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# Total Chemical Synthesis and Biological Activities of Glycosylated and Non-Glycosylated Forms of the Chemokines CCL1 and Ser-CCL1**

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***Abstract:*** *CCL1 is a naturally glycosylated chemokine protein that is secreted by activated T-cells and acts as a chemoattractant for monocytes.[1] Originally, CCL1 was identified as a 73 amino acid protein having one N-glycosylation site,[1] and a variant 74 residue non-glycosylated form, Ser-CCL1, has also been described.[2] There are no systematic studies of the effect of glycosylation on the biological activities of either CCL1 or Ser-CCL1. Here we report the total chemical syntheses of both N-glycosylated and non-glycosylated forms of (Ser-)CCL1, by convergent native chemical ligation. We used an N-glycan isolated from hen egg yolk together with the Nbz linker for Fmoc chemistry solid phase synthesis of the glycopeptide-$^{\alpha}$thioester building block.[3] Chemotaxis assays of these glycoproteins and the corresponding non-glycosylated proteins were carried out. The results were correlated with the chemical structures of the (glyco)protein molecules. To the best of our knowledge, these are the first investigations of the effect of glycosylation on the chemotactic activity of the chemokine (Ser-)CCL1 using homogeneous N-glycosylated protein molecules of defined covalent structure.*

Chemokines are chemotactic cytokine proteins, usually of vertebrate origin, that play an important role in host defense responses by regulating the migration of leukocyte cells.[4] These protein molecules are relatively small (8–10 kDa) and can be categorized as CC and CXC types depending on the position of two highly conserved cysteine residues near the N-terminal of the protein's polypeptide chain. Chemokines exert their biological function through binding to specific G protein-coupled receptor molecules on the cell surface. Because chemokines play a critical role in the pathogenesis of inflammatory diseases (e.g. asthma, atherosclerosis, rheumatoid arthritis), or of viral infection including by the human immunodeficiency virus-1 (HIV-1), there is wide-spread interest in understanding the molecular basis of chemokine function.[5] There are several common features of chemokine molecular structure: a flexible peptide chain at the N-terminus; three anti-parallel β-strands; and, a C-terminal α-helix. These secondary structural elements are formed into a common tertiary structural fold, which is usually supported by two disulfide bonds.[6] Considerable effort has been devoted to the analysis of chemokine structure–activity relationships, and has already revealed that the flexible N-terminal peptide chain invokes the activation of the G protein-coupled receptor, and that the loop region following the N-terminus is responsible for the initial binding to the receptor.[7]

Biological responses to the chemokine CCL1 are mediated through its binding to the G protein-coupled receptor CCR8.[8] It is noteworthy that CCL1 can bind only to CCR8, whereas most other chemokines bind to multiple receptor molecules. This specific binding of CCL1 to the CCR8 receptor causes unique responses, such as anti-dexamethasone-induced apoptosis of T-cell lymphomas,[9] or inhibition of HIV infection,[10] and neuropathic pain induction that was found very recently.[11] The variant Ser-CCL1 is a partial agonist for the CCR8 receptor.[2] NMR structural analysis of Ser-CCL1 was also reported, and revealed that the CCL1 protein possesses the secondary structures common to all chemokines, as described above, with an unusual third disulfide bond that makes the C-terminal α-helix shorter than that of the other chemokines.[12] Because of the small size of the CCL1 chemokine protein molecule, incorporation of a complex glycan could have considerable impact on biological function.[13]

Previously it was shown that the N-glycosylated form of CCL2 (MCP-1) showed less potency than the non-glycosylated form, using recombinantly prepared glycoprotein that has heterogeneous glycan structure.[14] In the work reported here, we set out to explore the effect of N-glycosylation on the chemotactic activity of (Ser-)CCL1 using glycosylated proteins of defined chemical structure.

The 73 or 74 residue (Ser-)CCL1 polypeptide chains has 6 cysteines: $Cys^{10}$, $Cys^{11}$, $Cys^{26}$, $Cys^{34}$, $Cys^{50}$, and $Cys^{68}$ (Figure 1). Synthesis of the non-glycosylated proteins CCL1

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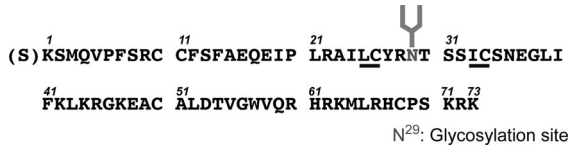
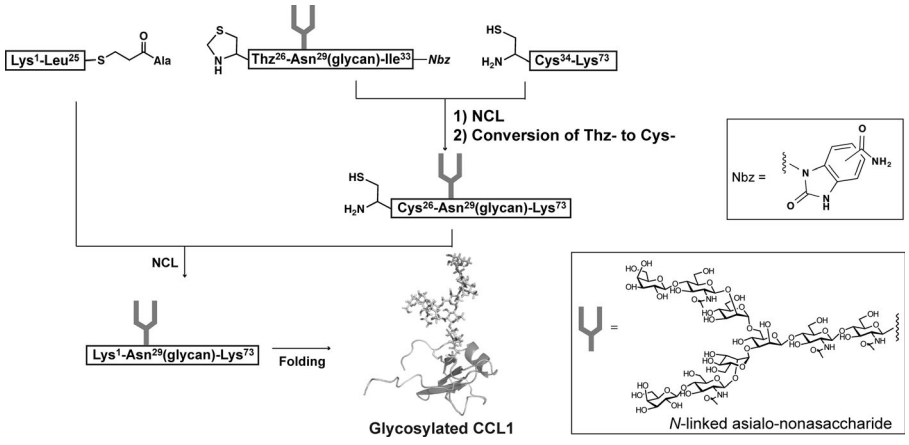



***Figure 1.*** Sequence of CCL1. $N^{29}$ is the natural glycosylation site.[1a] Ser-CCL1 has an additional Ser residue at the N-terminus (in parentheses). Ligation sites are underlined.

and Ser-CCL1 was expected to be straightforward, using Boc SPPS (*tert*-butoxycarbonyl solid-phase peptide synthesis) of peptide thioesters and a Cys-peptide, and convergent native chemical ligation of unprotected synthetic peptides to give the full-length polypeptide chain followed by folding with concomitant formation of the three native disulfide bonds.[15]

In the total chemical syntheses of CCL1 and Ser-CCL1 glycoproteins, the key consideration was introduction of the glycan moiety. One practical method for glycoprotein synthesis is to prepare a suitable glycopeptide-thioester segment for use in a convergent synthetic strategy based on native chemical ligation. Considering the inherent difficulties of glycopeptide synthesis,[16] we wanted this segment to be as short as possible to minimize exposure of the glycan moiety to the conditions of chemical peptide synthesis. Since the natural N-glycosylation site is at $Asn^{29}$, we chose $Leu^{25}$-$Cys^{26}$ and $Ile^{33}$-$Cys^{34}$ as ligation sites, and designed three peptide segments for use in a convergent synthesis: $Lys^{1}$-$Leu^{25}$-$^{\alpha}$thioester (for Ser-CCL1 we used the one residue longer Ser-$Lys^{1}$-$Leu^{25}$-$^{\alpha}$thioester), $Thz^{26}$-$Ile^{33}$-$^{\alpha}$thioester (where Thz is a protected form of cysteine), and $Cys^{34}$-$Lys^{73}$ (Scheme 1).




***Scheme 1.*** Synthetic strategy using convergent native chemical ligation (NCL) reactions. Non-glycosylated Ser-CCL1 PDB coordinates (1L0) were used for the model of glycosylated CCL1. The glycan model was created on Glycoscience.de (http://www.glycosciences.de).

This synthetic design enabled us to use a glycopeptide segment consisting of just eight amino acids, i.e. $Thz^{26}$-$Asn^{29}$(glycan)-$Ile^{33}$-thioester.

The exact structure of the glycan moiety found in CCL1 isolated from nature has not been determined. It has been reported that the N-linked glycan found in recombinantly prepared CCL1 exhibits heterogeneity but is non-sialylated.[1b,8b,17] In principle, an N-linked glycan possesses a consensus core pentasaccharide at the reducing end, which is covalently linked to an Asn side chain.[13] Thus, at a minimum, the N-glycan we would use should have that core structure and be non-sialylated. Based on this reasoning, we decided to introduce a dibranched complex-type asialo-nonasaccharide as the glycan moiety (Scheme 1). This complex type N-glycan is one of the mature N-linked glycan moieties found in human glycoproteins, and is frequently observed in biological systems.[13]

Conventional Boc SPPS cannot be used for the synthesis of peptides containing a complex glycan moiety because of the instability of the glycan moiety in strong acid media particularly the anhydrous HF used in the final deprotection step.[18] Thus, Fmoc (9-fluorenylmethoxycarbonyl) SPPS, which is compatible with glycopeptide synthesis, is used to make such glycopeptides. However, the synthesis of peptide-$^{\alpha}$thioesters is not compatible with Fmoc SPPS because the thioester moiety is not stable to the repeated treatments with concentrated piperidine solution used to remove the *N*-$^{\alpha}$Fmoc group at each stage of the synthesis.[19] Recently, Dawson et al. reported the use of diaminobenzoic acid (Dbz) as a linker for Fmoc SPPS of peptide-$^{\alpha}$thioesters by using peptide-*N*-acylbenzimidazolinone (peptide-$^{\alpha}$Nbz).[3] Since this method is a simple and feasible alternative for the synthesis of glycopeptide-$^{\alpha}$thioesters, we set out to make the glycopeptide $Thz^{26}$-$Asn^{29}$(glycan)-$Ile^{33}$-$^{\alpha}$Nbz by Fmoc SPPS.

The N-glycan moiety was obtained as $N^{\alpha}$-Fmoc-Asn-(glycan)-OH from hen egg yolk as previously reported.[20] For the synthesis, we used amino-PEGA-resin[21] and we combined use of the Rink amide linker with the diaminobenzoic acid (Dbz) linker as reported by Dawson.[3] Coupling of the linker moieties and the four C-terminal amino acids was performed using the standard Fmoc SPPS protocol reported by Dawson et al. Subsequent couplings of amino acid derivatives were conducted according to previously reported methods. Coupling of the $N^{\alpha}$-Fmoc-Asn(glycan)-OH was performed by using $N^{\alpha}$-Fmoc-Asn(glycan)-OH, 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DPEBT), and *N*,*N*′-diisopropylethylamine. Since the glycan moiety possessed completely free hydroxy groups, the last three amino acid residues (Fmoc-Arg-(Pbf), Fmoc-Tyr(*t*Bu), and Boc-Thz) following $Asn^{29}$(glycan) were coupled using dilute reaction conditions to avoid undesired esterification of glycan hydroxy groups.[20b]

A potentially challenging step in the synthesis of the glycopeptide $Thz^{26}$-$Asn^{29}$(glycan)-$Ile^{33}$-$^{\alpha}$Nbz, was the final activation of the Dbz linker moiety to convert it into the Nbz linker. This step required formylation with *p*-nitrophenylchloroformate followed by a base treatment to promote

intramolecular attack of the anilide to form the resin-bounded benzimidazoline.[3] In the current synthesis, the glycopeptide has an unprotected glycan, so unwanted formylation of the many hydroxy groups was a concern. However, the formylation step was performed by using only *p*-nitrophenylchloroformate in dichloromethane, that is, non-basic conditions, under which the glycan hydroxy groups would not be expected to act as nucleophiles. In the event, we were able to perform the conversion of the Dbz moiety to Nbz without significant problems, and we could obtain sufficient amount of the glycopeptide-$^{\alpha}$Nbz after cleavage from the resin with simultaneous deprotection of side-chains by trifluoroacetic acid (TFA) treatment, followed by HPLC purification (43% isolated yield, Figure 2). The successful synthesis of the glycopeptide [Thz$^{26}$-Asn$^{29}$(glycan)-Ile$^{33}$]-$^{\alpha}$Nbz, demonstrated that Dawson's Nbz linker strategy is compatible with glycopeptide synthesis even using an unprotected glycan.

After successfully obtaining the glycopeptide-$^{\alpha}$Nbz segment, we set out to make the full-length CCL1 glycosylated polypeptide chain by convergent native chemical ligation (Figure 3). Non-glycosylated peptides were prepared by standard methods (Supporting Information). Cys$^{34}$-Lys$^{73}$ and a 1.1-fold excess amount of Thz$^{26}$-Asn$^{29}$(glycan)-Ile$^{33}$-$^{\alpha}$Nbz

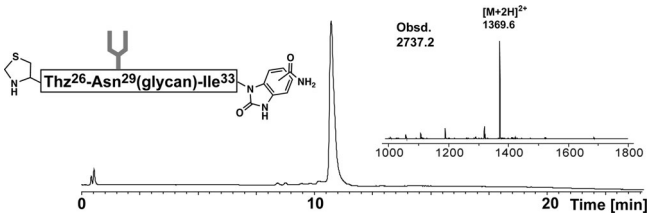



***Figure 2.*** Analytical LC-MS of purified Thz$^{26}$-Asn$^{29}$(glycan)-Ile$^{33}$-$^{\alpha}$Nbz. Inset is on-line ESI-MS spectra taken at the top of major peak (obsd mass: 2737.2 Da; calcd mass: 2737.7 Da (average isotopes). Chromatographic separation was performed by using a linear gradient (1–41% of buffer B in buffer A over 20 min after an initial isocratic phase of 1% buffer B in buffer A for 3 min. Buffer A: 0.1% (vol/vol) TFA in water; buffer B: 0.08% (vol/vol) TFA in acetonitrile).

were reacted at pH 7.2 in aqueous phosphate buffer containing 6 M guanidine hydrochloride (GuHCl), 200 mM 4-mercaptophenylacetic acid (MPAA) as thiol catalyst, and 20 mM triscarboxyethylphosphine hydrochloride (TCEP) as a reducing agent. The high concentration of the MPAA thiol catalyst, as well as high concentrations of both peptide segments (4.0 mM and 4.4 mM, respectively) were essential for this first native chemical ligation step. Even so, the sterically hindered amino acid (Ile) at the Xaa-Cys ligation site gave only slow

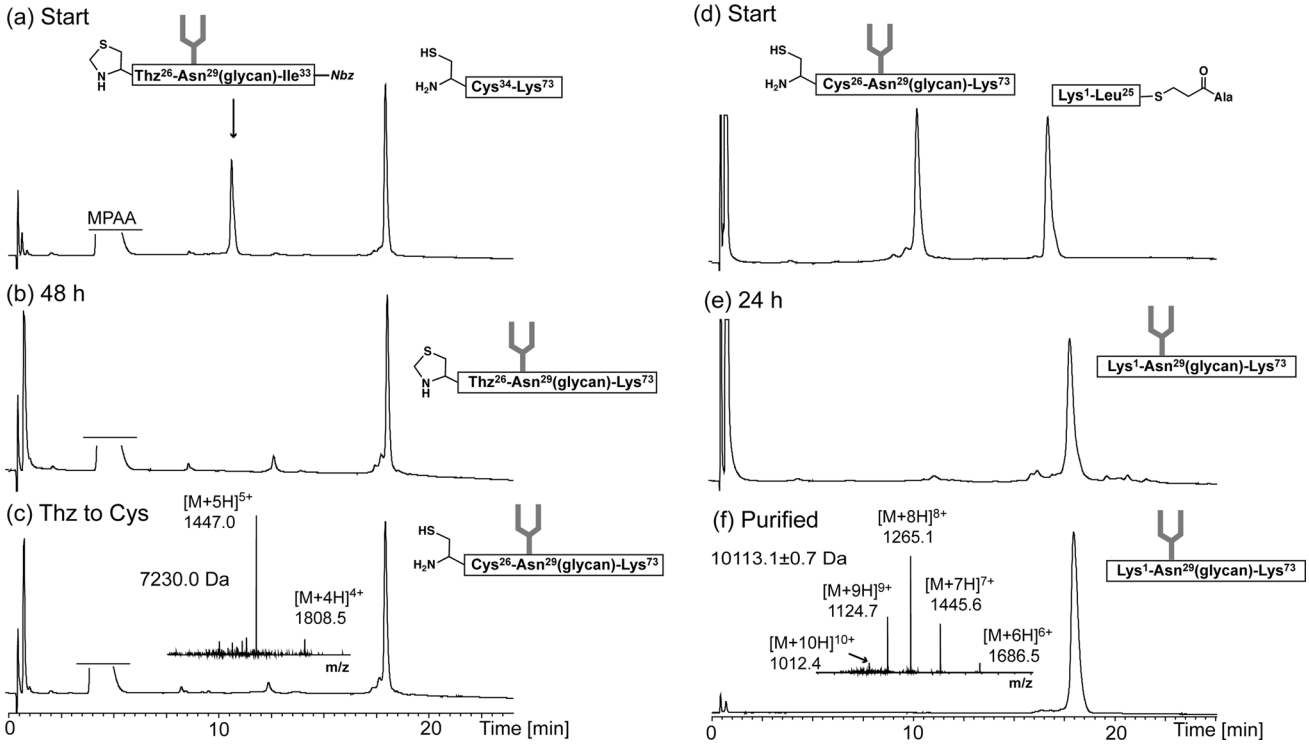



***Figure 3.*** Synthesis of the full-length glycosylated-CCL1 glycopeptide chain by convergent native chemical ligations. Left side: The 1st ligation between Thz$^{26}$-Asn$^{29}$(glycan)-Ile$^{33}$-$^{\alpha}$Nbz and Cys$^{34}$-Lys$^{73}$: a) reaction mixture starting point ($t<1$ min); b) after 48 h; c) Thz to Cys conversion was complete after overnight reaction ($\approx$15 h). Inset: Observed ESI-MS of the main peak (obsd mass: 7230.0 Da; calcd. mass for Cys$^{26}$-Asn$^{29}$(glycan)-Lys$^{73}$: 7230.6 Da (average isotopes)). Right side: HPLC monitoring of the 2nd ligation between Lys$^{1}$-Leu$^{25}$-$^{\alpha}$thioester and Cys$^{26}$-Asn$^{29}$(glycan)-Lys$^{73}$: d) reaction mixture starting point ($t<1$ min); e) after 24 h; f) product after purification. Inset: Observed ESI-MS of the main peak (obsd mass: 10113.1$\pm$0.7 Da; calcd mass for Lys$^{1}$-Asn$^{29}$(glycan)-Lys$^{73}$: 10113.5 Da (average isotopes)). Isolated yield 45% based on peptide Cys$^{34}$-Lys$^{73}$. Chromatographic separations were performed using linear gradients: for (a)–(c) 1–41% of buffer B in buffer A over 20 min after an initial isocratic phase of 1% buffer B in buffer A for 3 min; for (d)–(f) 20–40% of buffer B in buffer A over 20 min after an initial isocratic phase of 20% buffer B in buffer A for 3 min. Buffer A: 0.1% (vol/vol) TFA in water; buffer B: 0.08% (vol/vol) TFA in acetonitrile.

reaction, even under the optimized conditions derived from our previous work on the synthesis of IGF-1.[22] After 48 h, the reaction was essentially complete and gave a near-quantitative yield of the desired reaction product (Figure 3b). The resulting Thz$^{26}$-Asn$^{29}$(glycan)-Lys$^{73}$ glycopeptide was converted to the Cys-glycopeptide by treatment at pH 4.0 with 0.2 M methoxyamine-HCl; this afforded Cys$^{26}$-Asn$^{29}$(glycan)-Lys$^{73}$.

The crude Cys$^{26}$-Asn$^{29}$(glycan)-Lys$^{73}$ product was then reacted with Lys$^{1}$-Leu$^{25}$-$^{\alpha}$thioester, in pH 7.0 buffer solution consisting of 0.1 M phosphate containing 6 M GuHCl, 150 mM MPAA, and 20 mM TCEP. The MPAA used in this buffer solution was carried over from the first ligation step and the peptide concentrations were adjusted to 3.0 mM for the Cys-peptide and 3.3 mM for the peptide-$^{\alpha}$thioester. Reaction was essentially completed in 24 h at room temperature (Figure 3e). Purification of the reaction mixture by preparative HPLC afforded the desired Lys$^{1}$-Asn$^{29}$(glycan)-Lys$^{73}$ full-length glycosylated polypeptide (Figure 3f), with obsd mass of 10113.1 ± 0.7 Da (calcd 10113.5 Da (average isotopes)). The isolated yield was 45% over the two ligation steps, based on the limiting amount of starting peptide Cys$^{34}$-Lys$^{73}$.

In essentially the same manner, we obtained the following full-length polypeptides: [Lys$^{1}$-Lys$^{73}$]CCL1 (obsd mass 8491.4 ± 0.2 Da, calcd 8490.0 Da); glycosylated Ser-[Lys$^{1}$-Asn$^{29}$(glycan)-Lys$^{73}$]CCL1 (obsd mass 10200.4 ± 0.3 Da, calcd 10200.5 Da), and non-glycosylated Ser-[Lys$^{1}$-Lys$^{73}$]CCL1 (obsd mass 8576.2 ± 0.3 Da, calcd 8577.1 Da). Full experimental details and analytical data are reported in the Supporting Information.

In order to form the three disulfide bonds present in the native CCL1 protein molecule, the obtained glycosylated Lys$^{1}$-Asn$^{29}$(glycan)-Lys$^{73}$ polypeptide was subjected to folding by using a redox system containing 8 mM cysteine and 1 mM cystine at pH 8.0. The reaction was monitored by LC-MS, and was completed in 3 h with the appearance of an earlier-eluting final product on LC-MS analysis (Figure 4), with an observed mass of 10107.1 ± 0.2 Da. This was consistent with the mass of the desired product (calcd 10107.4 Da, average isotope composition) and was 6.0 ± 0.5 Da lower mass compared with the starting polypeptide, consistent with the formation of three disulfide bonds with concomitant loss of 6 protons. The isolated yield was 17.7 milligrams of purified synthetic glycoprotein corresponding to a yield of 68%, based on the initial amount of reduced polypeptide. In essentially the same manner, we obtained comparable amounts of the other folded protein products: glycosylated Ser-CCL1, CCL1, and Ser-CCL1, all with correct masses within experimental uncertainty (Figure 5 and Supporting Information). All the folding/disulfide bond formation reactions were completed within 3 h, and there were no significant differences observed among the folding reactions. The folded structures of Ser-CCL1 and glycosylated Ser-CCL1 were confirmed by high-resolution X-ray crystallography.[23]

Each glycosylated protein product eluted earlier on analytical reverse phase HPLC compared to the corresponding non-glycosylated protein, because of the presence of the additional hydrophilic glycan moiety. These results are consistent with the previously reported results for the chemical syntheses of chemokines, as well as the reported synthesis of a glycosylated chemokine: glycosylated-MCP3.[24]

It is well known that chemokines can induce specific leukocyte chemotaxis. In the case of CCL1, this biological function is exerted through binding to the receptor CCR8.[8] We performed chemotaxis assays on the synthetic CCL1 (glyco)proteins using a CCR8-transfected cell line. As shown in Figure 6, the most potent synthetic protein was the non-glycosylated 73 amino acid form of CCL1, which invoked cell migration with a typical bell-shaped dependence on concentration. The maximum migration was at a concentration of 10 nM. In five replicates of the chemotaxis assay, the mean peak chemotactic index for this form of CCL1 ranged from 6.4–19.3 at this concentration (data not shown). Glycosylated-CCL1 was also active on CCR8 transfectants, but with less potency than the non-glycosylated form: CCL1 has approximately double the activity of glycosylated-CCL1. On the other hand, for Ser-CCL1 both glycosylated and non-glycosylated forms exhibited much less potency than either form of CCL1—barely above background—and there was no significant difference between glycosylated and non-glycosylated forms.

The binding mode of chemokines to receptors has been extensively investigated, and it has been found that the N-terminal peptide preceding the first disulfide bond as well as the loop region following that disulfide bond are essential for receptor binding.[7a] In the present work, the glycosylation site is on neither of those regions of the CCL1 polypeptide chain. However, since the volume and the mobility of the glycan may be considerable,

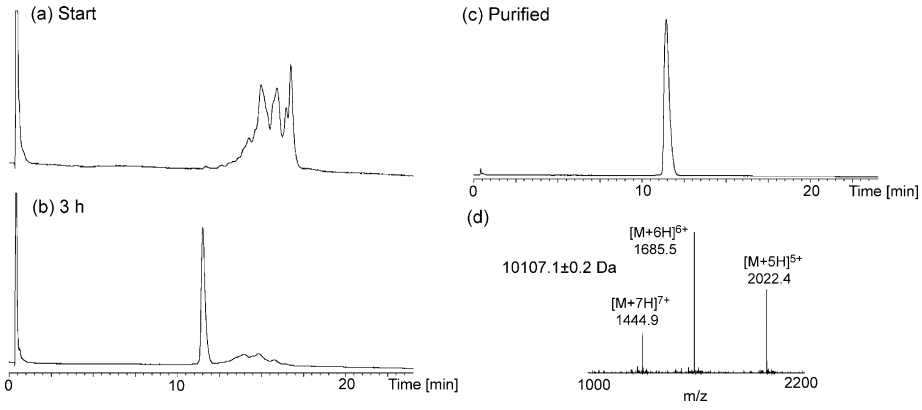



***Figure 4.*** Folding of Lys$^{1}$-Asn$^{29}$(glycan)-Lys$^{73}$. Folding was conducted using a redox system containing 8 mM cysteine and 1 mM cystine, at pH 8.0. LC-MS analysis of the folding reaction: a) starting point ($t$ < 1 min); b) after 3 h; c) after purification; d) Observed ESI-MS of the main peak (obsd mass: 10107.1 ± 0.2 Da; calcd mass for for folded glycosylated-CCL1: 10107.4 Da (average isotopes). Chromatographic separations were performed using linear gradients for (a)–(c) of 20–40% of buffer B in buffer A over 20 min after an initial isocratic phase of 20% buffer B in buffer A for 3 min. Buffer A: 0.1% (vol/vol) TFA in water; buffer B: 0.08% (vol/vol) TFA in acetonitrile.

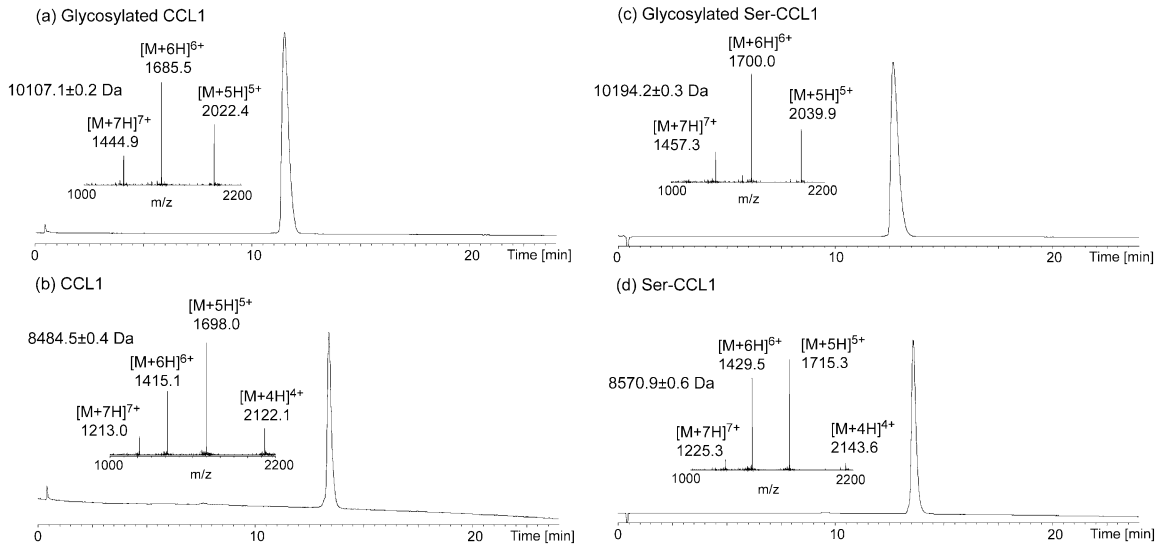



***Figure 5.*** Analytical LC-MS characterization of the synthetic proteins. Calculated masses (average isotope composition): a) glycosylated-CCL1: 10107.4 Da; b) CCL1: 8483.9 Da; c) glycosylated-Ser-CCL1: 10194.6 Da; d) Ser-CCL1: 8571.1 Da. Chromatographic separations were performed using linear gradients for (a)–(d) of 20–40% of buffer B in buffer A over 20 min after an initial isocratic phase of 20% buffer B in buffer A for 3 min. Buffer A: 0.1% (vol/vol) TFA in water; buffer B: 0.08% (vol/vol) TFA in acetonitrile.

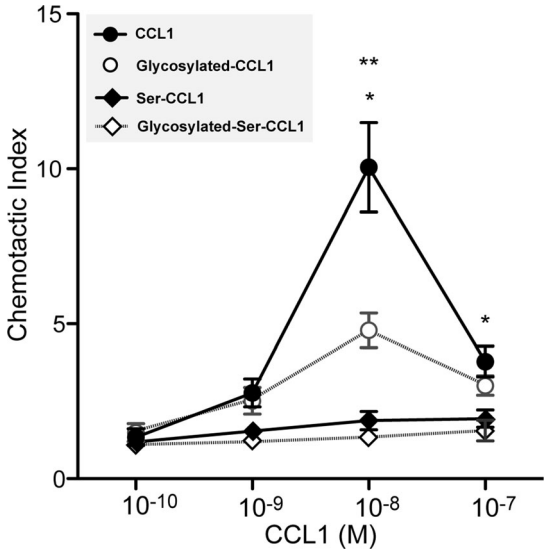



***Figure 6.*** Chemotaxis of hCCR8-transfected 4DE4 cells in response to varying concentrations of Ser-CCL1, Glycosylated-Ser-CCL1, CCL1, and Glycosylated-CCL1 (*, $p < 0.05$ by 2-way ANOVA with Bonferroni correction for CCL1 compared to Ser-CCL1 and Glycosylated-Ser-CCL1; **, $p < 0.05$ for CCL1 compared to Glycosylated-CCL1). The figure summarizes results from five independent experiments (mean ± SEM).

glycosylation may disturb receptor-binding by the CCL1 chemokine. In fact, chemokines are known to be retained on endothelial cells through binding to heparane-sulfate proteoglycan for recruiting leucocytes to the site of inflammation in vivo.[4,25] It could be that in vivo the incorporation of an N-glycan on CCL1 changes the binding affinity to glycosaminoglycan and consequently enhances leukocyte recruitment, a more important physiological effect than the negative effect on CCR8-mediated chemotaxis.

In conclusion, we have devised total chemical syntheses of glycosylated and non-glycosylated forms of the chemokine proteins CCL1 and Ser-CCL using a convergent synthetic strategy based on native chemical ligation (Scheme 1). The glycan moiety was obtained from hen egg yolk and was prepared as $N^{\alpha}$-Fmoc-Asn(asialo-nonasaccharide). Using this glycan, we carried out the synthesis of an unprotected glycopeptide-$^{\alpha}$Nbz thioester-equivalent as the key building block for the total synthesis of the glycoproteins. Combining this reagent equivalent of a glycopeptide-$^{\alpha}$thioester with the other peptide and peptide-$^{\alpha}$thioester segments, we obtained tens-of-milligram amounts of homogeneous synthetic glycoproteins as highly pure materials of defined chemical structure. Chemotaxis assays using a CCR8-transfected cell line were used to evaluate the impact of glycosylation on the biological function of CCL1 and Ser-CCL1. Non-glycosyalted CCL1 invoked chemotaxis at a concentration of 10 nM, while Ser-CCL1 had only minimal activity.

Intriguingly, we found that glycosylation of CCL1 negatively impacted CCR8-mediated chemotaxis in this assay. Thus we could conclude that glycosylation may not contribute directly to the chemotaxis function of CCL1 but may affect some other aspect of the molecular properties of CCL1 that are important for its biological functions in vivo. With convenient synthetic access to the structurally defined glycoprotein CCL1 and its non-glycosylated form, we will be able to further explore this question in a systematic fashion.

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