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# Furin Is a Chemokine-modifying Enzyme

## *IN VITRO* AND *IN VIVO* PROCESSING OF CXCL10 GENERATES A C-TERMINALLY TRUNCATED CHEMOKINE RETAINING FULL ACTIVITY*

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**Chemokines comprise a class of structurally related proteins that are involved in many aspects of leukocyte migration under basal and inflammatory conditions. In addition to the large number of genes, limited processing of these proteins by a variety of enzymes enhances the complexity of the total spectrum of chemokine variants. We have recently shown that the native chemokine CXCL10 is processed at the C terminus, thereby shedding the last four amino acids. The present study was performed to elucidate the mechanism *in vivo* and *in vitro* and to study the biological activity of this novel isoform of CXCL10. Using a combination of protein purification and mass spectrometric techniques, we show that the production of C-terminally truncated CXCL10 by primary keratinocytes is inhibited *in vivo* by a specific inhibitor of pro-protein convertases (*e.g.* furin) but not by inhibition of matrix metalloproteinases. Moreover, CXCL10 is processed by furin *in vitro*, which is abrogated by a mutation in the furin recognition site. Using GTPγS binding, $Ca^{2+}$ mobilization, and chemotaxis assays, we demonstrate that the C-terminally truncated CXCL10 variant is a potent ligand for CXCR3. Moreover, the inverse agonist activity on the virally encoded receptor ORF74 and the direct antibacterial activity of CXCL10 are fully retained. Hence, we have identified furin as a novel chemokine-modifying enzyme *in vitro* and most probably also *in vivo*, generating a C-terminally truncated CXCL10, which fully retains its (inverse) agonistic properties.**

Chemokines comprise a class of structurally related proteins that are involved in many aspects of leukocyte migration under basal and inflammatory conditions (1). Their action is primarily achieved through ligand-specific activation of seven transmembrane G-protein-coupled receptors expressed on target cells.

In addition to the large number of genes encoding chemokines, processing of these proteins by (a limited number of) enzymes increases the complexity of chemokine signaling. Besides the large body of evidence on chemokine processing by dipeptidylpeptidase IV (CD26) (2), several novel chemokine processing enzymes have been identified in the past few years, *e.g.* matrix metalloproteinase 2 and 9, cathepsin D and G, and elastase (3–8). Apart from a range of biological effects on chemokine receptor activation, processed chemokines occasionally gain very unexpected, sometimes not even chemokine receptor-related, activities (9, 10).

In general, alterations at the N terminus down-regulate chemokine receptor potencies and are therefore considered important mechanisms to modulate inflammatory responses. Several naturally occurring truncations of chemokines have been identified from which it is still unknown which system is involved in their formation. Interestingly, several of these represent C-terminal truncations, as in the case of CXCL7 (11, 12) and CCL20 (13). One specific C-terminal truncation of CXCL7, found in platelets, involves the removal of the last two amino acids. In contrast to intact CXCL7, this form has potent antibacterial activity (10), pointing to a role central role of the C-terminal amino acids in the innate immune activity of this chemokine.

For the chemokine receptor CXCR3 three ligands are known (CXCL9, -10, and -11). The CXCR3 receptor is highly expressed on activated T-lymphocytes and CXCR3+ T-cells have been implicated in several pathological conditions. For example, CXCR3 and its ligands are involved in allograft rejection (14, 15), wound healing, and angiogenesis (16). In the skin, expression of CXCR3 targeting chemokines by keratinocytes, fibroblasts, and endothelial cells contributes to the influx of T-lymphocytes (17, 18). A recent study has shown that the CXCR3 ligands also possess direct antibacterial activity (19), suggesting a role for these chemokines in the innate immune response.

All three CXCR3 ligands are sensitive to N-terminal truncation by dipeptidylpeptidase IV (20, 21). In addition, we have recently shown that native CXCL10 that is produced upon stimulation of primary human keratinocytes with interferon-γ is partially processed to a CXCL10 molecule that lacks the last four C-terminal amino acids (22). The aim of the present study was to identify the enzymatic system responsible for the *in vivo* cleavage of CXCL10 and to determine the biological activity of this novel CXCL10 variant using several different biological assays. We demonstrate that furin can cleave CXCL10 *in vitro*

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and that an inhibitor of pro-protein convertases (*e.g.* furin) prevents processing of CXCL10 *in vivo*. We therefore identify furin as a novel chemokine-processing enzyme. The resulting CXCL10 variant retains full (inverse) agonistic activities.

## EXPERIMENTAL PROCEDURES

*Materials*—Human CXCL9, -10, and -11 and murine CXCL10 were obtained from R&D Systems Inc. (Minneapolis, MN). Human CXCL11 and CXCL10 were obtained from Peprotech (Rocky Hill, NJ). Purified furin and the pro-protein convertase inhibitor decanoyl-RVKR-chloromethylketone were purchased from Alexis (Lausen, Switzerland). Porcine pancreas carboxypeptidase B was obtained from Calbiochem (San Diego, CA). Recombinant human IFN-$\gamma$[1] and TNF-$\alpha$ were from Strathmann Biotech GmbH (Hannover, Germany). Poros 50 R2 was obtained from Applied Biosystems (Foster City, CA). DEAE-dextran (chloride form) was obtained from Sigma. Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen, fetal bovine serum was purchased from Integro B. V. (Dieren, The Netherlands) and penicillin-streptomycin was from BioWhittaker Europe (Verviers, Belgium). pNF-$\kappa$B-Luc was obtained from Stratagene (La Jolla, CA.)

*Cell Culturing and Stimulation*—Keratinocytes were obtained from human foreskin and grown in single cell suspensions in low calcium (0.1 mM) serum-free medium (keratinocyte-SFM, Invitrogen) as described earlier (22). The culture of primary human dermal fibroblasts was established as described earlier (23).

To induce chemokine expression, cells were stimulated with 2000 units/ml human rIFN-$\gamma$ (keratinocytes) or 500 units/ml human rIFN-$\gamma$ + 500 units/ml human rTNF$\alpha$ (fibroblasts), for 6 h. Following this stimulation, cells were washed with phosphate-buffered saline and transferred to MCDB153 medium (Invitrogen) and allowed to secrete chemokines for 18 h at 37 °C.

*Application of Proteinase Inhibitors in Keratinocyte Cultures*—Human keratinocytes were cultured and stimulated as described above. After stimulation and washing of the cells, keratinocyte-derived chemokines were produced in the presence of either 25 $\mu$M decanoyl-RVKR-chloromethylketone or 20 $\mu$g/ml Marimastat.

*Purification of CXCR3-targeting Chemokines*—Supernatants from chemokine expressing cells were filtered through a 0.22-$\mu$m filter and applied to a 1-ml HiTrap heparin column (Amersham Biosciences) equilibrated with 10 mM sodium phosphate, pH 7.1. The column was subsequently washed with 5 ml of 10 mM sodium phosphate (pH 7.1) containing 0.35 M NaCl. This was followed by elution of heparin-binding proteins using 2 ml of 1.5 M NaCl in 10 mM phosphate (pH 7.1).

Chemokines were further purified using reverse phase high-performance liquid chromatography (HPLC) on a 2.1 × 250 mm Nucleosil C-18 column (Machery-Nagel, Düren, Germany) with a flow rate of 0.3 ml/min. Proteins were eluted using a continuous gradient from 0 to 60% acetonitrile (in 0.02% HCl) over 60 min and collecting 1-min fractions.

*In Vitro Processing and Mass Spectrometric Analysis of Chemokines*—Recombinant chemokines at a concentration of 2 $\mu$M were incubated with furin (5 units/ml) alone, or in combination with carboxypeptidase B (0.5 units/ml) in a buffer containing 100 mM HEPES and 1 mM $CaCl_2$ (pH 7.6) at 37 °C for 3 h.

To establish the time course of furin-mediated cleavage of human CXCL10, recombinant CXCL10 (0.5 $\mu$M) was incubated with 0.1 units of furin in 20 $\mu$l of 100 mM HEPES and 1 mM $CaCl_2$, pH 7.6, for 0, 0.5, 1, 5, 30, and 60 min at 37 °C.

After digestion, chemokines were desalted using Poros 50 R2, packed in a pipette tip. Peptides were eluted in 60% acetonitril/0.02% HCl and measured by MALDI-MS (Reflex, Bruker Daltonics, Bremen) using dihydroxybenzoic acid as a matrix.

To determine kinetic parameters of CXCL10 conversion by furin, different concentrations of CXCL10 were incubated with 0.1 units of furin and incubated for 15 min. The ratio of the abundance of the intact and the cleaved forms was measured by MALDI-MS and used to calculate the amount of substrate converted. Data were subsequently fitted to the Michaelis-Menten equation.

For large scale production of C-terminally truncated CXCL10, *in vitro* digestions were carried out using 25 $\mu$g recombinant CXCL10 using 10 units of furin in combination with 0.3 units of carboxypeptidase B, and used this material in several bioassays. As a control, intact recombinant CXCL10 was treated identically, though without the enzymes.

*GTP$\gamma$S Binding*—CXCR3-expressing CHO cells (24) were scraped in their medium, pelleted, and resuspended in GTP$\gamma$S binding buffer (20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% bovine serum albumin, pH 7.4), containing 1 mM EDTA. Cells were kept on ice for 20 min and subsequently sonicated (2 × 15 s, with 2-min interval). Large cellular debris was removed by centrifugation at 200 × *g*. Cell membranes were pelleted from the supernatant at 48,000 × *g* and resuspended in GTP$\gamma$S binding buffer and incubated (1 $\mu$g) for 20 min at 30 °C with 1 $\mu$M GDP in a total volume of 200 $\mu$l. Following incubation with various concentrations of chemokines for 10 min at 30 °C, membranes were incubated with 0.3 nM [$^{35}$S]GTP$\gamma$S (1250 Ci/mmol, PerkinElmer) for 20 min at 30 °C. The reaction was terminated by filtering the membranes through a GF/B filter (Whatman) using ice-cold buffer (20 mM HEPES, 10 mM sodium pyrophosphate ($Na_4P_2O_7$), pH 7.4). Filters were transferred to counting vials and membrane-bound [$^{35}$S]GTP$\gamma$S was measured by liquid scintillation in a Wallac 1410.

*$Ca^{2+}$ Mobilization*—Intracellular calcium mobilization was measured as described previously (24). In brief, CHO cells stably transfected with human CXCR3 were loaded with 1 mM Fura-2 acetoxymethyl esther (Molecular Probes, Leiden, The Netherlands). Upon receptor stimulation, fluorescence recordings were made in a continuously stirred 1-ml cuvette at 37 °C using a fluorescence spectrophotometer (Photon Technology International, Inc., Lawrenceville, NJ).

*Chemotaxis*—Human peripheral blood T-cells were isolated from buffy coats of healthy donors and stimulated with IL-2 (100 units/ml) and PHA (1 mg/ml) to induce CXCR3 expression, as described previously (24). Chemotaxis of these cells toward chemokines was studied using a Transwell assay starting with 5 × $10^6$ cells/100 $\mu$l for each individual assay.

*ORF74 Constitutive Activity*—The cDNA of HHV-8-encoded ORF74 (GenBank™ accession number U71368 with a silent G-to-T mutation at position 927 (a gift from Dr. T. W. Schwartz)) was inserted into $pcDEF_3$ (A gift from Dr. J. A. Langen) after PCR amplification. COS-7 cells were grown at 5% $CO_2$ and 37 °C in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 50 units/ml penicillin and 50 $\mu$g/ml streptomycin. COS-7 cells were transiently cotransfected with $pcDEF_3$-ORF74 or $pcDEF_3$ (mock) and pNF-$\kappa$B-Luc using the DEAE-dextran method. After transfection, cells were seeded in 96-well white plates (Costar, Corning, NY) in serum-free culture medium in the presence of indicated ligands. 48 h after transfection, cells were assayed for NF-$\kappa$B-driven luciferase expression. Luminescence was measured in a Wallac Victor (PerkinElmer).

*Antibacterial Activity*—Radial diffusion assays (RDAs) were performed as previously described (19), using *Escherichia coli* ATCC 054 and *Listeria monocytogenes* EGD. The underlay consisted of 1% agarose and 1:100 dilution of trypticase soy broth in 10 mM sodium phosphate, pH 7.4, containing 1 × $10^6$ bacteria. Within the solidified agarose, a series of 3-mm diameter wells were punched and 5 $\mu$l of chemokines were added. Plates were incubated for 3 h at 37 °C to allow peptide diffusion. The underlay was then covered with overlay (6% trypticase soy broth agar and 1% agarose in $H_2O$), and the plates were allowed to solidify. Antimicrobial activity was determined as a clear zone around the well after 16-h incubation at 37 °C, and are represented in radial diffusion units: (diameter of clear zone in millimeters − well diameter) × 10.

## RESULTS

*Production of CXCL10 by IFN-$\gamma$-stimulated Primary Human Keratinocytes*—We have previously shown that stimulation of human keratinocytes with IFN-$\gamma$ induces the production of the CXCR3 targeting chemokines CXCL9, -10, and -11 (24). Following heparin affinity chromatography these chemokines can be purified by reversed-phase chromatography (Fig. 1*A*). Interestingly, upon mass spectrometric analysis of HPLC-purified keratinocyte-derived CXCL10, we found two different molecules of CXCL10, one complete form (1–77) and a C-terminally truncated form (1–73) that lacks the last four amino acids Lys-Arg-Ser-Pro (Fig. 1*B*).

The sequence -Lys[70]-Glu-**Arg**-Ser-**Lys-Arg**-Ser-Pro[77] (KE**R**-S**KR**SP) found in the C-terminal part of human CXCL10 contains a consensus sequence (Arg-Xaa-(Lys/Arg)-Arg-Xaa) for the pro-protein convertase furin. This tempted us to speculate that human CXCL10 is processed *in vivo* by furin convertase.

[1] The abbreviations used are: IFN-$\gamma$, interferon $\gamma$, CHO, Chinese hamster ovary; GTP$\gamma$S, guanosine 5′-3-*O*-(thio)triphosphate; ORF, open reading frame; TNF, tumor necrosis factor; PC, pro-protein convertases.

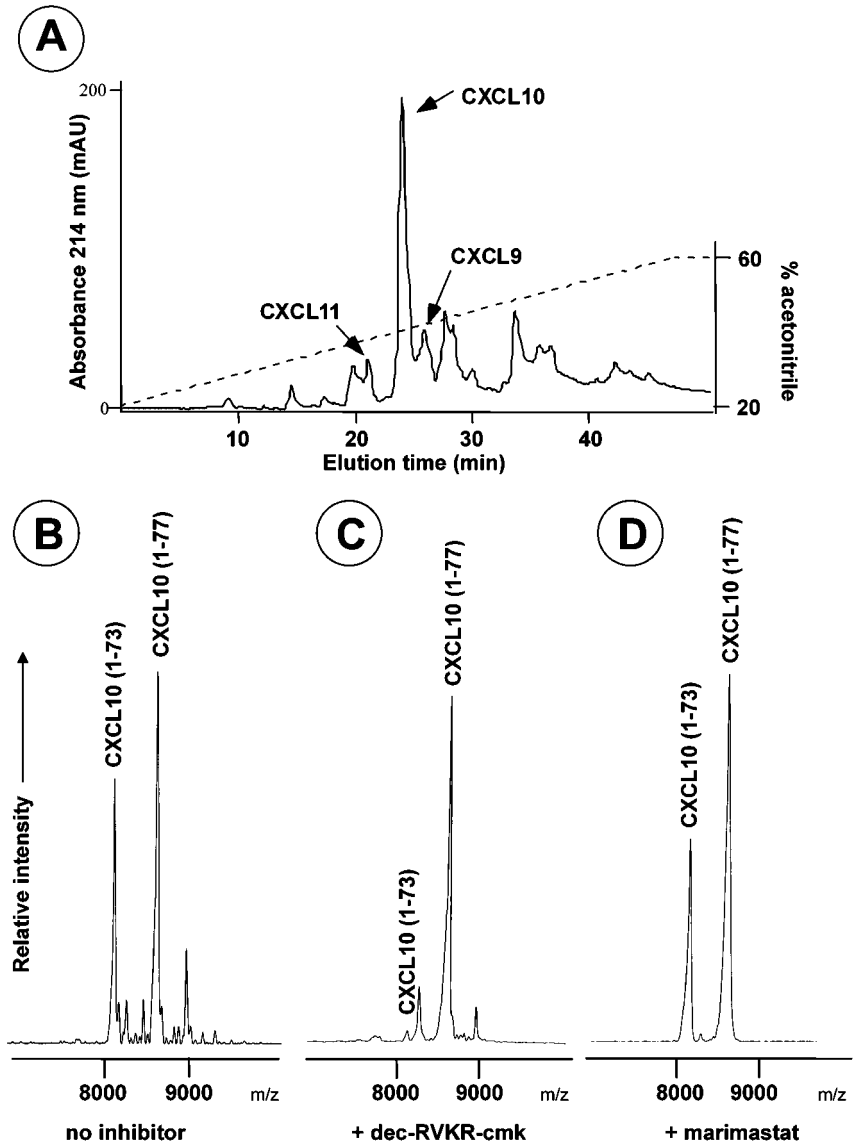



FIG. 1. **Influence of protease inhibitors on the production of different CXCL10 variants by primary human keratinocytes.** Primary human keratinocytes were stimulated with IFNγ (2000 units/ml) and allowed to excrete chemokines for 16 h. Following heparin affinity chromatography of the culture medium, CXCR3 targeting chemokines were purified using reversed phase chromatography (*A*). MALDI-MS was used to characterize the CXCL10 variants produced by keratinocytes cultured without inhibitor (*B*), treated with the pro-protein convertase inhibitor dec-RVKR-cmk (*C*) and the matrix metalloproteinase inhibitor marimastat (*D*).

In analogy with other systems (*e.g.* insulin and neuropeptides), additional trimming to serine by a carboxypeptidase would yield fully processed CXCL10-(1–73).

To explore the possibility that CXCL10 is processed by such a system, we first treated IFN-γ-stimulated keratinocytes with the cell permeable pro-protein convertase inhibitor decanoyl-RVKR-chloromethylketone and determined the form of CXCL10 produced by these cells. As predicted, the production of CXCL10-(1–73) was almost completely inhibited by decanoyl-RVKR-chloromethylketone (Fig. 1*C*).

It has recently been shown that several chemokines are substrates for matrix metalloproteinases (MMP) (3, 25). Several of these MMPs are activated upon cleavage by furin (26). This raised the possibility that inhibition of furin could have indirectly inhibited the production of CXCL10-(1–73) by preventing activation of MMPs. We therefore also treated IFN-γ-stimulated keratinocytes with the broad range MMP inhibitor Marimastat (20 μg/ml). This treatment did not have any effect on the relative amounts of CXCL10-(1–77) and CXCL10-(1–73) that were produced by these cells (Fig. 1*D*).

To exclude the possibility that the appearance of CXCL10-(1–73) in the medium of IFN-γ stimulated keratinocytes was an artifact of culturing conditions, we also cultured keratinocytes in medium (Defined SFM, Invitrogen) that did not contain bovine pituitary extract. After stimulation with IFN-γ these cells produced similar amounts of CXCL10-(1–73) as shown in Fig. 1*B*.

*In Vitro Cleavage of Human CXCL10 with Furin and Carboxypeptidase B*—To directly demonstrate that human CXCL10 is a substrate for furin, we performed *in vitro* digestions with commercially available human CXCL10. This CXCL10 contains an extra N-terminal methionine (Met-CXCL10-(1–78)). We first treated a 2 μM solution of Met-CXCL10-(1–78) with 5 units/ml of furin at 37 °C for 3 h and compared this with CXCL10 left at 37 °C without furin. The mass of Met-CXCL10-(1–78) is 8773 Da (Fig. 2*A*, Table I). After treatment with furin, the mass of the recombinant CXCL10 shifted to 8589 Da (Fig. 2*B*, Table I). This matches with a Met-CXCL10-(1–76) molecule lacking the last two amino acids Ser-Pro, resulting from cleavage after the dibasic sequence Lys-Arg preceding these two C-terminal amino acids. This is exactly as would be expected from furin-mediated cleavage of CXCL10. Upon treatment of the furin processed CXCL10 with the basic specific carboxypeptidase B *in vitro* (0.5 units/ml), this molecule was further processed to Met-CXCL10-(1–74) with a mass of 8305 Da (Fig. 2*C*). This matches with the removal of the basic amino acids Lys-Arg, the two C-terminal amino acids of CXCL10 after treatment with furin (Table I). Intact human CXCL10 was insensi-

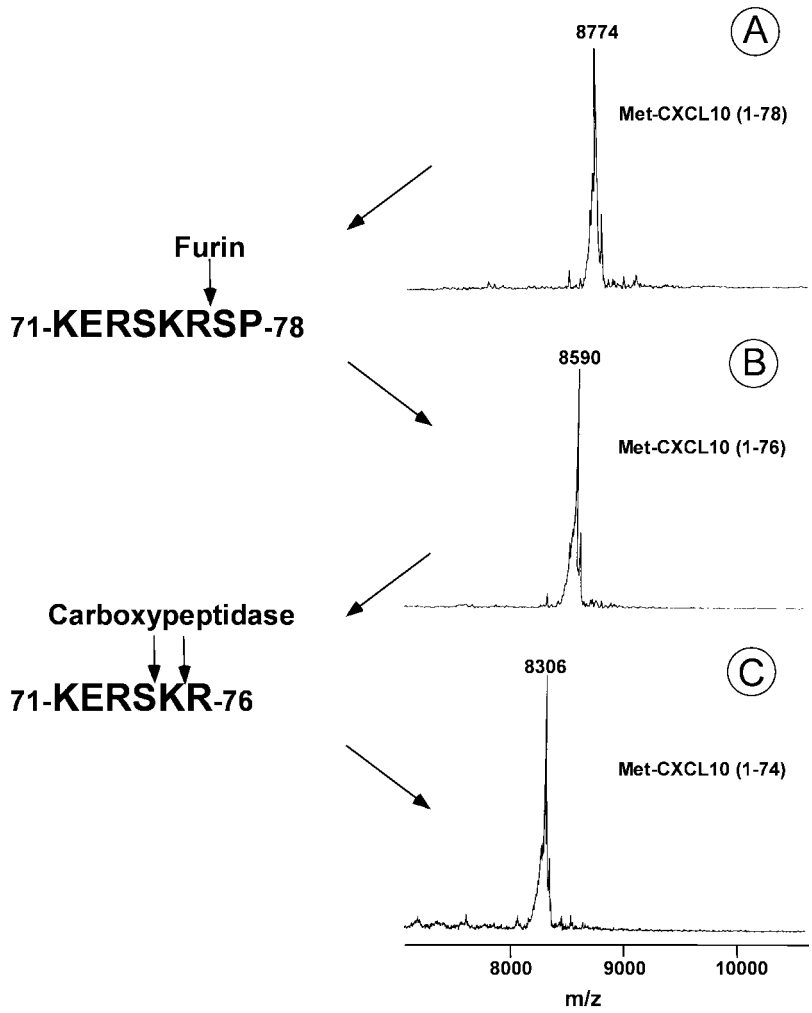



FIG. 2. ***In vitro* cleavage of CXCL10 by furin and carboxypeptidase B.** Recombinant human Met-CXCL10 (2 $\mu$M) was incubated for 3 h at 37 °C in the presence or absence of different enzymes. Samples were subsequently measured using MALDI-MS. *A*, untreated Met-CXCL10. *B*, Met-CXCL10 incubated with furin (5 units/ml). *C*, Met-CXCL10 incubated with furin (5 units/ml) + carboxypeptidase B (0.5 units/ml). The C-terminal amino acids of CXCL10 and the cleavage site of the respective enzymes are shown.

TABLE I
*Characterization of intact and furin cleaved CXCR3 targeting chemokines*

Recombinant chemokines were treated with furin with or without carboxypeptidase B, and their masses were determined using MALDI-MS. Theoretical masses for the furin-processed chemokines are only specified whenever the furin consensus sequence Arg-Xaa-(Lys/Arg)-Arg is present within the primary structure of the chemokine. CXCL10 (R72M) refers to the CXCL10 molecule that has a methionine at position 72 instead of an arginine and therefore lacks the furin consensus site.

| Chemokine | AA[a] | Theoretical mass (Da) | | | Experimental mass (Da) | | |
|---|---|---|---|---|---|---|---|
| | | Intact | +Furin | +Furin+CPB[b] | Intact | +Furin | +Furin+CPB[b] |
| Human | | | | | | | |
| Met-CXCL10 | 78 | 8773.5 | 8589.3 | 8304.9 | 8773.8 | 8588.9 | 8305.5 |
| CXCL10 (R72M) | 77 | 8617.3 | | | 8617.5 | 8617.5 | |
| CXCL9 | 103 | 11721 | | | 11722 | See Fig.5 | |
| CXCL11 | 73 | 8303.0 | | | 8303.6 | 8303.3 | |
| Mouse | | | | | | | |
| Met-CXCL10 | 78 | 8828.6 | 8660.3 | 8376.0 | 8828.3 | 8660.3 | 8375.7 |

[a] AA, amino acids.
[b] CPB, carboxypeptidase B.

tive to treatment by carboxypeptidase B alone (data not shown). Altogether, these data show that sequential treatment of CXCL10 with furin followed by a basic specific carboxypeptidase results in the formation of CXCL10 lacking the last four amino acids.

*Kinetics of Furin-mediated Cleavage of Human CXCL10*—To determine the time dependence of the furin-mediated cleavage of human Met-CXCL10 *in vitro*, 0.5 $\mu$M solutions of human Met-CXCL10 were incubated with 5 units/ml of furin at 37 °C for different periods of time. MALDI-MS was used to determine the relative amount of Met-CXCL10-(1–78) and Met-CXCL10-(1–76). As can be seen in Fig. 3*A*, cleavage was completed within 1 h of incubation with furin. In addition, the concentration dependence of the conversion rate of CXCL10 was determined (Fig. 3*B*). This analysis revealed a $K_m$ of 5 $\mu$M.

*A Mutation in the Furin Consensus Site Prevents Cleavage of Human CXCL10*—A cDNA encoding the human CXCL10 protein has long been used as a template for production of human CXCL10 protein (27). This predicted protein, however, differs from that identified on the basis of mass spectrometric analysis of the native protein (22), because it contains a methionine at position 72 instead of an arginine (CXCL10 R72M). A commercially available variant of recombinant human CXCL10 has this methionine at position 72 (PeproTech) and therefore has

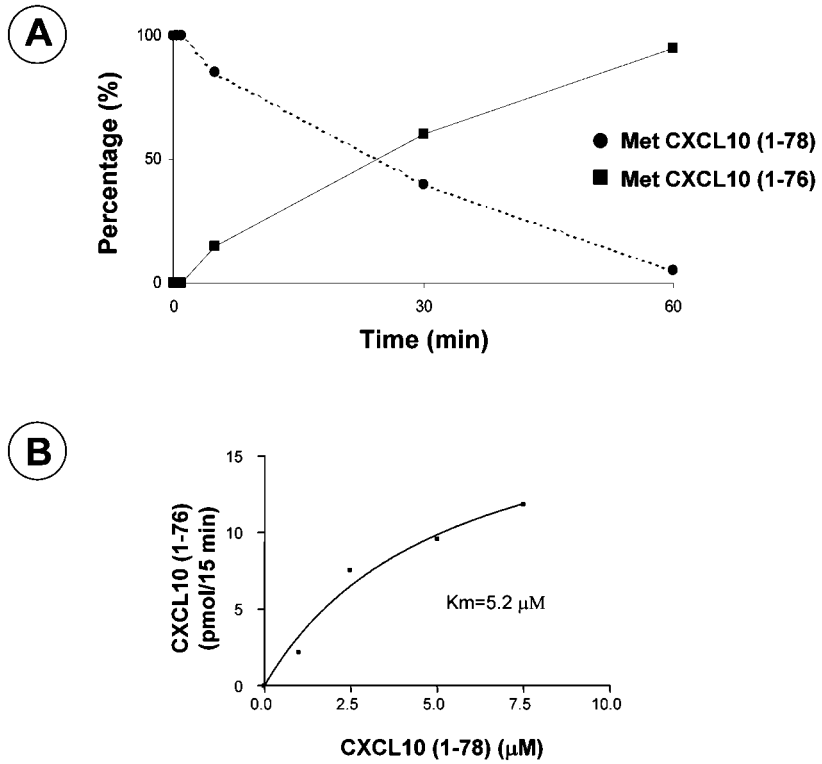



FIG. 3. **Kinetics of furin-mediated cleavage *in vitro*.** *A*, recombinant human CXCL10 (0.5 $\mu$M) was incubated with 5 units/ml of furin at 37 °C for different periods of time. MALDI-MS was used to determine the relative amount of intact CXCL10 (Met-CXCL10-(1–78)) and processed CXCL10 (Met-CXCL10-(1–76)). *B*, different concentration of recombinant human CXCL10 (20 $\mu$l) were incubated with 5 units/ml of furin for 15 min. MALDI-MS was used to determine the relative amount of intact CXCL10 and processed CXCL10. Data were fitted according to Michaelis-Menten kinetics.

the C-terminal sequence -Lys-Glu-**Met-**Ser-**Lys-Arg-**Ser-Pro. Because this sequence does not comply with the furin consensus sequence shown above, we predicted that this recombinant human CXCL10 is not sensitive to furin cleavage. Upon treatment of this mutant recombinant protein with furin, we indeed found that it was not processed (Table I). This confirms that the arginine at position 72 is essential for furin-mediated cleavage of human CXCL10.

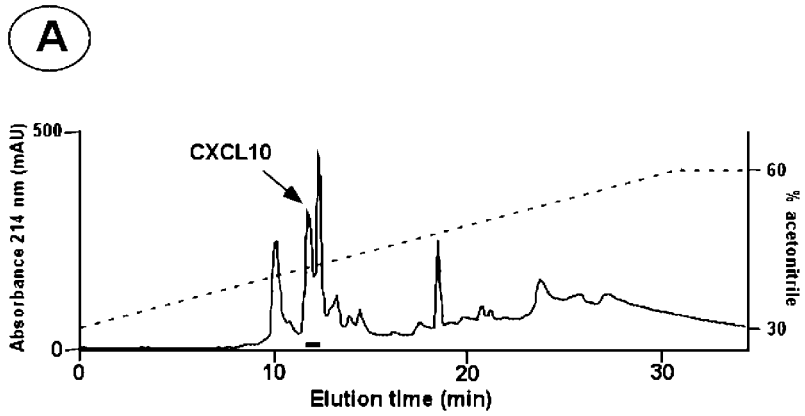
*CXCL10 Production in Cytokine-stimulated Primary Fibroblasts*—Unlike other pro-protein convertases, furin is widely expressed in different cells and tissues. To determine whether processing of CXCL10 is keratinocyte specific or also occurs in another cell type, we stimulated primary human dermal fibroblasts with IFN-$\gamma$ and TNF-$\alpha$ at concentrations that have previously been shown to highly induce mRNA levels of the CXCR3 targeting chemokines in these cells (28). Following heparin affinity chromatography, chemokines were purified using reversed phase chromatography (Fig. 4*A*). Mass spectrometric analysis of a CXCL10-containing fraction (indicated with a bar in the chromatogram), showed that also in these cells CXCL10 was (partially) processed to a molecule with a mass of 8173 Da (Fig. 4*B*), corresponding to CXCL10-(1–73). In contrast to keratinocyte-derived CXCL10, however, most of the fibroblast produced CXCL10 was intact (1–77). In addition, masses corresponding to N-terminally truncated CXCL10-(3–77) and CXCL10-(3–73) were identified, most probably reflecting N-terminal processing of CXCL10 by dipeptidylpeptidase IV.

In addition to CXCL10, we identified masses matching CCL5-(1–68) and CCL5-(3–68) in this sample (Fig. 4*B*), the latter most probably representing the dipeptidylpeptidase IV processed form of CCL5 (20). The identity of CCL5 was confirmed by sequence analysis of a tryptic fragment of this protein (EYFYTSGK, amino acids 26–33 of CCL5). No masses were identified that would match with furin-mediated cleavage of CCL5, which is in agreement with the fact that CCL5 does not contain a furin consensus sequence.

*Furin-mediated Cleavage of Other CXCR3-targeting Chemokines*—In contrast to human CXCL10, human CXCL9 and -11 do not contain the furin consensus sequence Arg-Xaa-(Lys/Arg)-Arg, although they contain many basic residues in their C-terminal region. As it has been shown that also proteins containing sequences that do not fully fit the consensus sequence can be substrates for furin, we also incubated 2 $\mu$M solution of recombinant human CXCL9 and -11 with 5 units/ml furin at 37 °C for 3 h. This demonstrated that human CXCL11 is not a substrate for furin (Table I). On the other hand, human CXCL9 was cleaved at five different positions in the C-terminal part of the molecule, all after a basic amino acid residue (Fig. 5). Under identical conditions used for the complete processing of human CXCL10 (Fig. 2), however, human CXCL9 was only partially processed.

Close inspection of the protein sequence of CXCL10 from species other than human showed that the furin consensus sequence is highly conserved. Mouse CXCL10 for example, contains the C-terminal furin consensus sequence (in bold) Ser-Gln-Lys-**Arg-**Ser-**Lys-Arg**-Ala-Pro, suggesting it could also be processed after the second dibasic site. In agreement with this, we found that upon treatment with furin, the mass of recombinant mouse Met-CXCL10 shifted from 8828 to 8660 Da (Table I). This corresponds to the loss of the C-terminal amino acids Ala-Pro. No other masses were identified in the mouse CXCL10 treated with furin, showing that the other di-basic sequence in the C terminus of this CXCL10 (see above) is not a substrate for furin.

*Activation of CXCR3*—The ability of the novel CXCL10 variant to activate CXCR3 was tested using three different bioassays. First, Met-CXCL10 stimulated GTP binding to G$\alpha$ in CXCR3-transfected CHO cells was determined using a GTP$\gamma$S binding assay (Fig. 6*A*). This revealed that Met-CXCL10-(1–74) retained full activity on CXCR3. Similar results were obtained in $Ca^{2+}$ mobilization in CXCR3-transfected CHO cells (Fig. 6*B*) and chemotaxis of primary human T-cells (Fig. 6*C*). Overall, the above results show that the agonistic properties of Met-CXCL10-(1–78) and -(1–74) for CXCR3 are similar, indicating that the C-terminal part of CXCL10 is not essential for proper CXCR3 activation.



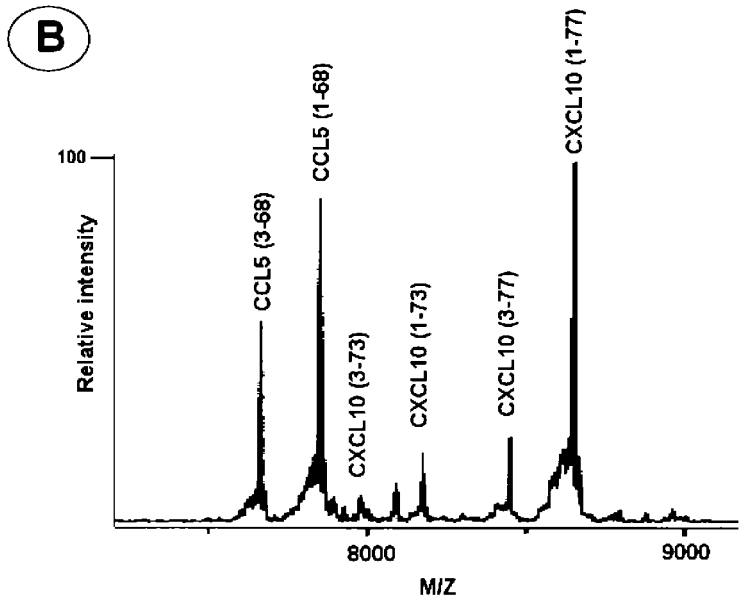




FIG. 4. **Purification and characterization of CXCL10 produced by primary human fibroblasts.** Primary human fibroblasts were stimulated with IFN$\gamma$ (500 units/ml) and TNF$\alpha$ (500 units/ml) and allowed to excrete chemokines for 16 h. Following heparin affinity chromatography of culture medium, chemokines were purified using reversed phase chromatography (*A*). CXCL10-containing fractions were measured using MALDI-MS (*B*). *Bar* indicates fractions measured by MALDI-MS.

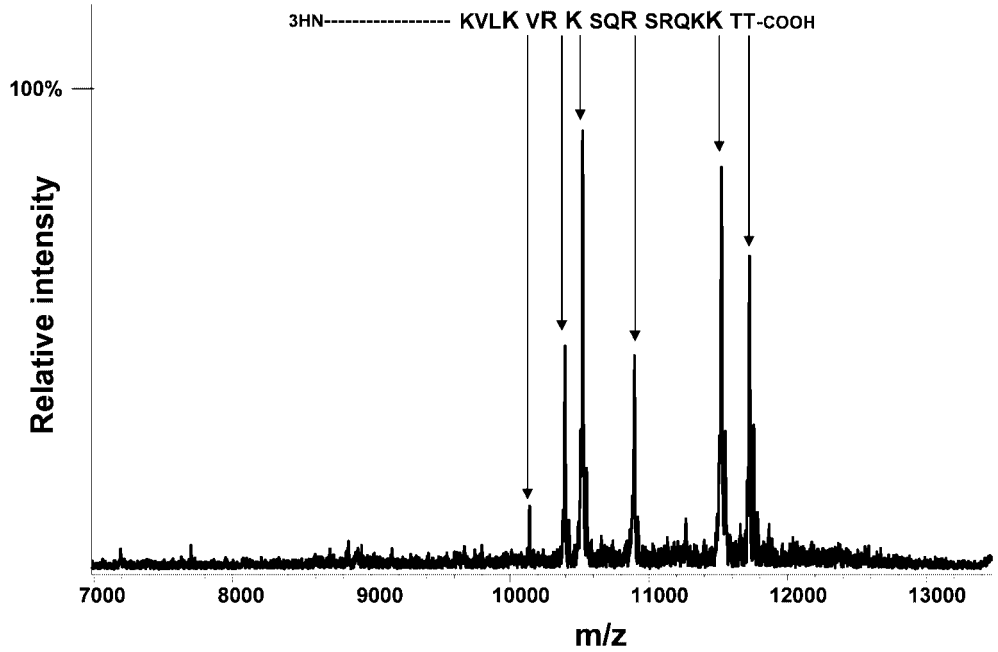



FIG. 5. ***In vitro* cleavage of human CXCL9 by furin.** Recombinant human CXCL9 (2 $\mu$M) was incubated with furin (5 units/ml) at 37 °C for 3 h. MALDI-MS was used to determine the different processed variants of CXCL9. The C terminus of CXCL9 is shown with the cleavage sites indicated by *capital letters*.

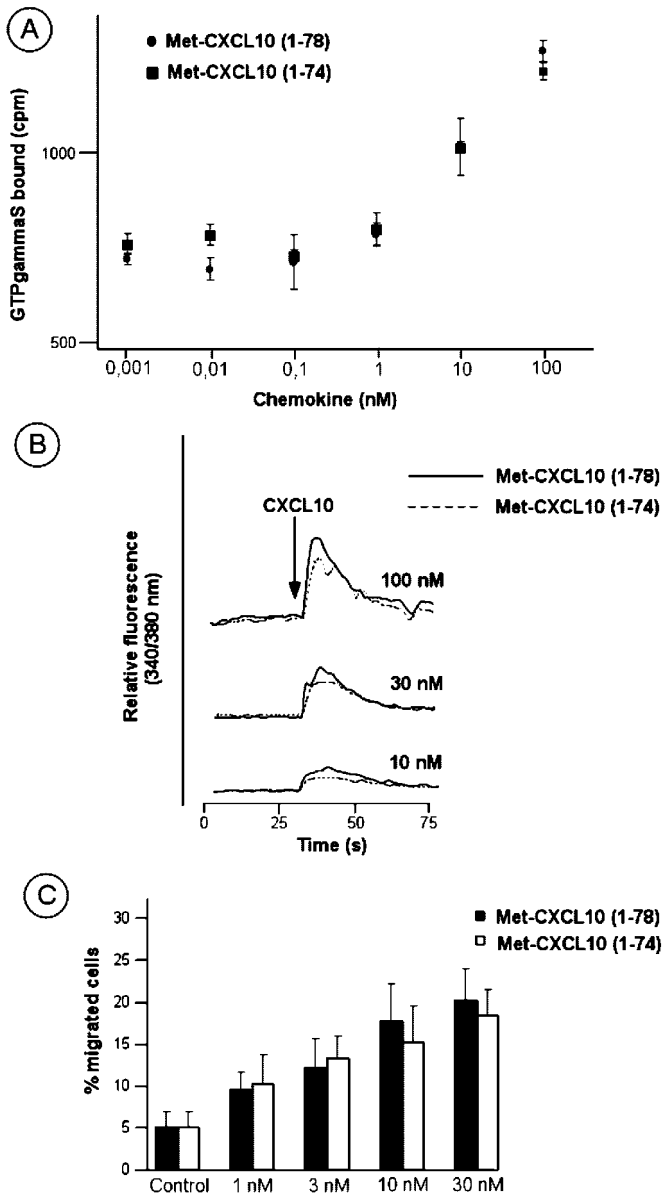



FIG. 6. **CXCR3 activating potency of CXCL10 variants.** Full-length (Met-CXCL10-(1–78) and C-terminally truncated CXCL10 (Met-CXCL10-(1–74) were tested for their activity on CXCR3. *A*, GTPγS binding in CXCR3-transfected CHO cells. *B*, $Ca^{2+}$ mobilization in CXCR3-transfected CHO cells loaded with Fura-2. *C*, chemotaxis of PHA and IL-2 expanded primary human T-cells. For the experiments with CHO cells, a representative experiment of three is shown. Chemotaxis experiments were performed with T-cells from three different donors.

*Inverse Agonism of ORF74*—The genomes of several viruses contain sequences that show a high similarity with chemokine receptors. The Kaposi's sarcoma associated herpes virus (human herpesvirus 8), for example, contains a gene (ORF74) encoding a seven-transmembrane receptor that shows the highest similarity with human CXCR1, 2 and 3. This viral receptor shows a high level of constitutive activity, which is most probably causally related to the pathogenesis of Kaposi's sarcoma (29).

Interestingly, CXCL10 has been shown to be an inverse agonist on this receptor (30). Although the N-terminal amino acids of CXCL10 have been shown to be essential for CXCR3 activation (31), this is not the case for the inhibitory effect on the constitutive activity of ORF74 (30), indicating that other determinants might be involved in this activity. Similarly, we found that Met-CXCL10-(1–74) has still potent inverse agonistic activities on ORF74 (Fig. 7).

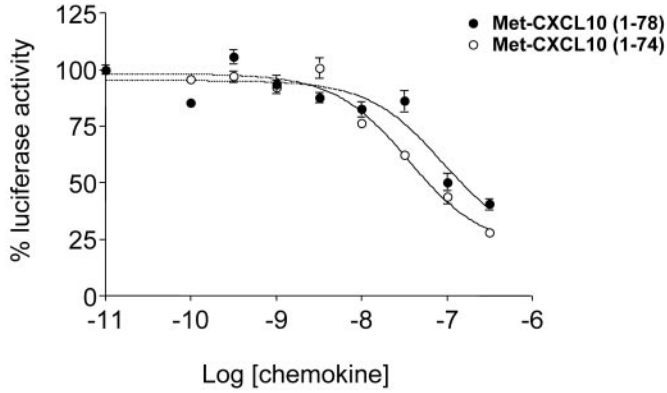



FIG. 7. **Inverse agonism activity of CXCL10 on ORF74.** COS-7 cells were transiently transfected with a cDNA encoding the HHV-8 encoded ORF74. Cells were incubated with different concentrations of full-length CXCL10 (Met-CXCL10-(1–78) and C-terminally truncated CXCL10 (Met-CXCL10-(1–74). After 48 h, NF-κB-driven luciferase expression was measured. A representative experiment performed in triplicate is shown, and the experiment was repeated two times.

*Antibacterial Activity of CXCL10*—It has recently been shown that several chemokines, including the ligands for CXCR3, show direct microbicidal activity (19, 32). Because it is believed that the C-terminal region plays an essential role for this activity, we determined the potency of intact and C-terminally truncated CXCL10 in inhibiting the growth of *E. coli* and *L. monocytogenes*, which revealed no apparent difference between the two CXCL10 variants (Fig. 8).

## DISCUSSION

Chemokines comprise a class of more than 50 proteins that control the migration and activation of leukocytes through activation of their concomitant receptors. The complexity of this system is not only controlled at the level of expression but also as a result of post-translational processing. A limited number of studies have shown restricted truncation of chemokines.

It is at present well established that the endopeptidase dipeptidylpeptidase IV (CD26) cleaves many chemokines having a penultimate proline or alanine at the N terminus, including the CXCR3 ligands (2, 22, 31). Recently, the N-terminal region of chemokines has been shown to be sensitive to cleavage by matrix metalloproteinases (MMP). MMP-9- and MMP-2-mediated cleavage of CCL7 and CXCL12, respectively, negatively modulates their chemokine receptor-mediated activity (3, 33) while CXCL8 is highly potentiated after cleavage by MMP-9 (25). Other proteases involved in processing of chemokines include cathepsins (4, 6), elastase (5) urokinase plasminogen activator, and plasmin (8).

In this study we have described furin as a novel chemokine-modifying enzyme. Furin cleaves CXCL10 *in vitro* at a furin consensus site, resulting in a CXCL10 molecule that is sensitive to a basic amino acid-specific carboxypeptidase. Upon trimming by such a carboxypeptidase *in vitro*, a CXCL10 molecule is created that lacks the last four amino acids. This C-terminally truncated CXCL10 molecule is also produced *in vivo* by cytokine stimulated primary keratinocytes and fibroblasts. A pro-protein convertase inhibitor, decanoyl-RVKR-chloromethylketone, prevents the production of this C-terminally truncated CXCL10, indicating that furin is also involved in the *in vivo* cleavage of CXCL10.

Furin belongs to the group of pro-protein convertases (PCs). These enzymes are involved in the production of many biologically active proteins like *e.g.* growth factors, hormones, receptors, and matrix metalloproteinases (26, 34). In general, these

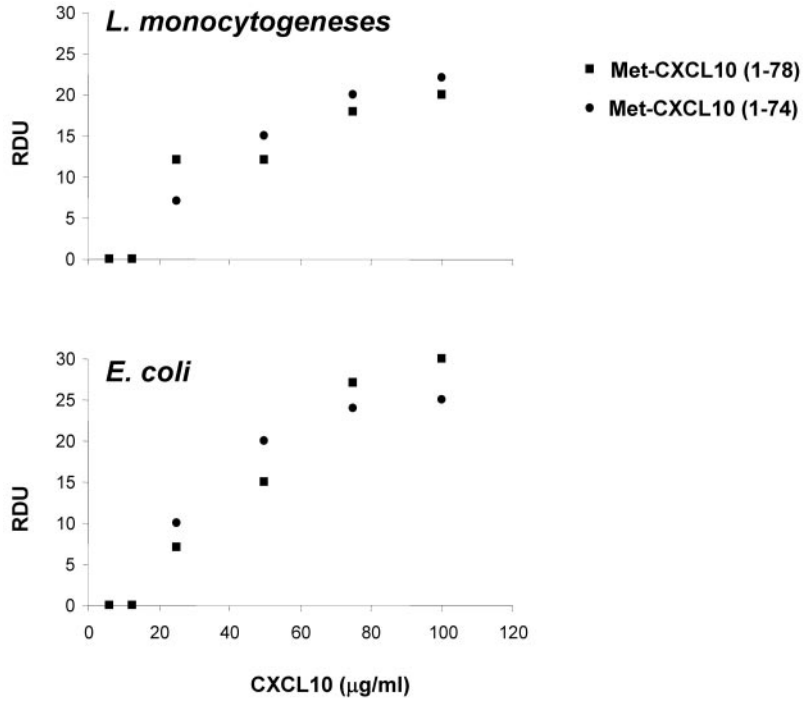



FIG. 8. **Antibacterial activity of CXCL10.** The antibacterial activity of full-length CXCL10 (Met-CXCL10-(1–78)) and C-terminally truncated (Met CXCL10-(1–74)) was tested on *L. monocytogeneses* (*upper part*) and *E. coli* (*lower part*) using a radial diffusion assay. For this purpose, within solidified agar containing the representative bacteria, 3-mm wells were punched and filled with 5 $\mu$l of chemokine solution. Antimicrobial activity was determined as a clear zone around the well after 16-h incubation at 37 °C, and are represented in radial diffusion units: (diameter of clear zone in millimeters − well diameter) × 10. A representative experiment of three is shown. *RDU*, radial diffusion units.

active proteins result from limited processing at paired basic residues within higher molecular mass precursors. In the case of neuropeptides, proteolysis often occurs in secretory vesicles that are secreted upon the proper stimulus. The expression of furin is found in the *trans*-Golgi network of many cells and tissues and plays a role in the processing of precursors for constitutively secreted proteins. Some PCs are specific for certain cells and tissues. In the epidermis, expression of furin, PACE4, PC5/6, and PC7/8 has been identified (35). Because the pro-protein convertase inhibitor used in our studies also inhibits other PCs, we cannot exclude the possibility that other PCs are involved in the CXCL10 processing *in vivo*.

Human and murine CXCL10 (but also *e.g.* hamster, rat, sheep, goat, and monkey) contain the furin consensus sequence $Arg^{-4}$-Xaa-$Lys^{-2}$-$Arg^{-1}$, and we demonstrated that both are processed by furin. In a survey of the primary structures of other human chemokines we found no perfect match with a furin consensus sequence in the C-terminal region. Our results with the *in vitro* processing of human CXCL9 however showed, that also sequences that do not fully comply with the consensus sequence are potential furin cleavage sites, though only partial processing was observed. Consequently, more chemokines than solely predicted on this consensus sequence may be substrates for furin *in vitro*. Using cytokine-stimulated keratinocytes, we only detected full-length CXCL9 (22) and did not find evidence for furin-mediated processing of CXCL9 *in vivo*. Others have found C-terminal truncations of CXCL9 (36) and although the mechanism by which these are formed is unknown, it most likely does not reflect furin-mediated cleavage.

In the case of CXCL9, furin cleavage *in vitro* was most efficient at paired basic residues. Interestingly, cleavage also occurred after lysine residues, although site-directed mutagenesis experiment with several substrates and structural analysis of furin suggested that an arginine at this position is essential (26, 37). A basic amino acid at position −4 is also favorable for furin-mediated cleavage. In the case of human CXCL10, the importance of this residue is underscored by the observation that the mutant CXCL10 with the Arg to Met substitution at this position was not cleaved by furin *in vitro* (Table I). Moreover, mouse CXCL10 contains two pairs of dibasic residues in the C-terminal part of the protein, with $Lys^{71}$-$Arg^{72}$ not having a basic residue at position −4. This dibasic sequence was insensitive to furin cleavage *in vitro*, while the $Lys^{74}$-$Arg^{75}$ was fully processed (Table I).

The fact that only ~40% of CXCL10 from cytokine-stimulated keratinocytes is processed indicates that the activity of furin in these cells is limited. In contrast, the fact that we have never identified a native CXCL10 molecule corresponding to CXCL10-(1–75) indicates that the trimming of the basic residues by a carboxypeptidase is not a rate-limiting step *in vivo*. The cleavage of CXCL10 by furin seems to be very efficient as the $K_m$ (5 $\mu$M) is much lower then identified for other substrates (38).

In contrast to the N-terminal truncations, there are only a few examples of natural occurring C-terminal truncations of chemokines having effect on chemokine receptor activation. In general, the N terminus and the N-loop are considered to form the most important chemokine binding and activation domains (39). An exception to this rule is CXCL7, for which a more potent CXCR2 agonist was identified after cleavage of several C-terminal amino acids by an as yet unknown mechanism (11). In the case of CXCL10, full activity on CXCR3 was found for the C-terminally truncated variant (Fig. 6). This indicates that furin-mediated processing of CXCL10 does not impair nor enhance the activity on CXCR3. This is in concordance with recent data on the interaction of CXCL10 with its receptor. Mutational analysis studies have focused on specific residues in CXCL10 that are important in the CXCR3 activating activity of CXCL10 (40). Among others, especially the arginine at position 8 was shown to be very important.

Another G-protein-coupled receptor with CXCL10 binding properties is encoded by the Kaposi's-associated human herpes virus 8. This receptor displays a high level of constitutive activity (41), which appears an important determinant in the pathogenesis of Kaposi's sarcoma. Although several chemokines (*e.g.* CXCL1) are agonists for this receptor, CXCL10 has been shown to be a potent inverse agonist (30). In this case, the N terminus of CXCL10 has been shown to be of minor importance (30) and our studies have now shown that also the C-terminal four amino acids are not essential for this inverse agonistic activity.

Some chemokines possess activities that are not mediated through G-protein-coupled receptor activation. It has for example recently become apparent that certain chemokines possess a direct antibacterial activity, pointing to the fact that these proteins play a role in both the innate and acquired immunity. Similar dual activities have also been assigned to a group of peptides, the β-defensins, which were at first defined as antibacterial proteins (42). Several overlapping structural domains between chemokines and defensins have been implicated in their common antibacterial activity, which showed that especially the α-helix is of major importance (32). Fascinatingly, in the case of CXCL7, a yet undefined mechanism in platelets can release the last two C-terminal amino acids resulting in a very potent antibacterial protein, previously known as the thrombocidins. This points to an essential role of the C terminus in the microbicidal activity of this chemokine (10). Direct antibacterial activity for the CXCR3-targeting chemokines has only recently been recognized (19). In contrast to the results with CXCL7, deletion of amino acid residues at the C terminus of CXCL10 does not alter its antibacterial activity toward *E. coli* and *L. monocytogeneses*.

Because the stretches of basic amino acids in the C terminus of chemokines are generally believed to be involved in the affinity for heparin, one possibility could be that the truncated CXCL10, that has lost two basic amino acids, has altered heparin binding affinities. Recent studies performed with a large set of CXCL10 mutants however, suggests that the last four amino acids are not essential for heparin binding (40). Moreover, in our own chemokine purification procedure we have also applied salt gradients to elute chemokines from the heparin affinity column. In these cases, both CXCL10 variants always co-eluted (data not shown).

Truncated CXCL10 might have an altered affinity for the recently described CXCR3B receptor (43), which is implicated in the angiogenic activities of the CXCR3 ligands. However, CXCR3B differs from CXCR3 only in its N-terminal part and as this region is probably only involved in recognizing the N terminus of the ligands this possibility seems less plausible. Moreover, genomic data from the CXCR3 locus in mouse indicates that there is no CXCR3B in this species and therefore a similar mechanism, expected for the conserved processing site, would be absent in this species.

Since all ligands for CXCR3 are natural antagonists for CCR3 (44), truncated CXCL10 might have an increased or impaired affinity for CCR3.

Finally, truncated CXCL10 might affect hitherto unexpected biological responses like recently shown for CXCL12, where MMP-2-mediated cleavage gives rise to a highly neurotoxic variant implicated in (CXCR4-independent) neurodegeneration (9). Interestingly, there is evidence for a CXCL10 receptor that is not CXCR3 on a set of different cell lines (45). Its cloning and characterization would open the possibility to study the role of the C-terminally processed CXCL10 in activating this novel receptor.

In conclusion, we have identified furin as a novel chemokine-modifying enzyme *in vitro* and most probably also *in vivo*. This conversion generates a CXCL10 variant that retains full agonistic activity for CXCR3, inverse agonistic activities on ORF74 and antibacterial properties toward *E. coli* and *L. monocytogenes*. The biological significance of furin-mediated CXCL10 truncation is as yet not elucidated. The high conservation of the furin consensus site in CXCL10 from different species implies a specific function for furin-processed CXCL10.

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