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Lymphotactin structural dynamics

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Abstract

Lymphotactin/XCL1, the defining member of the C class of chemokines, undergoes a conformational change that involves the complete restructuring of all stabilizing interactions. Other chemokines are restricted to a single conformation by a pair of conserved disulfide crosslinks, one of which is absent in lymphotactin. This structural interconversion is entirely reversible, and the two-state equilibrium is sensitive to changes in temperature and ionic strength. One species adopts the conserved chemokine fold as a monomer and functions as an agonist for XCR1, the specific G protein-coupled receptor for lymphotactin. Rearrangement to the other conformation produces a novel four-stranded sheet that dimerizes to form a beta sandwich with high affinity for cell-surface glycosaminoglycans. We developed methods for resolving the two species, and investigated the dynamics of human lymphotactin structural interconversion using NMR spectroscopy, heparin affinity chromatography, and time-resolved fluorescence on the wild-type protein and a panel of amino acid substituted lymphotactin variants. Our results show that the lymphotactin structural rearrangement occurs at a rate of $\sim 1/s$, and that mutation of residues required for glycosaminoglycan binding shifts the conformational equilibrium toward the chemokine-like fold. We speculate that charge repulsion between arginines 23 and 43 destabilizes the chemokine fold and promotes conversion to the novel lymphotactin dimer, while binding of chloride or another anion stabilizes the chemokine fold by neutralizing the repulsive effect.

Introduction

Lymphotactin (Ltn) is a chemokine that recruits T and NK cells and is produced mainly by activated CD8⁺ T cells and activated NK cells. Like other chemokines, Ltn induces intracellular calcium mobilization and chemotaxis by binding a specific G protein-coupled receptor (GPCR) (Yoshida et al., 1998). When it was originally cloned from a mouse progenitor T cell cDNA library in 1994 (Kelner et al., 1994), comparisons with other chemokines highlighted two novel structural features in the Ltn sequence. At 93 residues in length, the mature, secreted form of Ltn contains a C-terminal extension of ~ 25 amino acids relative to the CXC and CC chemokines. Moreover, it lacks the first and third of four conserved cysteine residues found in all other chemokines, and thus possesses only a single disulfide bond. While the CXC and CC chemokine genes cluster on human chromosomes 4 and 17, respectively (Modi and Chen, 1998; Naruse et al., 1996), human lymphotactin is located on chromosome 1 (Kelner et al., 1994). On the basis of these distinctions, lymphotactin was taken to define a novel type of chemokine, the ‘C’ family (Kelner et al., 1994), and given the systematic designation XCL1.

Ltn binds the GPCR XCR1 with low nanomolar affinity to induce CD8⁺ T cell and NK cell chemotaxis, and chemoattracts CD4⁺ T cells with lower efficiency (Kelner et al., 1994). This may be due in part to the ability of Ltn to costimulate the apoptosis of CD4⁺ T cells but

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not CD8⁺ T cells (Cerdan et al., 2001). Ltn is produced mainly through T cell receptor activation in CD4⁺ and CD8⁺ T cells (Kelner et al., 1994; Tikhonov et al., 2001), but also by NK cells (Kelner et al., 1994) and $\gamma\delta$ T cells (Boismenu et al., 1996). Together, the data suggest that Ltn produced by T cells can regulate or modulate T cell-mediated immune responses.

Lymphotactin expression or activity is associated with a number of T cell-mediated disease states. Data from rheumatoid arthritis patients (Blaschke et al., 2003) and animal models for acute allograft rejection (Wang et al., 1998), Crohn's disease (Scheerens et al., 2001), and glomerulonephritis (Natori et al., 1998) are consistent with an immunomodulatory role for Ltn. Inhibitors or mimics of this chemokine may therefore have therapeutic value in the context of autoimmune and inflammatory diseases. In fact, Ltn has already been employed in the development of novel cancer immunotherapies. A number of animal studies have shown that Ltn can recruit T cells to the site of a tumor (Dilloo et al., 1996; Huang et al., 2005), and combined expression of Ltn and interleukin-2 in a neuroblastoma tumor vaccine induced measurable antitumor immune responses, including complete remission in two patients (Rousseau et al., 2003).

Chemokines share a highly conserved tertiary structure consisting of a flexible N-terminus, three-stranded β -sheet, and C-terminal α -helix, stabilized by two disulfide bonds (Clare and Greonenborn, 1995). Despite its small size (<10 kDa), the surface of each chemokine must support specific high affinity binding to two different targets to function *in vivo*. In addition to specific recognition and activation of a GPCR on the target cell, chemokines also bind glycosaminoglycans (GAGs) immobilized in the extracellular matrix (Proudfoot, 2006; Proudfoot et al., 2003). While the fundamental structural features of the family are now well-established, novel aspects of chemokine structure and recognition continue to be revealed. The ability to form homodimers may also be essential for chemokine function (Campanella et al., 2006; Proudfoot et al., 2003), and chemokine heterodimers have recently been characterized (Crown et al., 2006; Nesmelova et al., 2008). No chemokine receptor structures have yet been solved, but sulfotyrosine modifications in the GPCR N-terminus enhance their recognition by chemokine ligands (Bannert et al., 2001; Farzan et al., 2002; Fong et al., 2002; Seibert et al., 2002; Seibert et al., 2008), as illustrated recently for a complex between SDF-1/CXCL12 and its receptor CXCR4 (Veldkamp et al., 2008; Veldkamp et al., 2006).

Unlike all other chemokines, lymphotactin is constrained by only one disulfide bond permitting it to access two completely different native state structures. A series of NMR studies showed that Ltn is conformationally heterogeneous in solution (Kuloglu et al., 2001), interconverting between the conserved chemokine fold (Kuloglu et al., 2002) and an unrelated dimeric structure (Tuinstra et al., 2008). This metamorphic folding behavior is unlike any previously described protein conformational rearrangement (Murzin, 2008). The chemokine-like Ltn conformation is a functional XCR1 agonist, but has no high-affinity glycosaminoglycan- (GAG) binding site. In contrast, the alternative structure binds GAGs with high affinity but fails to activate XCR1. Because each structural species displays only one of the two functional properties essential for activity *in vivo*, the conformational equilibrium is likely to be essential for the biological activity of lymphotactin.

Despite its ability to orchestrate T cell-mediated immune responses, a specific biological role for lymphotactin remains obscure. Some investigators have reported difficulty obtaining reproducible lymphotactin activity, and these challenges may derive from its unusual conformational plasticity. We investigated the dynamics of the native state equilibrium using a number of different methods as described in this report, with the ultimate goal of

understanding how interconversion between two distinct lymphotactin structures contributes to its functional role in the immune system.

Production of biologically active lymphotactin

Despite the challenge associated with large-scale production of disulfide-containing proteins, many biologically active chemokines have been prepared by chemical synthesis or bacterial expression (Clark-Lewis et al., 1997). A key consideration is to mimic the mature protein secreted from mammalian cells after processing of an amino acid signal peptide, since like most chemokines, lymphotactin can be inactivated by any modifications to the amino terminal sequence (Tuinstra et al., 2007). Amino acid sequences for human lymphotactin and its orthologs are shown in Figure 1A. We have used two different approaches for bacterial expression of Ltn for structural and functional studies. Our original expression system employed Factor Xa cleavage of an N-terminal fusion protein to release Ltn with the native N-terminus (Kuloglu et al., 2001). This material was biologically active in assays that measure XCR1 activation by chemotaxis or intracellular calcium flux, but nonspecific proteolysis of the Ltn C-terminus led us to develop a more robust method based on cyanogen bromide (CNBr) cleavage of an N-terminal hexahistidine tag. CNBr cleaves the peptide bond following methionine residues, but human Ltn contains two nonconserved Met residues (M63 and M73) which we changed to valine and alanine, respectively (Tuinstra et al., 2007). Ltn proteins produced by the two methods are indistinguishable in terms of structure and biological activity (Tuinstra et al., 2007; Tuinstra et al., 2008).

Final purification of chemokines is typically achieved by reverse phase HPLC after disulfide formation in an oxidation/folding step. Lymphotactin refolding can be achieved either in solution or using an on-column buffer exchange process to catalyze disulfide formation (Veldkamp et al., 2007). On-column refolding is considerably faster and can improve the yield of protein with the correct disulfide pattern for some chemokines. However, since Ltn contains only a single disulfide bond, solid-phase refolding presents no particular advantage and we typically perform disulfide oxidation in solution as previously described (Peterson et al., 2004).

An unusual feature of the lymphotactin structure is glycosylation of the extended C-terminus in a portion of the protein purified from cultured mammalian cells (Dorner et al., 1997). Investigations into the functional significance of glycosylation used Ltn synthesized with carbohydrate modifications (Marcaurelle et al., 2001) or expressed in insect cells using a baculovirus system (Dong et al., 2005). Glycosylation had no detectable effect on XCR1 activation (Marcaurelle et al., 2001), and our own measurements confirm that the Ltn C-terminus (residues 73–93) is dispensable for both calcium-flux (Tuinstra et al., 2007) and T cell chemotaxis (S. Kutlesa, F. C. Peterson, and B. F. Volkman, unpublished results). A purpose for Ltn glycosylation thus remains unknown, and the functional role of the novel C-terminal sequence is a focus of our ongoing studies.

Ltn folds into two unrelated native state structures

NMR spectroscopy provided the first evidence of conformational duality in the lymphotactin structure. A 2D ^1H - ^{15}N HSQC spectrum acquired in typical conditions for protein NMR (20 mM sodium phosphate buffer, 200 mM NaCl, pH 6.0, 37 °C) contains roughly twice the number of peaks expected for a stably folded 93 residue protein (Figure 1B). Intense signals in a narrow ^1H chemical shift range (~7–8 ppm) suggested that some of the protein was unfolded, while other more dispersed peaks corresponded to one or more folded states, but the spectrum was otherwise uninterpretable. We verified the identity, purity, and disulfide oxidation state by analytical HPLC and electrospray ionization mass spectrometry, and reproduced the same ^1H - ^{15}N HSQC spectrum in multiple preparations. Initially, we varied

sample pH, temperature and buffer composition with no obvious improvement in the HSQC spectrum, however larger changes in temperature and solution ionic strength had a surprisingly dramatic effect. After systematic NMR screening of both parameters, we obtained a vastly simplified HSQC spectrum at 10 °C in 200 mM NaCl (Figure 1C) and solved the NMR structure under those conditions (Kuloglu et al., 2001). We refer to the conformation observed in low temperature/high salt conditions as Ltn10 (Figure 1D), a monomeric state that closely resembles other chemokines with the addition of a highly flexible C-terminal extension consisting of residues 70–93.

A very different HSQC pattern was observed at higher temperature in low ionic strength buffer (Figure 1E) (Kuloglu et al., 2002). Analytical ultracentrifugation revealed that the Ltn structure at 40 °C in the absence of salt (Ltn40) is dimeric with a K_d in the low micromolar range, but the structure of the Ltn40 dimer proved difficult to resolve. Our initial NMR studies of Ltn40 revealed a dramatic change from the typical pattern of secondary structure elements observed in the chemokine-like Ltn10 structure. Nuclear Overhauser effect (NOE) crosspeaks identified long-range contacts between a new β -strand comprised of residues 11–14 (β_0) and the β_3 strand, and showed that the α -helix had become disordered with the rest of the C-terminal domain (residues 53–93), but they failed to identify the Ltn40 dimer interface (Kuloglu et al., 2002). In an effort toward selective detection of intermolecular NOEs linking aliphatic protons from one subunit to amide protons of the other subunit, we acquired a F1 ^{13}C -edited/F3 ^{15}N -edited 3D NOESY-HSQC spectrum on a equimolar mixture of $[\text{U-}^{15}\text{N}]$ -Ltn and $[\text{U-}^{13}\text{C}]$ -Ltn, but it was devoid of signals.

Heteronuclear filtering can be technically difficult in protein NMR spectroscopy, particularly the ^{13}C filtering of proton signals exhibiting a wide range of ^1H - ^{13}C coupling constants. We successfully detected unambiguous intermolecular NOEs only after implementing an F1 $^{13}\text{C}/^{15}\text{N}$ -filtered/F3 ^{13}C -edited 3D NOESY-HSQC pulse sequence originally described by Palmer and coworkers (Stuart, 1999). Their pulse scheme compensates for the normal variations in ^1H - ^{13}C scalar couplings in proteins, and, most importantly, is both robust and simple to use, requiring no complex pulse calibrations or empirical optimizations. We routinely use this filtered NOESY experiment to solve structures for homodimer (Peterson et al., 2006) and heterodimer (Veldkamp et al., 2008) complexes.

A total of 31 intermolecular NOEs were assigned in the filtered NOESY spectrum of Ltn40, each of which gave rise to a pair of symmetry-related intermolecular distance restraints. These restraints enabled us to solve the structure of the Ltn40 dimer (Tuinstra et al., 2008), which adopts a novel β -sandwich fold with four strands in each monomer subunit and no helices (Figure 1F). While the β_1 , β_2 and β_3 strands may seem unchanged, the rearrangement from Ltn10 to Ltn40 creates a completely different pattern of β -sheet hydrogen bonding and hydrophobic packing as described previously (Kuloglu et al., 2002; Tuinstra et al., 2008). Thus, Ltn folds into two distinct, unrelated structures depending on temperature and solution conditions.

Kinetics of interconversion in the Ltn conformational equilibrium

For both native state Ltn structures to contribute to its chemokine activity *in vivo*, we postulated that individual molecules must be capable of converting from the Ltn10 state to the Ltn40 state and vice versa. A series of HSQC spectra collected on a single sample from 10 to 40 °C confirmed that the Ltn10–Ltn40 transition is fully reversible, and spectra at intermediate temperatures contain distinct signals for each conformational state (Kuloglu et al., 2002; Tuinstra et al., 2007). Thus, changes in the Ltn conformational equilibrium induced by temperature, ionic strength, mutations or binding interactions can be monitored by 1D ^1H and 2D ^1H - ^{15}N NMR spectroscopy. It is also of interest to measure the kinetics of

conformational exchange, and the choice of technique depends on the timescale. The presence of distinct HSQC signals for the two species in equilibrium indicates that the frequency of Ltn10–Ltn40 interconversion is significantly lower than the observed chemical shift differences (~50–100 Hz).

We initially tried to assess the rate of structural interconversion by monitoring amide H/D exchange rates. We reasoned that since the conformational rearrangement breaks every hydrogen bond and replaces it with a different one (Kuloglu et al., 2002), the lifetime of the Ltn10 and Ltn40 species would set an upper limit on the protection factor of all amide hydrogens. If the interconversion is very slow (minutes or longer), the observed H/D exchange rates would be dominated by this rate. We acquired 1D ^1H and 2D ^1H - ^{15}N HSQC spectra on a sample of lyophilized Ltn dissolved in 100% D_2O . Regardless of pH, temperature or salt concentration, we failed to detect any amide ^1H signals in the first spectrum, which was acquired less than 5 minutes after dissolving the sample.

Knowing that the interconversion rate was on the order of seconds (not milliseconds or minutes), we measured 2D longitudinal ^{15}N zz-exchange spectra (Farrow et al., 1994) to detect exchange peaks that appear at the intersection of ^1H and ^{15}N shifts for the same residue in the Ltn10 and Ltn40 conformations. We acquired a series of 2D exchange spectra with different exchange mixing periods at 37 °C in low salt buffer conditions where Ltn40 is more abundant than Ltn10 but both species are present at detectable levels (Figure 2A) (Tuinstra et al., 2008). No exchange crosspeaks were detected until the mixing time exceeded 50 ms with maximal intensities observed at ~350 ms. Nonlinear fitting to equations accounting for a two-state exchange and ^{15}N T_1 relaxation (Tollinger et al., 2001) yielded rate constants for the forward ($k_{\text{forward}} = 0.4 \text{ s}^{-1}$) and reverse ($k_{\text{reverse}} = 1.2 \text{ s}^{-1}$) reactions consistent with an overall exchange rate, k_{ex} , of $\sim 1.5 \text{ s}^{-1}$.

The single tryptophan in Ltn, W55, is another useful sensor of the Ltn10–Ltn40 conformational equilibrium (Kuloglu et al., 2002; Tuinstra et al., 2008). Transfer of the W55 sidechain from a constrained environment in the Ltn10 hydrophobic core to a mobile, solvent-exposed state in Ltn40 shifts the tryptophan fluorescence emission maximum to a longer wavelength and reduces the intensity as illustrated in Figure 2B (Kuloglu et al., 2002). Thus, changes in the conformational equilibrium can be detected by monitoring the fluorescence intensity at ~335 nm. We speculated that time-resolved optical spectroscopy could be used to monitor the response of an Ltn10/Ltn40 mixture to an increase in salt concentration or the addition of a glycosaminoglycan like heparin.

To demonstrate the feasibility of this approach, we performed a series of salt-jump experiments using the intrinsic fluorescence emission of W55. While monitoring the emission at 335 nm, we injected buffer, concentrated NaCl or low molecular weight heparin into a sample of 15 μM Ltn (Figure 2C). The jump in NaCl concentration from 0 to 250 mM produced an increase in fluorescence intensity consistent with a shift toward Ltn10. In contrast, injection of heparin produced a decrease in intensity which we interpreted as an increase in the relative concentration of Ltn40. As described below, this results is consistent with a model in which cell-surface GAGs bind preferentially to the Ltn40 species. Rates for the Ltn40→Ltn10 and Ltn10→Ltn40 reactions at 30 °C (0.22 and 0.73 s^{-1} , respectively) obtained from nonlinear fitting are slightly lower than those measured by 2D exchange NMR at 37 °C. While the fluorescence approach may be improved by using a stopped flow apparatus with a shorter dead time for sample mixing, the general agreement of the preliminary results from fluorescence titrations and 2D exchange NMR validates both methods for kinetic analysis of the Ltn conformational change. Future studies of the temperature dependence of k_{ex} will permit us to define the height of the energetic barrier (activation energy, E_a) separating the Ltn10 and Ltn40 species.

Engineering conformationally-restricted lymphotactin variants

Conveniently, the Ltn structure is highly sensitive to variations in temperature and salt concentration within the ranges typically used for biophysical measurements on proteins (e.g. 10–45 °C, 0–200 mM NaCl), and this allowed us to resolve the two conformations present at physiological conditions. However, assays for biological activity using animals or cell cultures impose constraints on the buffer composition and temperature. These limited our ability to assign specific functional roles to the Ltn10 and Ltn40 species, since both would be present in significant amounts when attempting to measure intracellular calcium flux, chemotaxis or cell recruitment *in vivo*. To solve this problem, we generated a panel of Ltn variants designed to favor either the Ltn10 or Ltn40 structure and prevent interconversion.

While the 3D structure of Ltn40 had yet not been solved, its secondary structure was known (Kuloglu et al., 2002), and we used the structures of Ltn10 and the chemokine HCC-2 to design two different disulfide crosslinks that would prevent the rearrangement to Ltn40 as described previously (Tuinstra et al., 2007). Amino acid substitutions corresponding to the CC1-Ltn and CC3-Ltn variants are shown in Figure 3. We took a different approach in designing an Ltn40-restricted variant: selectively destabilize the Ltn10 fold. Since residues 52–93 are disordered in Ltn40, we identified W55 as an essential hydrophobic core residue in Ltn10 that makes no contribution to the Ltn40 structure and changed it to an alanine or aspartic acid (Figure 3) (Tuinstra et al., 2008). All of the mutants were successfully expressed and purified, and we examined their 2D ¹H-¹⁵N HSQC spectra. For an objective measure of their similarity to one structure or the other we plotted the ¹H and ¹⁵N shifts for each mutant against those of Ltn10 and Ltn40 (Figure 4A). The W55D mutant matches the Ltn40 shifts very closely and shows a poor correlation with Ltn10, and HSQC spectra of the mutant proteins acquired at different temperatures show no evidence of interconversion to the other conformation (Figure 4B). Conversely, the nearly perfect correlation with Ltn10 shifts for the CC1 and CC3 variants show convincingly that the disulfide-locked proteins retain the chemokine-like fold, and we confirmed this by solving the NMR structure of CC3 (Figure 4C) (Tuinstra et al., 2007). We next employed the CC3-Ltn and W55D-Ltn proteins in a series of functional studies designed to assign functional roles to the Ltn10 and Ltn40 states.

Functional analysis of distinct Ltn native state conformations

Chemokine function depends on dual biochemical activities, GAG binding and GPCR activation. In the case of lymphotactin, the two functional roles may be segregated by a conformational barrier corresponding to the activation energy for Ltn10↔Ltn40 interconversion. Treating the CC3 and W55D variants described above as ‘pure’ versions of Ltn10 and Ltn40, respectively, we compared their ability to activate the Ltn receptor XCR1 stably expressed on the surface of HEK293 cells (generously provided by Joseph Hedrick, Schering-Plough Research Institute) (Shan et al., 2000). As detailed by Tuinstra *et al.* (Tuinstra et al., 2007), changes in intracellular calcium levels in response to each protein at a concentration of 200 nM were monitored using cells loaded with the Fluo-3-AM dye at 37 °C using a PTI spectrofluorometer. CC3-Ltn and wild-type Ltn induced similar calcium flux responses (Tuinstra et al., 2007), while W55D-Ltn was completely inactive (Tuinstra et al., 2008).

To compare relative GAG binding activity for the Ltn10 and Ltn40 species, we eluted CC3-Ltn and W55D-Ltn from a heparin-Sepharose column using a sodium chloride concentration gradient (Tuinstra et al., 2008). Wild-type Ltn elutes in two broad fractions at ~450 and 700 mM NaCl (Figure 5 and Table 1), suggesting that the two conformations bind heparin with significantly different affinities. CC3-Ltn elutes at a single sharp peak at 450 mM NaCl,

while the broader W55D-Ltn elution resembles the high-affinity fraction of wild-type Ltn with a peak at 750 mM NaCl.

Not surprisingly, our results showed that only the chemokine-like Ltn10 structure is capable of XCR1 signaling, and we learned that the Ltn40 dimer encodes tight GAG binding while the Ltn10 species binds heparin with low but detectable affinity. To show that cell surface GAGs interact preferentially with Ltn40, we attempted to use 2D NMR as a the equivalent of a pull-down or co-precipitation assay. Initially, we added low molecular weight heparin to an NMR sample of [U-¹⁵N]-wild-type Ltn at conditions where both conformations are present, expecting to see an effect on the Ltn40 peaks only. Instead, HSQC signals for both species diminished and disappeared with increasing amounts of heparin. As illustrated by fluorescence spectroscopy in Figure 2C, the addition of heparin to an equilibrium mixture of Ltn10 and Ltn40 causes a shift toward the Ltn40 state. At NMR concentrations, heparin forms insoluble complexes with Ltn40 and probably shifts the conformational equilibrium until all the protein has precipitated.

To allow for selective heparin binding to either the Ltn10 or Ltn40 species without the potential for interconversion, we performed the same titration on a mixture of ¹⁵N-labeled CC3-Ltn and W55D-Ltn (Tuinstra et al., 2008). HSQC spectra of wild-type Ltn and the CC3/W55D mixture HSQC are virtually identical, but upon addition of a heparin tetrasaccharide to the mixed sample only the Ltn40 signals were lost (Figure 6). Collectively, these studies support a model for lymphotactin activity in which the native state is coupled to two different binding equilibria, either of which can alter the Ltn10-Ltn40 conformational equilibrium (Figure 7). In this model, cell surface GAGs can inhibit Ltn activity by shifting the equilibrium toward the Ltn40 state and reducing the concentration of Ltn10 available for XCR1 activation. Presumably, there must be a mechanism for shifting the equilibrium toward the Ltn10 state when conditions require XCR1-mediated signaling and T cell chemotaxis. Whether the XCR1 receptor can shift the conformational equilibrium directly has not been investigated, and other factors that alter the Ltn10-Ltn40 balance may be identified in future *in vivo* studies of lymphotactin function.

GAG binding residues are linked to the Ltn conformational equilibrium

In an previous study, we substituted most of the lysine and arginine residues in Ltn with alanine and used surface plasmon resonance (SPR) to measure heparin binding affinities. We identified R23 and R43 as the residues that contributed most to GAG binding, since each alanine substitution reduced the affinity for heparin by ~30-fold (Peterson et al., 2004). R23 and R43 are adjacent in the Ltn10 structure (Figure 1C), but as described earlier, heparin-Sepharose binding studies showed that Ltn40 supplies the GAG binding function. Since SPR measurements provide no information on the relative affinities of the two Ltn structural species, we ran the panel of arginine and lysine mutants over the heparin column and noted the elution of the high- and low-affinity fractions. The results are illustrated schematically in Figure 5 and summarized in Table 1. For substitutions that have little or no effect on GAG binding (e.g. R65S and K66A), the position and relative intensities of the two peaks are similar to wild-type. As expected, the high-affinity fractions of R23A and R43A elute at significantly lower salt concentrations (557 and 458 mM NaCl, respectively) than wild-type Ltn40 (694 mM NaCl). Interestingly, the peak ratio for R43A was skewed toward the low affinity fraction, in comparison to wild-type Ltn (Table 1), and the effect was even more pronounced for the R23A/R43A double mutant which favored the low-affinity fraction by a ratio of more than 2:1.

Elimination of key residues for high affinity GAG binding seems to shift the conformational equilibrium toward Ltn10, but this could simply reflect a diminished capacity for heparin to stabilize Ltn40 and shift the equilibrium in the opposite direction. To determine whether

substitutions at R23 and R43 alter the relative stabilities of Ltn10 and Ltn40 independent of heparin, we compared the HSQC spectra of wild-type Ltn and the R23A, R43A and R23A/R43A mutants. Except for signals from the substituted residues, the HSQC spectrum of each mutant is superimposable with the spectrum of wild-type Ltn, thus the tertiary structure is unchanged. As shown in Figure 8, wild-type Ltn readily converts from Ltn10 to Ltn40 as the temperature is increased from 10 to 45 °C. However, substitution of either R23 or R43 with alanine appears to stabilize the Ltn10 species and shift the Ltn40 transition to higher temperatures (data not shown). The effect is more pronounced for the R23A/R43A double mutant which remains predominantly in the Ltn10 form even at 45 °C (Figure 8).

How does alanine substitution of one or two solvent-exposed loop residues affect the conformational equilibrium so dramatically? A recent molecular dynamics study suggests the close proximity of R23 and R43 in the Ltn10 conformation may be a destabilizing factor in low ionic strength solution. In a 14 ns MD simulation performed at 45 °C in the presence of 200 mM NaCl, Cui and coworkers found that the positively charged R23 and R43 sidechains coordinate a chloride ion that also forms hydrogen bonds with the T41 hydroxyl and backbone amides from the 40's loop (Formanek et al., 2006). Chloride association with R23, R43 and the 40's loop persisted for the last 5 ns of the simulation, illustrating a specific protein-ion interaction that would favor the Ltn10 conformation. In the absence of a stabilizing anion, electrostatic repulsion between the nearby R23 and R43 sidechains is relieved by the Ltn10→Ltn40 rearrangement which separates the two positively charged residues by a distance of ~20 Å (Figure 9). Replacement of either R23 or R43 reduces the repulsive effect, diminishing the need for anion stabilization and favoring the Ltn10 structure relative to Ltn40.

Conclusions

Unique features of the lymphotactin sequence distinguish it from other chemokines. Structural analysis revealed an unprecedented native-state interconversion between two unrelated structures, each of which contributes to the biological activity of Ltn. In this work, we showed that the Ltn10 and Ltn40 structures interconvert with a frequency of ~1 s⁻¹ using fluorescence and NMR experiments. Data from mutagenesis, heparin affinity chromatography and NMR interpreted in the context of published MD simulations suggested that electrostatic repulsion in the Ltn10 structure between key GAG-binding residues can be stabilized by anion binding in solutions of high ionic strength. Our long term goals are to understand each step in the process of structural interconversion between the Ltn10 and Ltn40 states and to relate this unusual conformational equilibrium to lymphotactin activity *in vivo*. The experimental approaches and results described here serve as a foundation for more detailed studies of lymphotactin dynamics using a combination of mutagenesis, optical and NMR spectroscopy and MD simulations.

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Figure 1. Lymphotactin adopts two unrelated folds in equilibrium

(A) Alignment of Ltn sequences from human, mouse, rat, cow and chicken. (B) ^1H - ^{15}N HSQC spectra acquired for wild-type human Ltn at 37 °C and (C) 10 °C in 20 mM sodium phosphate (pH 6.0) containing 200 mM sodium chloride. (D) Structure of wild-type Ltn at 10 °C showing that under these solution conditions Ltn adopts the canonical chemokine fold, subsequently referred to as Ltn10. Unstructured residues (1–7, 76–93) were omitted for clarity. (E) ^1H - ^{15}N HSQC spectrum of wild-type Ltn at 40 °C acquired in 20 mM sodium phosphate (pH 6.0) in the absence of salt. (F) Structure of wild-type Ltn at 40 °C (Ltn40) adopts a novel four-stranded antiparallel β -sheet that self-associates as a head-to-tail dimer. Disordered N- and C-termini (1–7, 56–93) were omitted for clarity.

Figure 2. Spectroscopic detection of structural interconversion

(A) Exchange peaks between the Ltn10 and Ltn40 ^1H - ^{15}N resonances of Gly⁴⁴ were detected using a 2D ^{15}N zz-exchange spectrum acquired on 250 μM wild-type Ltn in 20 mM sodium phosphate, pH 6.0, 150 mM NaCl. Exchange spectra were measured using mixing times of 50, 150, 200, 250, 300, 350 (shown), 400, 500, 750, and 900 ms. Intensities of the peaks were fit to a two-state exchange model yielding rates of 0.4 and 1.2 s^{-1} for the forward (Ltn40 $\rightarrow$ Ltn10) and reverse (Ltn10 $\rightarrow$ Ltn40) reactions, respectively. (B) Tryptophan emission spectrum of wild-type Ltn (15 μM) at 30 °C in 0 mM NaCl, where the Ltn10:Ltn40 ratio is roughly 1:1. Arrows indicate how fluorescence intensity increases and the emission maximum shifts to lower wavelengths as the tryptophan is buried in the Ltn10 core, while increased solvent exposure and flexibility in Ltn40 decreases the intensity and causes a red shift. (C) Time dependent fluorescence intensity at 335 nm for Ltn samples (15 μM , 30 °C, 0 mM NaCl) injected at time 0 with buffer (●), 15 μM low molecular weight heparin (▲), or 200 mM NaCl (○). Equilibration curves were fit to a simple exponential decay using ProFit software yielding Ltn40 $\rightarrow$ Ltn10 and Ltn10 $\rightarrow$ Ltn40 exchange rates of 0.22 and 0.73 s^{-1} , respectively.

Figure 3. Ltn variants stabilize either the Ltn10 or Ltn40 structure

The introduction of a second disulfide bond to stabilize the Ltn10 conformation was based on alignment of Ltn10 with CC chemokines. Construction of CC1-Ltn (T10C + dipeptide insertion G³²-AC-S³³) is based upon alignment with the first disulfide with RANTES, while the positions of the cysteine substitutions in CC3-Ltn (V21C/V59C) mimic the unusual third disulfide in HCC-2. Stabilization of the Ltn40 conformation is accomplished by introducing an aspartate or alanine point mutation at W55. Loss of the tryptophan sidechain disrupts the hydrophobic core and destabilizes the Ltn10 conformation. Mutated residues are shown in white or black outline.

Figure 4. CC3-Ltn and W55D-Ltn are structurally indistinguishable from Ltn10 and Ltn40
 (A) Amide chemical shifts for CC1-, CC3-, and W55D-Ltn plotted against the corresponding values for wild-type Ltn at 10 and 40 °C. The excellent correlation of the Ltn10 chemical shifts with CC1-Ltn and CC3-Ltn compared to the poor correlation with the Ltn40 chemical shifts suggests that CC1-Ltn and CC3-Ltn adopt the chemokine-like fold. The converse is true for W55D-Ltn demonstrating its similarity to Ltn40. Outliers in each plot are labeled and correspond to residues at or adjacent to the mutated positions in the amino acid sequence. (B) ^1H - ^{15}N HSQC spectra of CC3-Ltn and W55D-Ltn acquired at 10 and 45 °C in 20 mM sodium phosphate (pH 6.0). Comparison of the low and high temperature spectra indicates that CC3-Ltn only adopts the Ltn10 conformation and W55D-

Ltn only adopts the Ltn40 conformation. (C) Ribbon diagram of CC3-Ltn showing the canonical chemokine fold. The structure was determined at 25 °C in 20 mM sodium phosphate, a solution condition that normally contains a mixed population of the two Ltn conformers. The disulfide bond incorporated to restrict the conformation is shown.

Figure 5. Dual Ltn conformations bind heparin with different affinities

Elution of wild-type Ltn from a heparin-Sepharose column yields a biphasic profile with a low- and high-salt peak. In contrast, CC3-Ltn and W55D-Ltn elute as a single fraction corresponding to the low- and high-salt wild-type Ltn peaks, respectively. Alanine substitution of two arginines involved in GAG binding, R23 and R43, shifts the elution of the high-affinity (Ltn40) fraction. The high/low affinity peak ratio for R43A is skewed toward the Ltn10 conformation. This effect was more pronounced in the R23A/R43A double mutant, favoring the Ltn10 conformation by a ratio of 2:1. In contrast, mutation of residues that do not participate in heparin binding (R65A and K66S) had a minimal effect on the elution profiles or peak ratios when compared with wild-type Ltn.

Figure 6. Selective heparin binding monitored by 2D NMR

HSQC spectra recorded on an NMR sample containing 250 μ M each of CC3-Ltn and W55D-Ltn at 37 $^{\circ}$ C in 20 mM sodium phosphate (pH 6.0). Prior to the addition of heparin tetrasaccharide (left panel), ^1H - ^{15}N HSQC signals are observed for W55D-Ltn (gray, Ltn40 conformation) and CC3-Ltn (black, Ltn10 conformation). Addition of heparin tetrasaccharide results in broadening (center panel) and disappearance of the W55D-Ltn resonances (right panel) while the CC3-Ltn resonances are unaffected.

Figure 7. Functional relevance of the Ltn conformational equilibrium

Under physiological conditions, Ltn partitions equally between the Ltn10 and Ltn40 conformations. The conformational equilibrium may shift in response to interactions with cell-surface GAGs or XCR1. Cell-surface GAGs stabilize Ltn40 relative to Ltn10 while XCR1 binding may shift the equilibrium towards the Ltn10 conformer.

Figure 8. Mutation of GAG binding residues alters the Ltn10-Ltn40 equilibrium
 Alanine substitution of R23 and R43 stabilize the Ltn10 conformer. (A) ^1H - ^{15}N HSQC spectra of wild-type and R23A/R43A-Ltn acquired at 10 and 45 °C in 20 mM sodium phosphate (pH 6.0). Comparison of the low- and high-temperature HSQCs indicates that the R23A/R43A double mutant stabilizes the Ltn10 conformation in the absence of salt. (B) Relative intensities of the W55 $^1\text{H}^{\text{e1}}$ peak in the Ltn10 and Ltn40 conformations is plotted as a function of temperature. The plots indicate the equilibrium mid-point for wild-type Ltn is ~25 °C. In contrast, the equilibrium mid-point for R23A/R43A is shifted to ~50 °C under conditions that typically favor the Ltn40 conformation.

Figure 9. Ionic strength dependence in the Ltn structural equilibrium

Electrostatic repulsion in Ltn10 is relieved by salt or conversion to Ltn40 conformer. In the Ltn10 conformation R23 and R43 are close enough ($< 8 \text{ \AA}$) for their guanidinyll groups to coordinate a chloride ion (left panel). In the absence of salt, electrostatic repulsion between the positively charged sidechains destabilizes the Ltn10 conformation (center panel). Alternatively, the R23-R43 repulsion can be eliminated by conversion to the Ltn40 conformer (right panel).

Table 1

Elution from heparin-Sepharose and relative intensities for lymphotactin variants.

Ltn mutant	Peak 1 Elution (mM NaCl)	Peak 2 Elution (mM NaCl)	Fractional Population Peak 1	Fractional Population Peak 2
WT	452	694	40	60
CC3	430	N.D.	100	0
W55D	N.D.	753	0	100
R23A	382	557	41	59
R43A	376	458	53	47
R23A/R43A	309	344	71	29
R65S	415	658	29	71
K66A	408	659	38	62