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Sequential processing of the transmembrane chemokines CX3CL1 and CXCL16 by α- and γ-secretases ☆

A. Schulte [a], B. Schulz [a], M.G. Andrzejewski [b], C. Hundhausen [a], S. Mletzko [a], J. Achilles [a], K. Reiss [a], K. Paliga [a], C. Weber [b], S. Rose John [a], A. Ludwig [b,*]

[a] *Institute of Biochemistry, Christian-Albrechts-University, Kiel, Germany*
[b] *Institute for Molecular Cardiovascular Research (IMCAR), University Hospital, RWTH Aachen, Pauwelsstr. 30, 52074 Aachen, Germany*

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Abstract

The chemokines CX3CL1/Fractalkine and CXCL16 are expressed as transmembrane molecules and can mediate cell–cell-adhesion. By proteolytic processing, CX3CL1 and CXCL16 are released from the cell surface by proteolytic shedding resulting in the generation of soluble chemoattractants. This ectodomain release is mediated by the α-secretase-like activity of the two disintegrins and metalloproteinases ADAM10 and ADAM17. Using CX3CL1 and CXCL16 constructs C-terminally fused to two Z-domains of Protein A (2Z-tag) we detect C-terminal fragments (CTFs) of both chemokines resulting from ADAM10-mediated cleavages at multiple sites as examined by inhibitor studies. Furthermore, inhibitor studies as well as genetic studies using presenilin 1/2-deficient cell lines suggest the involvement of γ-secretase- but not-β-secretase-like activity in the processing of transmembrane chemokines. The combination of α- and γ-secretase and proteasomal inhibitors points towards a sequential processing of transmembrane chemokines by first ADAM10 and then γ-secretases and possible further degradation. This proteolytic processing cascade of transmembrane chemokines is similar to that described for Notch and E-cadherin where CTFs generated by γ-secretase serve as intracellular signal transmitters.

Keywords: Chemokines; Metalloproteinases; Shedding; Secretases; Regulated intramembrane proteolysis; Cell migration; Inflammation

Chemokines are usually small, secreted proteins modulating cell migration and cell–cell communication [1]. Among more than 40 known human chemokines, CXCL16 and CX3CL1/Fractalkine are special as they are expressed as type I-transmembrane molecules consisting of an N-terminal chemokine domain followed by a highly *O*-glycosylated, mucine-like stalk, a single transmembrane α-helix and a short cytoplasmic tail. CX3CL1 and CXCL16 are expressed by several different cell types either constitutively or upon induction by proinflammatory stimuli [2–4]. As transmembrane molecules they mediate firm adhesion to cells carrying their corresponding heptahelical receptors CXCR6 or CX3CR1, respectively [5,6].

CX3CL1 and CXCL16 can be transferred into soluble forms by limited proteolysis at the cell surface. Experiments using specific inhibitors, knock-out cell lines and siRNA-knock-down have shown that the constitutive release of both CX3CL1 and CXCL16 is mainly brought about by the disintegrin and metalloproteinase ADAM 10 [7–10]. Shedding of CX3CL1 via ADAM10 can be further enhanced by ionomycin (Hundhausen et al., unpublished), whereas phorbol-12-myristate-13-acetate (PMA)-induced shedding is attributed to the induced activity of ADAM17 [11,12]. Shedding generates soluble forms of CX3CL1 and CXCL16 that induce chemotaxis of receptor-positive leukocytes [2,4]. However, the generation and fate of the C-terminal chemokine fragments that remain associated with the cells has not been investigated.

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* Corresponding author. Fax: +49 241 8082716.
E-mail address: aludwig@ukaachen.de (A. Ludwig).

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Generation of C-terminal cleavage fragments (CTFs) by shedding has been intensively studied for the amyloid precursor protein (APP) [13]. Cleavage of APP occurs at multiple sites and the involved proteases have been termed as α-, β- and γ-secretases. Cleavage by α-secretases occurs at the cell surface proximal to the cell membrane and is mediated by ADAM10 and ADAM17 while β-secretase cleavage occurs more distal from the membrane and is mediated by the aspartylprotease β-site APP-cleaving enzyme (BACE). Extracellular cleavage generates CTFs residing in the cell membrane that undergo further proteolysis within the cell membrane by the γ-secretase complex. The γ-secretase complex is composed of several subunits including either presenilin 1 or 2 (PS1/2), which are required for proteolytic activity as shown by embryonic fibroblasts generated from PS1/2-deficient mice [14]. Since proteolytic processing of APP by β- and γ-secretases is critically involved in the pathology of Alzheimer's disease the secretases have been recognized as promising drug targets. However, vital type 1 surface molecules like Notch [15–17], E-cadherin and N-cadherin are also sequentially cleaved by α- and γ-secretases [18–20] and even further processing events may depend on these secretases.

To further delineate the cleavage mechanism of the two transmembrane chemokines CX3CL1 and CXCL16, we examined the C-terminal fragments generated by ectodomain shedding in detail. By cleavage studies with defined inhibitors and by using cell lines derived from PS1/2-deficient mice we provide evidence that several C-terminal fragments are generated by the α-secretase ADAM 10 and are further cleaved by the γ-secretase complex.

Materials and methods

Antibodies and inhibitors. Unconjugated goat anti-human CXCL16 Ab, normal rabbit IgG, unconjugated, biotinylated and phycoerythrin (PE)-conjugated monoclonal antibodies to human CX3CL1 were obtained from R&D Systems (Wiesbaden, Germany). Unconjugated and biotinylated rabbit anti-human CXCL16 antibodies were from PeproTech (London, UK). The rabbit antiserum against the chemokine domain of human CX3CL1 was characterized previously [21]. Secondary fluorescein-conjugated swine-anti rabbit antibody was from Dako (Hamburg, Germany). The monoclonal IgM-antibody against human CXCL16 was raised against the chemokine domain of recombinant CXCL16 (Asn49- Pro137).

GW280264X and GI254023X were synthesized and assayed for inhibition of recombinant human ADAM17 and ADAM10 as described [10]. Marimastat was from GlaxoSmithKline. The γ-secretase-inhibitors DAPT (*N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester) and Inhibitor X, the proteasome inhibitor MG132 and the calpain inhibitor ALLN (*N*-acetyl-Leu-Leu-Nle-CHO) and the β-secretase inhibitor OM99-2 were from Calbiochem (Darmstadt, Germany).

DNA constructs. 2Z-cDNA was amplified by PCR using pQE60-2Z [22] as a template and ligated to CXCL16 or CX3CL1 cDNA, respectively. Subsequently, a PCR using the CXCL16/CX3CL1 sense primer and the 2Z antisense primer was performed and the resulting cDNA was ligated into pcDNA 3.1 (+) (Invitrogen, Karlsruhe; Germany) and sequenced.

Cell culture and transfection. HEK293 cells were cultured in DMEM containing 10% FCS (all cell culture media and reagents were from PAA, Pasching, Austria). Murine embryonic fibroblast cell lines deficient for presenilin 1 and 2 (PS1/2) were characterized previously and kindly provided by Paul Saftig, Institute for Biochemistry, University of Kiel [23]. For transfection, cells were seeded at 3×10^4 cm^2 in six-well dishes (Sarstedt, Nuembrecht, Germany) and incubated for 24 h to 80% confluency. Medium was exchanged with 1 ml fresh medium and 120 μl serum-free medium/well was added containing 1 μg cDNA encoding for CXCL16-2Z or CX3CL1-2Z, respectively and 2.5 μl Lipofectamin2000® (Invitrogen), prepared according to the manufacturer's instructions. After an incubation period of 6 h, medium was exchanged to 2 ml fresh DMEM and cells were incubated for another 42 h. For the generation of HEK293 cells stably expressing CXCL16-2Z, CX3CL1-2Z cells were selected with geniticin (500 μg/ml, PAA, Pasching, Austria) and subcloned after 7 d by limiting dilution. Clones were tested for expression of 2Z-tagged CXCL16 and CX3CL1 by Western blotting of cell lysates and in parallel by ELISA of conditioned media. The primate kidney fibroblast cell line COS7 was cultured and transfected essentially as described previously [8].

Deglycosylation. To remove *O*-glycosylation from CX3CL1-2Z and CXCL16-2Z, cell lysates (100 μg protein) were treated for 2 h either with recombinant α-2-3,6,8,9-neuraminidase (5 mU) from Arthrobacter ureafaciens, recombinant endo-α-*N*-acetylgalactosaminidase from *Streptococcus pnemoniae* (2 mU, both Calbiochem, Darmstadt, Germany) or both according to the manufacturer's instructions. Subsequently, reducing Laemmli-buffer was added and the lysates analyzed by Western blotting.

CXCL16/CX3CL1 cleavage assays. Cells expressing CX3CL1 or CXCL16 were grown to 80–90% confluency in fully supplemented DMEM in six-well-dishes for 24 h before stimulation. Cells were washed once with sterile PBS and 750 μl of serum-free medium with or without inhibitors was added. Cells were incubated for varying periods of time and conditioned media were harvested and supplemented with a protease inhibitor mixture (Complete, Roche). Conditioned media were cleared by centrifugation and analyzed by ELISA (see below). Cells were washed twice with ice-cold PBS and either lysed in PBS containing 1% Triton X-100 and a protease inhibitor mixture (Complete, Roche) and analyzed for CXCL16-2Z and CX3CL1-2Z by Western blotting (see below) or scraped off in 1 ml ice-cold PBS and examined for CXCL16-2Z and CX3CL1-2Z surface expression by flow cytometry.

ELISA for CX3CL1 and CXCL16. The ELISAs specific for CX3CL1 or CXCL16, respectively, were essentially carried out as described before [7,8]. The specificity of both ELISAs was demonstrated by their positive reaction with lysates and conditioned media of CX3CL1 or CXCL16-transfected HEK-293 cells but not with wild type cells.

Membrane preparation. To enrich membranes and separate them from the cytosolic fractions, 5×10^6 stably transfected CX3CL1-2Z or CXCL16-2Z-HEK293 cells were suspended in 1 ml hypotonic 5 mM Hepes buffer, pH 7.4, passed through a 0.4 mm needle for 15 times and centrifuged at 700*g* for 20 min. Subsequently, 100 μl 1.4 M NaCl was added to 900 μl supernatant and membranes were sedimented at 20,000*g* for 60 min. Pellets were solved in twofold concentrated reducing Laemmli buffer, supernatants were concentrated fivefold using 3 kDa cut-off ultracentrifuge devices (Sartorius, Goettingen, Germany) and fivefold concentrated Laemmli buffer was added.

Western blotting. Western blot analysis was carried out as described previously [7,8]. CX3CL1-2Z and CXCL16-2Z were detected using a rabbit-antiserum raised against the human CX3CL1 chemokine domain (1:2000) or an unspecific rabbit antiserum (1 μg/ml), respectively. After 3 washes, bound antibody was detected with a secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (Jackson) diluted 1:10,000 in PBS with 0.01% Tween. Bound anti-rabbit IgG was detected using enhanced chemiluminescence substrate (ECL Plus, Amersham). Signals were recorded using a luminescent analyser (FujiFilm Image Reader LAS 1000; Fuji, Tokyo, Japan) and image analyser software (AIDA 3.2.1; Raytest, Staubenhardt, Germany).

Flow cytometry. Stably transfected HEK293 cells were scraped off in 1 ml ice-cold PBS, spun down and resuspended in PBS containing 1% BSA and 0.01% NaN_3 at 3×10^6 cells/ml. For detection of cell surface CX3CL1-2Z, cells were incubated with a phycoerythrin (PE)-conjugated monoclonal antibody (1.25 μg/ml) for 1 h on ice. For CXCL16-2Z detection, cells were incubated with a monoclonal mouse anti-human CXCL16 IgM antibody and subsequent detection with fluorescein-conjugated anti-mouse $F(ab')_2$-fragment. The fluorescence signal of the cells

was analyzed by flow cytometry (FACScanto, Becton Dickinson, Heidelberg, Germany).

Results

Proteolytic processing of CXCL16 and CX3CL1 leads to the formation of several distinct C-terminal fragments

To examine C-terminal fragments resulting from cleavage of CX3CL1 and CXCL16, we generated expression plasmids coding for CX3CL1 or CXCL16, both C-terminally linked to a 2Z-tag consisting of two identical repeats of the IgG-binding Z-domain of Protein A (CX3CL1-2Z and CXCL16-2Z) [22] (Fig. 1A). FACS analysis of tansfected HEK293 cells, demonstrated that both fusion proteins were expressed on the cell surface (Fig. 1B). Analysis of the conditioned media by ELISA specific for the chemokine domain of CX3CL1 and CXCL16, respectively, revealed that they were shed to a soluble form similar to the wild type chemokines (Fig. 1C). Therefore the chosen cell line and the fusion constructs were considered suitable to study the processing of transmembrane chemokines.

Full size molecules as well as C-terminal cleavage fragments were then detected in the cell lysates. For Western blot analysis of CX3CL1-2Z-transfected cells a rabbit antiserum to the chemokine domain of CX3CL1 reacting with wild type molecule via the chemokine domain as well as C-terminal fragments via the 2Z-tag was used. As shown in Fig. 2A several distinct proteins were detected in CX3CL1- and CX3CL1-2Z-transfected HEK293 cells, but not in mock-transfected cells (not shown). Consistent with previous reports, two protein bands at 90 and 55 kDa were found in CX3CL1-transfected cells, representing full size CX3CL1 and a less glycosylated precursor [11]. By contrast, in CX3CL1-2Z-transfected cells four distinct proteins were detected. The protein band at 105 kDa very likely represents the full size molecule consisting of transmembrane CX3CL1 together with the 2Z-tag (16 kDa). A smaller band migrating at 70 kDa corresponds to the less glycosylated proform of CX3CL1 plus the 2Z-tag. CXCL16-2Z was detected with an irrelevant rabbit antise-

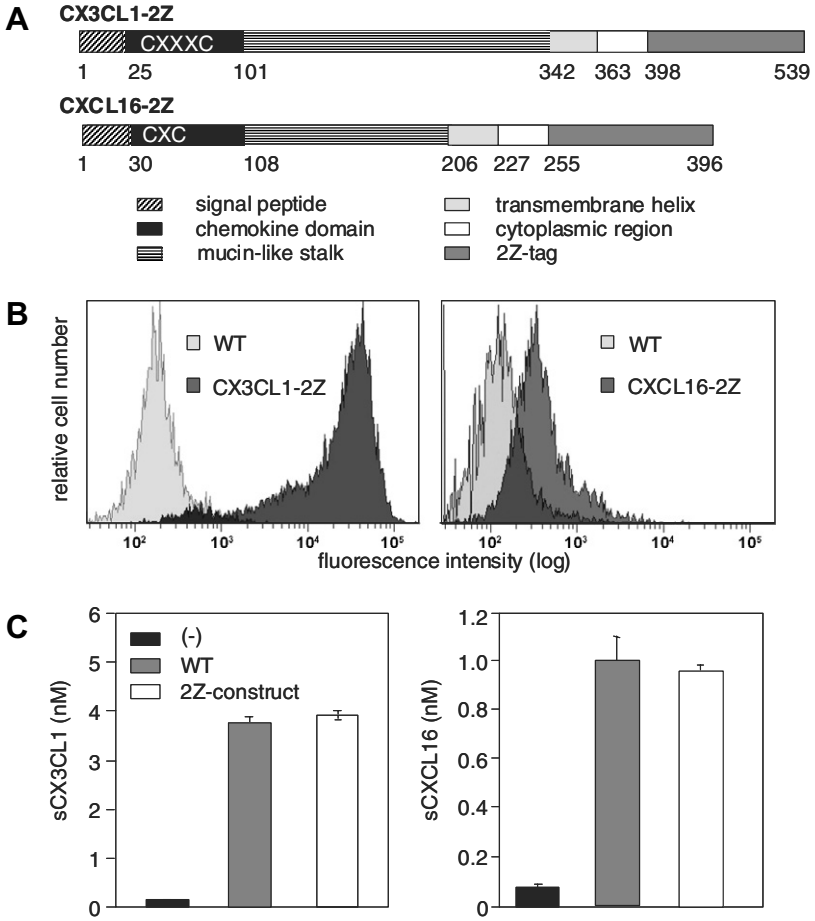

Fig. 1. Expression and shedding of CX3CL1- and CXCL16-2Z fusion proteins. (A) Schematic representation of the domain structure of CX3CL1 and CXCL16. CX3CL1 and CXCL16 were C-terminally fused to two repeats of the Z-domain from Protein A (2Z-tag). Numbers denote positions of the first amino acid residues for each domain. (B) HEK293 cells were transfected with CX3CL1-2Z or CXCL16-2Z, respectively, after 48 h cells were analyzed for surface expression of the fusion proteins by flow cytometry using monoclonal antibodies to CX3CL1 (left) and CXCL16 (right), respectively. (C) HEK293 cells transfected with CX3CL1-2Z and CXCL16-2Z-fusion proteins or respective wild type molecules were analyzed for the release of soluble CX3CL1 (left) and CXCL16 (right) ectodomains by ELISA specific for either chemokine.

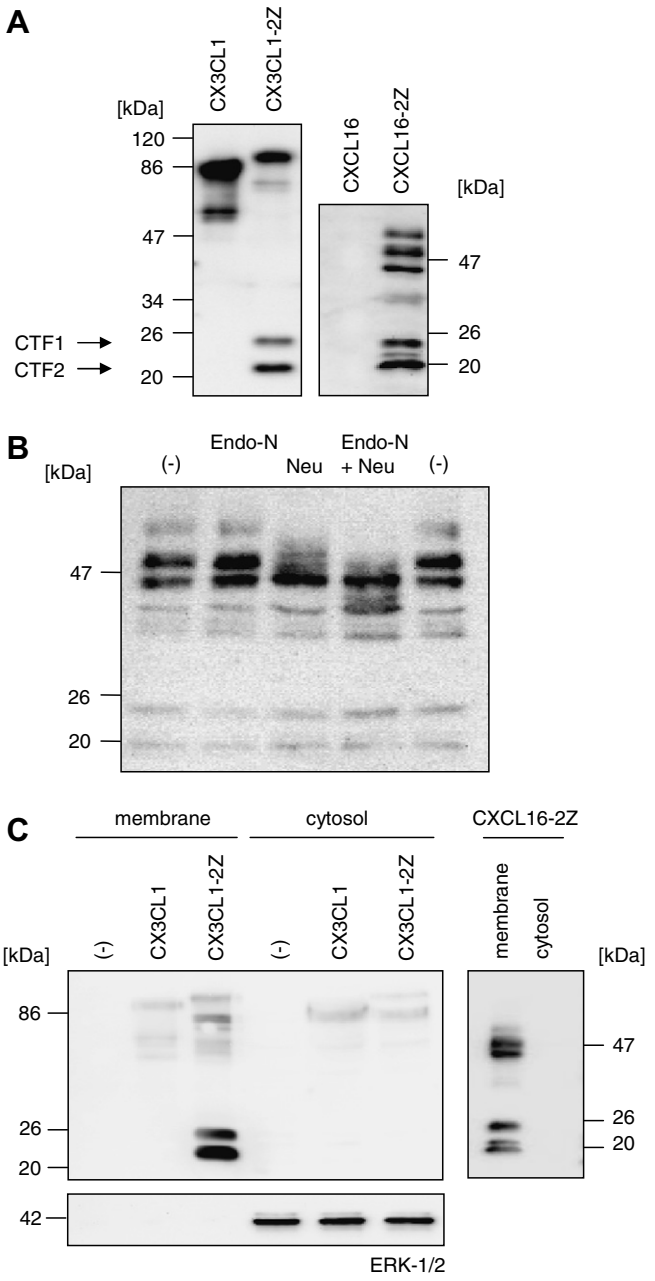

Fig. 2. Detection of CX3CL1 and CXCL16 C-terminal cleavage fragments. (A) HEK293 cells were transfected with CX3CL1, CX3CL1-2Z, CXCL16-2Z or empty vector and analyzed by Western blotting using rabbit antiserum to CX3CL1 or purified rabbit IgG, for detection of full length and C-terminal cleavage fragments (CTFs) of CX3CL1 and CXCL16, respectively. Arrows indicate prominent CTFs of CX3CL1- and CXCL16-2Z. (B) Western blot analysis of CXCL16-2Z from transfected HEK293 cells after deglycosylation with endo-α-*N*-acetylgalactosaminidase (Endo-N), neuraminidase (Neu) or both for 2 h. (C) CX3CL1, CX3CL1-2Z, CXCL16-2Z or mock-transfected HEK293 cells were lysed with hypotonic buffer and membranes were separated from the cytosol by centrifugation. CTFs were visualized by Western blotting using an antiserum to CX3CL1 (left) or purified rabbit IgG, respectively (right). The cytosolic MAP-kinase Erk was used as a marker for successful separation of cytosol and membrane fraction.

rum, which only recognized proteins containing the 2Z-tag but not wild type CXCL16. As expected, CXCL16-2Z and its precursor molecules migrated at a lower molecular weight than CX3CL1, reflecting that CXCL16 has a smaller stalk region than CX3CL1 (Fig. 2A).

In addition to the full size molecules and their precursors we detected smaller variants of CX3CL1-2Z and CXCL16-2Z. These smaller variants migrating at 25 and 22 kDa for CX3CL1-Z were not detectable by the antiserum to the chemokine domain of CX3CL1 and therefore represent no N-terminal but rather C-terminal fragments (CTFs). For CXCL16 slightly smaller fragments of 24 and 20 kDa were found and in some experiments an additional protein band was detected between the 24 and 20 kDa variant with varying intensity and becoming more prominent upon repeated freezing and thawing of the cell lysates suggesting that it is a degradation product that originated from the sample preparation. Transfection of CX3CL1-2Z and CXCL16-2Z into COS7 cells also revealed the existence of two major CTFs with the same apparent sizes as that determined with transfected HEK293 cells (data not shown) indicating that the generation of CTFs was not cell line specific.

Since CXCL16 is known to carry several *O*-glycosylations [4] a combination of neuraminidase and endo-α-*N*-acetylgalactosaminidase was applied. In contrast to the 60 kDa full size protein and the 50 kDa precursor, the 20 and 24 kDa CTFs were not affected by the enzyme treatment suggesting that they did not represent differentially glycosylated forms but rather distinct cleavage fragments (Fig. 2B). In the following, the suspected fragments are therefore termed CTF1 and CTF2. Both fragments of CX3CL1-2Z and CXCL16-2Z were present in membrane preparations but not in the cytosolic fractions, indicating that they were anchored in the cell membrane presumably by a still intact transmembrane domain (Fig. 2C).

C-terminal cleavage fragments are generated by the activity of the α-secretase ADAM10

To examine which protease may be implicated in the generation of the two C-terminal fragments, we incubated CX3CL16-2Z-HEK293 cells with different protease inhibitors (10 μM pepstatin, 100 μM E-64, 1 mM PMSF, 10 μM marimastat, or 2 μM β-secretase inhibitor OM99-2) for 2 h. Only the broad spectrum metalloproteinase inhibitor marimastat showed an inhibitory effect, which was more prominent on CTF2 than on CTF1 (data not shown). A time course experiment revealed that longer incubation periods were required to efficiently suppress the generation of CTF1 which indicates that the turnover of this fragment is slower than that of the 22 kDa CTF2 (Fig. 3A). Inhibition of CTF generation was associated with the accumulation of the full size molecule.

Since ADAM10 and ADAM17 have been implicated in the shedding of transmembrane CX3CL1 and CXCL16 [7,8,11,12] we examined the role of the two α-secretases in the generation of CTFs using the preferential ADAM10

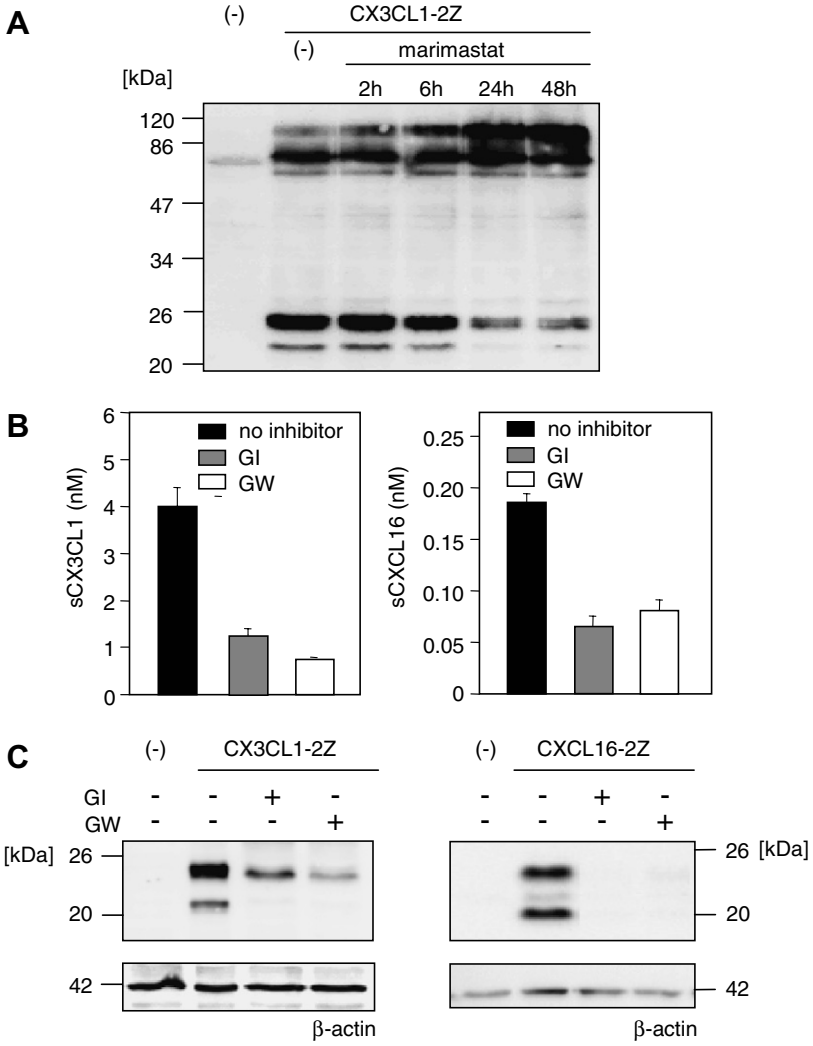

Fig. 3. Cleavage of CX3CL1 and CXCL16 by α-secretase-like activity. (A) Stably transfected CX3CL1-2Z-HEK293 cells were treated with the broad band metalloproteinase inhibitor marimastat (10 μM) for different periods of time, lysed and subsequently analyzed by Western blotting for the presence or absence of CTFs. (B) CX3CL1-2Z- and CXCL16-2Z HEK293 cells were treated with the preferential ADAM10 inhibitor GI254023X and the combined ADAM10/17 inhibitor GW280264X (both 10 μM) in serum-free medium for 4 h and inhibition of CX3CL1 and CXCL16 release by the inhibitors was monitored by ELISA specific for either chemokine. (C) Cells treated with GI254023X or GW280264X were lysed and examined for the generation of CTFs by Western blotting.

inhibitor GI254023X and the combined ADAM10 and ADAM17 inhibitor GW280264X [10]. As determined by ELISA both inhibitors profoundly reduced the generation of soluble CX3CL1 and CXCL16 (Fig. 3B), which was associated with a marked decrease of CTF1 and CTF2, suggesting that both fragments were generated by the activity of ADAM10 (Fig. 3C).

C-terminal cleavage fragments are degraded by γ-secretase activity

We next investigated whether α-secretase-like cleavage is followed by further processing of the CTFs by γ-secretase activity. In the presence of specific γ-secretase inhibitors (DAPT or Inh. X), more CTFs were detectable in the cell lysates of CX3CL1-2Z cells. This effect was not due to enhanced CX3CL1- or CXCL16-protein synthesis or increased α-secretase cleavage as confirmed by Western blot analysis of the full size molecules (Fig. 4A) as well as by ELISA measurement of the shed soluble variants (Fig. 4B) which were not upregulated in the presence of either inhibitor. When α-secretase and γ-secretase inhibitors were applied in combination, accumulation of CTFs was much less pronounced (Fig. 4C) and CTF2 was even completely absent. These data suggest that the cleavage fragments but not the full size molecules are further degraded by γ-secretase. We next investigated cell lysates of CXCL16-2Z-HEK293 cells for the presence of further cleavage fragments resulting from γ-secretase mediated cleavage. Upon long exposure of the Western blots, we detected a small fragment of 18 kDa which was not found when cells were treated

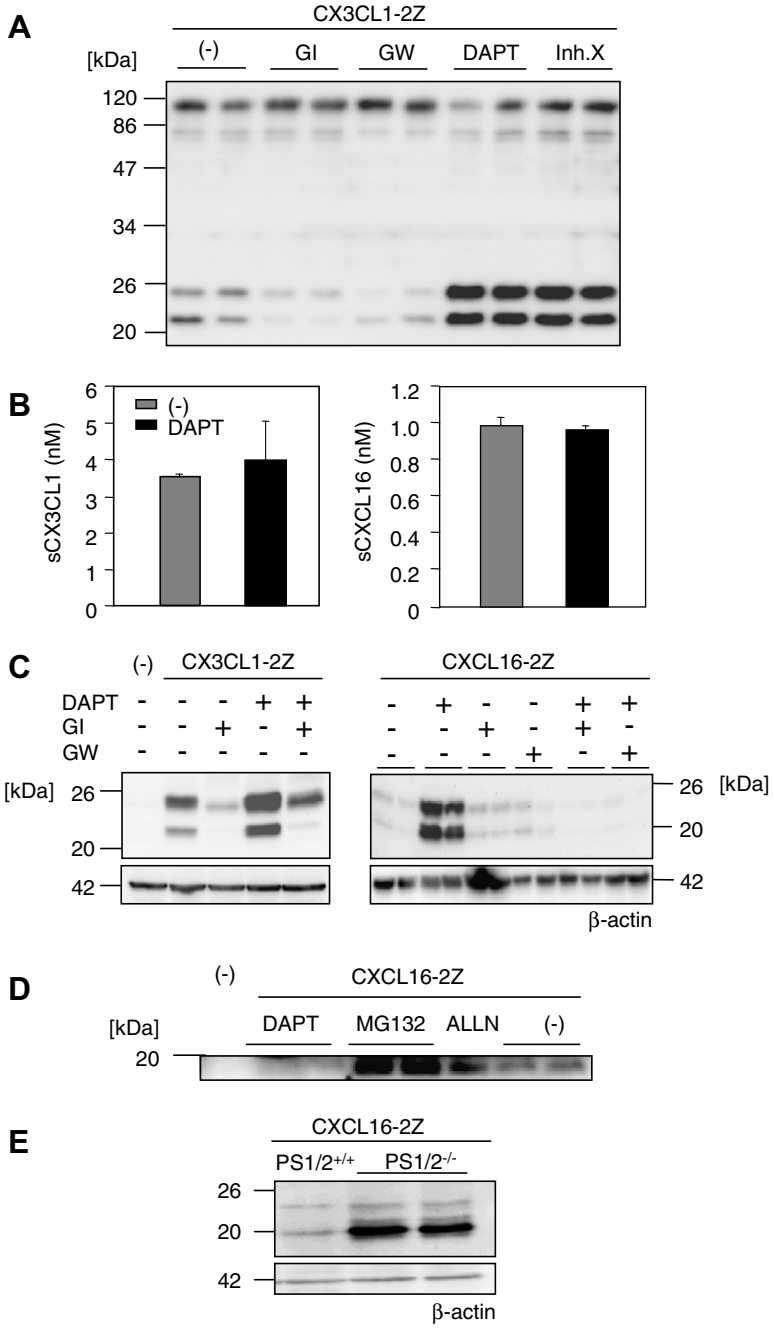

Fig. 4. Cleavage of CX3CL1 and CXCL16 by γ-secretase-like activity. (A) CX3CL1-2Z-HEK293 cells were treated with GI254023X, GW280264X (both 10 μM), the γ-secretase inhibitors DAPT (2 μM) or Inh. X (0.5 μM) for 4 h, followed by lysis and Western blot analysis. (B) CX3CL1-2Z- and CXCL16-2Z-HEK293 cells were treated with DAPT (2 μM) for 4 h and subsequently conditioned media were analyzed for release soluble chemokine variants by ELISA. (C) Combined inhibition of the γ-secretase complex and α-secretases in stably transfected CX3CL1-2Z-HEK293 cells (left) and CXCL16-2Z-HEK293 cells (right). Cells were treated with inhibitors for 4 h, lysed and examined by Western blotting. (D) CXCL16-2Z-HEK293 cells were treated with the proteasome inhibitor MG132 (2 μM), the inhibitor ALLN (10 μM) or left untreated for 4 h, subsequently lysed, and analyzed by Western blotting using long exposure times to detect small cleavage products. Overexposed bands for CTF1 and CTF2 are not shown. (E) PS 1/2-deficient fibroblasts were transfected with a cDNA coding for CXCL16-2Z and after 48 h cell lysates were analyzed CTFs visualized by Western blotting using IgG.

with DAPT but clearly became prominent when cells were treated with the proteasomal inhibitor MG132 or with ALLN (Fig. 4D). These findings indicate that CTFs are degraded by intramembraneous cleavage via γ-secretase activity and possibly further proteasomal proteolysis. Finally, transfection of CXCL16-2Z into murine embryonic fibroblasts generated from PS1/2-deficient mice [23] confirmed that a lack of γ-secretase activity led to the accumulation of both CTFs (Fig. 4E). Again, this effect was more pronounced for CTF2 suggesting that γ-secretase-mediated degradation of this fragment is more rapid than that of CTF1.

Discussion

In previous studies we and others have demonstrated the involvement of ADAM10 in the constitutive cleavage of transmembrane chemokines [7–9]. The current study provides further insight into the cleavage mechanism of transmembrane chemokines by the α-secretase ADAM 10 and discovers cleavage products of transmembrane chemokines as novel substrates for the γ-secretase complex. By fusion of CX3CL1 and CXCL16 to a C-terminal tandem repeat of the IgG-binding domain of Protein A (2Z-tag) we provide evidence for the generation of several C-terminal fragments (CTFs) for CX3CL1 and CXCL16. The fusion technique employed in this study has been successfully used for the identification of cleavage products of other type I-transmembrane proteins including APLP1 [22] and IL6R (Paliga et al., unpublished) and turned out to be a suitable tool allowing to study CTFs of CX3CL1 and CXCL16 without affecting surface expression of the fusion constructs or release of their soluble ectodomains. We here identify for the first time multiple CTFs of transmembrane chemokines that are sensitive to inhibition or lack of ADAM10 and presenilins, suggesting that multiple proteases and cleavage events are involved in the processing of CX3CL1 and CXCL16 as it has been described for APP, E-cadherin and Notch [14,15,17–19].

Proteolytic shedding of CX3CL1 and CXCL16 generated two major C-terminal cleavage fragments. As shown by enzymatic deglycosylation, the two CTFs do not represent differentially glycosylated variants. CX3CL1 and CXCL16 constructs are rather cleaved at two different sites leading to two different CTFs. Notably, the size of each fragment generated from cleavage of the CX3CL1 construct was about 1 kDa larger than that of the CXCL16 fusion protein, most likely reflecting that the predicted cytoplasmic domain of CX3CL1 is 6 amino acids longer than that of CXCL16. The molecular size of the small CTF determined for CXCL16 was 20 kDa, which would comprise the 2Z-Tag of 15 kDa and a chemokine-derived portion of 5 kDa. Notably, the calculated size of the intracellular plus transmembrane domains of CXCL16 would account for approximately 5 kDa. This would be sufficient to anchor even the small CTF of CXCL16 in the cell membrane which is consistent with our finding that the CTFs are present in membrane preparations but not in the cytosol. Therefore, extracellular cleavage of the chemokine stalk in very close proximity to the cell membrane is responsible for the generation of the small CTF, while the larger fragments very likely originate from another cleavage more distal from the cell membrane. We here demonstrate that the activity of ADAM10 is involved in both cleavages within CX3CL1 and CXCL16. This underlines our previous studies using ADAM10-deficient cell lines to show that ADAM10 is the major protease responsible for the release of soluble CX3CL1 and CXCL16 [7,8] and therefore correlates the shedding process with the generation of both CTFs. CX3CL1 and CXCL16 may not be the only substrates of disintegrin-like metalloproteinases that undergo several cleavages. Also for IL-6R two cleavage sites have been found [24], while other substrates such as E-cadherin and N-cadherin appear to be cleaved at one preferential site only [18,19].

Shedding of transmembrane chemokines leads to the generation of fragments residing in the cell membrane. By the use of two different inhibitors for the γ-secretase complex as well as by presenilin 1/2-deficient embryonic fibroblasts we demonstrated that γ-secretase activity is implicated in the degradation of both CTFs. Since γ-secretase has no influence on the shedding or cellular expression of the full size molecule, we conclude that both chemokines are sequentially cleaved, first by the α-secretase ADAM10 and then by γ-secretase activity. As demonstrated for various other substrates including APP, Notch and E-cadherin, γ-secretase cleavage occurs within the cell membrane and leads to the generation of smaller fragments that are released from the cell membrane [14,15,20]. Also for CXCL16 and CX3CL1 smaller fragments became detectable upon inhibition of proteasomal degradation, but were absent when γ-secretase activity was blocked. Therefore, cleavage of the CTFs for CX3CL1 and CXCL16 by γ-secretase may be rapidly followed by proteasomal degradation of the resulting fragments in the cytoplasm. This sequence of proteolytic cleavage events could represent a mechanism for clearing the cell membrane from of the C-terminal fragments of transmembrane chemokines which would otherwise accumulate in the cell membrane upon continuous shedding of the full size molecules. As shown for Notch and also E-cadherin, cleavage of CTFs by the γ-secretase complex could possibly result in the generation of intracellular fragments implicated in intracellular signalling, reviewed in [13].

In summary, we provide novel evidence for sequential processing of transmembrane chemokines by the α-secretase ADAM10 and then by the γ-secretase complex which process leads to removal of the CTFs from the membrane. Within the cytosol the released cleavage products could be further degraded but possibly also fulfil yet unknown functions as signalling molecules.

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