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NMR indicates the N-termini of PSGL1 and CCR7 bind competitively to the chemokine CCL21

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ABSTRACT

Chemokines are from a family of secreted cytokines that direct the trafficking of immune cells to coordinate immune responses. Chemokines are involved in numerous disease states, including responding to infections, autoimmune disorders, and cancer metastasis. Ther are chemokines, like CCL21, that signal for cellular migration through the activation of G protein-coupled receptors, like CCR7, through interaction with the receptor's extracellular N-terminus, loops, and core of the receptor. CCL21 is involved in routine immune surveillance but can also attract metastasizing cancer cells to lymph nodes. P-selectin glycoprotein ligand 1 (PSGL1) has a role in cellular adhesion during chemotaxis and is a transmembrane signaling molecule. PSGL1 expression enhances chemotactic responses of T cells to CCL21. Here NMR studies indicate the binding sites on CCL21 for the N-termini or PSGL1 and CCR7 overlap, and binding of the N-termini of PSGL1 and CCR7 is competitive.

1. Introduction

Chemokines, also called chemotactic cytokines, are a family of secretory proteins that are a vital part of the immune system and directing the trafficking of immune cells as part of an immune response or homeostasis. They can also play roles in many disease states from autoimmune and inflammatory diseases to infections or cancer [1–4]. Chemokines, like CCL19 or CCL21, signal by activating chemokine G protein-coupled receptors, like CCL19 and CCL21's receptor CCR7 [5,6]. For example, the chemokines CCL19 and CCL21 help recruit antigen-presenting dendritic cells and naive T-cells to the lymph nodes to help prime the adaptive immune response. At the same time, CCL19 and CCL21 are also hypothesized to attract metastatic cancer cells expressing CCR7 to the lymph nodes for secondary tumor formation [4–8].

Activation of chemokine receptors, which are integral membrane heptahelical G-protein coupled receptors (GPCR), is thought to occur through a two-step, two-site model in which the N-terminus of the chemokine receptor binds the chemokine first followed by the chemokine's N-terminus binding and activating the receptor [9]. While some have moved to more complex models than two-step, two-site through proposing a 1.5 step and site or a beyond the two-site model [10,11], past and continued investigations of the interactions of various chemokines with N-terminal peptides derived from their receptor continues to provide important structural information [12–22].

The binding of a chemokine to its typical chemokine GPCR leads to intracellular signaling typically through heterotrimeric G-proteins and beta-arrestin generating responses like migration and chemotaxis towards higher chemokine concentrations [6,23–25]. The binding of a chemokine to its atypical chemokine receptor generally leads to signaling through beta-arrestin and, commonly, internalization that effectively scavenges the chemokine from the extracellular environment and does not result in cellular migration [6,23,24,26]. As secreted proteins, chemokines diffuse from the cells that produce them; usually a site of inflammation or injury, or a tissue or organ involved in homeostatic immune system function, while binding to extracellular

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glycosaminoglycans in concert with atypical chemokine receptors forms and shapes the concentration gradient for cells expressing typical chemokine receptors to follow [27–30]. CCL21's extended basic tail enhances its binding to glycosaminoglycans [27,31,32]. The canonical chemokine binding partners are their receptors, either typical or atypical, and glycosaminoglycans [9]. For example, CCL21 binds to the typical chemokine receptor CCR7 [33] and GPR174 [34] and the nontypical chemokine receptor ACKR4 [30,35]. Outside of these normal chemokine binding partners, P-Selecting Glycoprotein Ligand 1 (PSGL1) is reported to bind CCL21 and CCL19 and, through doing so, enhances the chemotactic response of PSGL1 and CCR7 expressing T-cells to these chemokines [14,36,37].

Classically, PSGL1's role in chemotaxis involves leukocyte rolling and tethering [36]. PSGL1, a type I membrane protein, is expressed as a disulfide-linked homodimer whose N-terminal O-glycan modifications, when bound by selectins, can induce signaling events that influence cell phenotype and function [36,37]. Furthermore, PSGL1's acidic N-terminus also contains tyrosines that can be posttranslationally modified to sulfotyrosines [36,37]. Chemokine receptor N-termini are acidic and can contain sulfotyrosines that increase affinity for chemokine ligands [17,38]. Veerman et al. [37] identified CCL21 as a PSGL1 binding partner and showed that PSGL1's presence can improve T-cell chemotaxis towards CCR7 ligands CCL19 and CCL21 and enhances homing to lymph nodes and is independent of PSGL1's interaction with selectins [37]. Previously, undergraduates at the University of Wisconsin-Whitewater as a part of CURE (Course-based Undergraduate Research Experience [39–41]) lab classes, contributed to experiments indicating that CCL19 bound the N-termini of both CCR7 and PSGL1 and that binding was competitive [14]. Here we, including undergraduates as a part of a CURE class, present evidence that CCL21 binds to the N-terminus of PSGL1, that the PSGL1 binding site overlaps with that of the N-terminus of CCR7, and that the N-termini of PSGL1 and CCR7 can compete for binding to CCL21.

2. Materials and methods

2.1. Protein expression and purification

Natural abundance and uniformly ^{15}N labeled [U–^{15}N] CCL21 was expressed and purified as described previously [13,14,42]. Residues 1–15 of PSGL1 ^{1}QTAEYEYLDYDFLPE^{15} were purchased as a purified peptide generated by a commercial solid phase peptide synthesis company. Natural abundance and [U–^{15}N] N-terminal PSGL1 residues 2–15 (^{2} TAEYEYLDYDFLPE ^{15}) and CCR7 residues 2–30C24A (^{2}DEVTDDYIGDNTTVDYTLFESLASKKDVR^{30}) peptides were expressed and purified recombinantly as previously described [14] and were used in the competition assays to ensure each peptide was uniform. The N-terminal residue of both mature PSGL1 and CCR7 is glutamine, which in solution partially reacts spontaneously to form pyroglutamate resulting in a mixture of peptide with either Gln1 or pGlu1.

2.2. Nuclear magnetic resonance

NMR data was collected at the Medical College of Wisconsin's NMR facility on either a 500 or 600 MHz NMR spectrophotometer equipped with a ^{1}H/^{15}N/^{13}C cryoprobe at 25 °C in NMR buffer, 25 mM deuterated MES pH 6.0. CCL21 and N-terminal CCR7 and PSGL1 peptide chemical shift assignments are from Love et al. and Veldkamp et al. [13,14].

2.3. Chemical shift mapping

2D ^{15}N–^{1}H HSQC spectra monitored a titration of [U–^{15}N] CCL21 in NMR buffer with increasing amounts of PSGL1 1–15 peptide and binding was in fast exchange allowing chemical shifts to be transferred by inspection. Combined amide chemical shift perturbations for CCL21 amino acids and the CCL21 PSGL1 N-terminal dissociation constant (K_d) were determined as previously described [13,14].

2.4. Competitive binding NMR assay

Titrations were monitored using 2D ^{15}N–^{1}H HSQC spectra and all samples were in NMR buffer. 100 μM [U–^{15}N] PSGL1 2–15 was titrated with additions of CCL21 followed by additions of CCR7 2–30C24A. Molar ratios of [U–^{15}N] PSGL1:CCL21:CCR7 for samples were as follows 1:0:0, 1:1:0, 1:2:0, 1:2:1, 1:2:3, 1:2:7, and 1:2:10. 100 μM [U–^{15}N] CCR7 2–30C24A was titrated with incremental additions of CCL21 followed by additions of PSGL1 2–15. Molar ratios of [U–^{15}N] CCR7:CCL21:PSGL1 for samples were as follows 1:0:0, 1:1:0, 1:2:0, 1:2:1, 1:2:3, 1:2:7, and 1:2:10.

3. Results

3.1. CCL21 binds the N-terminus of PSGL1 and the binding site overlaps with the site for binding the N-terminus of CCR7

To assess if CCL21 binds the PSGL1 N-terminus, [U–^{15}N] CCL21 was titrated with increasing amounts of PSGL1 peptide and monitored using protein NMR. Fig. 1A shows overlaid ^{15}N–^{1}H HSQC spectra of CCL21 with increasing concentrations of PSGL1 peptide. Chemical shift perturbations observed for some amino acid cross peaks indicate binding. Combined amide chemical shift perturbations in CCL21 induced by 1500 μM PSGL1 peptide are plotted versus CCL21 residue with residues showing a perturbation greater than 0.25 shown in red (Fig. 1B). Chemical shift perturbations for residues 12, 14, 19, 21, 22, 23, 46, 51, 63, 64, 67, 68, 69, 70, 74, and 75 showed dose-dependent changes with increasing PSGL1 peptide concentration and were used for determining a dissociation constant (K_d) of 300 ± 100 μM for CCL21 and the PSGL1 peptide through non-linear regression. The inset of Fig. 1B shows representative data and fits for three of these residues. Fig. 1C highlights CCL21 residues with a chemical shift >0.25 in red, which indicates potential binding residues for the PSGL1 N–terminus. The perturbed residues cluster in and between the N-loop and third beta-strand of CCL21 and extend up through the C-terminal end of and just past CCL21's alpha helix. A few of these regions of CCL21 are also perturbed when titrated with N-terminal peptides from the receptor CCR7 [13,38]. Fig. 1D indicates residues perturbed by the CCR7 N-terminus residues 1–30 shown in the blue map to the CCL21 N-loop and third beta-strand, indicating the binding sites for the CCR7 and PSGL1 N-termini may overlap. The perturbed residues for both PSGL1 and the CCR7 N-terminus are shown in Fig. 1E with the same color scheme as in Fig. 1C, and D. Residues that both PSGL1 and CCR7 perturbs are highlighted in purple. As expected, these residues cluster in the N-loop and the third beta-strand of CCL21. Shorter CCR7 N-terminal peptides, like CCR7 residues 5–11, also perturb the end of CCL21's alpha helix [38], indicating there may be more of an overlap in the binding sites than Fig. 1E indicates.

3.2. The N-termini of PSGL1 and CCR7 can compete for binding to CCL21

The overlapping binding sites on CCL21 for N-terminal peptides from CCR7 and PSGL1 suggest that these peptides could compete for binding to CCL21. To address this, HSQC spectra of the N-terminus of CCR7 or the PSGL1 alone indicate an unbound state (Fig. 2A and B, black spectra). Adding CCL21 to the CCR7 or the PSGL1 N-terminus and the resulting chemical shift perturbations for some residues suggests the peptides bind to CCL21 (Fig. 2A and B gray and light gray spectra). When the CCR7 N-terminus CCL21 complex is titrated with PSGL1 peptide, residues with chemical shift perturbations, for example, CCR7 I9 and S25, retreat towards their unbound chemical shifts with increasing PSGL1 concentrations (Fig. 2A, increasing red peaks). The displacement of the CCL21 bound CCR7 by the PSGL1 N-terminus

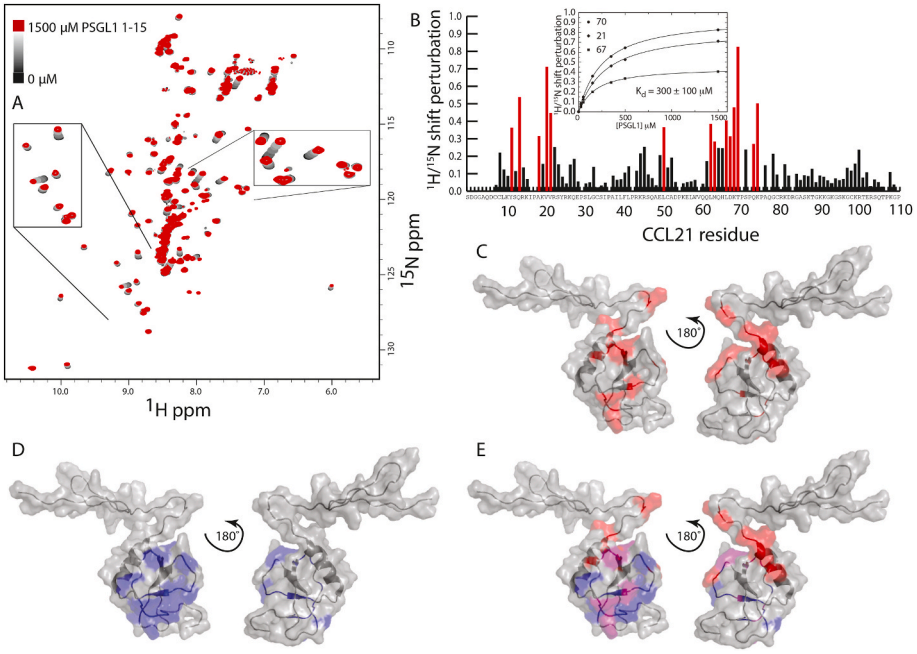

Fig. 1. PSGL1's N-terminus (QTAEYEYLDYDFLPE) binds CCL21 with a micromolar K_d potentially with a binding site that overlaps that of the CCR7 N-terminus. (A) ^{15}N–^{1}H HSQC spectra of [U–^{15}N] CCL21 alone (black) with increasing concentrations of PSGL1 (QTAEYEYLDYDFLPE) (increasing grays) and with 1500microM PSGL1 (red). (B) Combined CCL21 amide chemical shift perturbations induced by 1500microM PSGL1 plotted versus CCL21 residue. CCL21 residues with a combined chemical shift perturbation of greater than 0.25 are colored red. Residues without observable signal or prolines have a zero-perturbation value. Inset, combined amide chemical shift perturbations for CCL21 residues 21, 63 and 70 plotted versus PSGL1 concentration along nonlinear fitting used for the K_d determination. Fits are representative of those for all residues (12, 14, 19, 21, 22, 23, 46, 51, 63, 64, 67, 68, 69, 70, 74, and 75) used in the K_d determination. (C) Mapping the CCL21 residues (red) with chemical shift >0.25 onto the surface of CCL21 indicates potential PSGL1 N-terminus binding residues. (D) Perturbations induced by the CCR7 N-terminus (residues 1–30) taken from Love et al. mapped onto CCL21 (blue). (E) CCR7 perturbed residues (blue), PSGL1 perturbed residues (red) and residues perturbed by both the CCR7 and PSGL1 N-terminal peptides (purple). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

suggests competitive binding to CCL21. The PSGL1 CCL21 complex was titrated to confirm competitive binding with the CCR7 N-terminal peptide (Fig. 2B, blue peaks). PSGL1 residues with chemical perturbations, like PSGL1, D10, and L12, returned to their unbound state with increasing additions of CCR7 peptide. This, combined with CCL21 having overlapped binding sites for the N-termini of CCR7 and PSGL1 (illustrated in Fig. 1E), strongly indicates competitive binding to CCL21.

4. Discussion

Here we confirm the direct interaction of CCL21 and PSGL1 observed by Veerman et al. [37] and through chemical shift mapping have identified a likely binding site in CCL21 for the N-terminus of PSGL1. As observed for CCL19 [14], CCL21's PSGL1 binding site overlapped with that of the binding site for the CCR7 N-terminus and binding of these N-terminal CCR7 and PSGL1 peptides to CCL21 is competitive. Not unlike the N-terminus of CCR7, the PSGL1 N-terminus and the PSGL1 peptides used here contain polar and acidic amino acids. For example, the PSGL1 N-terminal peptides used here include three glutamic and two aspartic acid residues. CCL21 is a very basic protein and PSGL1 induced perturbations in CCL21 include basic amino acids or residues near basic amino acids, but perturbations are not found in the highly basic CCL21 extended C-terminus (Fig. 1B). This suggests the interaction between CCL21 and PSGL1's N-terminus is, in part but not in total, electrostatic in nature and that the interaction is specific in nature versus solely based on non-specific electrostatic interactions. Posttranslational modifications found in the N-terminus of PSGL1, like sulfotyrosines that were not included in any of the peptides used here, would also likely increase affinity for CCL21. Tyrosines in the PSGL1 N-terminus can be sulfated [36,43] and that would presumably increase the interaction with CCL21 similar to how N-terminal CCR7 sulfotyrosines enhances affinity for CCL21 [38]. In fact, sulfotyrosines in the N-terminus of PSGL1 are reported as essential for binding to the chemokine CCL27 [44].

While it is clear sulfotyrosines should enhance binding of CCL21 to PSGL1, how competition between PSGL1 and CCR7 for CCL21 could contribute to PSGL1's enhancement of chemotaxis induced by CCL21 activation of CCR7 and the increased trafficking of resting T-cells to secondary lymphoid organs observed by Veerman et al. is less clear [37]. When we observed similar competition between the N-terminal peptides of PSGL1 and CCR7 for CCL19, we speculated on how PSGL1's expression in CCR7 expressing T-cells resulted in enhanced chemotaxis [14]. We make similar speculations here. CCL19 and CCL21 could be signaling directly through activation of PSGL1 resulting in Veerman et al.'s [37] observed enhancements; or PSGL1 may be serving to protect CCL19 or CCL21 from chemokine sinks like atypical chemokine receptors allowing for increased activation of CCR7. Other potential explanations could be that PSGL1 expression in T-cells is serving as a reservoir for CCL19 or CCL21 for latter presentation to or release for continued activation of T-cell CCR7.

Yet another possibility for how PSGL1 could impact CCR7 directed chemotaxis is that for cells exposed to both CCL19 and CCL21, PSGL1 might select which chemokine has more opportunity to activate CCR7. This would be hypothesized to occur through PSGL1 binding more strongly to one chemokine leaving the other more available to activate CCR7. This could be biologically important as CCL19 and CCL21 are not equivalent agonists of CCR7; CCL19 and CCL21 are biased agonists whose signaling through CCR7 results in different outcomes [6,23,25,32,45,46]. Here we show that a N-terminal PSGL1 peptide not containing posttranslational modifications like sulfotyrosines binds to CCL21 with a K_d of 300 μM while CCL19 binds the same peptide with a K_d of about 20 μM [14]. Differing extents of post translational modifications, including sulfotyrosines or glycosylation [43], in the N-termini of full length, intact CCR7 and/or PSGL1 might impact which chemokine has more opportunity to activate CCR7 in cells expressing PSGL1.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

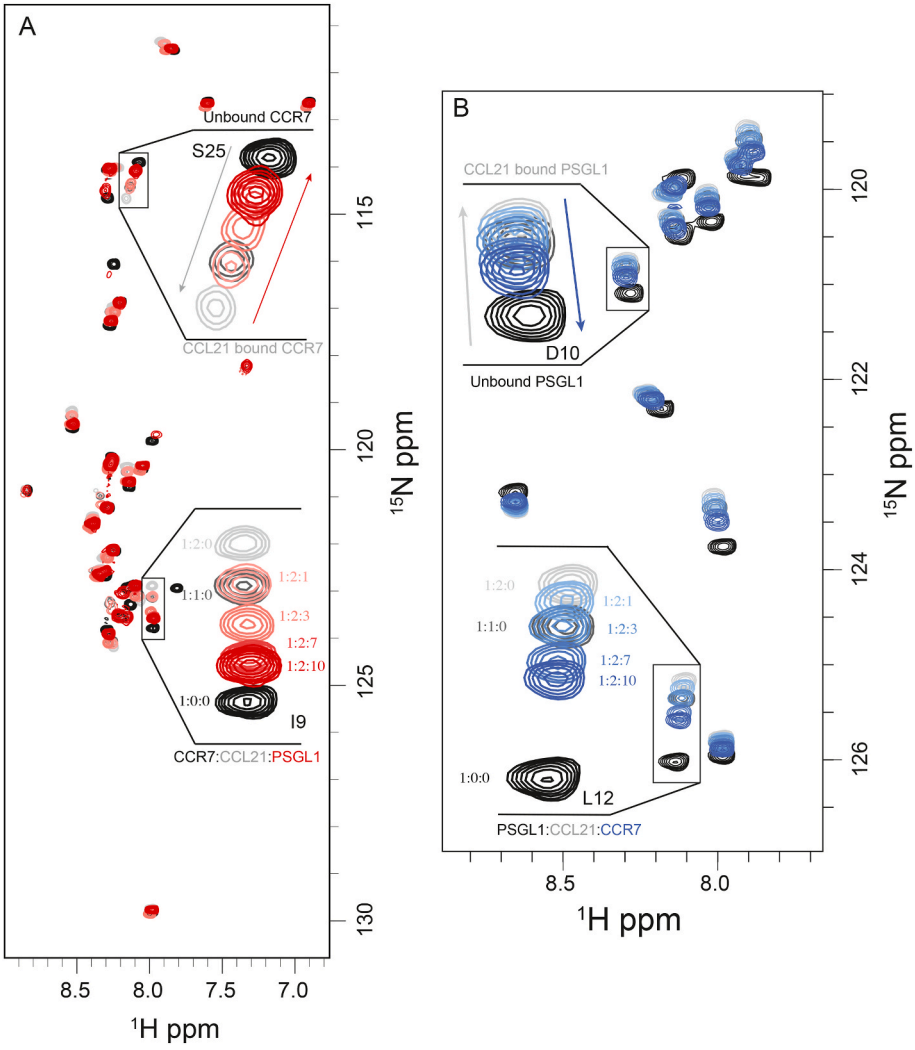

Fig. 2. The N-terminus of CCR7 and PSGL1 compete for binding to CCL21. A) ^{15}N – ^{1}H HSQC spectra of 100 μM [U–^{15}N] CCR7 with increasing concentrations of CCL21 and then PSGL1 overlaid. The black spectra show 1:0:0 [U–^{15}N]CCR7:CCL21:PSGL1 (no CCL21 and no PSGL1 and unbound CCR7). The gray and lighter gray spectra show 1:1:0 and 1:2:0 M ratios of [U–^{15}N]CCR7:CCL21:PSGL1. The spectra with increasing red tones are of molar ratios of 1:2:1 (light peach), 1:2:3, 1:2:7, and 1:2:10 (true red) of [U–^{15}N]CCR7:CCL21:PSGL1. B) ^{15}N – ^{1}H HSQC spectra of 100 μM [U– ^{15}N] PSGL1 (black) with increasing concentrations of CCL21 (gray) and CCR7 (blue) overlaid. The black spectra show the 1:0:0 [U–^{15}N] PSGL1:CCL21:CCR7 or unbound PSGL1 with 1:1:0 (gray) and 1:2:0 (lightest gray), indicating CCL21 binding. The spectra with increasing blue tones are of molar ratios of 1:2:1 (light blue), 1:2:3, 1:2:7, and 1:2:10 (darkest blue) of [U–^{15}N]PSGL1:CCL21:CCR7. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Francis Peterson has ownership interest in Protein Foundry LLC and XLock Biosciences LLC.

Data availability

Data will be made available on request.

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