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REVIEW

# The structural role of receptor tyrosine sulfation in chemokine recognition

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Tyrosine sulfation is a post-translational modification of secreted and transmembrane proteins, including many GPCRs such as chemokine receptors. Most chemokine receptors contain several potentially sulfated tyrosine residues in their extracellular N-terminal regions, the initial binding site for chemokine ligands. Sulfation of these receptors increases chemokine binding affinity and potency. Although receptor sulfation is heterogeneous, insights into the molecular basis of sulfotyrosine (sTyr) recognition have been obtained using purified, homogeneous sulfopeptides corresponding to the N-termini of chemokine receptors. Receptor sTyr residues bind to a shallow cleft defined by the N-loop and β3-strand elements of cognate chemokines. Tyrosine sulfation enhances the affinity of receptor peptides for cognate chemokines in a manner dependent on the position of sulfation. Moreover, tyrosine sulfation can alter the selectivity of receptor peptides among several cognate chemokines for the same receptor. Finally, binding to receptor sulfopeptides can modulate the oligomerization state of chemokines, thereby influencing the ability of a chemokine to activate its receptor. These results increase the motivation to investigate the structural basis by which tyrosine sulfation modulates chemokine receptor activity and the biological consequences of this functional modulation.

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## Tyrosine sulfation

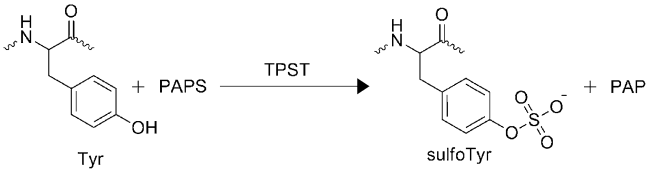
Tyrosine sulfation is a post-translational modification of secreted and transmembrane proteins, in which a sulfate group is transferred from 3′-phosphoadenosine 5′-phosphosulfate to the hydroxyl group of a tyrosine residue to form a tyrosine $O^4$-sulfate ester (Figure 1).

Tyrosine sulfation occurs in the trans-Golgi apparatus and is catalysed by the enzyme tyrosylprotein sulfotransferase (TPST). Mammals possess two TPST isoforms (TPST-1 and TPST-2); TPSTs have also been found in other vertebrates, several invertebrate species and plants (Moore, 2003; Komori *et al.*, 2009). TPST enzymes are single pass, integral membrane proteins with their catalytic domains luminally orientated. Nearly all TPSTs characterized to date are type I enzymes with their C-terminal regions oriented in the lumen, whereas the *Arabidopsis* TPST is a type II enzyme and has its N-terminus oriented in the lumen (Moore, 2009). The enzymes are thought to preferentially sulfate protein targets possessing exposed contiguous stretches of residues containing Glu/Asp clustered around tyrosines. Commonly, target proteins contain multiple tyrosine residues in these regions and sulfation is incomplete and heterogeneous. Several studies have investigated the mechanism of action and specificity of TPSTs (Mishiro *et al.*, 2006; Danan *et al.*, 2008; 2010; Jen *et al.*, 2009).

Tyrosine sulfation is essential in mammals. Mice deficient in both TPST isoforms have severely impaired post-natal survival and individual TPST-1 or TPST-2 knock-outs have severely impaired growth, reproductive function and immune responses (Westmuckett *et al.*, 2008). Similarly, severe phenotypic changes are observed in the *Arabidopsis* (plant) TPST knock-out (Komori *et al.*, 2009). Although the molecular basis of these phenotypic alterations remains to be determined, it is clear that tyrosine sulfation plays important roles in health and disease. Expression of both TPST isoform transcripts is detectable in all mammalian cell types and cell lines tested (Moore, 2003). However, expression levels are tissue and cell specific, presumably creating variability in sulfation profiles for target proteins which may have important functional consequences (Mishiro *et al.*, 2006). Unlike phosphorylation, there is no evidence, so far, for reversible or dynamic tyrosine sulfation, although it is plausible that uncharacterized tyrosine sulfatases exist or that sulfate

hydrolysis could occur spontaneously, particularly under low pH conditions such as occurring in endosomes.

Tyrosine sulfation is known to occur in numerous secreted and integral membrane proteins and this list is sure to grow as proteomics databases expand (Stone *et al.*, 2009). Some examples of mammalian proteins known to be tyrosine sulfated are: complement protein C4 (Karp, 1983; Hortin *et al.*, 1986; 1989); numerous blood coagulation proteins such as factor V (Hortin, 1990; Pittman *et al.*, 1994); hormones such as human choriogonadotropin α-chain (Bielinska, 1987); and cell-surface adhesion molecule P-selectin glycoprotein ligand-1 (Pouyani and Seed, 1995; Sako *et al.*, 1995; Wilkins *et al.*, 1995). In addition, many GPCRs are tyrosine sulfated including: C3a and C5a-anaphylatoxin chemotactic receptors and type 1 sphingosine 1-phosphate receptor (involved in complement activation, chemotaxis and T-cell activation) (Farzan *et al.*, 2001; Gao *et al.*, 2003; Fieger *et al.*, 2005); follicle-stimulating hormone receptor and luteinizing hormone receptor (involved in reproductive function) (Bonomi *et al.*, 2006); and thyroid-stimulating hormone (essential for proper endocrine function of the thyroid gland) (Bonomi *et al.*, 2006) as well as several chemokine receptors. We refer interested readers to other reviews that list all known tyrosine-sulfated proteins (Moore, 2003; Stone *et al.*, 2009). In this review, we focus on the structural roles played by tyrosine sulfation of chemokine receptors in their interactions with chemokine ligands.




## Figure 1

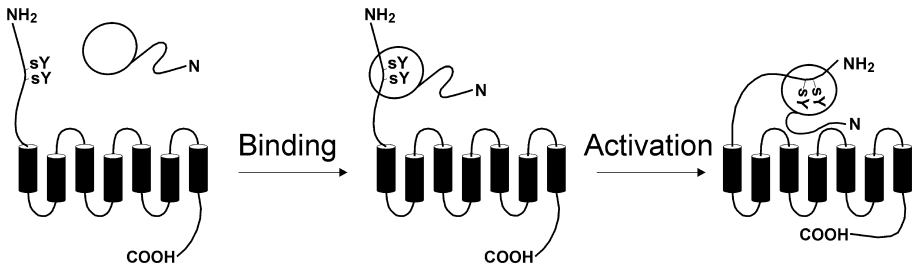
The reaction scheme for TPST-catalysed sulfation of tyrosine residues. A sulfate group within the PAPS substrate (3′-phosphoadenosine 5′-phosphosulfate) is transferred to the phenolic oxygen of the substrate tyrosine residue to form the product sulfotyrosine residue and PAP (3′-phosphoadenosine 5′-phosphate).

## Chemokine receptor structure and function

Chemokine receptors are a family of approximately 20 class 1 (rhodopsin-like) GPCRs that are primarily expressed in leukocyte membranes (Moser *et al.*, 2004; Szpakowska *et al.*, 2012). Activation of these receptors by chemokines leads to G-protein and arrestin-mediated signalling, cytoskeletal rearrangements, integrin activation, firm adhesion of the leukocyte to the endothelium and migration of the leukocyte to the surrounding tissue (Mellado *et al.*, 2001; Scholten *et al.*, 2012). Thus, chemokine–receptor interactions are essential in controlling leukocyte recruitment in inflammatory responses as well as normal lymphocyte homing. In addition, tumour cells expressing chemokine receptors tend to migrate to tissues expressing chemokine ligands, thus promoting tumour metastasis (Koizumi *et al.*, 2007). Furthermore, chemokine receptors are utilized by pathogens (HIV-1 and the malarial parasite *Plasmodium vivax*) to facilitate infection of leukocytes or reticulocytes respectively (Miller *et al.*, 1976; 2002; Farzan *et al.*, 1999).

Chemokine receptors possess an N-terminal tail (approximately 30–40 residues) and three extracellular loops that are poised for interaction with chemokines. Extensive mutational studies have concluded that the initial binding interaction of chemokine ligands occurs in the N-terminal tail of chemokine receptors and is followed by the formation of interactions of the chemokine with one or more extracellular loops and/or the α-helical transmembrane segments, thus inducing receptor conformational change and activation. This two-step model (Crump *et al.*, 1997), shown schematically in Figure 2, is consistent with the existence of chemokines that act as antagonists of certain receptors.

Within the N-terminal tails of all chemokine receptors are clusters of negatively charged residues (Asp and Glu) in the vicinity of tyrosine residues and these constitute the sequence motifs necessary for tyrosine sulfation. Tyrosine sulfation of several chemokine receptors has been demonstrated by biosynthetic incorporation of $^{35}$S-sulfate and the sulfated Tyr residues (within the acidic sequences) have been identified by mutational studies (Farzan *et al.*, 1999; 2002a; Preobrazhensky *et al.*, 2000; Choe *et al.*, 2005). Despite the




## Figure 2

The proposed two-step model for chemokine binding and activation of chemokine receptors (Crump *et al.*, 1997). During the first step, the chemokine (open circle with tail) binds to the N-terminal portion of the chemokine receptor, an interaction now known to be enhanced by tyrosine sulfation. In the second step, the tethered chemokine : receptor N-terminus complex interacts with extracellular loops and/or α-helical regions elsewhere within the receptor to induce structural changes at the cytoplasmic face of the receptor necessary for G-protein activation and subsequent signalling events.

considerable sequence differences among the N-terminal regions of chemokine receptors, almost all contain tyrosine residues close to acidic amino acids, suggesting that most chemokine receptors are tyrosine sulfated *in vivo*. Table 1 lists the chemokine receptors currently known to be sulfated and briefly summarizes the relevant research findings for each receptor. The general conclusion is that tyrosine sulfation in the N-terminal regions of chemokine receptor enhances the affinity and potency of chemokine ligands for these receptors and also promotes binding of pathogen proteins.

Because of their roles in normal immune function, inflammatory diseases, cancer and pathogen invasion, chemokine receptors are important targets for development of new therapeutic agents (chemokine and receptor nomenclature follows Alexander *et al*. 2013). Already small molecule drugs directed against the helical bundle of chemokine receptors are on the market. AMD3100 (Plerixafor, Mozobil), a selective CXCR4 antagonist, is used to promote stem cell mobilization prior to autologous transplantation in patients with lymphoma and multiple myeloma (De Clercq, 2010). A small molecule antagonist of CCR5, Maraviroc (Pfizer, New York, NY, USA), has been approved for treatment of individuals infected with CCR5-tropic HIV-1 (Emmelkamp and Rockstroh, 2007) and other CCR5 antagonists are in trials (Wood and Armour, 2005). Strategies for drug development targeting chemokine receptors are discussed in two recent reviews (Scholten *et al*., 2012; Szpakowska *et al*., 2012).

The recent crystal structure of CXCR4 (Wu *et al*., 2010) and solid state NMR structure of CXCR1 (Park *et al*., 2012), the first structures of chemokine receptors, will prove invaluable for rational design of further small molecule and perhaps even larger peptide-based drugs. However, these structures did not reveal details of the N-termini, presumably because this region is disordered. Indeed, considering its conformational flexibility, therapeutic targeting of the sulfated N-terminal region is likely to require the use of larger molecules such as antibodies or modified chemokines, as discussed elsewhere (Scholten *et al*., 2012; Szpakowska *et al*., 2012).

## Chemokine structure and function

Chemokines are a family of about 50 small (8–10 kDa), globular, soluble proteins that bind to (and usually activate) chemokine receptors. Some chemokines are expressed constitutively and function to direct B- and T-cell homing and maturation in lymph organs. However, the majority of chemokines are expressed in inflamed tissues under the control of other pro-inflammatory cytokines and function to stimulate the migration of leukocytes to the inflamed tissue (Fernandez and Lolis, 2002; Moser *et al*., 2004).

Chemokines are identified based on their distinct pattern of disulfide bonding and highly conserved tertiary structure. Moreover, they are divided into two major (CXC and CC) and two minor (C and $CX_3C$) subclasses based on the number and spacing of N-terminal cysteine residues near the protein N-terminus. Although most chemokine receptors can be activated by several chemokines they are generally selective for chemokines of one subclass. Therefore, a systematic nomenclature has been developed in which chemokines are designated as, for example, CCL or CXCL, and the corresponding receptors are designated as, for example, CCR or CXCR (Zlotnik and Yoshie, 2000). In this review, we refer to chemokines by their systematic names but also give the common names at first mention.

The conserved secondary and tertiary structure of chemokine monomers is depicted in Figure 3A and B. The N-terminus preceding the first cysteine residue is unstructured. Following the N-terminal cysteine pair is an irregularly structured and somewhat flexible loop of approximately 10 residues, called the N-loop, which terminates in a short $3_{10}$-helix. This is followed by three strands of an antiparallel β-sheet and a single α-helix. The α-helix associates with one face of the β-sheet, forming a hydrophobic core. The first N-terminal cysteine residue forms a disulfide bond with a cysteine residue in the 30 s loop (between the first two β-strands) and the second N-terminal cysteine residue forms a disulfide bond with a cysteine residue in the third β-strand.

Chemokine mutational studies are generally consistent with the two-step model (Figure 2). In the first step, the N-terminal region of chemokine receptors is thought to interact primarily with a shallow crevice between the N-loop and the third β-strand of the chemokine (as described further below). In the second step, the flexible N-terminus of the chemokine is believed to interact with the receptor extracellular loops or transmembrane helices resulting in receptor activation.

Chemokines homo-oligomerize at high concentrations and in the presence of glycosaminoglycans (GAGs) such as heparin (Fernandez and Lolis, 2002). Notably, dimers of the two main subclasses form different quaternary structures. The CC chemokines dimerize via their extended N-termini (Figure 3C) whereas CXC chemokines dimerize via residues within the first β-strand (Figure 3D), a difference thought to arise from differences in electrostatic surface charge and hydrophobicity between the two subfamilies (Fernandez and Lolis, 2002). The phenomenon of GAG-mediated oligomerization of chemokines is thought to play a pivotal role in the establishment of chemotactic gradients of chemokines *in vivo*, sequestering the chemokines at higher concentrations near their sites of release and providing a pool of dilutable chemokine that aids in leukocyte homing. However, chemokine dimerization can influence receptor interactions. In CXC chemokine dimers all the functionally important regions remain exposed so CXC chemokine dimers can retain the ability to activate receptors and, in some cases, induce different signalling responses relative to their monomeric forms (Veldkamp *et al*., 2008; Nasser *et al*., 2009; Drury *et al*., 2011; Gangavarapu *et al*., 2012; Ravindran *et al*., 2013). In contrast, CC chemokine dimerization causes burial of the functionally important N-terminus so CC chemokine dimers must dissociate to allow receptor activation (Jin *et al*., 2007; Tan *et al*., 2012).

## Sulfopeptides as models for chemokine receptor N-terminal regions

It is now well established that many chemokine receptors contain sulfated tyrosine residues in their N-terminal regions

## Table 1

Chemokine receptors known to be sulfated and their cognate chemokines

| Receptor | Chemokine ligands[1] | Receptor N-terminal amino acid sequence[2] | Key findings | References |
|---|---|---|---|---|
| CCR2 | CCL2/MCP-1<br>CCL7/MCP-3<br>CCL8/MCP-2<br>CCL11/eotaxin-1<br>CCL13/MCP-4<br>CCL16/HCC-4/LEC | $_{1}$MLSTSRSRFIRNTNESGEEVTTFFD**Y**D**Y**GAPC$_{32}$ | Y26 is sulfated<br>Y26A mutant has reduced receptor binding/activation.<br>Mutation of D25 reduces sulfation. | (Preobrazhensky *et al.*, 2000; Tan *et al.*, 2013) |
| CCR5 | CCL3/MIP-1α<br>CCL4/MIP-1β CCL5/RANTES<br>CCL8/MCP-2<br>CCL11/eotaxin-1<br>CCL14/HCC-1<br>CCL16/HCC-4/LEC | $_{1}$MD**Y**QVSSPI**Y**DIN**YY**TSEPC$_{20}$ | CCR5 is Tyr-sulfated.<br>Sulfated Tyr residues contribute to binding of CCL3, CCL4 and HIV-1 surface proteins. | (Farzan *et al.*, 1999) |
| CCR8 | CCL1/I-309<br>CCL4/MIP-1β<br>CCL16/ HCC-4/LEC<br>CCL17/TARC | $_{1}$MD**Y**TLDLSVTTVTD**YYY**PDIFSSPC$_{25}$ | N-terminal Tyr residues are sulfated.<br>Sulfated Tyr residues contribute to binding of I-309. | (Gutierrez *et al.*, 2004) |
| CXCR3 | CXCL9/Mig<br>CXCL10/IP-10<br>CXCL11/I-TAC | $_{1}$MVLEVSDHQVLNDAEVAALLENFSSS**Y**D**Y**GENESDSC$_{37}$ | Y27 and Y29 or CXCR3 are sulfated.<br>Mutation of Y27 or Y29 reduces binding and activation by CXCL9-11. | (Colvin *et al.*, 2006; Gao *et al.*, 2009) |
| CXCR4 | CXCL12/SDF-1 | $_{1}$MEGISI**Y**TSDN**Y**TEEMGSGD**Y**DSMKEPC$_{28}$ | N-terminal Tyr residues are sulfated.<br>Mutation of N-terminal Tyr residues reduces CXCL12 binding. | (Farzan *et al.*, 2002a) |
| $CX_3CR1$ | $CX_3CL1$/fractalkine | $_{1}$MDQFPESVTENFE**Y**DDLAEAC**Y**IGDIV$_{27}$ | Mutation of N-terminal Tyr residues or sulfatase treatment reduces fractalkine binding affinity. | (Fong *et al.*, 2002) |
| DARC | Many CC and CXC chemokines | $_{1}$MGNCLHRAELSPSTENSSQLDFEDVWNSS**Y**GVNDSFP DGD**Y**DANLEAAAPCHSCNLLDDS$_{60}$ | Y30 and Y41 are sulfated.<br>Mutation of Y30 and Y41 reduces binding to different chemokines.<br>Mutation of Y41 reduces binding to *Plasmodium vivax* Duffy binding protein. | (Choe *et al.*, 2005) |

[1]Chemokine ligands are those listed in Szpakowska *et al.* (2012).
[2]Potentially sulfated Tyr residues are shown in bold; acidic residues are underlined.

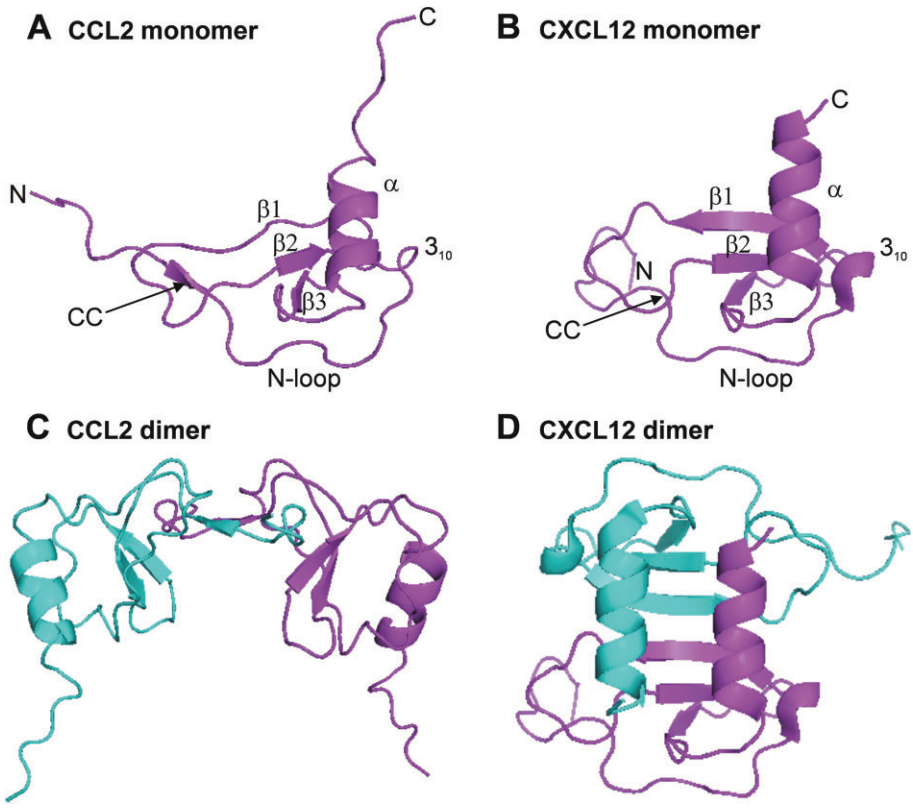



**Figure 3**

The structures of CC and CXC chemokines. Monomeric subunits of (A) CCL2 and (B) CXCL12 and non-covalent dimers of (C) CCL2 (protein databank file 1DOM) and (D) CXCL12 (protein databank file 2KO3) (Handel and Domaille, 1996; Veldkamp *et al.*, 2008). In the dimer structures, the different monomer subunits are coloured cyan and magenta.

and that these residues influence the interactions of chemokines with their receptors. However, the details of these interactions are just beginning to be characterized and many questions remain about the structural basis by which sulfation influences chemokine : receptor recognition. Three major factors make it difficult to address these questions using intact receptors. First, determination of chemokine receptor (or other GPCR) structures remains very challenging; the two published structures (Wu *et al.*, 2010; Park *et al.*, 2012) do not include a chemokine ligand and do not reveal the structure of the receptor N-terminus. Second, there are no well-established methods for controlling or determining the positions and extent of tyrosine sulfation in GPCRs expressed on cell surfaces. Third, receptor sulfation in mammalian cells is usually heterogeneous, greatly complicating the interpretation of cell-based binding and activation data. With time, the first two problems may be overcome. However, the heterogeneity is an intrinsic property of the receptors and cells and will remain a challenge.

To circumvent the difficulties inherent to studying intact receptors, we and others have used peptides containing sulfotyrosine (sulfopeptides) to begin to understand the influence of chemokine receptor tyrosine sulfation on chemokine recognition. Such sulfopeptides are likely to be reasonable mimics of the N-terminal regions of chemokine receptors, which are known to be highly flexible. Moreover, they have the advantage that chemokine interactions with sulfopeptides can be studied using a variety of rigorous biophysical and structural methods. Homogeneous sulfopeptides can be obtained by enzymatic sulfation of peptides (or expressed proteins) *in vitro*, then purification of specific sulfated forms by careful chromatography (Seibert *et al.*, 2008). Unfortunately, this approach is quite slow and laborious. Alternatively, it is now possible to synthesize specifically sulfated peptides using solid-phase methods and recently developed protection chemistry (Simpson *et al.*, 2009; Taleski *et al.*, 2011).

Table 2 summarizes the biophysical studies in which sulfopeptides have been used to investigate the structural and energetic basis of chemokine recognition by sulfotyrosine residues in chemokine receptors. The following sections focus on the major issues that have been addressed in these studies.

## The structural basis of sulfotyrosine recognition

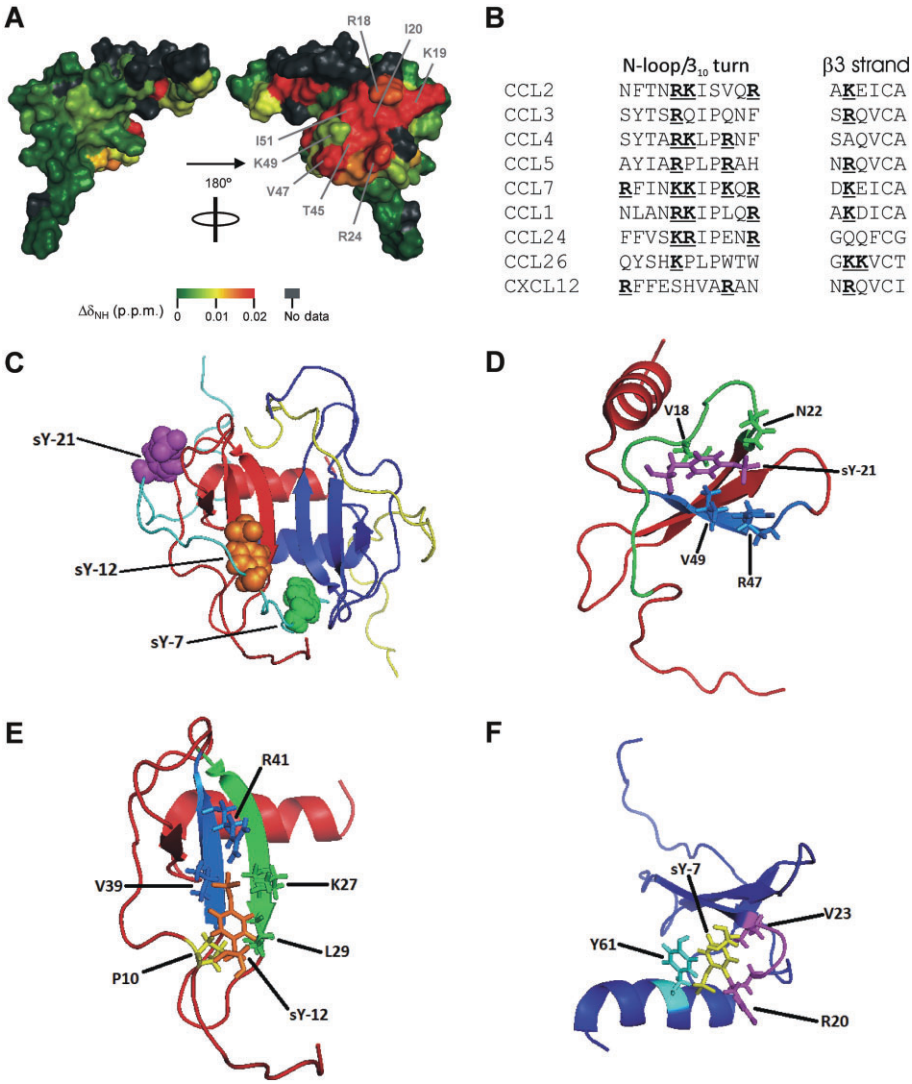
Due to the inherent flexibility and modest affinities of complexes between chemokines and receptor sulfopeptides, NMR spectroscopy has been the method of choice for characterization of these complexes. We and others have used NMR to

## Table 2

Biophysical studies of chemokine binding by receptor sulfopeptides

| Receptor | Chemokine(s) studied | Sulfopeptide sequences[1] | Key findings | References |
|---|---|---|---|---|
| CCR2 | CCL2/MCP-1<br>(wild type; obligate monomer P8A; obligate dimer T10C) | $_{18}$EEVTTFFD**Y**D**Y**GAP$_{31}$<br>(all four sulfation states) | All three forms of CCL2 bind CCR2 sulfopeptides.<br>Sulfation of single Tyr residues enhances affinity by 4- to 30-fold.<br>Sulfation of both Tyr residues enhances affinity additively for monomer and cooperatively for dimer.<br>Sulfopeptides destabilize dimeric CCL2 in favour of active monomer.<br>Sulfopeptides bind to N-loop/β3 site. | (Tan *et al.*, 2013) |
| CCR2 | CCL7/MCP-3 | $_{21}$TTFFD**Y**D**Y**GA$_{30}$<br>(non-sulfated, monosulfated and disulfated) | Single sulfation enhances CCL7 affinity ~4-fold.<br>Double sulfation enhances CCL7 affinity ~36-fold.<br>Binding affinities can be measured by electrospray mass spectrometry. | (Jen and Leary, 2010) |
| CCR3 | CCL11/eotaxin-1<br>CCL24/eotaxin-2<br>CCL26/eotaxin-3 | $_{8}$VETFGTTS**YY**DDVGLL$_{23}$<br>(all four sulfation states) | Single sulfation enhances binding to CCL11, 24 and 26 ~3- to ~30-fold.<br>Sulfation of Y16 and Y17 gives different affinity enhancements.<br>Double sulfation enhances affinity additively for CCL11 and CCL24, cooperatively for CCL26.<br>Sulfopeptides bind to N-loop/β3 site. | (Simpson *et al.*, 2009; Zhu *et al.*, 2011) |
| CCR5 | CCL5/RANTES | $_{1}$Nle**D**YQVSSPI**Y**D**I**N**YY**TSEPSQKINV$_{25}$<br>Nle = norleucine<br>(non-sulfated; Y10/Y14-disulfated) | Sulfation of Y10 and Y14 enhances affinity for CCL5 by >100-fold.<br>Sulfopeptide binds to N-loop/β3 site. | (Duma *et al.*, 2007) |
| CXCR4 | CXCL12/SDF-1 | GS$_{1}$MEGISI**Y**TSDN**Y**TEEMGSGD**Y**DSMKEPAFREENANFNK$_{38}$<br>(non-sulfated; Y21-sulfated; Y7/Y12/Y21-trisulfated) | Single and triple sulfation enhances affinity ~3- and ~30-fold respectively.<br>Sulfopeptides stabilize dimeric CXCL12.<br>NMR structures of complexes determined and sTyr binding sites identified (see text). | (Veldkamp *et al.*, 2006; 2008; Seibert *et al.*, 2008) |

[1]Potentially sulfated Tyr residues are shown in bold; acidic residues are underlined.




## Figure 4

Chemokine binding sites for sulfotyrosine residues. (A) Monomer structure of CCL2 (extracted from protein databank file 1DOM) with the left and right views related by a 180° rotation about the vertical axis. The protein surface is coloured to show the NMR chemical shift change of each backbone NH group induced by binding of a fully sulfated CCR2 N-terminal peptide (Tan *et al.*, 2013). (B) Sequence alignment of the regions defining the conserved sulfotyrosine binding pocket (N-loop/$3_{10}$-turn and β3-strand) for the chemokines listed in Table 2. Basic residues are underlined and in bold type. (C) Structure of cross-linked, dimeric CXCL12 bound to a triply sulfated peptide derived from its cognate receptor CXCR4 (Veldkamp *et al.*, 2008). CXCL12 subunits are coloured red and blue; the two CXCR4 sulfopeptides are shown as cyan and yellow ribbons with the three sTyr residues of the cyan sulfopeptide shown as spheres coloured magenta (sTyr-21), orange (sTyr-12) and green (sTyr-7). (D) Interactions of sTyr-21 (magenta sticks) with the conserved binding site of CXCL12, including the N-loop and $3_{10}$-turn (green) and the β3-strand (blue). (E) The CXCL12 binding site for sTyr-12 (orange sticks), showing the β1-strand (green) and β2-strand (blue). (F) The CXCL12 binding site for sTyr-7 (yellow sticks), showing the $3_{10}$-turn (magenta) and residue Tyr-61 from the α-helix (green).

identify the regions of chemokines that interact with receptor sulfopeptides. Typically these studies have involved comparison of two-dimensional $^{15}N$-$^{1}H$ correlation spectra of $^{15}N$-enriched chemokines in the presence and absence of sulfopeptides. Chemical shift changes for the backbone NH groups are mapped onto the chemokine structure, as shown for CCL2 (MCP-1) in Figure 4A, thus identifying the sulfopeptide binding site.

All the chemical shift mapping studies reported to date (listed in Table 2) have identified a conserved sulfopeptide binding site located within a shallow cleft on the chemokine surface defined primarily by residues of the N-loop and third β-strand (β3); in some cases, the neighbouring elements ($3_{10}$-turn and $\beta_2$-$\beta_3$ turn) also contribute to this site. There is substantial sequence variation in the N-loop and β3 regions of chemokines (Figure 4B), which is presumably important for receptor binding selectivity. However, in all cases the N-loop and β3 regions include some positively charged amino acids likely to be important for recognition of the negatively charged receptor sulfotyrosines. Notably, non-

sulfated peptides from the N-terminal regions of receptors also bind to this conserved site, albeit more weakly, indicating that other electrostatic and hydrophobic interactions also contribute to affinity. In addition to the conserved binding site, some sulfopeptide binding studies have revealed spectral changes in other regions of the chemokine structure, related to additional binding sites or changes in the oligomerization state of the chemokine (see below).

Volkman and co-workers have solved the structure of the chemokine CXCL12 (SDF-1) bound to three variants of an N-terminal sulfopeptide (residues 1-38) from receptor CXCR4: non-sulfated, singly sulfated (sTyr-21) and fully sulfated (sTyr-7, sTyr-12 and sTyr-21) (Veldkamp *et al.*, 2008). The sulfopeptides used in this study had been shown to enhance CXCL12 binding compared with the non-sulfated species (Farzan *et al.*, 2002a; Veldkamp *et al.*, 2006; Seibert *et al.*, 2008). Moreover, these peptides stabilize the dimeric state of CXCL12 (Veldkamp *et al.*, 2006). Therefore, structures were determined using a cross-linked dimeric form of the chemokine with two independent, symmetry-related peptide binding sites (Figure 4C). Each sulfopeptide molecule lies in an extended conformation across the dimer surface and forms interactions with both CXCL12 subunits.

In the complex with the fully sulfated peptide, sTyr-21 binds to the conserved binding site (Figure 4D) forming hydrophobic interactions with Val-18 (from the N-loop) and Val-49 (β3 strand) and electrostatic interactions with Asn-22 ($3_{10}$-turn) and Arg-47 (β3-strand). In most chemokines, there is an arginine or lysine residue in the β3-strand so the salt bridge between Arg-47 and sTyr-21 may represent a conserved recognition element (Veldkamp *et al.*, 2006; 2008; Ziarek *et al.*, 2011). Indeed, residues Val-18, Arg-47 and Val-49 are ~50% conserved among the 16 known CXC chemokines and a tyrosine corresponding to sTyr-21 of CXCR4 may likewise be found in all CXC family receptors. Nevertheless, there is considerable variation of amino acid residues in the conserved binding pocket consistent with the substantial variation in the number and spacing of potentially sulfated Tyr residues within receptor N-terminal regions. Thus, it is likely that chemokines and their receptors have co-evolved by utilizing sTyr recognition to achieve binding selectivity, as discussed below.

To explore the potential of chemokine inhibition by targeting the conserved binding site, Veldkamp *et al*. performed *in silico* screening of a virtual compound library and identified several compounds able to bind to the N-loop/β3-strand binding site of CXCL12 (Veldkamp *et al.*, 2010). Moreover, the tightest binder identified from this screen, a benzoic acid derivative with structural similarity to sTyr, inhibited the ability of CXCL12 to activate CXCR4 but did not affect the activity of CCL2. This result suggests that it may be possible to develop therapeutics that inhibit specific chemokines by targeting the sTyr-binding site.

In addition to revealing the structure of the conserved binding site, the complex of CXCL12 bound to the fully sulfated CXCR4 peptide includes distinct binding sites for the other sTyr residues. Residue sTyr-12 is positioned on the opposite face of the β-sheet in the same chemokine subunit as the conserved binding site and interacts with residues in the first and second β-strands (Figure 4E). Residue sTyr-7 interacts with residues on the other monomer subunit and is positioned on the opposite face of the $3_{10}$-turn from the conserved binding site, also interacting with residue Tyr-61 from the α-helix (Figure 4F). Notably, residues sTyr-7 and sTyr-12 and their CXCL12 binding sites are not conserved in the CXC receptors or chemokines respectively. However, a number of chemokine receptor N-termini contain widely spaced tyrosine residues that are potential candidates for sulfation, so similar multivalent complexes may be formed by other receptor : chemokine pairs.

## Receptor tyrosine sulfation enhances chemokine affinity in a site-specific manner

Considering that mutation of receptor tyrosine residues or inhibition of sulfation causes a reduction in chemokine binding affinity and potency, it is unsurprising that sulfated peptides derived from chemokine receptors bind to the cognate chemokines of those receptors with higher affinity than their non-sulfated analogues. The homogeneity of the sulfopeptides has made it possible to determine the specific affinity enhancements resulting from sulfation of each individual tyrosine residue. The observed affinity enhancements (Table 2) for sulfation of a single tyrosine residue have ranged from ~3-fold to as much as ~30-fold (Mayer and Stone, 2000; Ye *et al.*, 2000; Farzan *et al.*, 2002b; Veldkamp *et al.*, 2006; Duma *et al.*, 2007; Seibert *et al.*, 2008; Simpson *et al.*, 2009; Jen and Leary, 2010; Zhu *et al.*, 2011; Tan *et al.*, 2012; Tan *et al.*, 2013). A caveat of these binding studies is that they have commonly been performed under the low salt conditions at which modern NMR probes have the highest sensitivity, thus exaggerating the strength of electrostatic interactions. Nevertheless, the results highlight some general principles that are likely to be retained under physiological conditions.

A key finding is that sulfation of the same receptor peptide at different positions can have a markedly different effect on chemokine binding affinity. For example, sulfation of a CCR3-derived peptide at Tyr-17 enhances binding to CCL11 (eotaxin-1) by a factor of ~7, whereas sulfation of the same peptide at the adjacent Tyr-16 enhances binding to the same chemokine ~28-fold (Simpson *et al.*, 2009). At this stage, it is unknown whether the predominant sites of sulfation for a particular receptor differ according to the cell type expressing the receptor or additional environmental factors although this seems quite likely considering the variations in the expression levels of TPST isoforms among different cell types. The peptide binding data suggest that such variations would alter the affinities of chemokines for their receptors and thereby regulate cellular responses to chemokines.

Chemokine binding data for the decoy chemokine receptor DARC (Duffy antigen) support the possibility that differential receptor sulfation could modulate chemokine selectivity (Choe *et al.*, 2005). DARC is expressed on reticulocytes, the precursor cells of erythrocytes, and binds chemokines of both the CC and CXC families. DARC is not G-protein coupled but chemokine binding induces internalization of the receptor-chemokine complexes, thus down-regulating chemokine activity. DARC is tyrosine sulfated at

positions 30 and 41. Mutation of Tyr-41 to Phe suppresses binding of the chemokines CCL2, CCL5 (RANTES) and CXCL1 (MSGA) but not that of CXCL8 (IL-8), whereas mutation of Tyr-30 to Phe suppresses binding of CXCL8 but not the other three chemokines (Choe *et al.*, 2005). These data strongly suggest that the forms of DARC sulfated at different sites would also differ in their chemokine selectivity.

## Receptor tyrosine sulfation modulates chemokine selectivity

An important principle to emerge from studies of sulfopeptides binding to chemokines is that sulfation of a receptor peptide can change not only the affinity of chemokine binding but also the selectivity (i.e. relative affinity) of binding among different chemokine ligands of the same receptor. For example, as shown in Figure 5, the non-sulfated CCR3 peptide binds to the chemokines CCL11, CCL24 (eotaxin-2) and CCL26 (eotaxin-3) with similar affinities (Zhu *et al.*, 2011). However, the sTyr-16 sulfopeptide has approximately ~6- to ~8-fold higher affinity for CCL11 than for CCL24 and CCL26. The sTyr-17 sulfopeptide shows less discrimination, with ~2- to ~4-fold higher affinity for CCL11 than for the other chemokines. Most interestingly, the peptide sulfated on both sTyr-16 and sTyr-17 displays different chemokine selectivity from the non-sulfated or either of the singly sulfated variants, binding with similar affinities to both CCL11 and CCL26 but substantially more weakly to CCL24 (Zhu *et al.*, 2011). The main reason for this is that the two sulfate groups of the doubly sulfated CCR3 peptide appear to interact in a cooperative manner with CCL26 but an additive manner with the other two chemokines. Thus, in comparison to the peptide sulfated on only Tyr-16, the doubly sulfated peptide binds ~3 to ~4-fold more strongly to CCL11 and CCL24 but a dramatic ~80-fold more strongly to CCL26. The N-loop and β3-strand sequences of CCL26 differ substantially from those of CCL11 and CCL24 (Figure 4B) so presumably the binding cooperativity results from distinct structural interactions with these residues. While much work remains to understand the observed selectivity differences, the key conclusion to date is that addition of one or more sulfate group(s) to receptor Tyr residue(s) can modulate the binding selectivity among cognate chemokine ligands. Again, this is a potential mechanism for regulating differential cellular responses to chemokines.

## Receptor N-terminal sulfopeptides can modulate chemokine oligomerization

As discussed above, CC and CXC chemokines form distinct dimer structures. Whereas CXC chemokine dimers can maintain the ability to activate receptors, CC chemokine dimerization obscures the receptor activation elements. Three studies have shown that binding to receptor sulfopeptides can influence the dimerization states of chemokine ligands.

Veldkamp *et al.* have shown that binding to a singly sulfated (sTyr-21) N-terminal peptide from CXCR4 shifts the monomer-dimer equilibrium for the CXC chemokine CXCL12 towards the dimer form (Veldkamp *et al.*, 2006). Previous studies had shown that the CXCL12 monomer-dimer equilibrium is modulated by pH and salt and shifted towards the dimer by GAG binding (Veldkamp *et al.*, 2005). Stabilization of the dimer by the receptor sulfopeptide presumably results from the sulfopeptide forming binding interactions with residues on both monomer subunits, as described in detail above (Figure 4C–F).

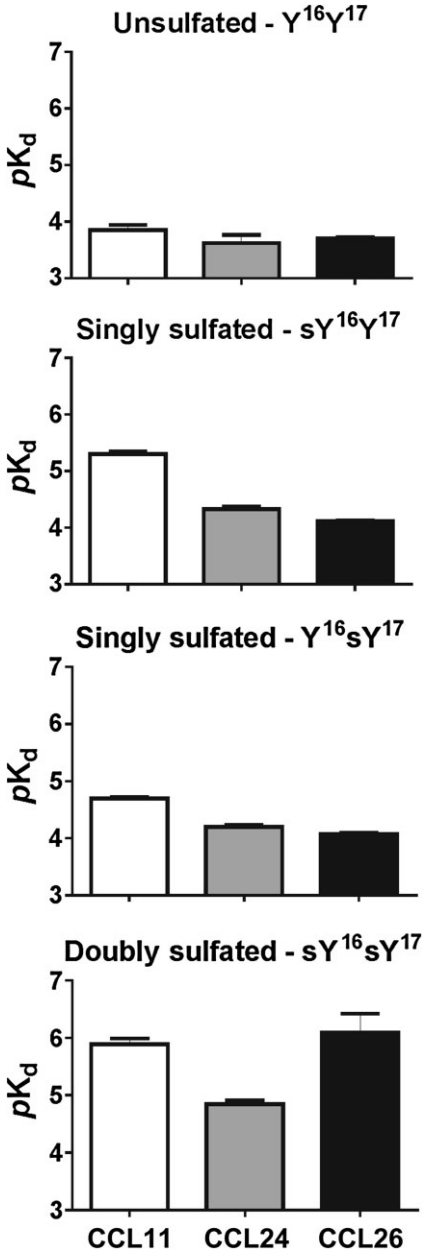
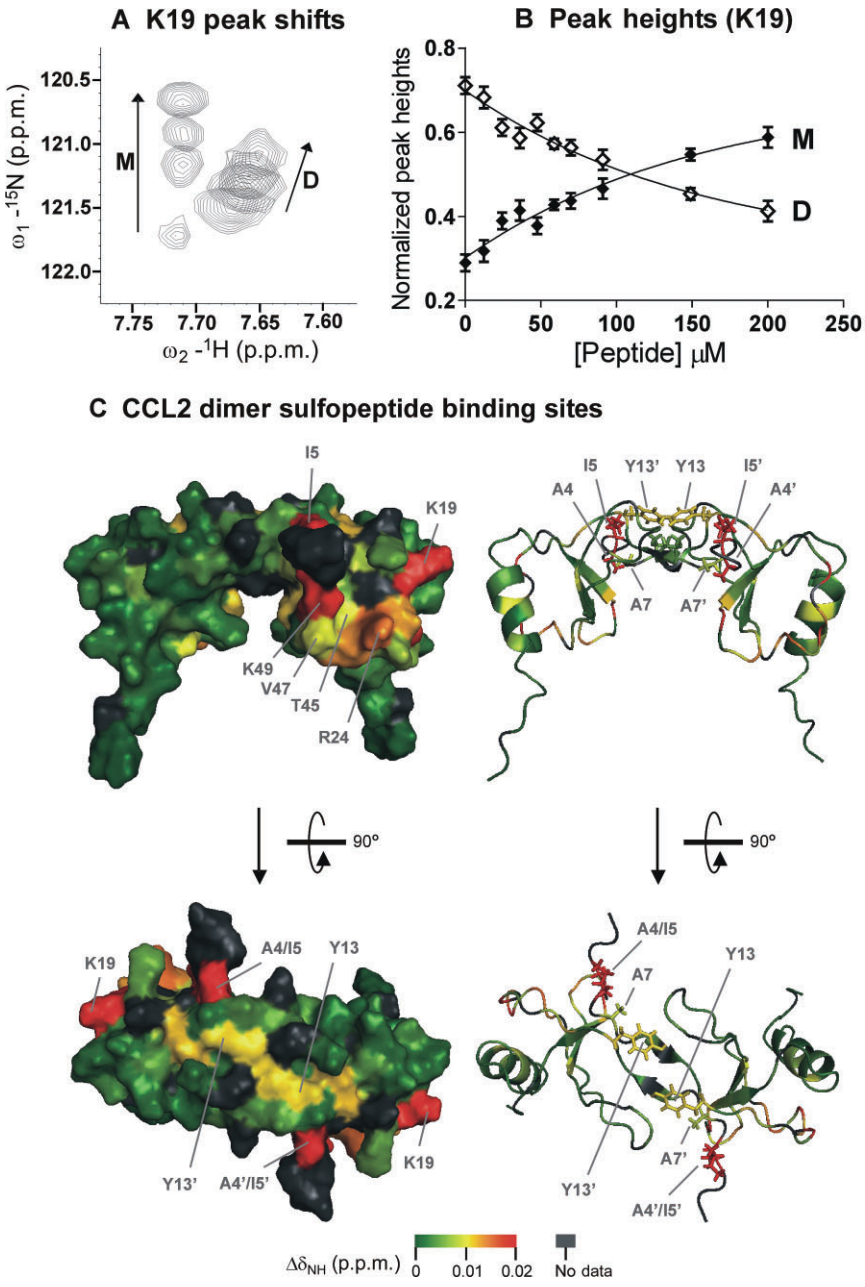



**Figure 5**

Tyrosine sulfation of a receptor peptide changes selectivity among different chemokines (Zhu *et al.*, 2011). Each panel shows the $pK_d$ for binding of a CCR3-derived peptide (residues 8-23) to the three chemokines CCL11, CCL24 and CCL26. The four panels show data for the four different sulfation states of the receptor peptide.




## Figure 6

Sulfopeptide binding modulates chemokine oligomerization equilibria (Tan *et al.*, 2013). Data are shown for wild-type CCL2 interacting with a CCR2 receptor peptide (residues 18-31) sulfated at (A, B) position Tyr-26 or (C) positions Tyr-26 and Tyr-28. (A) The NMR peaks for the amide NH signal of Lys-19 in both the monomer and dimer forms of the protein shift as the sulfopeptide concentration is increased (arrows). (B) Averaged normalized peak heights for five residues (K19, L25, I42, F43 and C52) in the monomeric (M) and dimeric (D) forms of CCL2; as the concentration of peptide increases monomer signals increase at the expense of dimer peaks. (C) Space filling and ribbon representations of dimeric CCL2 in two orthogonal orientations, coloured to show the NMR chemical shift change of each backbone NH group induced by binding of a fully sulfated CCR2 N-terminal peptide. Residues shown as sticks are influenced by peptide binding and also involved in dimerization.

In contrast to the dimer stabilization observed for CXCL12, studies of two CC chemokines have shown that binding to receptor sulfopeptides shifts the monomer-dimer equilibrium in favour of the monomeric state. For the chemokine CCL5, a doubly sulfated peptide from receptor CCR5 was shown to bind exclusively to the monomeric state, thereby shifting the monomer-dimer equilibrium towards the monomer (Duma *et al.*, 2007). The lack of observable binding interactions with the CCL5 dimer was suggested to arise from the considerable overlap between the dimer interface and sulfopeptide binding site.

Our recent study of interactions between CCL2 and sulfopeptides from receptor CCR2 (Tan *et al.*, 2013) revealed both similarities to and differences from the CCL5: CCR5

interaction. In addition to wild-type CCL2, for which both monomer and dimer signals were observable in NMR spectra, we used mutants of CCL2 that were trapped in either the monomeric or dimeric state. Whereas the monomer retains wild-type ability to activate CCR2, the dimer does not activate the receptor, consistent with burial of the chemokine N-terminus upon dimerization (Tan *et al.*, 2012). As shown in Figure 6A, addition of a CCR2 sulfopeptide to NMR samples of wild-type CCL2 causes both monomer and dimer peaks to shift, indicating that both species can bind to the receptor sulfopeptide; this was confirmed using the trapped monomeric and dimeric mutants. Moreover, as the sulfopeptide concentration was increased, the intensities of monomer peaks increased at the expense of dimer peaks (Figure 6B), clearly indicating that CCR2 sulfopeptides shift the equilibrium preferentially towards the monomeric form (Tan *et al.*, 2013). Using the trapped dimeric mutant, we showed that CCR2 sulfopeptides bind to the conserved N-loop/β3-strand binding cleft of dimeric CCL2 but that they also cause small perturbations in the N-terminal dimerization interface (Figure 6C). Apparently, these perturbations weaken the dimerization promoting dissociation of the inactive dimeric to the active monomeric species. Remarkably, the sulfopeptide whose sulfation pattern mirrored that of intact CCR2 expressed in HEK293 cells (singly sulfated at position Tyr-26) was the most effective at destabilizing the dimeric form in favour of the active monomer, whereas the non-sulfated CCR2 peptide did not bind observably to either monomeric or dimeric species. In a biological context, one could imagine that binding of dimeric CCL2 to the sulfated N-terminus of CCR2 (the first step in the two-step model; Figure 2) could lead to chemokine dimer dissociation on the receptor, thus allowing receptor activation by the newly exposed chemokine N-terminus (the second step in the two-step model). Thus, the sulfated receptor appears to promote activation of its own ligand!

## Summary and future directions

It is clear that tyrosine sulfation of chemokine receptors greatly influences their chemokine interactions. Sulfopeptides derived from the N-terminal regions serve as excellent models to understand the molecular basis of these interactions. Receptor sulfopeptides bind to a shallow cleft defined by N-loop and β3-strand elements of cognate chemokines. Although the location of this binding site is conserved across the chemokine family, the chemokine selectivity of receptor N-terminal regions results from the specific arrangement of basic and hydrophobic residues in this binding site. Sulfation of tyrosine residues in receptors and receptor peptides enhances the affinity of chemokine interactions in a site-specific manner and can furthermore control the selectivity of a given receptor among different cognate chemokines. Finally, binding to receptor sulfopeptides can modulate the oligomerization state of chemokines, thereby influencing the ability of a chemokine to activate its receptor.

Considering the various effects of chemokine receptor sulfation on chemokine interactions, there is strong motivation to understand the structural basis by which sulfation influences binding selectivity and chemokine oligomerization as well as the biological consequences of these effects. Additional biophysical and structural studies using sulfopeptides should reveal the general rules that govern chemokine binding interactions, thus paving the way for rational strategies to modulate these interactions pharmacologically. As mass spectrometry methods and sulfation-specific reagents are developed, it will become possible to characterize the specific sulfation states of chemokine receptors on cultured cells and in biological samples and to test hypotheses regarding the roles of specifically sulfated receptors in normal immune function and disease.

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## Conflict of interest

None.

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