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# Solution Structure of CCL21 and Identification of a Putative CCR7 Binding Site

Melissa Love,[†,#] Jamie L. Sandberg,[†,#] Joshua J. Ziarek,[‡] Kyle P. Gerarden,[†] Renee R. Rode,[†] Davin R. Jensen,[‡] Darrell R. McCaslin,[§] Francis C. Peterson,[‡] and Christopher T. Veldkamp*[,†]

[†]Department of Chemistry, University of Wisconsin-Whitewater, 800 West Main Street, Whitewater, Wisconsin 53190, United States

[‡]Department of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226, United States

[§]Department of Biochemistry, Biophysics Instrumentation Facility, University of Wisconsin-Madison, 433 Babcock Drive, Madison, Wisconsin 53706, United States

Supporting Information

**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 $\beta$-strand of CCL21's chemokine domain. Details of CCL21-receptor recognition may enable structure-based drug discovery of novel antimetastatic agents.

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 1990s[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 antitumorigenic because introduction of CCL21 and/or CCL19 into a primary tumor could activate the immune system.[5] Most primary tumor environments 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. 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.[5,8−13] 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-terminus.[14] Detailed methods for expression, purification, and structure determination of CCL21 along with other experiments are found in the Supporting Information. 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.

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,[15] while the chemokine domain functions as the CCR7 agonist. On the basis of structural homology and previous functional studies, we expected CCL21 to consist of an N-terminal chemokine domain and a C-terminal extension. Figure 1A,B shows the solution structure of residues 8−70 of CCL21 (PDB ID 2L4N 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 $\beta$-carbon chemical shifts indicating the presence of a disulfide bond between Cys 80 and Cys 99, the C-terminal extension of

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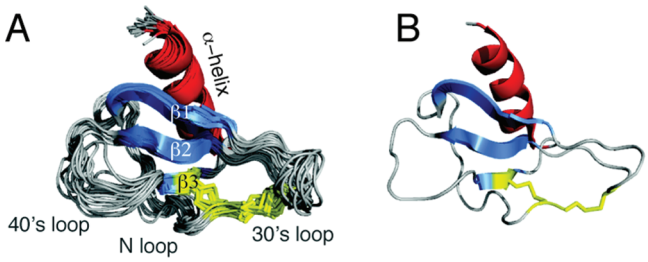


**Figure 1.** Solution structure of CCL21. (A) Ensemble of 20 CCL21 structures. CCL21 has the typical chemokine domain consisting of an unstructured N-terminus (residues 1−7, not shown for clarity) followed by the N-loop, a three-stranded $\beta$-sheet, and an $\alpha$-helix. CCL21 also contains an unstructured 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.

CCL21 is unstructured as indicated by the low $^{15}N-^{1}H$ heteronuclear NOE values and the lack of long-range NOEs. Additionally, Talos+[16] identified no regions of $\beta$-sheet or $\alpha$-helix in the C-terminus. Supplemental Figure 2 shows an ensemble of 20 full-length CCL21 structures and $^{15}N-^{1}H$ heteronuclear NOE values.

To define CCL21's oligmeric state sedimentation equilibrium analysis was performed. Three CCL21 samples of differing concentrations (19.6, 29.3, and 68.8 $\mu$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.

Chemokines are thought to bind and activate their receptors via a "two-step/two-site" mechanism.[17] First the receptor N-terminus binds 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.[18,19] The CCR7 N-terminal extracellular domain contains tyrosines that are near acidic amino acids, a motif that leads to sulfation by tyrosylprotein sulfotransferase enzymes.[19] 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.[20] Hence, we employed an unsulfated peptide to map the CCR7 N-terminus binding site on CCL21 by NMR. CCL21 was titrated with incremental additions of N-terminal CCR7 peptide and monitored by $^{15}N-^{1}H$ HSQC (Supplemental Figure 4A). Dose-dependent changes in CCL21 chemical shift perturbations upon titration with CCR7 peptide were fit using nonlinear regression giving a $K_d$ of 150 ± 30 $\mu$M (Supplemental Figure 4B). A plot of combined amide $^{15}N-^{1}H$ chemical shift perturbations (Figure 2A) suggests amino acids in CCL21 that are likely to participate

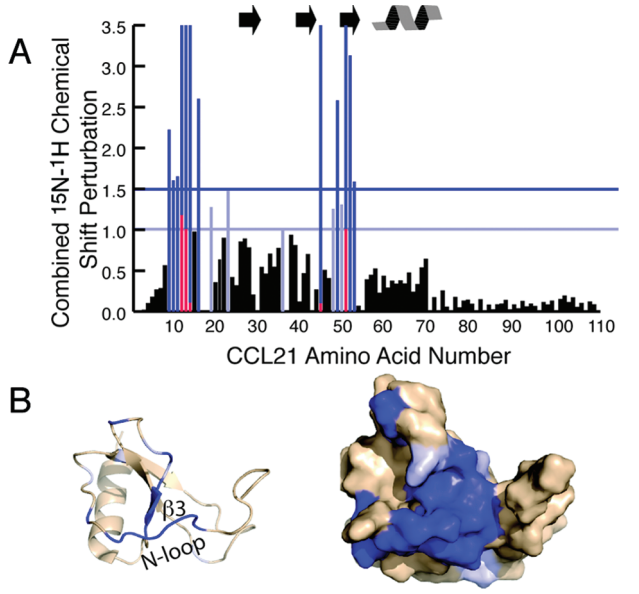


**Figure 2.** Putative CCR7 binding site. (A) Combined amide proton and nitrogen chemicals shift perturbations induced by the CCR7 peptide are plotted versus CCL21 residue number. Residues with significant perturbations whose signal ultimately broadened beyond detection (residues 12−14, 45, and 51) were 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. (B) 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.

in binding to the CCR7 N-terminus. These chemical shift perturbations are represented in shades of blue on the CCL21 structure (Figure 2B) and indicate that the N-terminus of CCR7 binds the chemokine domain of CCL21 near the N-loop and third $\beta$-strand.

CCL21 contains a tyrosine at position 12 in the N-loop. The signal from this amino acid broadens beyond detection upon addition of CCR7 peptide. Other amino acids with large chemical shift perturbations surround Y12 both sequentially and spatially (Figure 2). This is interesting as the N-loops of CCL4 and CCL2, also CC chemokines, have a F13 or Y13, respectively, that are essential for receptor binding.[21−24] Further, a CCL2 Y13A mutant shows a reduction in affinity for receptor and can function as an antagonist of chemotaxis.[21] We hypothesize that Y12 in CCL21's N-loop may serve a role similar to that observed for F13 and Y13 in the respective N-loops of CCL4 and CCL2.

In the structure of another chemokine, CXCL12, in this case bound to an N-terminal CXCR4 receptor peptide, the N-loop and third $\beta$-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.[20] In the complex of CXCL12 with the CXCR4 fragment, CXCL12 V18 and V49 have NOEs to sY21 of CXCR4, and CXCL12 R47 is positioned near the sulfate of sY21. A structure alignment[25] of CCL21 with the CXCL12−sY21 containing CXCR4 structure[20] (PDB ID 2K05) reveals


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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 (Supplemental Figure 5). CCL21 Q48 overlays most closely with R47 of CXCL12. Although the alignment indicates no basic residue in CCL21 that is 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 CCR7 peptide, and residues with large chemical shift perturbations surround K15 of CCL21. On the basis of 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.[20] If so, we speculate the same structure-based drug design methods already applied to target CXCL12's recognition of CXCR4 sY21[26] could be applied to CCL21.

## ■ ASSOCIATED CONTENT

### Supporting Information

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## ■ AUTHOR INFORMATION


### Corresponding Author

*E-mail: veldkamc@uww.edu.

### Author Contributions

#These authors contributed equally to this work.


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## ■ REFERENCES


(1) Baggiolini, M. (2001) *J. Intern. Med. 250*, 91−104.

(2) Yoshida, R., Imai, T., Hieshima, K., Kusuda, J., Baba, M., Kitaura, M., Nishimura, M., Kakizaki, M., Nomiyama, H., and Yoshie, O. (1997) *J. Biol. Chem. 272*, 13803−13809.

(3) Yoshida, R., Nagira, M., Kitaura, M., Imagawa, N., Imai, T., and Yoshie, O. (1998) *J. Biol. Chem. 273*, 7118−7122.

(4) Schumann, K., Lammermann, T., Bruckner, M., Legler, D. F., Polleux, J., Spatz, J. P., Schuler, G., Forster, R., Lutz, M. B., Sorokin, L., and Sixt, M. (2010) *Immunity 32*, 703−713.

(5) Ben-Baruch, A. (2008) *Clin. Exp. Metastasis 25*, 345−356.

(6) Forster, R., Davalos-Misslitz, A. C., and Rot, A. (2008) *Nat. Rev. Immunol. 8*, 362−371.

(7) Shields, J. D., Kourtis, I. C., Tomei, A. A., Roberts, J. M., and Swartz, M. A. (2010) *Science 328*, 749−752.

(8) Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verastegui, E., and Zlotnik, A. (2001) *Nature 410*, 50−56.

(9) Sancho, M., Vieira, J. M., Casalou, C., Mesquita, M., Pereira, T., Cavaco, B. M., Dias, S., and Leite, V. (2006) *J. Endocrinol. 191*, 229−238.

(10) Koizumi, K., Kozawa, Y., Ohashi, Y., Nakamura, E. S., Aozuka, Y., Sakurai, H., Ichiki, K., Doki, Y., Misaki, T., and Saiki, I. (2007) *Oncol. Rep. 17*, 1511−1516.

(11) Shields, J. D., Fleury, M. E., Yong, C., Tomei, A. A., Randolph, G. J., and Swartz, M. A. (2007) *Cancer Cell 11*, 526−538.

(12) Shields, J. D., Emmett, M. S., Dunn, D. B., Joory, K. D., Sage, L. M., Rigby, H., Mortimer, P. S., Orlando, A., Levick, J. R., and Bates, D. O. (2007) *Oncogene 26*, 2997−3005.

(13) Buonamici, S., Trimarchi, T., Ruocco, M. G., Reavie, L., Cathelin, S., Mar, B. G., Klinakis, A., Lukyanov, Y., Tseng, J. C., Sen, F., Gehrie, E., Li, M., Newcomb, E., Zavadil, J., Meruelo, D., Lipp, M., Ibrahim, S., Efstratiadis, A., Zagzag, D., Bromberg, J. S., Dustin, M. L., and Aifantis, I. (2009) *Nature 459*, 1000−1004.

(14) Fernandez, E. J., and Lolis, E. (2002) *Annu. Rev. Pharmacol. Toxicol. 42*, 469−499.

(15) Hirose, J., Kawashima, H., Swope Willis, M., Springer, T. A., Hasegawa, H., Yoshie, O., and Miyasaka, M. (2002) *Biochim. Biophys. Acta 1571*, 219−224.

(16) Shen, Y., Delaglio, F., Cornilescu, G., and Bax, A. (2009) *J. Biomol. NMR 44*, 213−223.

(17) Crump, M. P., Gong, J. H., Loetscher, P., Rajarathnam, K., Amara, A., Arenzana-Seisdedos, F., Virelizier, J. L., Baggiolini, M., Sykes, B. D., and Clark-Lewis, I. (1997) *EMBO J. 16*, 6996−7007.

(18) Farzan, M., Babcock, G. J., Vasilieva, N., Wright, P. L., Kiprilov, E., Mirzabekov, T., and Choe, H. (2002) *J. Biol. Chem. 277*, 29484−29489.

(19) Seibert, C., and Sakmar, T. P. (2008) *Biopolymers 90*, 459−477.

(20) Veldkamp, C. T., Seibert, C., Peterson, F. C., De la Cruz, N. B., Haugner, J. C., 3rd, Basnet, H., Sakmar, T. P., and Volkman, B. F. (2008) *Sci. Signaling 1*, ra4.

(21) Jarnagin, K., Grunberger, D., Mulkins, M., Wong, B., Hemmerich, S., Paavola, C., Bloom, A., Bhakta, S., Diehl, F., Freedman, R., McCarley, D., Polsky, I., Ping-Tsou, A., Kosaka, A., and Handel, T. M. (1999) *Biochemistry 38*, 16167−16177.

(22) Hemmerich, S., Paavola, C., Bloom, A., Bhakta, S., Freedman, R., Grunberger, D., Krstenansky, J., Lee, S., McCarley, D., Mulkins, M., Wong, B., Pease, J., Mizoue, L., Mirzadegan, T., Polsky, I., Thompson, K., Handel, T. M., and Jarnagin, K. (1999) *Biochemistry 38*, 13013−13025.

(23) Laurence, J. S., Blanpain, C., Burgner, J. W., Parmentier, M., and LiWang, P. J. (2000) *Biochemistry 39*, 3401−3409.

(24) Bondue, A., Jao, S. C., Blanpain, C., Parmentier, M., and LiWang, P. J. (2002) *Biochemistry 41*, 13548−13555.

(25) Shindyalov, I. N., and Bourne, P. E. (1998) *Protein Eng. 11*, 739−747.

(26) Veldkamp, C. T., Ziarek, J. J., Peterson, F. C., Chen, Y., and Volkman, B. F. (2010) *J. Am. Chem. Soc. 132*, 7242−7243.