokine binding relies on the receptor extracellular domains, the C–TM "pseudo-extracellular loop" is perhaps an important additional feature shaping the ligand interaction interface in receptors having relatively short, compared to other protein-binding GPCRs, N-terminus. This supposition may be substantiated by the observation that CXCR4 structure differs from other GPCRs in the location and the form of the ligand binding pocket, which is situated closer to the extracellular surface [16]. In this context, the existence of different C–TM sizes (11 or 18 residues) identified here that bear distinct signatures (K-X_3-K/R-X_7-PPLYS/W and E/$D_{+3/+4}$-K/$R_{+9/+10}$ respectively) is rather intriguing and the potential impact of these elements on the receptor functionality should be addressed in the near future. Furthermore, the conservation of these motifs may open new perspectives for phylogenetic studies of chemokine receptors and allow their alternative classification that, in contrast to the current system, would not be merely based on the recognized ligands.

More information is available on the flexible M–C part of the N-terminus since it had early been demonstrated to be directly involved in ligand binding. There has been growing interest in the post-translational modifications present in this region and one of the current central areas of concern in chemokine receptor interactions with ligands is sulfation of their N-terminal domains. For several receptors this post-translational modification has been demonstrated to be important for high-affinity binding to chemokines. Most chemokine receptors bear a potentially sulfated tyrosine about nine residues before the conserved cysteine, which may therefore interact with the sulfotyrosine-binding pocket suggested to be present on the surface of all chemokines [58]. Complementation between the negatively charged receptor N-terminus and the positive charges within this conserved binding site as well as hydrophobic interactions were proposed to facilitate the binding by proper positioning of the sulfotyrosine-bearing N-terminus and stabilization of the interaction. Although sulfotyrosine-driven binding mechanism seems to be shared by many chemokine-receptor pairs, the auxiliary residues involved in this interaction are highly variable and might have co-evolved in the binding partners determining, at least in part, their specificity. In several chemokine receptors, many other potentially sulfated tyrosines are present in the N-termini and appear to participate in high-affinity interactions with ligands. For CXCR4, their binding was shown to follow a mechanism similar to that proposed for the conserved sulfotyrosine indicating that other sulfotyrosine binding sites may exist at the surface of chemokines [29]. Sulfotyrosine-mediated recognition appears to be exploited not only in chemokine interactions but also by pathogen proteins and therefore sulfotyrosine-binding pockets represent valuable targets for drug development.

Furthermore, we propose that the presence of the multiple sulfate groups may provide repulsion forces that energetically favor an extended conformation of the N-terminus, exposing the residues that are critical for ligand binding.

Other features commonly found in the M–C part are the putative N-glycosylation sites. The presence of sugar chains has been experimentally determined in only a few receptors and their exact role has yet to be further investigated. It is nevertheless highly plausible that similarly to tyrosine sulfation, cell-dependent

glycosylation patterns result in structurally and functionally different receptor isoforms, like those observed for CXCR4 [77]. Such differences in the post-translational modifications may thus represent an additional level in the fine-tuning of the complex chemokine-receptor network.

Unfortunately, the recent resolution of the X-ray structure of CXCR4 failed to provide details on the flexible M–C part [16]. Nevertheless, alternative approaches exploiting chimeric, mutated or truncated receptors produced a compelling set of information on the critical roles of these N-terminus parts in ligand binding and receptor function [17–23]. In particular, NMR analyses of chemokines or viral proteins in complex with synthetic peptides derived from the receptor N-termini were a considerable steppingstone in the understanding of the receptor N-terminus biology and provided the first insights on the structural basis for site I interactions [27,29]. However, data from these studies should be interpreted with some caution. Among the problems to be taken into consideration is the fact that the peptides used do not always cover the full N-terminus sequence and often bear no post-translational modifications that are normally present in this receptor domain. Although studies with sulfated N-terminal fragments have been reported (mainly for CXCR4 and CCR5) the addition of this group is not a straightforward task [30,61,156,157].

To date, many questions on chemokine receptors remain under debate. It has become clear that post-translational modifications of the N-terminus should not be underrated in the role they play in receptor function but investigating it is somewhat challenging. The exact stoichiometry of chemokine-receptor interactions, including such aspects as receptor–receptor cross-talk, chemokine oligomerization and the biological relevance of receptor N-terminus-induced chemokine dimerization as observed for CXCR4–CXCL12 couple, also need to be further examined. Although, the development of chemokine receptor antagonist still remains a major challenge, the efforts made to unravel and characterize the structural and functional properties of chemokine binding mode will probably, in the future, enable the development of new specific chemokine-neutralizing molecules or N-terminus-targeting antibodies with high therapeutic potential [151,155,158].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bcp.2012.08.008.

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