N-terminal pyroglutamate formation in CX3CL1 is essential for its full biologic activity

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Abstract
CX3CL1 is the unique member of the CX3C chemokine family and mediates both adhesion and cell migration in inflammatory processes. Frequently, the activity of chemokines depends on a modified N-terminus as described for the N-terminus of CCL2 modified to a pyroglutamate (pGlu-) residue by glutaminyl cyclase activity. Here, we assess the role of the pGlu-modified residue of the CX3CL1 chemokine domain in human endothelial and smooth muscle cells.

For the first time, we demonstrated using mass spectrometry that glutaminyl cyclase (QC, QPCT) or its isoenzyme isoQC (QPCTL) catalyse the formation of N-terminal modified pGlu-CX3CL1. Expression of QPCT is co-regulated with its substrates CCL2 and CX3CL1 in human endothelial (HUVEC) and smooth muscle cells (HCASMC) upon stimulation with TNF-α and IL-1β whereas QPCTL expression is not affected. By contrast, inhibition of the NF-κB pathway using an IKK2 inhibitor decreased the expression of the co-regulated targets QPCT, CCL2, and CX3CL1. Furthermore, RNAi-mediated inhibition of QPCT expression resulted in a reduction of CCL2, CX3CL1 mRNA. In HCASMC, N-terminal modified pGlu1-CX3CL1 induced a significant stronger effect on phosphorylation of ERK1/2, Akt and p38 kinases than the immature Gln1-CX3CL1 in a time- and concentration-dependent manner. Furthermore, pGlu1-CX3CL1 affected the expression of CCL2, CX3CL1, and the adhesion molecule ICAM1/CD54 inducing in higher expression level compared to its Gln1-variant in both HCASMC and HUVEC.

These results strongly suggest that QC-catalysed N-terminal pGlu-formation of CX3CL1 is important for the stability or the interaction with its receptor and open new insights into the function of QC in inflammation.

Summary statement
The N-terminal pyroglutamate residue of fractalkine is formed by glutaminyl cyclase activities. This posttranslational modification is essential for full activity of this chemokine with respect to stimulation of kinase dependent signalling pathways and gene expression of chemokines and adhesion molecules.

Short Title:
Impact of N-terminal pyroglutamate formation on CX3CL1 activity

Keywords:
CX3CL1, CCL2, ICAM1/CD54, Glutaminyl Cyclase, signaling, gene expression

Abbreviation list:
Akt, protein kinase B; BSA, bovine serum albumin; CCL2/MCP-1, monocyte chemotactic protein-1; CX3CL1, fractalkine; CX3CR1, fractalkine receptor 1; ERK, extracellular signal-regulated kinase; HCASMC, human coronary artery smooth muscle cells; HUVEC, human umbilical vein endothelial cells; ICAM1/CD54, intercellular adhesion molecule-1; isoQC, isoglutaminyl cyclase; NTC, non-target control; p38, p38 mitogen-activated protein kinase; pGlu, pyroglutamate; QC, glutaminyl cyclase; QPCT, gene name of QC; QPCTL, gene name of isoQC
Introduction

Increased chemokine levels and monocyte activation are common components of the pathogenesis of inflammatory diseases including atherosclerosis and chronic lung diseases. Especially the chemokines CCL2 (MCP-1, monocyte chemoattractant protein-1) and CX3CL1 (fractalkine) are described as key players for the attraction and migration of monocytes into the inflamed tissue. In contrast to the secreted CCL2, CX3CL1 is synthesized as a transmembrane protein with its chemokine domain presented on an extended highly glycosylated mucin-like stalk [1,2]. The membrane-bound CX3CL1 promotes the integrin-independent adhesion by binding with its G protein-coupled, 7-transmembrane receptor CX3CR1 [3] and is a survival signal for circulating monocytes [4]. Soluble CX3CL1 can be cleaved from the cell surface-expressed CX3CL1 by metalloproteinases such as ADAM10, ADAM17 or MMP2 [5–8]. In contrast to the membrane-tethered chemokine, soluble CX3CL1 can act as a classical diffusible chemoattractant and can promote the formation of transepithelial dendrites [4,9]. Collectively in the periphery, CX3CL1/CX3CR1 interactions seem to play a critical role in inflammation both affecting leukocyte recruitment and local intercellular communication [4]. Interestingly, the N-terminus of both chemokines CCL2 and CX3CL1 possess a glutamine in the first position and could be post-translationally modified by glutaminyl cyclases to form a pyroglutamate (pGlu-) residue. Recently, we and others have shown that the chemotactic activity of CCL2 depends on a modified N-terminus of the polypeptide, particularly, the formation of a pGlu-residue protecting against proteolytic degradation in vivo [10,11]. The formation of the N-terminal pGlu-residue is an important maturation step during synthesis and secretion of not only CCL2 but also of hormones such as thyrotropin-releasing hormone (TRH) and gonadotropin-releasing hormone (GnRH) [12–14]. The N-terminal pGlu of CCL2 can be formed by both glutaminyl cyclase (E.C. 2.3.2.5, QC) and its iso-enzyme, the iso-glutaminyl cyclase (isoQC) [10,15]. Two distinct genes termed QPCT (QC; NM_012413) and QPCTL (isoQC; NM_017659) are coding for different proteins with QC activity. In contrast to the secreted QC, isoQC is exclusively localized within the Golgi complex. IsoQC shows 46 % sequence identity to the QC, and exhibit nearly identical substrate specificity in vitro [10,16]. Here, we describe CX3CL1 as a substrate of both enzymes QC and isoQC. We further demonstrate under inflammatory conditions a co-regulation of the substrates CCL2 and CX3CL1 with their modifying enzyme QC in human endothelial cells (HUVEC) and human coronary artery smooth muscle cells (HCASMC). Importantly, signalling of CX3CL1 depends on its modified N-terminus for activation of ERK1/2, p38, and Akt kinase as well as for induction of CCL2 and the adhesion molecule ICAM1/CD54 in HUVEC and HCASMC. Our results further support a role of QC in inflammatory processes.
Methods

Human glutaminyl cyclase (QC, EC 2.3.2.5) and its isoenzyme isoQC were expressed and purified as previously described [16].

For stimulation assays, recombinant human CX3CL1 chemokine domain (#300-31, PeproTech, Hamburg, Germany) was solubilised in aqua dest. (100 µg/ml). To generate the N-terminal pGlu-modification (pGlu1-CX3CL1), CX3CL1 (Gln1-CX3CL1; 1 mg/ml) was diluted 1:10 in phosphate buffered saline (PBS) and incubated with human recombinant QC (6 µg/ml) at 37 °C for 2 hours. Both CX3CL1 forms were aliquoted and stored at ≤-20°C until use. The pGlu-CX3CL1 was used without further depletion of QC.

Antibodies were purchased from Cell Signalling (Frankfurt am Main, Germany). The following antibodies were used: anti-pERK (Phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204, 20G11, rabbit mAb, #4376), anti-pP38 (Phospho-p38 MAPK, Thr180/Tyr182, 12F8, Rabbit mAb, #4631), anti-pAktSer473 (Phospho-Akt (Ser473), D9E, XP® Rabbit mAb, #4060), anti-ERK (p44/42 MAPK (Erk1/2), 137F5, Rabbit mAb, #4695), anti-P38 (p38 MAPK Antibody, #9212) and anti-Akt (Akt (pan), C67E7, Rabbit mAb, #4691). As secondary antibody goat anti-rabbit-IgG (HRP conjugated, #7074) was used.

Pooled QPCT-siRNA (ON-TARGETplus SMARTpool QPCT) and non-target control (NTC) were obtained from Dharmacon (Thermo Fisher Scientific, Karlsruhe, Germany), pool of 4 single FlexiTube siRNAs were from Qiagen (Hilden, Germany; for sequences see supplemental table 1).

MALDI-TOF mass spectrometry

A 25 µM CX3CL1 solution was prepared in Tris-buffer (20 mM, pH 8.0 adjusted with HCl), mixed to a final concentration of 10 µM with Tris buffer and 0.7 µg/ml of the enzymes QC or isoQC and incubated at 37 °C. After adding the enzyme, several samples were taken at indicated time points. The cyclisation of N-terminal glutamine residue was stopped with equal amounts of 0.1 % trifluoroacetic acid. Afterwards, samples were purified with ZipTip C18; Merck Millipore, Darmstadt, Germany) according to the instructor’s manual and mixed with the matrices α-Cyano-4-hydroxycinnamic acid (MW 500 – 3000 g/mol), ferulic acid (MW 3,000 – 4,000 g/mol) or sinapic acid (3,5-Dimethoxy-4-hydroxycinnamic acid, MW > 4,000 g/mol; all Sigma-Aldrich, Taufkirchen, Germany) at a ratio of 1:1. In the case of cyclisation of CX3CL1 in human serum, serum was diluted 1:10 with Tris buffer. MALDI-MS was performed using the Voyager De Pro (Applied Biosystems, Darmstadt, Germany) in a linear mode. One µl of the analyte/matrix mixture was spotted on the sample plate and air dried. The analytes were ionized by a nitrogen laser pulse (337 nm) and accelerated under 20 kV with a time delayed extraction before entering the time-of-flight mass spectrometer. Detector operation was in the positive ion mode ranging from 2,000 to 20,000 amu (atomic mass unit). Insulin and myoglobin (Sigma-Aldrich) were used for calibration in this range according to the instructor’s manual from Applied Biosystems. Each spectrum represents the sum of at least 6 × 100 laser pulses.

Cell culture and stimulation

Primary human HCASMC (human coronary artery smooth muscle cells) obtained from normal regions of human coronary arteries from two independent donors were obtained from Promocell (Heidelberg, Germany) and cultured according to manufacturer’s instructions using SMC Growth Medium 2 with supplements (SMC-2, Promocell, #C-22062). All experiments were performed following 24 h serum starvation in serum-free SMC-2 medium containing 0.1 % bovine serum albumin (BSA, Sigma Aldrich).

Primary human umbilical vein endothelial cells (HUVEC) (Lonza, Cologne, Germany) were isolated from the vein of the umbilical cord and pooled from up to four different donors. Cultivation occurred to manufacturer’s instructions using EGM™-2 medium including serum
supplements and growth factors (Lonza, #CC-3162). For stimulation experiments, cells were seeded in 24-well plates and treated with TNF-α (#300-01A) and IL-1β (#200-01B) (each 10 ng/ml; PeproTech, Hamburg, Germany) for 24 h. Compounds IKK2 inhibitor IV (#401481) and U0126 MEK inhibitor (#662005) (both Calbiochem) were added 30 min before cytokine treatment. For signalling experiments, stimulation with 300 ng/ml Gln1-CX3CL1 or pGlu1-CX3CL1 for the indicated time intervals occurred in serum-free media containing 0.1 % BSA. For RNAi studies, HUVEC were transfected with siRNA pools or NTC (each 100 nM) according to manufacturer’s instructions using transfection reagent DharmaFECT 1 (Dharmacon, #T-T-001). Until stated otherwise independent replicates for the experiments were done at different days with different cell preparations or different passages of the same preparation (passage 2 to 11 for HUVEC, passage 3 to 8 for HCASMC). Details including number of replicates are given in the figure legends.

Western blotting
Protein lysates (20 µg) were separated on a 4 - 12 % NuPage Bis-Tris gel (Life Technologies, Darmstadt, Germany) and transferred to nitrocellulose membrane (Roti-NC, 0.2 µm, Roth, Karlsruhe, Germany). Blots were blocked with blocking buffer (5 % w/v dried milk (Roth) / Tris buffered saline / 0.1 % Tween-20 (TBS-T)) for 60 min. Blots were incubated with primary antibody in blocking buffer overnight at 4 °C, then incubated for 60 min at room temperature with secondary antibodies which were detected using the SuperSignal West Femto kit (Thermo Fisher Scientific, #34095) and CCD-Imagingsysteme FUSION-FX7 (Peqlab, Erlangen, Germany). Western blots were stripped for 5 min at 37 °C and 15 min at room temperature in Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, #21059).

Quantitative PCR (qPCR)
RNA was isolated using the NucleoSpin RNA II kit (Macherey Nagel, Düren, Germany) according to the manufacturer’s instructions and RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Peqlab, Erlangen, Germany). RNA was reversely transcribed into cDNA using random primers (Roche, Mannheim, Germany), dNTPs (Peqlab) and Superscript III (Life Technologies). Quantitative PCR was performed in the Rotor-Gene RG3000 (Corbett Research, Sydney, Australia) using the Rotor-Gene SYBR green mastermix (Qiagen, Hilden, Germany) according to the manufacturer’s instructions and the QuantTect primer assays Hs_QPCT_1_SG (NM_012413) and QPCTL_1_SG (NM_017659) (Qiagen) or specific primers for CCL2 (NM_002982.3), ICAM1/CD54 (NM_000201.1), CX3CL1 (NM_002996.3), GAPDH (NM_002046.3), YWHAZ (NM_003406.2) all synthesized by Metabion ( Martinsried, Germany). The primer sequences are summarised in supplemental table 2. Relative amounts of gene expression were determined with the Rotor-Gene software version 6.1 using the comparative quantification method. GAPDH and YWHAZ were used as reference genes. Melt-curve analysis following PCR showed a single product for all amplicons.

Immunoassays
For determination of human total-CCL2 a specific ELISA was used as described previously [10]. CX3CL1 and soluble ICAM1/CD54 (sICAM1) concentrations in conditioned medium were determined using the MILLIPLEX MAP kit (Merck Millipore) and the analysing system Bio-Plex 200 (Bio-Rad, Munich, Germany). Calibration of the assay occurred via Bio-Plex Calibration kit in specific Bio-Plex MCV plates (Bio-Rad). After incubation of plates at 4 °C overnight, analytes were measured and concentrations were calculated using the Bio-Plex-Manager software (version 4.1.1).
ICAM1/CD54 detection by flow cytometry

Cells were detached with accutase (PAA, Cölbe, Germany), washed with PBS buffer and re-suspended in FACS staining buffer (PBS, 5% FCS, 2 mM EDTA). After blocking with FcR blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany, #130-059-901), cells were stained with the APC-conjugated mouse anti-human CD54 antibody (BD Bioscience, Heidelberg, Germany, #559771) or the isotype control antibody (eBioscience, Frankfurt am Main, Germany, #48-4301-80) for 30 min, at 4°C in the dark. Flow cytometry was performed using the FACS Calibur instrument with CellQuest software (BD Bioscience).

QC activity determination

After stimulation or siRNA transfection, conditioned medium was concentrated to a tenth of the original volume (Vivaspin 6 columns 10,000 MWCO; Sartorius, Göttingen, Germany). Cells were pelletized, sonicated in buffer (10 mM Tris, 100 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 10% Glycerol, pH 7.5) and centrifuged at 16,000 x g for 30 min and 4°C. The protein concentration of the resulting supernatant and the medium concentrate was determined using the method of Bradford (RotiQuant, Roth). The QC/isoQC activity was measured applying an HPLC assay as described previously [35,36].

Statistical analysis

All quantitative data are presented as mean ± SD. Differences between experimental groups were evaluated for significance using Student’s t-test for unpaired data or one-way ANOVA when appropriate by SigmaPlot software (Systat, Erkrath, Germany).
Results

**CX3CL1 is a substrate of glutaminyl cyclase**

The N-terminus of the chemokine CX3CL1 has a glutamine in the first position. To confirm whether QC and isoQC can catalyse N-terminal pGlu-formation, the recombinant chemokine domain of CX3CL1 was incubated with QC or isoQC and the resulting reaction products were analysed by mass spectrometry. Thirty minutes after adding the enzymes the peak of pGlu1-CX3CL1 had a much higher intensity than the peak of the non-modified form Gln1-CX3CL1 (Fig. 1 shows spectra with QC). After 45 min, the reaction was almost finished. Only a small signal of the original mass could be detected at this time point. Due to its high mass (theoretical mass of Gln1-CX3CL1: 8635 Da and of pGlu1-CX3CL1: 8618 Da) it comes to a mass drift of CX3CL1 during the MALDI-TOF-MS measurements resulting in a deviant mass of 8630 Da and 8613 Da for Gln1-CX3CL1 and pGlu1-CX3CL1, respectively. This deviation of 0.058% is near the mass accuracy of 0.05% specified by the manufacturer for the used equipment (Voyager DE Pro, linear mode, external calibration). However, the differences between theoretical and observed values were always constant. Similar results for the conversion of immature Gln1-CX3CL1 to pGlu1-CX3CL1 were seen with recombinant human isoQC (data not shown) or human serum as matrix (Supplementary Fig. 1). To summarise, we could show for the first time that human CX3CL1 is a substrate for both QC and isoQC. Both enzymes are capable to catalyse the posttranslational formation of an N-terminal pGlu residue.

**Co-regulation of QC and its substrates CCL2 and CX3CL1 upon stimulation with TNF-α/IL-1β**

To study the regulation of QC and its chemokine substrates, primary endothelial cells (Human Umbilical Vein Endothelial Cells, HUVEC) were treated with the pro-inflammatory cytokines TNF-α/IL-1β. Using qPCR, ELISA and flow cytometry for measuring gene and protein expression, we detected an increase of CX3CL1, CCL2 and ICAM1/CD54 levels (Fig. 2A, C-E). Furthermore, we found an increase of **QPCT** mRNA expression up to 2-fold \((P < 0.01)\), whereas **QPCTL** gene expression was not changed (Fig. 2A). A 3-fold increase of specific QC activity was measured in conditioned medium of stimulated cells \((P < 0.01, \text{Fig. 2B})\), but no change in QC/isoQC activity in cell extracts could be detected. This result is also indicative for the stimulation of the secreted QC but not of the Golgi resident isoQC.

To confirm the co-regulation of QC and its substrates, we studied the gene expression in a time dependent manner in human coronary artery smooth muscle cells (HCASMC). Whereas the increase of **CX3CL1**, **CCL2** and **ICAM1/CD54** levels was detectable already two hours after stimulation, **QPCT** and **QPCTL** transcripts were found to be increased only after 24 h (Fig. 3).

In conclusion, we demonstrated a co-regulation of QC and its substrates CX3CL1 and CCL2, as well as of the adhesion molecule ICAM1/CD54 in both primary endothelial and smooth muscle cells.

**NF-κB-dependent regulation of QPCT and its substrates CX3CL1 and CCL2**

NF-κB signalling is involved in rapid response to various stimuli, such as the cytokines TNF-α, IL-1β or infections, shear or oxidative stress. To assess whether NF-kB is involved in the regulation of QPCT and its substrates, we treated TNF-α/IL-1β-stimulated HUVEC with the inhibitor of the NF-kB pathway, IKK2 compound IV, or with the ERK/MAPK inhibitor U0126. The IKK2 inhibitor was able to abolish the TNF-α/IL-1β-induced gene expression of **QPCT** and its substrates CX3CL1 and CCL2 as well as of **ICAM1/CD54** in a concentration-dependent manner (Fig. 4). The ERK/MAPK inhibitor did not affect the **QPCT** and **CX3CL1** transcript levels, but did significantly reduce the **CCL2** as well as the **ICAM1/CD54** gene expression.
QPCT-gene silencing reduces CX3CL1, CCL2 and CD54 expression

Next, we studied the effect of QC knock down on the expression of its substrates. We transfected HUVEC with QPCT-siRNA pools or with a non-target control (NTC) for 72 h. As shown in Fig. 5A, QPCT-siRNA knock-down (reduction to 3 - 5 %; \( P < 0.001 \)) resulted in lowered mRNA amounts of CX3CL1 (reduction to 40 % of control, \( P < 0.01 \)), CCL2 (60 % of control, \( P < 0.01 \)), and ICAM1/CD54 (73 % of control, \( P < 0.05 \)). Treatment of HUVEC with NTC had no significant impact on the expression of genes investigated. RNAi of QPCT decreased QC activity in conditioned medium of transfected cells to the basal level of the culture medium, which contains activity from the serum supplement (horizontal line in Figure 5B). In cell extracts of siRNA-transfected HUVEC, we found a significant reduction of QC/isoQC activity compared to mock treated cells (\( P < 0.01 \), Fig. 5C). The remaining enzyme activity is certainly originated from the isoQC, which is located in the Golgi complex and was not affected by QPCT-siRNA transfection. Comparably to the decreased mRNA expression, protein levels of CCL2 and CD54 declined as well (Fig. 5D, E).

pGlu1-CX3CL1 induces ERK, p38 and Akt phosphorylation

White et al. published that mitogenic and anti-apoptotic effects of CX3CL1 require ERK and PI3K/Akt signalling in HCASMC [17]. Therefore, we studied the physiological relevance of pGlu1-formation on CX3CL1 for the activation of ERK, p38 and Akt signal transduction pathways. First, we confirmed a marginal CX3CR1 cell surface expression in HCASMC and HUVEC by flow cytometry. Only pGlu1-CX3CL1 but not Gln1-CX3CL1 was able to induce phosphorylation of kinases ERK1/2, p38 and Akt (Fig. 6A-C). Phosphorylation of ERK1/2 and p38 started within 10 min and peaked between 40 and 60 min after induction. Contrary, activation of Akt showed two peaks at 10 min and at 60 min. At any time, pGlu1-CX3CL1 induced a stronger phosphorylation of ERK1/2, p38 and Akt compared to Gln1-CX3CL1, which only activated the kinases at 40 and 60 minutes slightly. This indicates an important functional relevance of the pGlu-residue of CX3CL1 for induction of signalling in HCASMC.

pGlu1-CX3CL1 induces expression of CX3CL1, CCL2 and CD54

To prove the effect on gene expression as a subsequent result of CX3CR1 activation, HCASMC were treated with Gln1-CX3CL1 or pGlu1-CX3CL1 in time- and concentration-dependent assays. Using qPCR, the transcript levels of CX3CL1, CCL2, ICAM1/CD54, QPCT, and QPCTL were analysed. pGlu1-CX3CL1 was more effective in induction of gene expression than Gln1-CX3CL1. Gln1-CX3CL1 could only up-regulate the mRNA levels up to 2-fold whereas pGlu1-CX3CL1 induced mRNA expression in a concentration-dependent manner up to 7.5-fold for CX3CL1, 3.1-fold for CCL2, and 7.4-fold for ICAM1/CD54 (Fig. 7A, B, C). Noticeable, the mRNA expression of CX3CL1 and ICAM1/CD54 reached their maxima at 4 h, whereas that of CCL2 peaked later. After 24 h of incubation, QPCT gene expression presented a slight increase of 1.5-fold upon stimulation with 600 ng/ml pGlu1-CX3CL1 but not with Gln1-CX3CL1 (\( P < 0.05 \)). To confirm the differential gene expression on protein level, CCL2 protein was measured via CCL2 ELISA in the culture supernatant of HCASMC. After 4 h and 24 h, pGlu1-CX3CL1 increased significantly the CCL2 protein levels in a concentration dependent manner compared to Gln1-CX3CL1 (Fig. 7D, E).

To further verify the importance of the N-terminal pGlu-residue for CX3CL1 activity, we treated also primary endothelial cells (HUVEC) with both CX3CL1 variants in a concentration of 300 ng/ml for 6 h and 24 h. pGlu-CX3CL1 increased the transcript levels of CX3CL1 up to 3.3-fold, of CCL2 up to 2.6-fold, and of ICAM1/CD54 up to 2.4-fold, whereas Gln1-CX3CL1 did not affect transcript levels of these molecules at all (Fig. 8A). Using ELISA, we quantified the CCL2 concentration after 6 h and 24 h. Similar to HCASMC, the pGlu-form of CX3CL1 enhanced CCL2 protein concentrations in HUVEC supernatants 2.5-fold within 6 h (\( P < 0.001 \)) and 1.5-fold within 24 h (\( P < 0.01 \)) whereas Gln1-CX3CL1 shows...
no effect (Fig. 8B). In conclusion, we demonstrated for the first time that the pGlu-residue on CX3CL1 is essential for CX3CR1 signalling and subsequent gene and protein expression in HCASMC and HUVEC.
Discussion

Pyroglutamate formation catalysed by QCs is an important post-translational step in maturation of chemokines as CCL2, CCL7, CCL8, and CCL13 protecting against N-terminal proteolytic degradation, e.g. by dipeptidyl peptidase 4/CD26 [10,11,18]. Here, we could identify the chemokine domain of CX3CL1 as an additional new QC substrate. The recombinant Gln1-CX3CL1 was converted into its pGlu-form by recombinant QC or isoQC as well as by QC activity of human serum. Interestingly, in the human serum assays, CX3CL1 was cleaved C-terminally resulting in a truncation of the peptide by the C-terminal RNG triplet (Supplementary Fig. 1). This C-terminal cleavage of human CX3CL1 was independent of the N-terminal cyclisation reaction.

Besides the protection of the N-terminus against exopeptidase degradation, it is well known that the N-terminal pGlu residue can help stabilize the proper conformation of proteins for binding to their receptors [12,14]. Recently, we reported that pGlu1-CCL2 is more effective in a monocytic migration and a CCR2-internalisation assay compared to its immature Gln1-variant [10]. Here, we show the physiological relevance of pGlu1-formation on CX3CL1 for the activation of ERK, p38 and Akt signal transduction pathways in HASMC. At any time point investigated, pGlu1-CX3CL1 induced a significant stronger effect on phosphorylation of ERK1/2 and p38 kinase than the Gln1-CX3CL1. Similar results were observed for Akt\textsuperscript{Ser473} phosphorylation at time points 10, 20, and 40 min upon chemokine stimulation. A similar time course of kinase phosphorylation by CX3CL1 was described for monocytic cells, CX3CR1-transfected HEK293T cells, neuroblastoma, and osteoarthritis fibroblasts [19, 20–23]. Ryu and coworkers demonstrated in human aortic endothelial cells, that CX3CL1 activates ERK1/2 phosphorylation and subsequently the VEGF-A/KDR-1-induced angiogenesis [24]. Furthermore, in CX3CL1-stimulated human microvascular and umbilical endothelial cells changes in the phosphorylation status of ERK1/2 and JNK and alteration in the cytoskeleton were detected. Both processes are necessary for endothelial migration and angiogenesis in rheumatoid arthritis [23]. In rat aortic smooth muscle cells, CX3CL1 induced its own expression and was involved in cell-cell adhesion and cell proliferation via the PI3K/Akt\textsuperscript{Thr308}/NF-\kappaB pathway. Therefore, CX3CL1 was discussed as mediator in initiation and progression of atherosclerotic vascular disease [25]. Using human coronary artery smooth muscle cells, White et al. showed that CX3CL1-induced anti-apoptosis is mediated by cross-talk to the epidermal growth factor receptor pathway. CX3CL1 induced shedding of epiregulin, which acted in an autocrine/paracrine manner to activate the epidermal growth factor receptor, leading to PI3K activation and Akt phosphorylation [17].

To study the translation of the pGlu1-CX3CL1-mediated signalling into effects on differential gene expression, we quantified the RNA levels of CCL2, ICAM1/CD54, and CX3CL1 itself. pGlu1-CX3CL1 induced higher amounts of CCL2, ICAM1/CD54, and CX3CL1 mRNA than the immature Gln1-CX3CL1 in both HUVEC and HCASMC. In addition, we found significant higher CCL2 protein levels in the supernatant of HUVEC and HCASMC upon pGlu1-CX3CL1 stimulation compared to the appropriate stimulation with Gln1-CX3CL1. These results strongly suggest that the QC-catalysed pGlu-CX3CL1-formation is required for an effective activation of the CX3CL1/CX3CR1 axis and the induction of molecules important for adhesion and migration as CCL2, ICAM1/CD54, and CX3CL1. The up-regulation of ICAM1/CD54 by CX3CL1 was also reported in mouse hearts ex vivo and in HUVEC via the Jak/Stat5 pathway [26]. In aortic smooth muscle cells, both increased transcription of mRNA and higher mRNA stability contributed to the CX3CL1-induced CX3CL1 expression [25]. In the monocyte/endothelial cell cross-talk, endothelial CX3CL1 potentiated monocytic CCL2 release that might contribute to the recruitment of monocytes into inflamed areas [27].

Recently, synergistic induction of CX3CL1 in response to the combined stimulation with TNF-\alpha and IFN-\gamma was described for HUVEC [28] and human aortic smooth muscle cells [29].
Here, we demonstrated the co-regulation of the enzyme QC and its substrates CCL2 and CX3CL1 at the RNA and protein level upon stimulation with the proinflammatory cytokines TNF-α and IL-1β whereas the QPCTL gene expression was not affected. By contrast, inhibition of the NF-κB pathway using an IKK2 inhibitor decreased the expression of the co-regulated targets QPCT, CCL2, and CX3CL1. Furthermore, RNAi-mediated inhibition of QPCT expression resulted in a reduction of CCL2 as well as CX3CL1 mRNA. Recently, we described a similar NF-κB-dependent co-regulation of QPCT and CCL2 in epithelial cells of thyroid carcinomas upon TNF-α/IL-1β treatment [30]. QC is believed to be involved in the pathology of several diseases like osteoporosis [31], melanoma [32], Alzheimer’s disease [33], and septic arthritis [34]. In an animal model of atherosclerosis (cuff-induced accelerated atherosclerosis in ApoE3*Leiden mice) inhibition of QC activity reduced the number of adhering monocytes, down-regulated CCL2 in the media and the intima, and reduced neointima formation and lumen stenosis [10]. Furthermore, especially for the nervous system there is growing evidence that CX3CL1 is an important regulator of microglia-neuron crosstalk [37] and an imbalance of this interaction may be an important part of the pathology of Alzheimer’s disease (AD) and other neurodegenerative diseases. In this context the correlation found by Bridel and co-workers [38] in an AD biomarker study between QC activity and CSF levels of Aβ peptides, adhesion molecules (ICAM1, VCAM1) as well as members of the VEGF pathway (VEGFD and Flt1) is remarkable. Co-regulation of these molecules may be at least partially NF-κB or CX3CL1 dependent [24].

**Conclusion**

Posttranslational modification of N-terminal glutamine to pyroglutamate by QC/isoQC is used as a cellular strategy to fine-tune the activity of the chemokines CX3CL1 and CCL2. The data presented here provide evidence that in case of an inflammatory stimulus QC is co-regulated with its chemokine substrates CX3CL1 and CCL2. Furthermore, we demonstrated that the glutaminyl cyclase dependent formation of the N-terminal pGlu of CX3CL1 is required for full physiologic activity of this chemokine and a prerequisite for an effective activation of signalling pathways resulting in increase of expression of molecules, involved in inflammation, proliferation, cell migration and adhesion (Fig. 9) like CCL2, ICAM1 and CXCL3 itself. So, under inflammatory conditions where release of these chemokines could increase dramatically the coregulation of QC ensures that all of these chemokines are released in its fully active N-terminally cyclized form. If the missing N-terminal pyroglutamate results in an increased accessibility for N-terminal proteolytic degradation or directly affects receptor interaction or activity could not be answered by the available data and should be clarified in future experiments.

Nevertheless, data demonstrate the importance of the N-terminal pyroglutamyl modification also for CX3CL1 dependent signaling processes, and support the idea that reduction of pGlu-chemokine levels by inhibition of QC activity might result in a reduced inflammatory phenotype in chronic and acute diseases and provides a possible new treatment strategy.
Acknowledgements

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Declarations of interest
A.K., M.H., L.B., H.C., T.H., and H.-U.D. are present or former employees of Probiodrug AG. H.-U.D. is the former chief scientific officer, currently scientific advisor, and a stockholder of Probiodrug AG.
References


Figure legends

Figure 1: CX3CL1 is a substrate of human QC
After 0, 20, 30, and 45 min of incubation at 37 °C, N-terminal modification of Gln1-CX3CL1 by human recombinant QC was monitored using MALDI-TOF-MS. The product peak with a mass of 8,613 Da resulted from a loss of ammonia during cyclisation reaction (minus 17 Da) and exceeds the peak of the original mass of 8,630 Da after 30 min of incubation with QC.

Figure 2: Co-regulation of QC and its substrates CCL2 and CX3CL1 upon stimulation with TNF-α/IL-1β in HUVEC
HUVEC were treated with TNF-α/IL-1β (10 ng/ml) for 24 hours. (A) qPCR analysis was performed for the indicated genes. (B) Specific QC activity in conditioned medium was measured by HPLC and normalized to protein concentration. The basal activity of the culture medium without cells was subtracted. (C) CX3CL1 was quantified via immunoassay (MILLIPLEX MAP). (D) CCL2 concentration in supernatant was measured with specific human CCL2 ELISA. (E) Surface expression of CD54 was analysed by flow cytometry. Data from three independent experiments on different days are presented as mean ± SD (**P < 0.01, ***P < 0.001, n.d. – not detectable).

Figure 3: Co-regulation of QC and its substrates CCL2 and CX3CL1 in HCASMC is time dependent
HCASMC were serum-starved for 24 h and then treated with TNF-α/IL-1β (10 ng/ml) for the indicated times. qPCR was performed for the indicated genes (A) QPCT, QPCTL; (B) CX3CL1, CCL2, ICAM1/CD54. Data from eight independent experiments (cells from two different donors) are expressed as fold change over untreated and shown as mean ± SD (C) CCL2 was detected with immunoassay (MILLIPLEX MAP); (D) CX3CL1 was quantified with immunoassay (MILLIPLEX MAP); Data from three experiments performed in duplicates are shown as mean ± SD. (*P < 0.05, **P < 0.01, ***P < 0.001).

Figure 4: NF-κB-dependent co-regulation of QPCT and substrates CCL2 and CX3CL1 mRNA expression in HUVEC
After treatment with the appropriate inhibitor IKK2 or U0126 (each 10 µM) for 30 min, HUVEC were cultured for 6 h with cytokines TNF-α/IL-1β (100 ng/ml). Results of qPCR are shown relative to the basal control levels (A) QPCT, QPCTL; (B) CX3CL1, CCL2, ICAM1/CD54. Data from three independent experiments are shown as mean ± SD (*P < 0.05, **P < 0.01, ***P < 0.001).

Figure 5: Transfection of HUVEC with QPCT siRNA resulted in down-regulation of substrates CX3CL1 and CCL2
HUVEC were treated with siRNA over 72 hours. (A) qPCR was performed for the indicated genes. (B) Specific QC activity in conditioned medium was measured by HPLC and normalised to protein concentration. The activity of basal medium without cells is presented as horizontal line. (C) Specific QC/isoQC activity in cell extracts was measured by HPLC and normalised to protein concentration. (D) CCL2 was measured via hCCL2 ELISA. (E) ICAM1/CD54 was quantified via immunoassay (MILLIPLEX MAP). Data from 6 independent experiments are presented as mean ± SD (* P< 0.05, **P < 0.01, ***P < 0.001).
Figure 6: pGlu1-residue of CX3CL1 is important for phosphorylation of ERK1/2, Akt and p38 in HCASMC
HCASMC were serum-starved for 24 h prior to treatment with Gln1-CX3CL1 or pGlu1-CX3CL1 600 ng/ml for the indicated times. Cell lysates were prepared and western blotted. Membranes were stained with phospho-specific antibodies before stripping and re-probing with pan-specific antibodies. Quantification of the bands was done using FusionSoftware. Data from three independent experiments are shown as mean ± SD (*P < 0.05, **P < 0.01, ***P < 0.001; n.s. non significance). One blot is shown representatively. (A) Ratio of phospho ERK1/2/pan ERK1/2; (B) Ratio of phospho p38/pan p38; (C) Ratio of pAktSer473/total Akt.

Figure 7: Induction of mRNA and protein expression in HCASMC by CX3CL1: pGlu1-residue of CX3CL1 is important for induced expression of CX3CL1, CCL2 and ICAM1/CD54
HCASMC were serum-starved for 24 h prior to treatment with Gln1-CX3CL1 or pGlu1-CX3CL1 for the indicated concentrations and times. qPCR was performed for the indicated genes (A) CX3CL1; (B) CCL2; (C) ICAM1/CD54. Data from five independent experiments (cells from two different donors) are expressed as fold change over untreated. CCL2 levels were quantified with a specific hCCL2 ELISA 4 h (D) and 24 h (E) after treatment; Data from three independent experiments are shown as mean ± SD (*P < 0.05, **P < 0.01, ***P < 0.001).

Figure 8: Induction of mRNA and protein expression in HUVEC by CX3CL1: N-terminal pGlu1-residue of CX3CL1 is important for induction of CX3CL1, CCL2 and ICAM1/CD54 expression
HUVEC were treated with Gln1-CX3CL1 or pGlu1-CX3CL1 (300 ng/ml) for 6 hours and 24 hours. (A-C) Results of qPCR are shown relative to the basal control levels. CCL2 concentrations in supernatant were quantified with a specific hCCL2 ELISA 6 h (D) and 24 h (E) after treatment. Data from four independent experiments (triplicates) are shown as mean ± SD.

Figure 9: Role of pGlu1-CX3CL1 in HUVEC and HCASMC.
Activated NF-κB increase the expression of QC and its substrate CX3CL1. Pyroglutamate formation catalysed by QC is an important posttranslational step in CX3CL1 maturation. pGlu1-CX3CL1 can bind to the CX3CR1 expressed on the cell surface and activates the ERK1/2, p38 and Akt signal transduction pathways resulting in increased expression of chemokines and adhesion molecules as CX3CL1, CCL2, and ICAM1/CD54. Together, pGlu1-CX3CL1 functions as mediator and amplifier of cell migration, adhesion, and proliferation.
The diagram shows mass spectrometry profiles over time, with relative ion intensity on the y-axis and m/z on the x-axis. At 0 minutes, there are peaks for pGlu-Fraktalkin ([M+H]^+ = 8613 Da) and Gln-Fraktalkin ([M+H]^+ = 8630 Da). As time progresses (20 min, 30 min, 45 min), the intensity of these peaks changes, indicating a possible reaction or degradation over time.
Inflammation

- High levels of pE1-CX3CL1

Intra-cellular

- Increased expression of QC and CX3CL1
  - Activation of NF-κB

Extra-cellular

- CX3CR1
  - Phosphorylation of ERK1/2, p38, Akt
  - Increased expression of CX3CL1, CCL2, ICAM1/CD54

HUVEC, HCASMC

- Migration
- Adhesion
- Proliferation
Supplemental information

Supplemental Table 1.

Pooled siRNAs consisted of 4 single siRNAs with the following sequences

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Supplemental Table 2.

Primer sequences of the human genes

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**Fig 1 Supplement**

**Conversion of Q1-CX3CL1 to pE1-CX3CL1 in human serum**

At time point 0 min, the major peak corresponds to a mass of 8630, which is close to the theoretical molecular weight (8639) of Q1-CX3CL1. After incubating Q1-CX3CL1 with human serum for 30 min, the major peak corresponds to a mass of 8613, which is close to the theoretical molecular weight (8622) of pE1-CX3CL1. The doublet at 8286/8303 corresponds to a shortened peptide produced by limited proteolysis at the C-terminus (release of C-terminal RNG tripeptide). The C-terminal cleavage is independent of N-terminal cyclisation reaction.