

# Glycosylation enhances functional stability of the chemotactic cytokine CCL2

Paolo Ruggiero<sup>1\*</sup>, Silvio Flati<sup>2</sup>, Vito Di Cioccio<sup>1</sup>, Giovanni Maurizi<sup>2</sup>, Giovanni Macchia<sup>1</sup>, Alberto Facchin<sup>1</sup>, Roberto Anacardio<sup>1</sup>, Antonio Maras<sup>3</sup>, Marilena Lucarelli<sup>4</sup> and Diana Boraschi<sup>4</sup>

<sup>1</sup> Dompé Research Centre, L'Aquila

<sup>2</sup> Consorzio Biolaq, L'Aquila

<sup>3</sup> Università degli Studi di Roma « La Sapienza »

<sup>4</sup> ITB, CNR, Pisa, Italy

Correspondence: Diana Boraschi, Unit of Immunobiology and Cell Differentiation, Institute of Biomedical Technologies, National Research Council, Area della Ricerca di S. Cataldo, Via G. Moruzzi 1, I-56124 Pisa, Italy. Tel.: + 39/050/315.2790; Fax: + 39/050/315.3367; Email: borasc@tin.it

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**ABSTRACT.** The human chemokine CCL2 gene was expressed in the yeast *P.pastoris* and gave rise to a mixture of differently glycosylated recombinant proteins. In comparison to non-glycosylated *E.coli*-derived CCL2, glycosylated yeast CCL2L was 4-20 times less active in a chemotactic assay *in vitro*. However, CCL2L could maintain full activity upon prolonged incubation at 37°C, whereas the non-glycosylated chemokine readily lost activity. It could be hypothesized that glycosylation is a mechanism used by the organism to modulate CCL2 stability. The partial loss of specific activity due to glycosylation is balanced by the advantage of prolonging the effectiveness of chemokine. Thus, differential glycosylation allows one to obtain highly effective short-lived CCL2 or less-effective long-lived CCL2 and may thus represent a novel mechanism of adaptation to pathological *versus* physiological conditions.

Keywords: chemokines, CCL2, glycosylation, stability

## INTRODUCTION

Chemokines are small proteins (8-12 kDa) grouped into four subfamilies (CXC, CC, C, and CX3C) according to the organisation of positionally conserved cysteine residues [1]. The CC chemokine ligand 2 (CCL2), formerly known as monocyte chemoattractant protein-1 (MCP-1) [2], is a prototype CC chemokine, active towards monocytes, dendritic cells, and NK cells, thereby playing an important role in innate immunity by directing cell migration into inflammatory sites and activating their effector functions [3-8]. Recent data indicated a function for CCL2 in adaptive immunity as a regulator of T cell commitment to both T helper cell type 2 (Th2) and Th1 effector function [9-12].

The cDNA coding for CCL2 has been cloned and characterised [13]. CCL2 is expressed as an 8 kDa protein, containing a short signal peptide for secretion which is removed during processing and allows the N-terminal glutamate residue to be cyclised to pyroglutamate. The primary structure also contains two consensus glycosylation sequences, one for N-glycosylation

in the N-terminal region [14], the other one for O-glycosylation [15].

Native CCL2 is produced by a variety of cells under appropriate stimulation as a mixture of unglycosylated and glycosylated forms, the latter with different patterns of glycosylation [16]. Recombinant human CCL2 has been expressed in a range of expression systems including *Escherichia coli* [17], insect cells [18], COS-1 cells [19], and *Pichia pastoris* [20]. Recombinant CCL2 expressed in *E.coli* is active but unstable, suggesting that correct glycosylation can help CCL2 in assuming its proper conformation and stability. Glycosylated CCL2 produced by insect cells or *P.pastoris* is active but not to the same degree as *E.coli* CCL2, suggesting that glycosylation may impair activity.

This study was undertaken with the aim of elucidating the biological role of CCL2 glycosylation. To this end, the *P.pastoris* expression system has been used for the production of glycosylated CCL2. *Pichia pastoris* is a methylotrophic yeast, which has been used as a host for expression of recombinant proteins with excellent results [21]. The advantages of the system are the good levels of expression and good yields of recombinant products, the ease of culture and induction of expression, and a glycosylation pattern more similar to that of mammalian proteins, as compared with *Saccharomyces* [22]. Both

\* Present Address: IRIS Research Center, Chiron Vaccines S.r.l., Via Fiorentina 1, I-53100 Siena, Italy

CCL2 and CCL7 (previously named MCP-3) have been expressed in *P.pastoris*, yielding heterogeneously glycosylated proteins with modified N-terminus [20, 23]. The stability and biological activity of these forms is however not known.

Information gathered in this study about the role of glycosylation in CCL2 bioactivity will help the understanding of the heterogeneity of recombinant CCL2 activity. Indeed, the limited availability of correctly glycosylated CCL2 greatly impairs the possibility of *in vivo* testing, which would allow better characterisation of the physiological role of this protein and the possible development of CCL2-based therapeutics.

## METHODS

### Media

YPD medium is composed of 1% yeast extract, 2% peptone, 2% dextrose. RDB (regeneration dextrose) medium is 1 M sorbitol, 1% dextrose, 1.34% yeast nitrogen base (YNB),  $4 \times 10^{-5}$ % biotin, 0.005% amino acids. MD (minimal dextrose) medium is composed of 1.34% YNB, 1% dextrose,  $4 \times 10^{-5}$ % biotin. MM (minimal methanol) medium is composed of 1.34% YNB, 0.5% methanol,  $4 \times 10^{-5}$ % biotin. BMGY (buffered glycerol complex) medium is 1% yeast extract, 2% peptone, 1.34% YNB,  $4 \times 10^{-5}$ % biotin, 100 mM potassium phosphate, pH 6.0, and 1% glycerol. BMMY (buffered methanol complex) medium was the same as BMGY except for the addition of 0.5% methanol instead of 1% glycerol. Media were purchased from Difco (Detroit, MI, USA) and Sigma Chemical Co. (St. Louis, MO, USA).

### Cloning of the human CCL2 cDNA in pHIL-S1

A 228 bp cDNA fragment coding for the mature form of human CCL2 was PCR-amplified with the following oligonucleotides:

*Xho*I

**CCL2 F** GGTCTCGAGGTCAGCCAGATGCAATCAAT

Arg Gly Gln Pro Asp Ala Ile Asn

*Bam*HI

**CCL2 R** CAAAGGATCCTCAAGTCTTCGGAGTTTG

The amplified DNA fragment was restricted with *Xho*I and *Bam*HI and cloned in the same sites of plasmid pHIL-S1 (Invitrogen, San Diego, CA, USA). The final construct (pHIL-S1/HMCP-1) contained the human CCL2 coding sequence preceded by the PHO1 yeast signal, under the control of the yeast alcohol oxidase promoter (AOX-1). As a consequence of the use of the yeast signal sequence, in order to preserve the leader peptidase recognition sequence, two triplets were introduced downstream of the cleavage site. For this reason the sequence for human recombinant CCL2 also encodes for two extra amino acids at its N-terminus compared with the natural sequence (Arg-Gly-Gln-Pro-Asp- instead of (Pyro)-Gln-Pro-Asp-). The orientation and reading frame of the fragment were verified by sequence analysis.

### Expression of human recombinant CCL2 in *P.pastoris*

The *P.pastoris* strain GS115 (His<sup>4</sup>; Invitrogen) was transformed by electroporation with the plasmid pHIL-

S1/HMCP-1 following the manufacturer's instructions. Briefly, a culture of *P.pastoris* GS115 was grown in YPD medium at 30°C up to OD<sub>600</sub> 1.5. Cells were centrifuged, rinsed twice in ice-cold sterile water, and resuspended in ice-cold 1 M sorbitol in 1/20 and 1/40 of the volume of the starting culture. An aliquot of 80 µl of electrocompetent cells were electroporated together with approximately 15 µg of linearised plasmid DNA with the following parameters: 1500 Volts, 25 µF, 400 Ω. After plating on histidine-deficient medium (RDB), several colonies were selected and plated in duplicate on solid media containing dextrose (MD) or methanol (MM) as the sole carbon source. Two clones (51 and 69) showed a slow growth rate on minimal methanol medium, as a consequence of the integration in the AOX-1 locus of the pHIL-S1/HMCP-1 plasmid. These His<sup>+</sup>Mut<sup>-</sup> clones were cultured in BMGY liquid medium, and the expression of recombinant products was then induced with the addition of methanol (BMMY). Aliquots were collected at different times between 24 and 150 h from induction. Culture supernatants were analysed by SDS-PAGE. The presence of recombinant CCL2 was confirmed by Western blot analysis with rabbit anti-human CCL2 polyclonal antibodies (PeproTech, Rocky Hill, NJ, USA) or with mouse monoclonal anti-human CCL2 ascites (5D3; kindly provided by G. Peri, Mario Negri Institute, Milano, Italy) [24].

### Purification and analysis of recombinant CCL2

Culture supernatants were diluted 1:10 with 25 mM MES pH 5.0 (buffer A) and loaded on an S-Sepharose HP 35/100 column (Amersham Biosciences, Uppsala, Sweden) equilibrated in buffer A. After washing using the same buffer, the column was eluted by a linear gradient of NaCl, from 0 to 1 M in buffer A. The fractions were analysed by SDS-PAGE and Western blot as described below. The fractions with low molecular weight CCL2 (CCL2L) and high molecular weight CCL2 (CCL2H) were pooled separately, diluted 1:2 with 50 mM Tris pH 7.5 (buffer B), and affinity purified through a Heparin-Sepharose CL-6B column (Amersham Biosciences) equilibrated in buffer B and eluted with a linear gradient of NaCl, from 0 to 2 M in buffer B.

Samples were subjected to reducing 18% SDS-PAGE in a Mini-Protean II apparatus (Bio-Rad, Hercules, CA, USA). Broad molecular weight standards (Bio-Rad) were used to determine the apparent molecular weight of electrophoretic bands. To determine the protein concentration, serial dilutions of purified *P.pastoris* CCL2 and known amounts of *E.coli*-derived human recombinant CCL2 (PeproTech), ranging from 0.4 to 4 µg, were loaded in the same gel. After laser scanning of Coomassie blue-stained gels, the electrophoretic bands were quantified by Image Quant Software (Molecular Dynamics/Amersham Biosciences). The values obtained for *E.coli* CCL2 electrophoretic bands were used to create a reference curve, by which the protein concentration of *P.pastoris* CCL2 samples was calculated.

For Western blot analysis, samples run on 18% SDS-PAGE were transferred to a 0.22 µm nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany) at 1 mA/cm<sup>2</sup> for 90 min in a Multiphor II semidry apparatus (Amersham Biosciences). Membranes were stained with a Ponceau-S solution (Sigma), the relevant molecular

weight standards were marked, and complete destaining was achieved by rinsing in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS). Membranes were saturated for 1 h with a 3% BSA solution in TBS (BT), then incubated overnight with a 1:100,000 dilution of monoclonal 5D3 ascitic fluid anti-human CCL2 in BT containing 0.05% Tween 20 (BTT). After two washes in BTT, membranes were incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (Bio-Rad) diluted 1:15,000 in BTT, then washed once in BTT, once in 0.25% Tween 20 in TBS, and several times in TBS. All incubations were performed on a shaking platform at room temperature. The peroxidase activity was revealed either by IBI Enzygraphic Web film (Kodak, New Haven, CT, USA) or by the ECL detection system (Amersham International, Amersham, UK).

For de-glycosylation, approximately 10 µg of CCL2 derived from *P.pastoris* were heated at 100°C in the incubation buffer composed by 50 mM sodium acetate pH 5.0, 20 mM EDTA, SDS 2% w/v and 2-mercaptoethanol 1% v/v. After heating, N-octylglucoside (up to 1%) and 0.2 units of Endoglycosydase F (both from Boehringer Mannheim, Mannheim, Germany) were added sequentially and incubated overnight at 37°C.

#### Chemotaxis assay

Cells from the human myelomonocytic THP-1 cell line, obtained from ATCC (Rockville, MD, USA), were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 µM 2-ME and 50 µg/ml gentamicin sulfate (all from Life Technologies, Paisley, Scotland) and used as the responding cells.

THP-1 chemotaxis was measured using a 48-well chemotaxis chamber (Neuroprobe, Cabin John, MD, USA) housing a polycarbonate filter with 5 µm pores (Poretics Corp., Livermore, CA, USA). *E.coli*-derived human recombinant CCL2 (used as reference standard) and *P.pastoris* CCL2 samples were diluted in RPMI-1640 and placed in the lower wells. Aliquots of 10<sup>5</sup> THP-1 cells/well were placed above the filter in the upper wells. After 90 min at 37°C, the cells were scraped from the upper surface of the filter and cells on the lower surface were fixed in methanol and stained with Diff-Quik (Baxter, Dudinggen, Switzerland). Filters were mounted on glass slides and observed under a 100x oil-immersion objective (Axioplan; Zeiss, Oberkochen, Germany). The migration for each sample was expressed as the number of cells migrated in five randomly chosen high power fields. All measurements were performed in triplicate. For the stability assay, CCL2 samples were diluted at 1 µg/ml in saline buffer and incubated at 37°C for different times. After 1:10 dilution, the residual activity of samples was evaluated in the chemotaxis assay as described above. In parallel experiments, incubation at 37°C was performed directly in the lower wells of the chemotaxis chamber. To exclude LPS contamination, samples were routinely tested with the LAL test (Bio-Whittaker, Walkersville, MD, USA).

#### Mass spectrometry

A triple-stage quadrupole mass spectrometer (TSQ 700, Finnigan MAT, San Jose, CA, USA) equipped with an atmospheric pressure pneumatically assisted electrospray ion source was used. The interface capillary sprayer was

operated at a positive potential of 4.5 kV (spray current 1.4 µA). Ionised analyte was then transferred from atmospheric pressure to a vacuum ( $2.3 \times 10^{-6}$  Torr) through a heated metal capillary held at 200°C. Multiply charged molecule ions of the type  $M+nH^{n+}$  exciting the capillary were focussed by the tube lens through a skimmer and then focussed by an octapole lens into the mass analyser. Samples were delivered by direct infusion using a syringe pump (Harvard Apparatus, MA, USA) at a flow rate of 3 µl/min. Protein concentrations were typically 50 pmol/µl in water/methanol (50/50 vol/vol) containing 1% acetic acid. Full scan mass spectrometry experiments were performed by scanning the first quadrupole from  $m/z$  200 to 1800 in 1.5 sec. Data were acquired by a DEC station 5000/125 computer (Digital Equipment Corporation, Maynard, MA, USA) and were processed using the deconvolution software package BIOMASS (Finnigan MAT) to obtain molecular mass determinations from the multiply charged ion clusters.

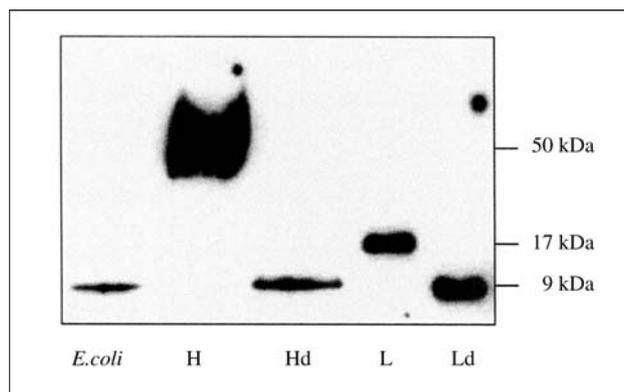
#### N-terminal sequencing

For N-terminal amino acid sequencing, the sample was subjected to a 15% SDS-PAGE in reducing conditions and electroblotted onto a polyvinylidene difluoride membrane (ProBlot™, Perkin Elmer, Norwalk, CT, USA) in 10 mM cyclohexylamino propane sulphonate, pH 11.0, containing 20% (vol/vol) methanol. The membrane was stained with 0.1% Coomassie blue R250 in 50% methanol and then destained in 50% methanol; bands of interest were excised. Automated Edman degradation of each band was performed using a Perkin Elmer model AB 476A sequencer.

## RESULTS

#### Expression and purification of human recombinant CCL2

Transformed yeast cells contained the gene for human CCL2 preceded by two extra triplets coding for Arg and Gly at the N-terminus. From selected transformants, two clones (51 and 69) were chosen for subsequent analysis. SDS-PAGE showed two recombinant products with the apparent molecular mass of 50 and 17 kDa, respectively, both recognised by specific antibodies raised against human CCL2. The two forms were termed CCL2H (high molecular weight; H) and CCL2L (low molecular weight; L) and probably represent differently glycosylated forms of the recombinant CCL2. The two forms were purified by ion exchange chromatography on S-Sepharose followed by affinity chromatography on Heparin-Sepharose (Figure 1; lanes H and L). Evidence that both CCL2H and CCL2L are glycosylated recombinant products has been obtained by treatment with Endoglycosydase F (Endo F), which cleaves Asn-linked glycans. Treatment with Endo F caused an increase of the electrophoretic mobility of the H and L forms, giving rise to digestion products (Figure 1; lanes Hd and Ld) that co-migrated with unglycosylated *E.coli* MCP-1 in SDS-PAGE. On the other hand, treatment with O-glycosidase, which cleaves O-linked sugars, had no effect on the electrophoretic mobility of CCL2H and CCL2L (data not shown). Sequence analysis of the *P.pastoris* CCL2 revealed, in addition to the expected product with two extra N-terminal amino acids (RG-



**Figure 1**

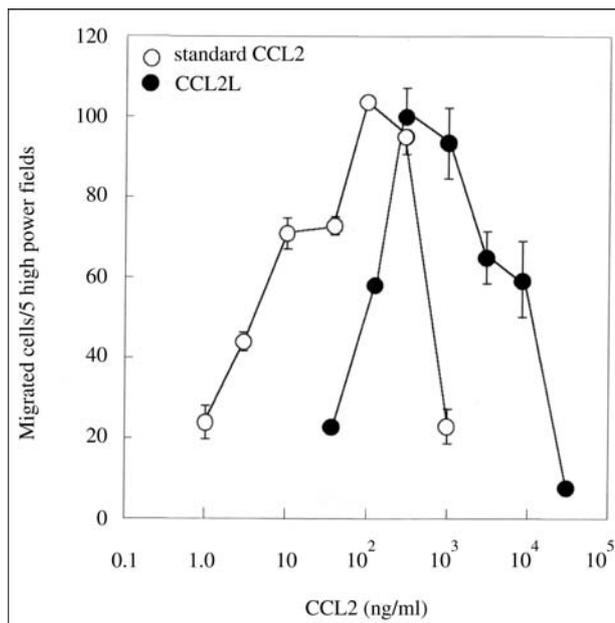
**Biochemical characterisation of human recombinant CCL2 expressed in *P.pastoris*.**

Samples of purified CCL2H and CCL2L were subjected to Western blot before (H and L) and after de-glycosylation with EndoF (Hd and Ld). CCL2 was detected using an anti-human CCL2 antibody and an HRP-conjugated secondary antibody. A reference standard of non-glycosylated *E.coli*-derived human recombinant CCL2 was used as control. On the right side the apparent molecular masses are indicated.

QPDAl), the presence of an amino-terminally truncated form starting at residue 3 (DAI; Table 1). The two proteins were present to approximately the same degree and, at variance with natural CCL2, the N-terminal end was not blocked. As expected, mass spectrometry of the mixture of CCL2 forms produced by *P.pastoris* revealed no trace of the natural 1-76 form of CCL2, with blocked N-terminal, usually produced by mammalian cells. By mass spectrometry, the molecular mass of non-glycosylated CCL2 from *P.pastoris* (N-terminally truncated form) was determined as 8,349 Da. Since the theoretical molecular mass of CCL2 3-76 (missing the first two amino acids) is 8,454 Da, it is clear that an additional truncation has occurred, conceivably at the C-terminal. Indeed, the molecular mass determined by mass spectrometry closely corresponds to that calculated for the 3-75 form of CCL2 (8,353 Da), i.e. a form lacking two amino acids at the N-terminal (QP) and one residue at the C-terminal (T).

**Biological activity of CCL2L**

The ability of CCL2L to induce chemotaxis of myelomonocytic cells was tested in a classical chemotaxis assay. CCL2L induced dose-dependent chemotaxis of THP-1 myelomonocytic cells (Figure 2) as well as of human peripheral blood monocytes (data not shown). The activity profiles followed a characteristic "bell shaped" dose-response curve with maximal effect observed around 300 ng/ml. CCL2L was about 4-20 fold less



**Figure 2**

**Chemotactic activity of human recombinant CCL2 expressed in *P.pastoris*.**

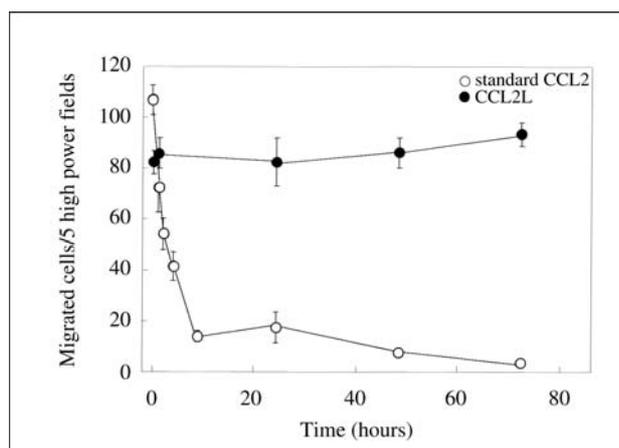
THP-1 chemotaxis was assessed in response to increasing concentrations of *P.pastoris* CCL2L (solid circles) or standard non-glycosylated *E.coli* CCL2 (open circles), and indicated as the net number (over background) of migrating THP-1 cells in five high-power fields. Data are expressed as the mean  $\pm$  SEM of triplicate filters. Background random migration to buffer alone was  $5 \pm 3$ . Data are from a single representative experiment.

active than non-glycosylated human recombinant CCL2 expressed in *E.coli* (used as reference standard) that showed a maximal response at approximately 100 ng/ml. From preliminary experiments, de-glycosylated CCL2L showed a chemotactic capacity slightly lower (2-5x) than that of *E.coli* CCL2 (data not shown). On the other hand, the hyper-glycosylated form CCL2H was almost completely inactive at all concentrations tested (data not shown), suggesting that hyper-glycosylation may cause loss of activity. To establish the role of glycosylation in the biological function of CCL2, the yeast CCL2L and the standard non-glycosylated *E.coli* CCL2 were kept at 37°C for different lengths of time before assay for chemotactic activity. As shown in Figure 3, standard non-glycosylated CCL2 rapidly lost its biological activity (about 50% in 2 h and 90% in 8 h), whereas the glycosylated CCL2L was able to retain full activity over 72 h at 37°C. Controls run with the purification buffer alone excluded any possible contribution of contaminating endotoxin (not shown).

**Table 1**  
**N-terminal sequence of CCL2 forms**

CCL2 form	Amino acids	N-terminal sequence	Presence in <i>P.pastoris</i>	Theoretical molecular mass
CCL2 wt	1-76	pyro-Gln-Pro-Asp-Ala-Ile	0%	8.662
CCL2L (a)	(-2)-75	Arg-Gly-Gln-Pro-Asp-Ala-Ile	50%	8.792
CCL2L (b)	3-75	Asp-Ala-Ile	50%	8.353

The N-terminal sequence of the two forms of *P.pastoris* CCL2L is shown, in comparison with that of the natural CCL2. Likewise, the theoretical molecular mass of the three CCL2 protein forms is indicated. The molecular mass of the N-terminally truncated CCL2L form of *P.pastoris* was measured and found to be 8,349, to indicate one additional C-terminal amino acid truncation. No trace of the intact 1-76 CCL2 form was found by mass spectrometry.



**Figure 3**

**Temperature stability of glycosylated versus non-glycosylated human recombinant CCL2.**

THP-1 chemotaxis was assessed in response to 100 ng/ml of *P.pastoris* CCL2L (solid circles) or standard non-glycosylated *E.coli* CCL2 (open circles), which had been pre-incubated at 37°C for the indicated times (horizontal axis). Chemotaxis was indicated as the net number (over background) of migrating THP-1 cells in five high-power fields. Data are expressed as the mean  $\pm$  SEM of triplicate filters. Background random migration to buffer alone was  $6 \pm 3$ . Data are from a single representative experiment.

## DISCUSSION

To study the biological function of glycosylation of human CCL2, recombinant human CCL2 was produced using the *Pichia pastoris* expression system [21]. This system produces oligosaccharidic structures in a fashion similar to that of mammalian cells, with a limited presence of hyper-glycosylated forms as compared to other expression systems [22]. Western blotting analysis of CCL2-transformed *P.pastoris* cell supernatants showed the presence of two CCL2 forms with different degrees of glycosylation. The ability of both forms to co-migrate with non-glycosylated CCL2 after digestion with Endoglycosidase F, but not with O-glycosydase, indicates that the polysaccharide chain is unique and Asn-linked, as already reported [20]. The N-terminal sequence of *P.pastoris* CCL2 revealed that two N-terminal variant forms of the protein are present in essentially equal amounts, the form with two extra *Pichia*-derived N-terminal amino acids (RG-QPDAI) and a truncated form missing the first two CCL2 amino acids (DAI). No trace of CCL2 with an intact N-terminal could be found by mass spectrometry. Mass spectrometry of the de-glycosylated CCL2 of *P.pastoris* indicated a molecular mass of 8,349 Da for the N-terminally truncated form. This corresponds to the theoretical mass of the 3-75 protein (8,353 Da), suggesting the presence of an additional truncation at the level of the C-terminal threonine. Since the N-terminally truncated form of CCL2 with the first two amino acids missing has been reported to be only weakly chemotactic [20, 25], it is conceivable that most of the biological activity observed in the CCL2L preparation could be attributed to the form with two extra N-terminal amino acids, which accounts for about 50% of the recombinant protein. C-terminal truncation, on the other hand, is not expected to significantly affect the chemotactic activity of CCL2. In fact, the natural CCL2 form 1-69 is fully active as compared to the entire form 1-76 [26]. Thus, CCL2L

would be expected to be about 2-fold less active than wild type CCL2. Preliminary experiments, limited by the insufficient recovery of material, suggest that de-glycosylated *P.pastoris* CCL2L has indeed 2-5 times lower activity than *E.coli* CCL2. On the other hand, the biological activity of CCL2L measured by chemotaxis assay is 4-20 fold lower than that of non-glycosylated *E.coli* CCL2. This may indicate that light glycosylation can partly reduce CCL2 specific activity [20, 27]. Conversely, heavy glycosylation, as in the CCL2H form, results in complete loss of activity. A significant functional difference between glycosylated and non-glycosylated CCL2 forms was evident when maintained at 37°C for a prolonged period. Incubation at 37°C caused the rapid loss of the chemotactic activity of the non-glycosylated CCL2, whereas the glycosylated yeast CCL2L protein maintained its biological effectiveness. Incubation experiments were performed directly in the wells of the chemotaxis chamber, to exclude loss of CCL2 by sticking on the vessel walls.

These results provide new information useful for the understanding of the functional role of CCL2 glycosylation [26]. Indeed, natural CCL2 is mainly glycosylated and shows a high degree of heterogeneity of glycosylation [26]. Current hypotheses about the role of glycosylation of CCL2 include enhanced resistance to protease attack (although N-terminal truncation can occur also in glycosylated forms [26]), modulation of affinity for the receptor, alteration of pharmacokinetic parameters of tissue distribution and clearance. A further hypothesis suggested by the present data is that the polysaccharidic chain might enhance stability of the protein, possibly by preserving native conformation and/or enhancing solubility. Thus, on one hand glycosylation impairs biological activity of CCL2 (the hyper-glycosylated CCL2H form is completely inactive; the CCL2L form is 4-20 fold less active than non-glycosylated CCL2), as also suggested in previous studies [28, 29]. On the other hand, glycosylated CCL2 has prolonged stability as compared to the highly labile non-glycosylated protein, and maintains its biological activity for up to 72 h at 37°C. The notion that natural CCL2 is glycosylated and that there is heterogeneity of glycosylation [26] leads to the hypothesis that glycosylation is a mechanism used by the organism to modulate the effectiveness of the chemokine. In fact, the partial loss of specific activity due to glycosylation is counterbalanced by the advantage of prolonging its effectiveness. It could be proposed that differential glycosylation may allow highly effective short-lived CCL2 or less-effective long-lived CCL2 to be obtained, and may thus represent a novel mechanism of adaptation to pathological versus physiological conditions.

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