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Solution structure of CCL21 and identification of a putative CCR7 binding site

Melissa Love^{1,^}, Jamie L. Sandberg^{1,^}, Joshua J. Ziarek², Kyle P. Gerarden¹, Renee R. Rode¹, Davin R. Jensen², Darrell R. McCaslin³, Francis C. Peterson², and Christopher T. Veldkamp^{1,*}

¹University of Wisconsin-Whitewater, Department of Chemistry, 800 West Main Street, Whitewater, WI 53190.

²Medical College of Wisconsin, Department of Biochemistry, 8701 Watertown Plank Road, Milwaukee, WI 53190.

³University of Wisconsin Madison, Department of Biochemistry, Biophysics Instrumentation Facility, 433 Babcock Drive, Madison, WI 53706.

Abstract

CCL21 is a human chemokine that recruits normal immune cells and metastasizing tumor cells to lymph nodes through activation of the G protein-coupled receptor CCR7. The CCL21 structure solved by NMR contains a conserved chemokine domain followed by an extended, unstructured C-terminus that is not typical of most other chemokines. A sedimentation equilibrium study showed CCL21 to be monomeric. Chemical shift mapping indicates that the CCR7 N-terminus binds to the N-loop and third β -strand of CCL21's chemokine domain. Details of CCL21-receptor recognition may enable structure-based drug discovery of novel anti-metastatic agents.

Keywords

Chemokines; CCL21; CCR7; NMR; cancer metastasis

Chemokines are a group of approximately 50 small, secreted proteins that function as chemoattractants, directing leukocyte trafficking in normal immune function and a variety of disease states(1). They target a family of approximately 20 seven transmembrane G-protein coupled receptors (GPCRs) that enable cells to migrate in response to a chemokine concentration gradient(1). CCL21 was cloned in the late nineties(2) and CCR7 was identified as the receptor for this chemokine(2, 3). CCL19 is a related chemokine that can also activate CCR7(4). Expression of CCL21 is localized to lymphoid organs, including lymph nodes, spleen and appendix(2).

CCL21 and CCL19 normally function to recruit CCR7 expressing antigen presenting cells, like dendritic cells, and naïve T-cells to the lymph nodes(5, 6). Hence, CCL21 and CCL19 bring these cell types in close proximity allowing for T-cell antigen-specific activation(5, 6). This may explain reports suggesting that CCL21 and CCL19 are anti-tumorigenic, because introduction of CCL21 and/or CCL19 into a primary tumor could activate the immune system generating a response against the tumor cells(5). Most primary tumor environments

*CORRESPONDING AUTHOR. Christopher T. Veldkamp, (262)-472-5267, veldkamc@uww.edu.

[^]These authors contributed equally to this work.

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lack expression of CCL21(5). However, when cancer tumors do express CCL21, as Shields et al. report for melanoma, CCL21 expression favors tumor progression by creating a tolerogenic microenvironment through the recruitment of cells that form a lymphoid-like environment(7). Lymph nodes produce CCL21 and CCL19 and are a common location for cancer metastases to form. Sentinel lymph nodes are tested to determine if cancers of various types have metastasized. Numerous cancer types including breast, colon, cervical, and skin cancers, have increased expression of CCR7 and migrate to the lymph nodes specifically in response to CCL21 during metastasis as originally shown by Muller and colleagues(8). Expression of CCR7 in some cancers is correlated with a poor prognosis and, although CCL21 and CCL19 are both ligands for CCR7, CCL21 appears to be the primary ligand involved in metastasis of solid tumors to the lymph nodes(5, 8–12) whereas CCL19 recruits T-ALL cells to the central nervous system(13). To enable the future development of CCL21-directed cancer therapies, we solved the solution structure of CCL21, determined its oligomeric state and identified CCL21 residues putatively involved in binding to the N-terminus of CCR7.

CCL21 is unusual in that it contains six cysteines versus the typical four found in almost all other chemokines, including CCL19(14). Additionally, while CCL19 is 77 residues in length, typical of most chemokines, CCL21 is 111 residues long due to the presence of an extended C-terminal domain(14). CCL21 was expressed as a Hexa-His tagged SMT3-CCL21 fusion, cleaved from the SMT3 fusion protein using ubiquitin like protease-1 and purified to > 98% homogeneity via reverse phase HPLC. Detailed methods for expression, purification, and structure determination are found in the supplemental materials. As shown in Supplemental Figure 1, equivalent activation of CCR7 in a calcium flux assay was observed for equal concentrations of recombinant CCL21 and commercial CCL21 indicating the formation of proper disulfide bonds. NMR samples for CCL21 structure determination contained 500 μ M U - ^{15}N , ^{13}C -CCL21 in 20 mM MES at pH 5.9 with 10% D_2O and 0.02% NaN_3 . Standard NMR techniques were used for chemical shift assignments and structure determination(15). Like all chemokines, CCL21 binds to a G-protein coupled receptor and to glycosaminoglycans. Normally the conserved chemokine domain accomplishes both of these functions. However, some have suggested the extended C-terminal region of CCL21 is responsible for binding glycosaminoglycans(16), while the chemokine domain functions as the CCR7 agonist. Based on structural homology and previous functional studies, we expected CCL21 to consist of an N-terminal chemokine domain and a C-terminal extension. Figure 1 A and B shows the solution structure of residues 8–70 of CCL21 (PDB ID 2I4n and BMRB ID 17245) and structure statistics are presented in Supplemental Table 1. As expected, residues 1–70 of CCL21 comprise the canonical chemokine fold. Despite the β -carbon chemical shifts indicating presence of a disulfide bond between Cys 80 and Cys 99, the C-terminal extension of CCL21 is unstructured as indicated by the low heteronuclear ^{15}N - ^1H NOE values (Figure 1C) and the lack of long-range NOEs. Additionally, Talos+(17) identified no regions of β -sheet or α -helix in the C-terminus. Supplemental Figure 2 shows an ensemble of 20 full-length CCL21 structures.

The unstructured, extended C-terminus of CCL21 complicated attempts to define CCL21's oligomeric state via comparing intrinsic fluorescence polarization values from tryptophan 59 of CCL21 and translational self-diffusion coefficient measurements to globular protein standards. It was difficult to draw any conclusions regarding the oligomeric state of CCL21 other than over the concentrations examined, 10 μ M to 500 μ M, the oligomeric state did not change. Thus sedimentation equilibrium analysis was performed which is a thermodynamically rigorous method not dependent on the use of standards. Detailed methods are found in the supplemental material. Three CCL21 samples of differing concentrations (19.6, 29.3 and 68.8 μ M in phosphate buffered saline at pH 7.4) were allowed to attain equilibrium at several speeds at 20°C. The complete data set showed no indication

of multiple species and thus was globally fit to a single species model. The ratio of the fitted weight average molecular weight to the sequence weight was 1.05 indicating CCL21 is a monomer. The results at two speeds for the three loading concentrations are shown in Supplemental Figure 3 as plots of the natural logarithm of absorbance versus squared radial position from the center of rotation. In such plots, a single homogeneous species appears as straight lines.

Chemokines are thought to bind and activate their receptors via a “two-step / two-site” mechanism(18). First the receptor N-terminus is thought to bind to the chemokine (site 1) followed by the chemokine N-terminus binding to a second site on the receptor causing receptor activation. Often one or more tyrosines in the receptor N-terminus are posttranslationally modified to sulfotyrosine. Sulfotyrosine residues function to increase the affinity of a receptor for its chemokine ligand(19, 20). The CCR7 N-terminal extracellular domain contains tyrosines that are near acidic amino acids, a motif that leads to sulfation by tyrosylprotein sulfotransferase enzymes(20). However, it is not yet known if these receptor tyrosines are sulfated on the surface of CCR7-expressing cells. Our previous structural analysis of CXCL12 bound to sulfated and unsulfated peptides corresponding to the CXCR4 N-terminus showed no difference in the position of sulfotyrosine or tyrosine residues in the chemokine-receptor complex(21). Hence, we employed an unsulfated, chemically synthesized peptide to map the CCR7 N-terminus binding site on CCL21 by NMR. A 200 μ M sample of U- 15 N CCL21 (20 mM MES pH 5.9) was titrated with incremental additions of synthetic N-terminal CCR7 peptide and monitored by 15 N- 1 H HSQC (Figure 2A). Dose dependent changes in CCL21 chemical shift perturbations upon titration with CCR7 peptide were fit using non-linear regression giving a K_d of $150 \pm 30 \mu$ M (Figure 2B). A plot of combined amide 15 N- 1 H chemical shift perturbations (Figure 2C) suggests amino acids in CCL21 that are likely to participate in binding to the CCR7 N-terminus. These chemical shift perturbations are represented in shades of blue on the CCL21 structure (Figure 2D) and indicate that the N-terminus of CCR7 binds to the chemokine domain of CCL21 in the region of the N-loop and the third β -strand.

CCL21 contains a tyrosine at position 12 in the N-loop. Signal from this amino acid broadens beyond detection upon addition of CCR7 peptide. Other amino acids with large chemical shift perturbations or that also have signal that broadens surround Y12 both sequentially and spatially (Figure 2). This is interesting as the N-loops of Mip-1 β /CCL4 and MCP-1/CCL2, also CC chemokines, have an F13 or Y13, respectively, that are essential for receptor binding (22–26). In the case of Mip-1 β /CCL4 an aromatic amino acid is essential for binding to CCR5 in this position(24), while in MCP-1/CCL2 a Y13A mutant shows a reduction in affinity for receptor and can function as an antagonist of chemotaxis(22). We hypothesize that Y12 in CCL21's N-loop may serve a similar role to that observed for F13 and Y13 in the respective N-loops of Mip-1 β /CCL4 and MCP-1/CCL2.

In the structure of another chemokine, CXCL12, in this case bound to an N-terminal CXCR4 receptor peptide, the N-loop and third β -strand are also involved in binding. The structure of CCL21 shows some similarities to a region of CXCL12 that forms contacts with sulfotyrosine 21 (sY21) of CXCR4, a residue that greatly enhances CXCL12 and CXCR4 interaction(21). In the structure of CXCL12 with the CXCR4 fragment, CXCL12 V18 and V49 have NOEs to sY 21 of CXCR4, and CXCL12 R47 is positioned near the sulfate of sY21. A structure alignment(27) of CCL21 with the CXCL12-sY21 containing CXCR4 structure(21) (PDB ID 2K05) reveals that CCL21 I17 and L51, both of which are found in regions of significant CCR7 induced chemical shift perturbations, overlay with CXCL12 V18 and V49 as shown in Supplemental Figure 4. CCL21 Q48 overlays most closely with R47 of CXCL12. Although the alignment shows there is no basic residue in CCL21 that is exactly analogous to CXCL12 R47, K15 in the N-loop of CCL21 and R44, K45 and R46 in

the 40's loop surround this region, and we speculate one of these may serve a role similar to CXCL12 R47. Indeed CCL21 K45's signal broadens beyond detection during titration with unsulfated CCR7 peptide and residues with large chemical shift perturbations also surround K15 of the N-loop. Based on sequence similarities to other chemokine receptors we expect tyrosines in CCR7 to be posttranslationally modified to sulfotyrosine. Future experiments will seek to determine if a sulfotyrosine in CCR7 binds to CCL21 in a fashion that is analogous to CXCR4 sY21(21). If sulfotyrosine recognition in CCL21 proves to be similar to CXCL12's recognition of CXCR4 sY21, perhaps broad spectrum inhibitors that target chemokine sulfotyrosine binding sites could be developed using the structure based drug design methods already applied to CXCL12(28). Or, one could speculate that if the binding mode of CCL21 and CCR7 is unique in any way, details of CCL21-receptor recognition could enable structure-based drug discovery of anti-metastatic agents with specificity for CCL21 directed metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Solution structure of CCL21. (A) Ensemble of 20 CCL21 structures. CCL21 has the typical chemokine domain consisting of an unstructured N-terminus (residues 1–7, not shown for clarity) followed by the N-loop, a three-stranded β -sheet and an α -helix. CCL21 also contains an unstructured C-terminus (residues 71–111, not shown for clarity) distinct from most chemokines. The conserved disulfide bonds, between C8 and C34 and C9 and C52 in CCL21, are shown in yellow. (B) Lowest energy structure of CCL21 showing residues 8–70. (C) Heteronuclear ^{15}N - ^1H NOE values plotted versus CCL21 amino acid number. The core chemokine domain of CCL21 is structured (residues 8–68) while the N-terminus and extended C-terminus are unstructured. The small positive values in the C-terminus are near C80 and C99, which form a disulfide bond.

Figure 2.

Putative CCR7 binding site. (A) Titration of CCL21 with a peptide corresponding to the N-terminus of CCR7 as monitored by ¹⁵N-¹H HSQC spectra. The spectrum of free CCL21 is shown in black with successive gray spectra indicating increasing concentrations of CCR7 peptide. CCL21 with a 5 molar excess of CCR7 peptide is shown in blue. (B) Non-linear fitting yields a K_d of $150 \pm 30 \mu\text{M}$ for CCL21 and the CCR7 N-terminus. (C) Combined amide proton and nitrogen chemical shift perturbations induced by the CCR7 peptide are plotted versus CCL21 residue number. Residues with significant perturbations whose signal ultimately broadened beyond detection (residues 12–14, 45 and 51) were given 3.5 ppm values with the last observable perturbation values for these residues shown as red bars. Residues 12, 14, and 51 were last observed at a molar ratio of 1:1.5 CCL21 to CCR7. Residues 13 and 45 were last detected at a molar ratio of 1:0.25 with each signal broadening beyond detection after the second peptide addition (1:0.5). Prolines and unobserved residues have values of 0. (D) Chemical shift mapping onto the surface of CCL21 suggests regions involved in CCR7 binding. Light blue indicates residues with chemical shift perturbations from 1.0 to 1.5 while those in blue have perturbations > 1.5.