Regulated Shedding of Transmembrane Chemokines by the Disintegrin and Metalloproteinase 10 Facilitates Detachment of Adherent Leukocytes[1]

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CX3CL1 (fractalkine) and CXCL16 are unique members of the chemokine family because they occur not only as soluble, but also as membrane-bound molecules. Expressed as type I transmembrane proteins, the ectodomain of both chemokines can be proteolytically cleaved from the cell surface, a process known as shedding. Our previous studies showed that the disintegrin and metalloproteinase 10 (ADAM10) mediates the largest proportion of constitutive CX3CL1 and CXCL16 shedding, but is not involved in the phorbolester-induced release of the soluble chemokines (inducible shedding). In this study, we introduce the calcium-ionophore ionomycin as a novel, very rapid, and efficient inducer of CX3CL1 and CXCL16 shedding. By transfection in COS-7 cells and ADAM10-deficient murine embryonic fibroblasts combined with the use of selective metalloproteinase inhibitors, we demonstrate that the inducible generation of soluble forms of these chemokines is dependent on ADAM10 activity. Analysis of the C-terminal cleavage fragments remaining in the cell membrane reveals multiple cleavage sites used by ADAM10, one of which is preferentially used upon stimulation with ionomycin. In adhesion studies with CX3CL1-expressing ECV-304 cells and cytokine-stimulated endothelial cells, we demonstrate that induced CX3CL1 shedding leads to the release of bound monocytic cell lines and PBMC from their cellular substrate. These data provide evidence for an inducible release mechanism via ADAM10 potentially important for leukocyte diapedesis. ***The Journal of Immunology,*** **2007, 178: 8064–8072.**

Leukocyte adhesion and migration through the vascular wall are regulated by soluble chemoattractants and membrane-bound adhesion molecules (1–3). Within the chemokine family of chemotactic peptides, CX3CL1, also termed fractalkine, and the CXC-chemokine CXCL16, are exceptional because they can function as either soluble chemoattractants or membrane-expressed adhesion molecules (4). CXCL16 and CX3CL1 are synthesized as type I transmembrane molecules (5, 6) 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. Both the chemoattractive and the adhesive function of CX3CL1 and CXCL16 are exercised by interaction with their corresponding seven-transmembrane, G protein-coupled receptors CX3CR1 or CXCR6, respectively (7–9). Notably, transmembrane CXCL16 also functions as a scavenger receptor for oxidized low density lipoprotein (10). CX3CL1 and CXCL16 were found on a number of different cell types, including neuronal cells, astrocytes, epithelial cells, hepatocytes, fibroblasts, smooth muscle cells, macrophages, and also endothelial cells (5, 6, 10–14). On endothelial cells and smooth muscle cells, both chemokines are strongly up-regulated by stimulation with proinflammatory cytokines (5, 11, 15, 16). When expressed on the surface of endothelial cells, they promote adhesion and firm arrest of receptor-expressing leukocytes under flow conditions (7–9). Both CX3CL1 and CXCL16 were found to be up-regulated in human atherosclerotic lesions (17, 18), and CX3CR1 has been implicated in monocyte recruitment into vascular lesions and increased risk for atherosclerosis in ApoE$^{-/-}$ mice (19, 20).

Soluble CX3CL1 as well as CXCL16 are generated by limited proteolysis at the cell surface, a process termed shedding. A variety of cell surface molecules with different functions are known substrates for shedding, among them cytokines, cytokine receptors, growth factors, and adhesion molecules (21, 22). In many cases, shedding occurs constitutively, but can be rapidly enhanced by cell stimulation with PMA, by cholesterol depletion (23), treatment with pore-forming toxins (24), or stimulation with the calcium-ionophore ionomycin (25). To date, the proteases responsible for shedding have been exclusively found within the zinc-dependent metzinkin family of metalloproteinases and in this study foremost among the disintegrin and metalloproteinases (ADAMs)[4] (21, 22). In particular, ADAM10 and the closely related ADAM17, which was originally identified as TNF-α-converting enzyme, have been described as sheddases of diverse cell surface proteins, such as TNF-α, TGF-α, IL-6R, notch, amyloid precursor protein, prion protein, L-selectin, E-cadherin, N-cadherin, γ-protocadherin C3,

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[4] Abbreviations used in this paper: ADAM, disintegrin and metalloproteinase; CTF, C-terminal cleavage fragment; FN, fibronectin; mβCD, methyl-β-cyclodextrin; MEF, mouse embryonic fibroblast; SLO, streptolysin O.

VCAM-1, CD44, L1 adhesion molecule, and CD30 (reviewed in Refs. 21 and 22). Interestingly, some of these molecules are exclusively shed by either ADAM10 (e.g., E-cadherin, N-cadherin, γ-protocadherin C3, and CD44) (25–27) or ADAM17 (L-selectin, VCAM-1, TNF-α) (28–30), whereas others are substrates for both proteases (IL-6R, L1, prion protein, and CD30) (23, 31–33).

ADAM10 is involved in most of the constitutive shedding of transmembrane chemokines, as demonstrated by the use of preferential ADAM10 inhibitors, embryonic fibroblasts generated from ADAM10-deficient mice, or ADAM10-specific small interfering RNA (14, 15, 34–36). Proteolytic cleavage of transmembrane chemokines appears to be essential for their switch from an adhesive to a chemoattractive function. On the cell surface, proteolytic cleavage leads to the reduced expression of the transmembrane variants and reduced adhesiveness for responsive leukocytes (9, 34). Once displaced from the cell surface, the soluble forms of CX3CL1 and CXCL16 can induce chemotaxis of receptor-positive leukocytes (5, 6). After binding of leukocytes to the membrane-anchored chemokines, however, shedding could also play an instrumental role in releasing the adhesive interaction, resulting in the detachment and onward transmigration of bound cells. This process could occur constitutively, but may be enhanced by cell stimulation. Such enhanced shedding of CX3CL1 can be observed following treatment with PMA and could be attributed to the activity of ADAM17 (37, 38). To date, other potent inducers of transmembrane chemokine shedding have not been described.

In this study, we introduce ionomycin as a more efficient and more rapid inducer of transmembrane chemokine shedding than PMA. Using ADAM10-deficient cell lines in combination with specific inhibitors, we identify ADAM10 as the most relevant sheddase of ionomycin-induced shedding. Finally, we demonstrate that induced shedding via activation of ADAM10 leads to the down-regulation of cellular adhesiveness under flow conditions and to a detachment of monocytic cells bound to CX3CL1.

Materials and Methods

Cytokines, Abs, enzymes, inhibitors, and vectors

Human extracellular domain rCX3CL1; human rIFN-γ; human rTNF-α; unconjugated, biotinylated, or PE-conjugated mAbs to human CX3CL1 (clones 81506, 51637, and 51637.11, respectively); as well as PE-conjugated mouse IgG1 isotype control (clone 11711.11) were obtained from R&D Systems. The antiserum against CX3CL1 was raised in rabbits and characterized previously (39). Expression vectors for human CX3CL1 and CXCL16 cDNA were described before (15, 34). For detection of C-terminal cleavage fragments (CTFs) of CX3CL1, a 2Z-tag (40) was fused to the C terminus of CX3CL1. The CX3CL1/gp130 chimera was generated by fusion of the CX3CL1 chemokine domain plus part of the stalk region (corresponding to aa 1–206) onto the C-terminal part of gp130 beginning with the second fibronectin (FN)-like domain (corresponding to aa 422–917). All constructs were inserted into pcDNA3.1. The metalloproteinase inhibitors GW280264X ((2R,3S)-3-(formyl-hydroxyamino)-2-(2-methyl-1-propyl) hexanoic acid [(1S)-5-benzyloxycarbamoylamino-1-(1,3-thiazol-2-yl carbamoyl)-1-pentyl] amide) and GI254023X ((2R,3S)-3-(formyl-hydroxyamino)-2-(3-phenyl-1-propyl) butanoic acid [(1S)-2,2-dimethyl-1-methylcarbamoyl-1-propyl] amide) were synthesized as described in U.S. Patents US 6 172 064, US 6 191 150, and US 6 329 400 and assayed for inhibition of human rADAM17 and rADAM10 ectodomains, as described (34, 35).

Cell culture and transfection

The human monocytic cell lines THP-1 and Mono Mac 6 were cultured as described (34, 41). All medium and reagents were from PAA Laboratories if not otherwise stated. The adherent cell line ECV-304 was stably transfected with CX3CL1 (CX3CL1-ECV-304) and characterized previously (39). CX3CL1-ECV-304 cells were cultured in M199 medium supplemented with 10% FCS and antibiotics up to 90% confluence before subculture. Mononuclear cells were prepared from peripheral blood of healthy volunteers by density centrifugation, as previously described (42). Human microvascular endothelial cells were provided by R. Sedlacek (Institute of Biochemistry, Kiel, Germany) and cultured in low serum Endothelial Cell Growth Medium MV (Promo Cell). Cells were costimulated with IFN-γ and TNF-α (10 ng/ml, respectively) for 24 h to express endogenous CX3CL1.

COS-7 cells were cultured in DMEM containing 10% FCS and antibiotics. For transfection of COS-7 cells with CX3CL1 or CXCL16, cells were grown to 70% confluence in 6-well dishes (Costar/Corning) or 60-cm^2 dishes (Greiner Bioscience). The medium was replaced with fresh medium (0.83 or 5 ml, respectively) containing 75 μM chloroquine. A total of 5 μg of either vector construct was mixed with 260 μg of 2-diethylaminoethyl-dextran in 500 μl of medium and then added to the cells (83 or 500 μl, respectively). After 5 h of incubation, cells were exposed to 10% DMSO in fresh medium for 6 min, which was then removed by normal culture medium.

The human embryonic kidney cell line HEK-293 was cultured in DMEM containing 10% FCS and antibiotics. Simian virus large T Ag-immortalized mouse embryonic fibroblast (MEF) cell lines from $ADAM10^{-/-}$ mice and respective wild-type animals were generated and characterized, as described elsewhere (43). Both MEF cell lines were cultured in DMEM containing 10% FCS and antibiotics. For transfection with CX3CL1 or CXCL16, HEK-293 of MEF cells was seeded at 1×10^4 cells/cm^2 in 6-well dishes (Costar/Corning) and incubated for 24 h to 70% confluence. The medium was replaced with 1 ml of fresh medium. Each well received 1 μg of vector DNA preincubated with 3 μl of lipofectamin (Invitrogen Life Technologies) in 100 μl of serum-free medium for 30 min. After 24 h of incubation, the medium was replaced. All transient transfections were performed in triplicates for each stimulatory condition. In all experiments, uptake of the vector was controlled by transfection with GFP in pcDNA3.1 in parallel and subsequent detection of expressed GPF by fluorescence microscopy.

CX3CL1 and CXCL16 cleavage assays

Cells expressing CX3CL1 or CXCL16 were grown to 70–90% confluence in complete medium in 6-well dishes (Costar) for 48 h before stimulation. The cells were washed with PBS, and FCS-free medium containing metalloproteinase inhibitors was added. After 10 min, the cells were stimulated with PMA, ionomycin, streptolysin O (SLO), or methyl-β-cyclodextrin (mβCD), or reacted to THP-1 cells (1×10^6 cells/well), as specified. The conditioned medium was harvested, and a protease inhibitor mixture (Complete; Roche) was added, according to the instructions of the manufacturer. The supernatants were cleared by centrifugation at $10{,}000 \times g$ and analyzed for the presence of released CX3CL1 or CXCL16 by ELISA or by Western blotting. The cells were washed with 2 ml of PBS and removed from the vessel by scraping in 1 ml of ice-cold PBS. CX3CL1 surface expression on intact cells was determined by flow cytometry. For Western blot analysis of cleavage fragments and for quantification of cell-associated CX3CL1 or CXCL16 by ELISA, the cells were centrifuged and resuspended in 500 μl of PBS, containing 0.1% Triton X-100 and a protease inhibitor mixture (Complete; Roche). After 30 min of incubation on ice under agitation, the cell lysates were centrifuged at $12{,}000 \times g$ for 10 min.

ELISAs

Quantification of CX3CL1 and CXCL16 was performed, as described before (15, 34). Briefly, a 96-well plate (Microlon; Greiner Bioscience) was coated overnight with 6 μg/ml mouse anti-CX3CL1 (clone 81506) in 50 mM Na_2CO_3 (pH 9.3), subsequently blocked with PBS-T containing 2% BSA for 2 h. Samples (50 μl/well) and a standard prepared as eight serial 1/2 dilutions of 3.9 nM full-size CX3CL1 in either medium or cell lysis buffer were added to the plate for 2 h. Following washing, 300 ng/ml biotinylated anti-CX3CL1 mAb (clone 51637) in PBS-T containing 1% BSA was added to each well, and the plate was incubated at room temperature for 1 h. After 1-h incubation with 100 mU/ml streptavidin-peroxidase conjugate (Roche) in PBS-T with 1% BSA, the bound enzymatic activity was quantified using a chromogenic peroxidase substrate (BM blue; Roche).

For detection of CXCL16, a similar protocol was used. Goat anti-human CXCL16 Ab (2 μg/ml) was coated as capture Ab, a standard was prepared as serial 1/2 dilutions of 6.25 ng/ml human rCXCL16, and 200 ng/ml biotinylated rabbit anti-human CXCL16 Ab was used for detection.

SDS-PAGE and Western blotting

Cell lysates and medium samples were subjected to reducing SDS-PAGE (7 and 10% polyacrylamide gels, respectively) and transferred onto polyvinylidene difluoride membranes (Hybond; Amersham). The membranes were incubated in blocking buffer (PBS containing 5% milk powder) at room temperature for 1 h and probed for 1 h with dilutions of rabbit antiserum to human CX3CL1 (1:2000) in blocking buffer. After three washes

with PBS-T, the membranes were incubated with a HRP-linked goat serum against rabbit Ig (Jackson ImmunoResearch Laboratories) diluted 1/10,000 in PBS-T for 1 h. After three washes, detection of bound anti-rabbit Ig was conducted using ECL substrate (ECLplus; Amersham) and imaging of chemiluminescence (LAS1000; Fuji).

Flow cytometric analysis

Adherent cells were harvested from culture flasks by treatment with ice-cold PBS containing 0.05% NaN_3 and 0.2% BSA, subsequent scraping, and centrifugation. The cells were washed and incubated at 2×10^6 cells/ml with a PE-conjugated mAb to CX3CL1 or a PE-conjugated IgG1 isotype control (both at 1.25 μg/ml in PBS with 0.2% BSA and 0.05% NaN_3) for 1 h on ice. Following 2-fold washing, cells were suspended in PBS containing 2% paraformaldehyde. The fluorescence signal of the labeled cells was then analyzed by flow cytometry (FACScan; BD Biosciences).

Cell adhesion assay

Wild-type and CX3CL1-expressing ECV-304 cells or cytokine-stimulated endothelial cells were seeded at 6×10^4 cells/well into 24-well dishes (Microlon; Greiner Bioscience). The cells were cultured to full confluence in M199 medium containing 10% FBS. Subsequently, the cells were assayed for adhesion of fluorescently labeled THP-1 cells or mononuclear cells freshly prepared from peripheral blood. For fluorescent labeling, cells were suspended at 2×10^6 cells/ml in RPMI 1640 containing 10% FCS and incubated with 2.5 μM fluorescent dye (calcein A; Molecular Probes) at 37°C for 30 min. Excess dye was removed by washing with 50 ml of PBS. Labeled cells were resuspended in serum-free RPMI 1640 and added to the ECV-304 or endothelial cells at 3×10^5 cells/well. Following 30 min of incubation at 37°C, the plate was repeatedly washed. One wash step comprised inversion of the plate, addition of PBS (1 ml/well), subsequent inversion, and addition of a minimum volume of PBS to prevent cells from running dry. After each wash step, the fluorescence signal from the adherent cells was measured using a fluorescence plate reader (λ Fluoro 230; MWG Biotec) at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

To investigate the effect of CX3CL1 shedding on cell adhesion, 0.5 ml of RPMI 1640 with or without the preferential ADAM10 inhibitor GI254023X (10 μM) was added to the monocytic cells adherent to the ECV-304 or endothelial cell layer. Subsequently, the cells were stimulated with ionomycin (1 μM) and incubated at 37°C for 15 min. Cells that detached from the ECV-304 or endothelial cell layer were then removed by washing with 1 ml of PBS and inversion of the plate. The remaining adherent cells were then quantified by fluorescence measurement.

Laminar flow assays were performed, as previously described (44). WT- and CX3CL1-ECV-304 cells were grown to confluence and pretreated with ionomycin or PMA in the absence or presence of metalloproteinase inhibitors, as specified, in 35-mm dishes that were assembled as the lower wall in a parallel wall flow chamber and mounted on the stage of an Olympus IMT-2 microscope. Mono Mac 6 or THP-1 cells (0.5×10^6/ml) were suspended in HBSS containing 10 mM HEPES (pH 7.4), 0.5% human serum albumin, and 1 mM Mg^{2+} and Ca^{2+} (added shortly before the assay), kept in a heating block at 37°C during assays, and perfused into the flow chamber at a rate of 1.5 dyn/cm^2 for 5 min. Subsequently, the number of firmly adherent cells was quantified in multiple fields by analysis of images recorded with a long integration JVC 3CCD video camera and a SR L900 E video recorder. Cell numbers were determined for at least five fields per experiment and expressed as cells/mm^2.

Statistical analysis

Data were analyzed by unpaired two-tailed *t* test or one-way ANOVA with Tukey's test for multiple comparisons using GraphPad Prism 4.0. Two populations of data were considered significantly different at *p* values smaller than 0.05.

Results

Inducible release of the transmembrane chemokines

We tested different stimuli that have been implicated in the induction of shedding events on the cell surface for their potential to stimulate shedding of CX3CL1 and CXCL16. The protein kinase C agonist PMA, which is known to induce shedding of CX3CL1 (37, 38), induced only minimal, but significant release of soluble CX3CL1 after 45 min of stimulation (Fig. 1*A*). Treatment with the cholesterol-extracting agent mβCD or with the pore-forming toxin

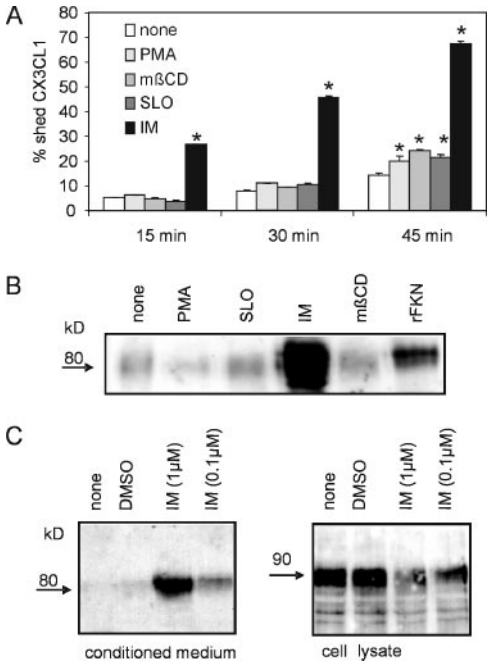

FIGURE 1. Stimulation of CX3CL1 release from COS-7 cells by ionomycin. COS-7 cells were transfected to express CX3CL1, medium was replaced, and the cells were treated with ionomycin (1 μM) PMA (100 ng/ml), SLO (1 μg/ml), or mβCD (10 μM) for 15, 30, or 60 min. *A*, Cell lysates and conditioned medium were then cleared by centrifugation and assayed for the presence of CX3CL1 by ELISA. The amount of released CX3CL1 was expressed as percentage of the total CX3CL1 content in the medium and cell lysates. Data are shown as mean ± SD ($n = 3$) and were reproduced in three independent experiments. Statistical differences in CX3CL1 release induced by PMA, mβCD, SLO, or ionomycin are indicated by asterisks. *B*, Released CX3CL1 within 15 min of stimulation was analyzed by Western blotting of the conditioned medium using an antiserum against the chemokine domain of CX3CL1. The extracellular domain of human CX3CL1 was used as standard protein for ELISA and for Western blotting. *C*, CX3CL1-transfected COS-7 cells were treated with ionomycin (0.1 and 1 μM) for 15 min, vehicle control (DMSO), or left untreated. Subsequently, released and cell-associated CX3CL1 was determined by Western blot analysis of the conditioned medium and cell lysates, respectively. One representative of three experiments is shown. IM, ionomycin.

SLO, both of which have been implicated in the induction of other shedding events, e.g., that of IL-6R (23, 24), stimulated release of CX3CL1 to a similar extent and with similar time kinetics as PMA. By contrast, stimulation with the ionophore ionomycin led to a very rapid and much more efficient release of the chemokine. These findings were confirmed by Western blot analysis of the conditioned medium following stimulation with either PMA, mβCD, SLO, or ionomycin, demonstrating a profound increase in the 80-kDa protein band previously identified as soluble CX3CL1 (11) (Fig. 1*B*). Analysis of the cell lysates revealed that the increase in soluble CX3CL1 upon ionomycin treatment was associated with the decrease of the cell-expressed full-size molecule of 90 kDa (Fig. 1*C*).

ADAM10 is implicated in ionomycin-induced shedding of transmembrane chemokines

The different shedding kinetics in response to ionomycin or PMA points toward the involvement of distinct shedding mechanisms. Because we and others have found that PMA-induced shedding of CX3CL1 is predominantly mediated by ADAM17 (37), whereas the constitutive shedding is mediated by ADAM10 (34), we next

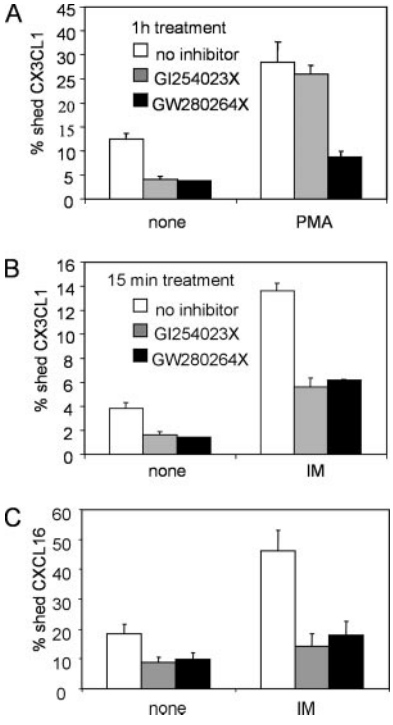

FIGURE 2. Inhibition of ionomycin-induced CX3CL1 and CXCL16 release by metalloproteinase inhibitors. COS-7 cells were transfected to express CX3CL1 (*A* and *B*) or CXCL16 (*C*), and fresh medium containing metalloproteinase inhibitor GI254023X or GW280353X or no inhibitor was added. Subsequently, cells were treated with PMA (100 ng/ml) for 1 h (*A*), or ionomycin (1 μM) for 30 min (*B* and *C*), or left untreated. Released CX3CL1 or CXCL16 in the medium was then determined by ELISA. Data are shown as mean ± SD ($n = 3$) and were reproduced in three independent experiments. IM, ionomycin.

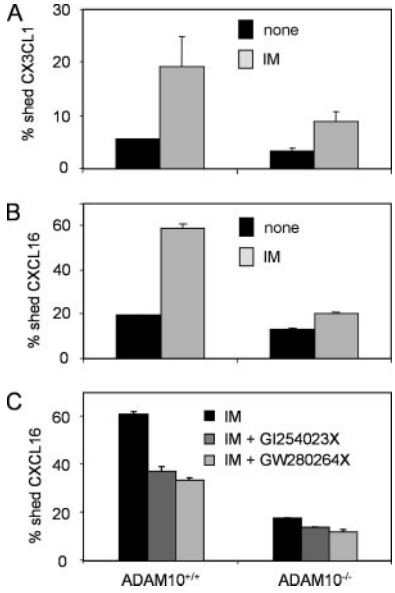

FIGURE 3. Effect of ionomycin on CX3CL1 and CXCL16 shedding in ADAM10-deficient embryonic fibroblasts. *A* and *B*, ADAM10-deficient MEFs and respective wild-type control cells were transfected to express CX3CL1 or CXCL16. Subsequently, cells were treated with ionomycin for 30 min. Cell lysates and medium were then investigated for cell-associated and released CX3CL1 (*A*) or CXCL16 (*B*), respectively, by ELISA. To normalize variations in the transfection efficiency, the amount of shed chemokine was calculated as percentage of the total chemokine content in the cell lysates and medium. *C*, CXCL16-transfected ADAM10$^{-/-}$ and wild-type MEFs were stimulated with ionomycin in the absence or presence of metalloproteinase inhibitor GI254023X or GW280264X (3 μM) for 30 min, and subsequently analyzed for their CXCL16-shedding activity, as described for *A* and *B*. All data were reproduced in three independent experiments and are shown as mean ± SD ($n = 3$). IM, ionomycin.

addressed the involvement of the two enzymes in ionomycin-induced shedding using hydroxamate-based inhibitors that differentially block ADAM10 and ADAM17 (35). As expected, the combined ADAM10/ADAM17 inhibitor GW280264X reduced the constitutive and PMA-induced shedding activity, whereas the preferential ADAM10 inhibitor GI254023X exclusively reduced the constitutive, but not the PMA-induced release, confirming the involvement of ADAM17 in the latter process (Fig. 2*A*). The blockade of ionomycin-induced shedding, however, was equally efficient with both inhibitors, arguing against the involvement of ADAM17 in this process (Fig. 2*B*).

We then examined whether ionomycin would also up-regulate the shedding of the other transmembrane chemokine, CXCL16. Indeed, its release from CXCL16-transfected COS-7 cells was markedly increased by treatment with ionomycin (Fig. 2*C*). As described for CX3CL1, this effect was dependent on inhibition of ADAM10, but not affected by additional inhibition of ADAM17, suggesting that ADAM10 is mediating not only the majority of constitutive, but also ionomycin-induced shedding of both transmembrane chemokines CX3CL1 and CXCL16.

The relevance of ADAM10 for the ionomycin-induced shedding of CX3CL1 and CXCL16 was further investigated by the use of MEFs generated from ADAM10-deficient mice (41). Released and cell-associated forms of the transfected chemokines were quantified by ELISA, and the percentage of shed chemokine was calculated. In the absence of ADAM10, constitutive and ionomycin-induced shedding were profoundly reduced, demonstrating that ADAM10 does not only contribute to the constitutive, but also to the ionomycin-induced shedding (Fig. 3, *A* and *B*). Notably, the effect of ionomycin was not completely suppressed in ADAM10-deficient fibroblasts and could not be prevented by additional inhibition of ADAM17 (Fig. 3*C*), suggesting that a minor proportion of ionomycin-induced shedding is independent of the two metalloproteinases.

ADAM10-mediated cleavage of transmembrane chemokines occurs at multiple sites

To gain further insight into the process of proteolytic cleavage, we analyzed the CTFs residing in the cell membrane. For this purpose, CX3CL1 was C-terminally tagged with two Fc-binding domains of protein A (2Z-tag) (40). This fusion protein (Fig. 4*A*) was transfected in HEK-293 cells and detected with an antiserum recognizing the chemokine domain of CX3CL1, but also binding to the 2Z-tag. The full-size molecule migrated at a molecular mass of 105 kDa. A smaller protein at 70 kDa most likely represents a CX3CL1 precursor, as previously described by others (37). Additionally, an intense band at 25 kDa and a slightly weaker band at 22 kDa were detectable via the 2Z-tag (Fig. 4*B*). The latter bands are likely to represent CTFs of CX3CL1 arising from proteolytic cleavage because treatment with either the preferential ADAM10 inhibitor or the combined ADAM10/17 inhibitor for 48 h reduced both protein bands (Fig. 4*B*). Notably, the generation of the larger fragment was only partially suppressed, whereas the smaller fragment was almost completely absent. This differential inhibition efficiency was even more pronounced when the cells were treated with the inhibitors for only 2 h, suggesting that the two CTFs differ in their degradation and turnover rate. Stimulation with ionomycin led to the preferential accumulation of the smaller 22-kDa fragment (Fig.

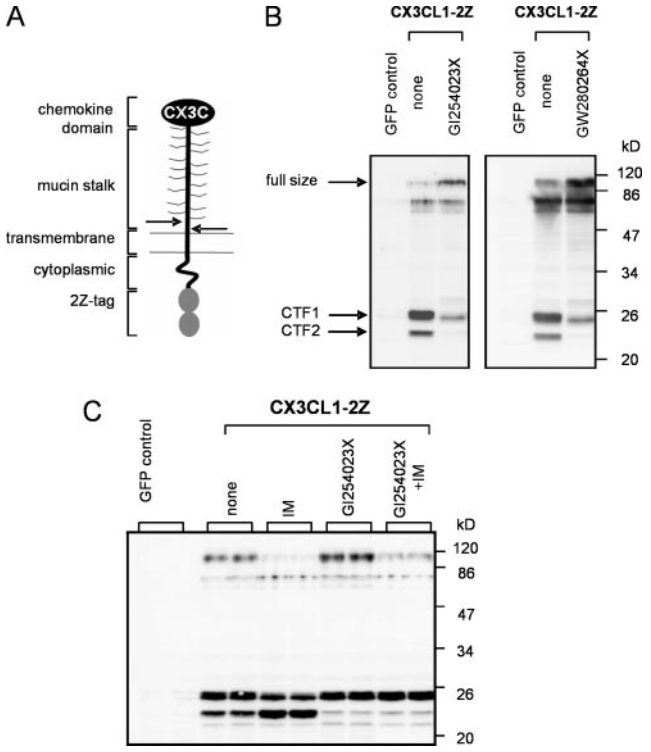

FIGURE 4. Generation of CTFs in ionomycin-treated HEK-293 cells expressing CX3CL1–2Z. *A*, CX3CL1 was C-terminally fused to a 2Z-tag containing two Fc binding sites for Ig. Potential cleavage sites are indicated by arrows. *B*, CX3CL1–2Z was transfected into HEK-293 cells. GFP-transfected cells served as a control. After 48 h of incubation in the presence or absence of the metalloproteinase inhibitor GI254023X or GW280264X (10 μM), cell lysates were analyzed for intact CX3CL1 and CTFs by Western blot analysis using a rabbit antiserum to CX3CL1. *C*, CX3CL1–2Z-transfected HEK-293 cells were stimulated with ionomycin (1 μM) in the presence or absence of GI254023X or left untreated for 30 min, and subsequently, cell lysates were investigated for the presence of cleavage fragments. Data are shown as representative experiments reproduced more than three times. IM, ionomycin.

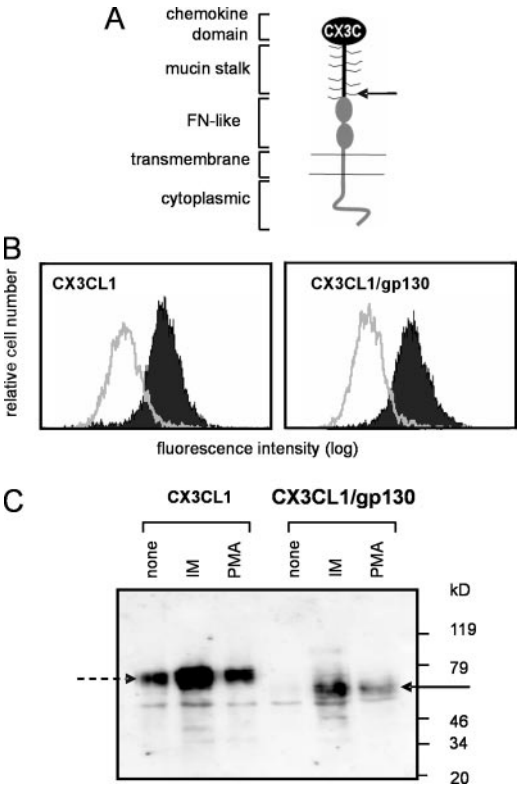

FIGURE 5. Constitutive and inducible cleavage of the CX3CL1/gp130 chimera. *A*, Part of the ectodomain of CX3CL1 comprising aa 1–206 was fused on top of the second FN-like domain of the signal transducer gp130, and the CX3CL1/gp130 chimera was investigated for its potential cleavage (arrow). HEK-293 cells were transiently transfected with the CX3CL1/gp130 chimera or WT-CX3CL1. *B*, Surface expression of CX3CL1 and CX3CL1/gp130 was controlled by flow cytometry. *C*, CX3CL1 and CX3CL1/gp130-transfected HEK-293 cells were stimulated for 30 min with PMA or ionomycin or left unstimulated, and the conditioned medium was then analyzed for the presence of soluble CX3CL1 by Western blotting: the protein bands representing released CX3CL1 from CX3CL1- and CX3CL1/gp130-transfected HEK-293 are indicated by arrows with broken and straight lines, respectively. Data are shown as representative experiments reproduced more than three times. IM, ionomycin.

4*C*), whereas the full-size molecule as well as the 25-kDa fragment were diminished. These data suggest that ionomycin treatment stimulates the rapid generation of the 22-kDa CTF from both the full-size molecule and the 25-kDa CTF. As expected, inhibition of ADAM10 was sufficient to prevent this proteolytic conversion. The 70-kDa precursor was not converted upon ionomycin treatment. Similar results were obtained with 2Z-tagged CXCL16 (data not shown), supporting the view that both chemokines undergo regulated cleavage via the same protease ADAM10.

Taking into account that the 22- and 25-kDa CTFs still contain a 2Z-tag of 16 kDa, the CX3CL1 proportion of the CTFs should be relatively small (6 and 9 kDa, respectively). Because the intracellular and transmembrane proportion of CX3CL1 accounts for 6 kDa, both CTFs must be generated by extracellular cleavage in the membrane-proximal region of the chemokine. We next removed the membrane-proximal extracellular part containing the potential cleavage sites as well as the subsequent C-terminal proportion of CX3CL1 by fusing the upper N-terminal part of the CX3CL1 ectodomain to the second FN-like domain of the signal transducer molecule gp130, which is not shed from the cell surface (45) (Fig. 5*A*). The CX3CL1/gp130 fusion construct was expressed in HEK-293 cells and detected on the cell surface by FACS analysis (Fig. 5*B*); however, it was not detected by Western blotting of the conditioned medium with an Ab to the chemokine domain of CX3CL1 (Fig. 5*C*). In contrast, upon ionomycin treatment or stimulation with PMA, a 55-kDa soluble form of CX3CL1 was released.

Induced shedding by ADAM10 regulates surface expression of transmembrane chemokines

To investigate the functional consequences of induced shedding for cell adhesion, we used ECV-304 cells forming tightly attached monolayers uniformly expressing the chemokine upon stable transfection. To verify that the induced release of soluble CX3CL1 by these cells was associated with a reduction of its surface-expressed variant, flow cytometry was performed. Treatment with ionomycin decreased the surface expression of CX3CL1 in CX3CL1-transfected ECV-304 cells. Exposure to either GI254023X or GW280264X led to an increase in surface expression of the chemokine and completely suppressed the down-regulation by ionomycin (Fig. 6*A*), confirming the involvement of ADAM10 in this process. As reported previously, treatment with PMA also induced the down-regulation of the chemokine from the cell surface (34, 37). However, as compared with ionomycin, down-regulation by PMA required longer exposure times (60 min) and was insensitive to GI254023X (data not shown), indicating a different mechanism with slower kinetics without involving

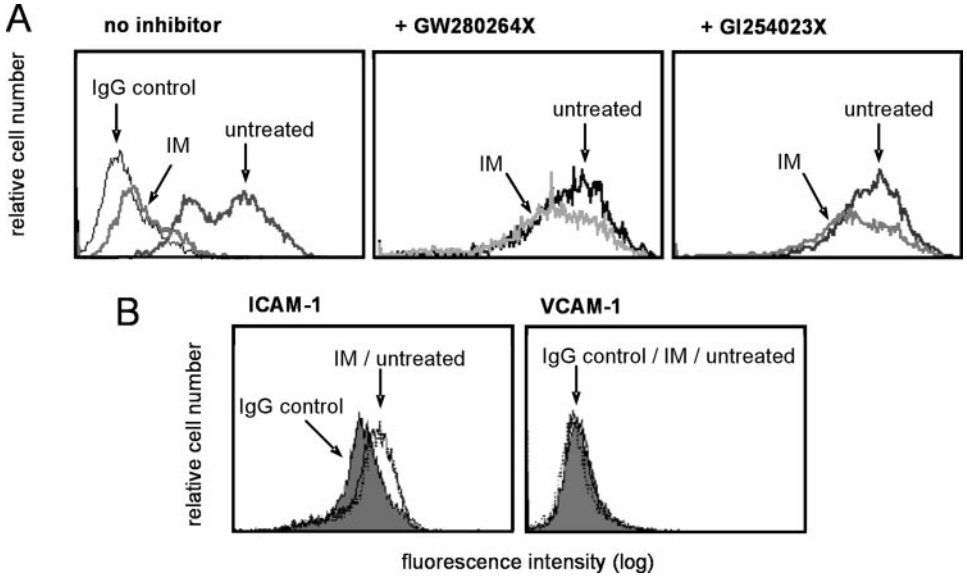

FIGURE 6. Regulation of CX3CL1 surface expression by ionomycin and metalloproteinase inhibitors. ECV-304 cells stably expressing CX3CL1 were pretreated for 5 min with fresh medium containing either metalloproteinase inhibitor GW280353X or GI254023X (3 μM each) or no inhibitor. Subsequently, cells were treated with ionomycin (1 μM) for 30 min or left untreated. Surface expression of CX3CL1 (*A*), ICAM-1 (*B*, *left*), and VCAM-1 (*B*, *right*) was then determined by flow cytometry. One representative of three experiments is shown. IM, ionomycin.

ADAM10. After 60 min of stimulation with PMA or ionomycin, the shedding of CX3CL1 was comparable. Transwell cell culture experiments demonstrated that CX3CL1 was released by cultured ECV-304 cells not only to the apical side, but also and even more pronounced (1.8-fold more) to the basal side (data not shown), suggesting that shedding does not only occur at the apical cell surface, but also at basal and possibly lateral sites.

Because ECV-304 cells may express and shed other adhesion molecules that contribute to cell adhesion, cell surface analysis of VCAM-1 and ICAM-1 was performed by flow cytometry. VCAM-1, which has been reported to undergo regulated shedding (29), was not expressed, whereas ICAM-1 was detected on the surface, but not shed upon treatment with ionomycin (Fig. 6*B*). Therefore, ECV304 cells constitute a suitable model to study the functional consequences of CX3CL1 shedding on cell adhesion without interference by ICAM-1 or VCAM-1 shedding.

Induced shedding by ADAM10 resolves cell adhesion

Next, flow adhesion experiments with the monocytic cell line THP-1 were performed. When ECV-304 cells were transfected to express CX3CL1, the arrest of monocytic cells was clearly enhanced, as previously reported (7, 46). This arrest was profoundly down-regulated by pretreatment of the ECV-304 cells with either PMA (200 ng/ml) or ionomycin (1 μM) for 60 or 30 min, respectively (Fig. 7), correlating with the down-regulation of CX3CL1 from the cell surface (compare Fig. 6). GW280264X and GI254023X clearly differed in their potential to block the PMA-induced effect, i.e., only the combined ADAM10/17 inhibitor GW280264X, but not the preferential ADAM10 inhibitor GI254023X restored cell adhesion. By contrast, both inhibitors were equally effective and partially restored THP-1 cell arrest to ionomycin-treated cells (Fig. 7). Very similar findings were made when the monocytic cell line Mono Mac 6 was used instead of THP-1 cells (data not shown), indicating that these findings were not restricted to one cell line. These data suggest that PMA and ionomycin down-regulate adhesive properties of CX3CL1-producing cells via distinct ADAM proteases.

We then questioned whether regulated shedding may even resolve cell to cell binding via CX3CL1. When THP-1 cells were seeded onto CX3CL1-expressing ECV-304 cells and washed repeatedly, most THP-1 cells remained adherent (Fig. 8*A*), but a considerable amount of cells was washed off. This effect could be caused by enhanced cleavage of the chemokine, as suggested by Western blot analysis of CX3CL1–2Z-expressing cells, which contained increased CTFs of the chemokine when reacted with THP-1 cells for 30 min (Fig. 8*B*). Moreover, when cleavage was further enhanced, upon the addition of ionomycin, the detachment of bound THP-1 cells from CX3CL1-ECV-304 cells was clearly increased (Fig. 8*C*), leaving only a few THP-1 cells adherent to the cell layer that had remained intact until the end of the washing procedure (Fig. 8*D*).

When washing was performed in the presence of the preferential ADAM10 inhibitor GI254023X, more cells remained adherent and, importantly, ionomycin-induced cell detachment was abolished (Fig. 8*E*). These data support the notion that ADAM10 is instrumental in resolving cellular interactions via membrane-bound CX3CL1 and its receptor, and that this process can be enhanced by activation of ADAM10, leading to enforced detachment of bound leukocytes.

To investigate the relevance of endogenous CX3CL1 shedding for cell adhesion to primary endothelial cells, THP-1 cells were

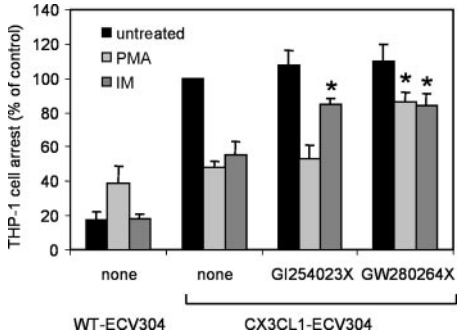

FIGURE 7. THP-1 cell arrest to ionomycin-treated CX3CL1-ECV-304 cells. WT- and CX3CL1-ECV-304 cells were pretreated with 200 ng/ml PMA for 60 min or 1 μM ionomycin for 30 min in the presence or absence of metalloproteinase inhibitors GI254023X and GW280264X (10 μM). Control cells were left untreated. Cells were then assayed for their ability to arrest THP-1 cells under flow conditions. Results were expressed as percentage of monocytic cell adhesion to the untreated CX3CL1-expressing control cells. Data are shown as mean ± SD of three independent experiments. Statistical differences in cell adhesion induced by the inhibitors in PMA- or ionomycin-treated cells are indicated by asterisks. IM, ionomycin.

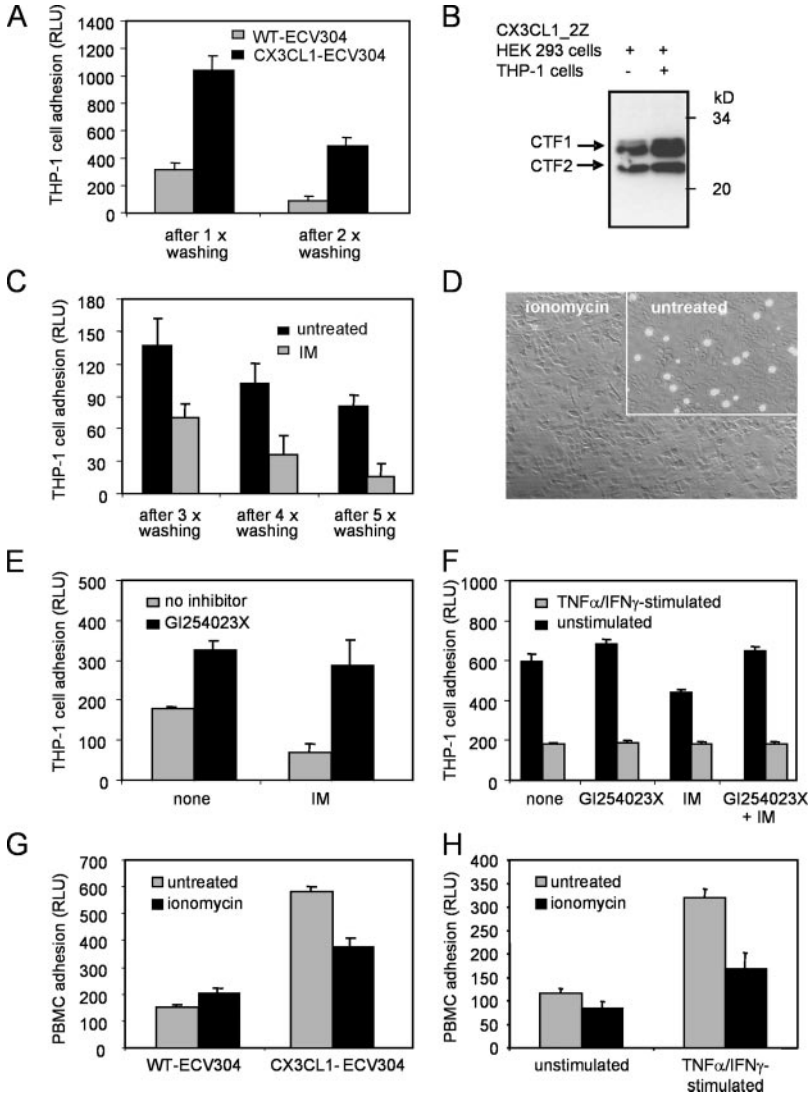

FIGURE 8. Detachment of THP-1 cells or mononuclear cells bound to CX3CL1. *A*, Calcein-labeled THP-1 cells were seeded onto CX3CL1-expressing ECV-304 cells or on respective wild-type control cells for 30 min and washed 2-fold. After each wash step, cell adhesion was determined by recording the fluorescence signal of adherent THP-1 cells. *B*, CX3CL1–2Z-transfected COS-7 cells were incubated for 30 min in the absence or presence of THP-1 cells and subsequently analyzed for the generation of C-terminal CX3CL1 cleavage fragments by Western blotting using a rabbit antiserum to CX3CL1. *C*, Labeled THP-1 cells were seeded onto CX3CL1-ECV-304 cells and washed, as described in *A*. Cells were then stimulated with ionomycin (1 μM) or left untreated for 30 min, and additional wash steps were conducted. After each wash step, the fluorescence signal of adherent cells was recorded. *D*, Finally, the integrity of the ECV-304 cell layer with adherent THP-1 cells was controlled by microscopic analysis. *E*, CX3CL1–2Z-expressing HEK-293 cells were incubated in the absence and presence of THP-1 cells and subsequently analyzed for the generation of cleavage fragments by Western blotting. CX3CL1-expressing ECV-304 cells with adherent calcein-labeled THP-1 cells were treated with ionomycin (IM; 1 μM) in the presence or absence of GI254023X (10 μM) and, after 2-fold washing, cell adhesion was determined. *F*, Endothelial cells were stimulated with TNF-α and IFN-γ (20 μg/ml) or left unstimulated for 24 h and subsequently assayed for adhesion of THP-1 cells. Following cell adhesion, ionomycin (IM; 1 μM) was added for 15 min. Subsequently, cells that had detached from the endothelial cell layer were washed off, and the fluorescence of the cells that remained adherent was determined. *G*, Mononuclear cells prepared from peripheral blood (PBMC) were seeded on WT- or CX3CL1-ECV-304 cells, subsequently ionomycin (1 μM) was added for 30 min, and after washing PBMC adhesion was determined. *H*, Endothelial cells were stimulated with TNF-α and IFN-γ (20 μg/ml) or left unstimulated for 24 h and subsequently analyzed for PBMC adhesion and detachment in response ionomycin (1 μM, 30 min). Data are shown as mean $\pm$ SD ($n = 3$). All data were reproduced in three independent experiments. IM, ionomycin.

seeded onto endothelial cells that were stimulated to express CX3CL1 by treatment with TNF-α and IFN-γ. Treatment with ionomycin induced detachment of bound THP-1 cells, which was prevented by the preferential ADAM10 inhibitor GI254023X (Fig. 8*F*). This effect was only seen with endothelial cells that were stimulated with TNF-α and IFN-γ, but not with unstimulated cells expressing no endogenous CX3CL1. Because other adhesion mechanisms different from CX3CL1 are known to contribute to THP-1 cell adhesion to primary vascular cells under the conditions used (11, 39), it was conceivable that a considerable amount of cells still remained adherent despite ionomycin treatment.

To finally demonstrate the role of CX3CL1 shedding for adhesion of primary leukocytes, PBMC were incubated with CX3CL1-ECV-304 cells or cytokine-stimulated endothelial cells. PBMC adhesion to these cells was ~3-fold higher than that determined for WT-ECV-304 or unstimulated endothelial cells, respectively (Fig. 8, *G* and *H*). Treatment with ionomycin resulted in considerable detachment of leukocytes bound to CX3CL1-ECV-304 cells (Fig. 8*G*) or cytokine-stimulated endothelial cells (Fig. 8*H*), but did not affect the basal PBMC binding to the wild-type control or to unstimulated endothelial cells.

Discussion

In this study, we have for the first time identified ADAM10 as an inducible sheddase of the transmembrane chemokines CX3CL1 and CXCL16, and have elucidated the functional role of this shedding event in leukocyte adhesion.

We and others have previously reported that ADAM10 is involved in the constitutive shedding of CX3CL1, whereas a different protease identified as ADAM17 mediates the enhanced cleavage in response to PMA. In this study, we introduce ionomycin as another very effective stimulus to trigger shedding and provide several lines of evidence that ionomycin-induced shedding differs from PMA-induced shedding: ionomycin-induced cleavage occurs within minutes, whereas the PMA-induced effect requires at least 45 min of stimulation. Specific inhibitors discriminating between ADAM10 and ADAM17 (35) revealed distinct inhibition profiles for ionomycin- and PMA-induced shedding, respectively. Finally, by the use of ADAM10-deficient cell lines (41), we confirmed that, in contrast to the shedding triggered by PMA, the majority of ionomycin-induced shedding is due to the activity of ADAM10 and not to that of ADAM17.

We demonstrate that both the constitutive and the ionomycin-induced shedding are mediated to a large extent by the activity of ADAM10. However, there appears to be a fundamental difference with respect to the cleavage site used. Although constitutive shedding occurs at two different sites, as indicated by the generation of two different C-terminal fragments of 22 and 25 kDa, ionomycin treatment leads to accumulation of only the 22-kDa fragment, suggesting preferential usage of the membrane-proximal site within

CX3CL1. It is very likely that ADAM10 is implicated in both cleavage events, as they are both sensitive to the preferential ADAM10 inhibitor. From these findings, one may conclude that mutation of either cleavage site is not sufficient to prevent cleavage. Indeed, others have found that small deletions within the membrane-proximal region do not affect cleavage, whereas the exchange of a membrane-proximal 30-aa stretch with that of CD4 reduces PMA-induced shedding (37). In a different approach, we have substituted the complete C terminus of CX3CL1, including the two potential cleavage sites, with that of the signal transducer gp130, leading to considerably reduced shedding. However, ionomycin was still capable of inducing shedding at an alternative site within the remaining stalk of CX3CL1. Therefore, multiple cleavage events can occur within the stalk of CX3CL1, depending on the type of cellular stimulation. These findings are in line with the view proposed for ADAM17 substrates, such as IL-6R (45), questioning the existence of highly specific sequence motifs for cleavage.

Although CX3CL1 and CXCL16 show some similarities in their shedding behavior, the constitutive CXCL16 shedding is much stronger and only slightly enhanced by PMA stimulation. We and others have found that ADAM10 is responsible for most of the constitutive shedding, but not for the PMA-induced cleavage of CXCL16 (15, 36). A role of ADAM17 in the latter process has been suggested by inhibitor studies (15), but needs to be confirmed by ADAM17-deficient cell lines. We report in this study that CXCL16 can be also shed in an ionomycin-inducible fashion that is mediated by ADAM10, suggesting that both transmembrane chemokines are regulated by similar constitutive and ionomycin-inducible pathways via ADAM10. Notably, constitutive and ionomycin-induced shedding of both chemokines was not completely suppressed by either inhibition or knockout of ADAM10, indicating a contribution of other proteases in both processes.

Our studies with CX3CL1 and CXCL16 revealed that shedding induced by ionomycin and PMA involves different proteases, namely ADAM10 in the first case and most likely ADAM17 in the latter. Several other surface molecules such as L1 adhesion molecule and CD44 have been found to undergo a comparable pattern of regulated shedding by these two proteases 32. By contrast, N-cadherin, E-cadherin, and γ-protocadherin C3 are exclusively shed by ADAM10, and even their PMA-induced shedding is mediated by this protease (25–27). Therefore, it is unlikely that PMA and ionomycin simply use two stimulatory pathways leading to the activation of either protease. Instead, it appears more likely that PMA and ionomycin modulate the interaction of either protease with only a particular group of substrates.

For most substrates, the signaling pathways leading to enhanced shedding upon cell activation by ionomycin or PMA remain unknown. The calcium regulatory protein calmodulin has been implicated in the shedding mechanism of L-selectin. Inhibition of calmodulin blocks L-selectin-dependent neutrophil rolling by enhancing the cleavage of L-selectin. On the molecular level, the inhibition of calmodulin induces its dissociation from L-selectin, which may then lead to increased access of the latter molecule by the cleaving metalloproteinase (47). To investigate the potential involvement of calmodulin in CX3CL1 shedding, we used the calmodulin inhibitor W7. Indeed, shedding of CX3CL1 is enhanced by the inhibitor that can be blocked by the ADAM10 inhibitor GI254023X (C. Hundhausen and A. Ludwig, unpublished data). Our finding that ionomycin and W7 enhance shedding of the transmembrane chemokine may suggest that calcium signaling is an important event in the regulation of CX3CL1 shedding. Such calcium signals may arise in endothelial cells upon interaction with adherent cells (48, 49).

CX3CL1 mediates flow-resistant adhesion of monocytes, T cell subsets, and NK cells simply by interaction with its specific receptor CX3CR1 on the leukocyte surface, which is to a large extent independent from further signaling (7, 8). Although there is growing in vivo and in vitro evidence for their involvement in promoting monocyte recruitment into inflamed tissue such as atherosclerotic lesions (17, 18), there is little knowledge of how shedding can influence the activity of transmembrane chemokines. By shedding of transmembrane chemokines, ADAM proteases could in general decrease the adhesiveness of endothelial cells for leukocytes. This was underscored by the ionomycin-induced and ADAM10-mediated reduction of monocyte arrest to CX3CL1-expressing ECV-304 cells under flow conditions. Moreover, the proteases could generate an excess of soluble ligand that would potentially antagonize the receptors on opposing or adjacent cells and thereby prevent adhesion. However, shedding could also be relevant during the adhesion process itself. This idea is supported by our adhesion studies demonstrating that CX3CR1-expressing cells detach from CX3CL1-expressing cell layers upon activation of ADAM10. It appears likely that the resolution of the interaction between CX3CL1 and CX3CR1 is not only required for cell detachment, but also allows firmly adherent leukocytes to proceed with lateral migration or diapedesis. Importantly, CX3CL1 shedding does only occur at the apical site, but would also be relevant within the intraepithelial junctions where ADAM10 is highly expressed (K. Reiss, J. Pruessmeyer, and P. Saftig, unpublished observations) to allow leukocyte transmigration. This may help to establish a novel paradigm, according to which regulated shedding may be instrumental in limiting firm arrest and promoting emigration, but also in enabling sequential responses of patrolling immune cells to subsequent migratory cues within tissues under both physiological and inflammatory conditions.

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Disclosures

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