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The Transmembrane CXC-Chemokine Ligand 16 Is Induced by IFN-γ and TNF-α and Shed by the Activity of the Disintegrin-Like Metalloproteinase ADAM10[1]

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The novel CXC-chemokine ligand 16 (CXCL16) functions as transmembrane adhesion molecule on the surface of APCs and as a soluble chemoattractant for activated T cells. In this study, we elucidate the mechanism responsible for the conversion of the transmembrane molecule into a soluble chemokine and provide evidence for the expression and shedding of CXCL16 by fibroblasts and vascular cells. By transfection of human and murine CXCL16 in different cell lines, we show that soluble CXCL16 is constitutively generated by proteolytic cleavage of transmembrane CXCL16 resulting in reduced surface expression of the transmembrane molecule. Inhibition experiments with selective hydroxamate inhibitors against the disintegrin-like metalloproteinases a disintegrin and metalloproteinase domain (ADAM)10 and ADAM17 suggest that ADAM10, but not ADAM17, is involved in constitutive CXCL16 cleavage. In addition, the constitutive cleavage of transfected human CXCL16 was markedly reduced in embryonic fibroblasts generated from ADAM10-deficient mice. By induction of murine CXCL16 in ADAM10-deficient fibroblasts with IFN-γ and TNF-α, we show that endogenous ADAM10 is indeed involved in the release of endogenous CXCL16. Finally, the shedding of endogenous CXCL16 could be reconstituted by retransfection of ADAM10-deficient cells with ADAM10. Analyzing the expression and release of CXCXL16 by cultured vascular cells, we found that IFN-γ and TNF-α synergize to induce CXCL16 mRNA. The constitutive shedding of CXCL16 from the endothelial cell surface is blocked by inhibitors of ADAM10 and is independent of additional inhibition of ADAM17. Hence, during inflammation in the vasculature, ADAM10 may act as a CXCL16 sheddase and thereby finely control the expression and function of CXCL16 in the inflamed tissue. *The Journal of Immunology,* 2004, 172: 6362–6372.

Chemokines are key mediators of cell migration. Most of the over 40 members of the chemokine family are synthesized and released as small soluble molecules building up chemotactic concentration gradients for responsive cells (reviewed in Ref. 1). Cell surface-expressed glycosaminoglycans are thought to play an important role in the immobilization of soluble chemokines for presenting them to responsive cells (reviewed in Ref. 2). On endothelial cells (ECs),[5] immobilized chemokines induce cell adhesion and transmigration of leukocytes. There are two exceptional chemokines that are already synthesized as surface-expressed molecules: CX3C ligand 1 (CX3CL1) (1) (also termed fractalkine or neurotactin) (3, 4) and CXC-chemokine ligand 16 (CXCL16). CXCL16 was discovered recently as a ligand for the CXC-chemokine receptor CXCR6 (5) and, independently, it was described as scavenger receptor for oxidized low density lipoprotein (LDL) and therefore was also termed as SR-PSOX (6). Both, CX3CL1 and CXCL16 are expressed on the cell membrane as multidomain molecules consisting of a chemokine domain followed by a glycosylated mucin-like stalk and a single transmembrane helix followed by a short cytoplasmic tail. CX3CL1 has been described on a number of different cell types, either constitutively expressed or inducible by proinflammatory mediators (3, 7, 8). To date, CXCL16 protein expression has been reported for APCs only (5, 9). CX3CL1 as well as CXCL16 also occur as soluble molecules lacking the transmembrane domain. They then function as chemoattractants, but activate different target cells. Although CX3CL1 acts through its specific receptor CX3CR1 which is expressed on monocyte subsets, NK and T cells (10–12), CXCL16 induces chemotaxis of activated T cells and bone marrow plasma cells via its receptor CXCR6 (5, 13). Transmembrane CX3CL1 also functions as adhesion molecule by establishing shear-resistant physical contact to its receptor CX3CR1 on monocytes and NK cells (10, 11). Likewise, transmembrane CXCL16 is thought to act

as adhesion molecule for CXCR6-expressing leukocytes as suggested by a recent report that CXCL16 immobilized to plastic surfaces is able to capture bone marrow plasma cells under physiologic flow conditions (13).

The two closely related disintegrin-like metalloproteinases a disintegrin and metalloproteinase domain (ADAM)17, also known as TNF-α-converting enzyme, and ADAM10/Kuzbanian have been implicated in a number of cleavage (shedding) processes leading to the conversion of transmembrane molecules into soluble mediators (for reference see Refs. 14 and 15). Mice with targeted disruption in the *ADAM17* or *ADAM10* gene have been generated (16, 17). The general importance of both proteases is underlined by the fact that the ADAM17-deficient mice die in uteri or soon after birth and that the ADAM10-deficient embryos die at day 9.5 of embryogenesis. From ADAM17- or ADAM10-deficient embryos, cell lines of murine embryonic fibroblasts (MEF) have been generated to study the effect of ADAM10 or ADAM17 deficiency on the cleavage of transmembrane cytokines and adhesion molecules. Under normal cell culture conditions, ectodomain shedding may occur spontaneously, but in many cases it is enhanced by stimulation with phorbol esters such as PMA. ADAM17 is a key player in the PMA-induced shedding of a number of cell surface molecules involved in cell adhesion or cellular communication such as TNF-α (18), IL-6R (19), or L-selectin (16).

The existence of either transmembrane or soluble variants of CXCL16 and CX3CL1 with distinct biological functions raised the question of how the generation of either form could be regulated. For CX3CL1, it has been demonstrated that the release from neuronal cells and smooth muscle cells is sensitive to broad spectrum inhibitors of metalloproteinases (7, 8). Using a series of different inhibitors against metalloproteinases, we have shown that potent inhibitors of ADAM10 effectively block CX3CL1 release. This was further supported by our finding that ADAM10$^{-/-}$ MEFs display a considerably reduced potential to shed CX3CL1 (20). Two other groups had previously reported that CX3CL1 release from ECs can be rapidly enhanced by PMA and that this release is to a large extent mediated by ADAM17 (21, 22). The view that disintegrin-like metalloproteinases could play a role in cell adhesion mediated by transmembrane chemokines is supported by our recent finding that CX3CL1 cleavage by ADAM10 results in the detachment of leukocytes bound to a CX3CL1-expressing cell layer (20).

To date the mechanism involved in the release of soluble CXCL16 remains unknown. In this study, we show that cell-expressed CXCL16 is released from the cell membrane by proteolytic cleavage. Using inhibitors of metalloproteinases with defined potency for distinct members of the disintegrin-like metalloproteinase family and with embryonic fibroblasts from ADAM10$^{-/-}$ mice, we provide several lines of evidence that the disintegrin-like metalloproteinase ADAM10 is relevant for the majority of the constitutive, but not for the PMA-inducible, cleavage of cell-expressed murine or human CXCL16. We demonstrate that the expression of endogenous CXCL16 is induced by IFN-γ and TNF-α in ECs, vascular smooth muscle cells, and in embryonic fibroblasts. Using ADAM10-deficient fibroblasts, we show that endogenous ADAM10 is indeed the most relevant sheddase of endogenous CXCL16.

Materials and Methods
Cytokines, Abs, and inhibitors

Recombinant human extracellular domain and chemokine domain CXCL16, recombinant human IFN-γ, TNF-α, and IL-4 as well as unconjugated goat anti-human CXCL16 Ab were obtained from R&D Systems (Wiesbaden, Germany). Unconjugated and biotinylated rabbit anti-human CXCL16 Ab and goat anti-murine CXCL16 Ab were from PeproTech

(London, U.K.). The rabbit antiserum B 42.1 against murine ADAM10 was characterized previously (17). The rabbit anti-human ADAM10 Ab was from Chemicon International (Hofheim, Germany).

The metalloproteinase inhibitors GW280264X ((2R,3S)-3-(formylhydroxyamino)-2-(2-methyl-1-propyl) hexanoic acid [(1S)-5-benzyloxycarbamoylamino-1-(1,3-thiazol-2-ylcarbamoyl)-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. Batimastat (BB94) was synthesized at GlaxoSmithKline (7). The compounds were assayed for inhibition of recombinant human ADAM17 and ADAM10 ectodomains as described (20). Cycloheximide was from Sigma-Aldrich (Munich, Germany).

Generation of CXCL16 vectors

Human CXCL16 (GenBank accession number AF275260) was amplified by PCR from cDNA generated by reverse transcription of mRNA isolated from PMA-stimulated THP-1 cells that were reported to express CXCL16 (6). The forward primers included a restriction site for *Eco*RI, followed by a Kozak consensus sequence and the start codon (5′-CCG GAA TTC CGG AGC ATG TCT GGG AGT CAG AGC GAG G-3′). The reverse primer included a sequence coding for a hemagglutinin (HA) tag inserted in front of the CXCL16 stop codon and a restriction site for *Xho*I (5′-T CCG CTC GAG TCA AGC GTA ATC TGG AAC ATC GTA TGG GTA GGT ATT AGA GTC AGG TGC CAC AGG TAT ATA ATG AAC CG-3′). The PCR product was first cloned into pCR4blunt-TOPO (Invitrogen, Karlsruhe, Germany). The correct sequence was then inserted in the expression vector pcDNA3.1 (Invitrogen) using *Eco*RI and *Xho*I.

Murine CXCL16 (GenBank accession number AF301017) was amplified by PCR from cDNA generated from RNA of murine spleen using primers 5′-CC GGA ATT CGG CCG GAG ATG AGG CGG GG-3′ and 5′-T CCG CTC GAG CTA GGG TCT TGG TTC AAC AGG AGT GTA CC-3′ with restriction sites for *Bam*HI and *Xho*I, respectively. The PCR product was ligated into pCR4Blunt-TOPO and sequenced. Subsequently, it was inserted into the gateway vector pENTR2b (Invitrogen) and finally into the bicistronic expression vector pEBS-IRES-GFP that was kindly donated by Dr. H. Gascan (Institut National de la Santé et de la Recherche Médicale, Angers, France) and that was supplemented with an additional gateway cassette for the clonase reaction (Invitrogen).

Cell culture and transfection

Cryopreserved ECs from human umbilical vein and smooth muscle cells (SMC) from human aorta were obtained from BioWhittaker (Berkshire, U.K.). Cells were cultured in six-well plates (Nunc, Roskilde, Denmark) in complete medium as supplied by BioWhittaker. SMCs were made quiescent by starvation in basal medium in the absence of FCS and growth factors for 24 h before stimulation. ECs and quiescent SMCs were stimulated with IFN-γ and TNF-α (both 20 ng/ml) for different periods of time. The primate kidney fibroblast cell line COS-7 was cultured and transfected essentially as described previously (20).

The ADAM10$^{-/-}$ murine embryonic fibroblast cell line ADAM10 40$^{-/-}$ and the wild-type fibroblast cell line PS1$^{+/+}$ were generated from ADAM10$^{-/-}$ mice and respective wild-type animals as described elsewhere (17, 23). Cells were cultured in DMEM containing 10% FCS (all cell culture media and reagents were from PAA (Coelbe, Germany) if not otherwise stated). For retransfection of ADAM10 in ADAM10-deficient MEFs, cDNA of bovine HA-tagged ADAM10 (20) was inserted into a retroviral expression vector based on pCXbsr (accession number AB041927). Ecotropic BOSC23 packaging cells were transfected with the retroviral vector using FuGENE (Roche, Mannheim, Germany). At day 2 after transfection, supernatants were collected, cleared by centrifugation, and added to ADAM10$^{-/-}$ MEFs in the presence of 10 ng/ml sequabrene (Sigma-Aldrich). After 6 days, transfected cells were subcloned by limiting dilution and positive colonies were selected by the expression of green fluorescent protein (GFP) encoded by the vector. For transient transfection with CXCL16, MEFs were seeded at 1×10^4 cells/cm^2 in six-well dishes (Costar/Corning, Koolhaven, The Netherlands) and incubated for 24 h to 70% confluence. The medium was replaced with 1 ml of fresh medium. Each well received 100 μl of serum-free medium containing 2 μg of CXCL16 pcDNA3.1 and 3 μl of FuGENE (Roche). Cells were cultured for 48 h and subsequently assayed for their capacity to cleave membrane-bound CXCL16. All transient transfections were performed in triplicates for each stimulatory condition. In all experiments, efficiency of transient transfections was controlled in parallel by transfection with GFP in pcDNA3.1 and subsequent detection of expressed GFP by fluorescence microscopy.

The adherent human cell line ECV-304 was transfected with CXCL16 (CXCL16-ECV-304) using the same protocol. CXCL16-ECV-304 cells were cultured in M199 medium supplemented with 10% FCS and antibiotics up to 90% confluence before subculture. Stably transfected ECV cells were generated by selection with 600 μg/ml geneticin and made monoclonal 6 days after transfection by subculture of the growing colonies.

Relative quantification of CXCL16 mRNA by TaqMan RT-PCR

RNA was isolated from cultured ECs or SMCs using TRIzol reagent (Life Technologies, Grand Island, NY). Total RNA (1 μg) was treated with DNaseI (Life Technologies) and reverse transcribed to cDNA using Superscript II polymerase (Life Technologies) according to the manufacturer's instructions. For each sample, a control without Superscript II was run parallel to allow assessment of genomic DNA contamination. Each cDNA sample was analyzed for expression of CXCL16 and GAPDH by real-time quantitative RT-PCR using the fluorescent TaqMan 5′-nuclease assay as previously described (8). Oligonucleotide primers and probes were designed using Primer Express software version 1.0 (PE Biosystems, Warrington, U.K.) and synthesized by PE Biosystems. Sequences of forward primer, reverse primer, and probe for CXCL16 were 5′-GAG CTC ACT CGT CCC AAT GAA-3′, 5′-TCA GGC CCA ACT GCC AGA C-3′, and 5′-FAM CAC CAT TCA CAC TGC GGG CCA C TAMRA-3′. Levels of CXCL16 and GAPDH mRNA were quantified by comparison of the fluorescence of each sample with that of a serially diluted standard of human genomic DNA (Promega, Southampton, U.K.).

CXCL16 cleavage assays

CXCL16-expressing cells were grown to 70–90% confluence in complete medium in six-well dishes (Nunc) for 24 h before stimulation. The cells were washed with PBS and 0.75 ml of FCS-free medium with or without metalloproteinase inhibitors was added. After 10 min, the cells were stimulated with PMA (200 ng/ml) for various periods of time. The conditioned media were harvested, and 0.75 ml of protease inhibitor mixture (Complete; Roche) was added according to the instructions of the manufacturer. The supernatants were centrifuged to remove nonadherent cells and, if necessary, concentrated 10-fold using 10-kDa cut-off filtration units (Vivaspin; Vivascience, Hannover, Germany). The presence of released CXCL16 in the conditioned media was demonstrated by Western blotting and quantified by ELISA (see below). The cells were washed with 2 ml of PBS and removed from the vessel by scraping in 1 ml of ice-cold PBS. CXCL16 surface expression of intact cells was determined by flow cytometry. To quantify cell-associated CXCL16 by ELISA, the cells were centrifuged and resuspended in 0.75 ml of PBS containing 0.1% Triton X-100 and a protease inhibitor mixture (Complete; Roche). After 30 min of incubation on ice under agitation, lysates were centrifuged at 12,000 $\times g$ for 10 min. The presence of equal amounts of protein in the lysates of differently treated cells was controlled by a bicinchoninic acid-based protein assay (Pierce, Rockford, IL).

CXCL16-specific ELISA

The ELISA for human CXCL16 was conducted in Microlon 96-well plates (Greiner, Nurtingen, Germany) at room temperature, the reaction volume was 50 μl. The plate was coated overnight with 2 mg/ml goat anti-human CXCL16 in 50 mM Na$_2$CO$_3$ (pH9.3), washed three times with 0.05% Tween in PBS (PBS-T), and blocked with PBS-T containing 2% BSA for 2 h. The plate was dried and the samples were added for 2 h. A standard prepared as nine serial 1/2 dilutions of 1.25 nM recombinant human CXCL16 in either PBS-T with 1% BSA, serum-free medium, or cell lysis buffer was run in parallel. Following washing, 200 ng/ml biotinylated rabbit anti-human CXCL16 in PBS-T/1% BSA were added to each well and the plate incubated at room temperature for 1 h. After washing, 100 mU/ml streptavidin-peroxidase conjugate (Roche) in PBS-T/1% BSA was added for 1 h. After washing, chromogenic peroxidase substrate (BM blue; Roche) was added. The reaction was stopped after a 20-min incubation by addition of 1.8 M H$_2$SO$_4$ before the OD was determined at 450 nm. The specificity of the ELISA was demonstrated by its positive reaction with lysates and conditioned media of CXCL16-transfected COS-7 cells or ECV-304 cells without showing any reaction with that of the respective wild-type cells or that of cells that were transfected to express murine CXCL16 or human CX3CL1 (20). The detection limit of the ELISA was 12 pM of either the extracellular domains or only the chemokine domain of CXCL16. The ELISA specific for murine CXCL16 was purchased from R&D Systems and performed following the manufacturer's instructions. The ELISA reacted with lysates of COS-7 cells transfected with murine CXCL16 but not with that of cells transfected with human CXCL16 or empty vector.

Western blot analysis of CXCL16 and ADAM10

Western analysis was performed as described previously (20). In brief, concentrated media samples were subjected to SDS-PAGE under reducing conditions using 10 or 7.5% Tris-glycine gels for subsequent detection of CXCL16 or ADAM10, respectively. Proteins were then transferred onto polyvinylidene difluoride membranes (Hybond-P; Amersham, Piscataway, NJ) that were probed with dilutions of rabbit anti-HA-tag (0.4 μg/ml), rabbit anti-human CXCL16 (0.2 μg/ml), goat anti-murine CXCL16 Ab (0.2 μg/ml), or rabbit antiserum to murine ADAM10 (1/10,000). Bound Ig was detected with either goat anti-rabbit Ig or pork anti-goat Ig, (both from Pierce, coupled with HRP, diluted 1/10,000) and subsequent addition of ECL substrate (ECLplus; Amersham). Signals were recorded using a luminescent image analyzer (Fujifilm Image reader, LAS1000; Fuji, Tokyo, Japan).

Analysis of CXCL16 surface expression by flow cytometry

Cells were resuspendend in ice-cold PBS containing 0.1% BSA and 0.01% NaN$_3$ at 3×10^5 cells/ml and incubated with purified rabbit anti-human CXCL16 or rabbit IgG control (both at 2 μg/ml in PBS with 0.1% BSA and 0.01% NaN$_3$) for 1 h on ice. Following 2-fold washing, cells were incubated with secondary fluorescein-conjugated goat anti-rabbit IgG (DK; DAKO, Carpinteria, CA) for 1 h on ice. Cells were washed twice and suspended in ice-cold PBS containing 2% paraformaldehyde. The fluorescence signal of the labeled cells was then analyzed by flow cytometry (FACScan; BD Biosciences, Heidelberg, Germany) and calculated as median fluorescence intensity (MFI) of the cell population.

Statistical analysis

Data were statistically analyzed using the unpaired two tailed t test. Two populations of data significantly different at p values smaller than 0.05 or 0.01 were indicated by one or two asterisks, respectively.

Results

Soluble CXCL16 is generated by proteolytic cleavage of a cell-expressed precursor

To examine the mechanism of CXCL16 cleavage, CXCL16 cDNA was generated from PMA-stimulated THP-1 cells and C-terminally tagged with an HA tag (Fig. 1A). Human CXCL16-HA cDNA was transiently transfected into COS-7 cells followed by Western blot analysis of the cell lysates. An HA-specific Ab revealed the presence of a broad band migrating at 49 kDa and a faint band of ~16 kDa (Fig. 1B). None of the bands was detected in nontransfected cells suggesting that they represent CXCL16 proteins of different size but with an intact C terminus. ECV-304 cells that were stably transfected with human CXCL16-HA consistently produced one major protein band at 48 kDa and two faint bands at 30 and 16 kDa (Fig. 1B). Concentrated media of CXCL16-transfected COS-7 cells were then analyzed for the presence of soluble CXCL16 with an Ab directed against the chemokine domain of CXCL16. The Ab recognized a 32-kDa protein in the supernatant that was not present in untransfected cells and therefore most likely represents soluble CXCL16 (Fig. 1C). To obtain first information about the mechanism of CXCL16 release, cells were treated with inhibitors of metalloproteinases that differed in their ability to block the two disintegrin-like metalloproteinases ADAM10 and ADAM17 which have both been implicated in the shedding of other transmembrane cytokines or receptors such as IL-6R and CX3CL1 (20, 21, 24). A sandwich ELISA using anti-CXCL16 Abs from goat and rabbit was used to quantify the amount of released and cell-associated CXCL16 in the conditioned media and cell lysates of CXCL16-transfected COS-7 cells, respectively. The ELISA was specific for CXCL16 because it did not cross-react with supernatants or cell lysates of untransfected COS-7 cells (data not shown). As shown in Fig. 1D, the release of soluble CXCL16 was effectively blocked by the broad spectrum inhibitor of metalloproteinases batimastat (BB94) which was associated with an increase in cellular CXCL16 detectable in the cell lysates. The two hydroxamates GW280264X and

FIGURE 1. Transient expression and release of human and murine CXCL16. *A*, Schematic structure of the human CXCL16 construct used for transfection. The transmembrane molecule consists of an N-terminal chemokine domain on a highly glycosylated mucin-like stalk, a transmembrane domain, and a short cytoplasmic tail. Human CXCL16 was C-terminally fused with an HA tag for detection of the transmembrane molecule in the cell lysates. The number of amino acids encompassed by each single domain is given in parentheses. *B*, Human HA-tagged CXCL16 or empty vector were transiently transfected into COS-7 cells or into ECV-304 cells. Cell extracts were analyzed by Western blotting with an anti-HA Ab. *C*, COS-7 cells were transiently transfected with human CXCL16 or treated with empty vector. Subsequently, cells were incubated for 8 h in serum-free medium. The conditioned media were concentrated 30-fold and analyzed by Western blotting using a rabbit Ab to the chemokine domain of CXCL16. *D*, COS-7 cells transfected with CXCL16 were incubated for 8 h in serum-free medium with 10 or 3.3 μM batimastat (BB94); 10, 3.3, 1.1, 0.37, 0.15, or 0 μM GW280264X (mixed ADAM10/ADAM17 inhibitor); or 10, 3.3, 1.1, 0.37, 0.15, or 0 μM GI254023X (preferential ADAM10 inhibitor). The conditioned media and cell lysates (both 0.75 ml per dish) were then analyzed for the presence of CXCL16 by ELISA and results were calculated as mean and SD of one representative experiment performed in triplicates. *E*, COS-7 cells were transiently transfected with murine CXCL16 or control vector, washed, and incubated for 8 h in serum-free medium. Subsequently, murine CXCL16 was detected in the cell extracts and 10-fold concentrated media samples using a goat Ab against the chemokine domain of murine CXCL16. *F*, COS-7 cells transfected with murine CXCL16 were incubated in serum-free medium in the absence or presence of GI254023X for 8 h and assayed for the expression and release of CXCL16 in the cell lysates and conditioned media, respectively. Results are representative of three independent experiments.
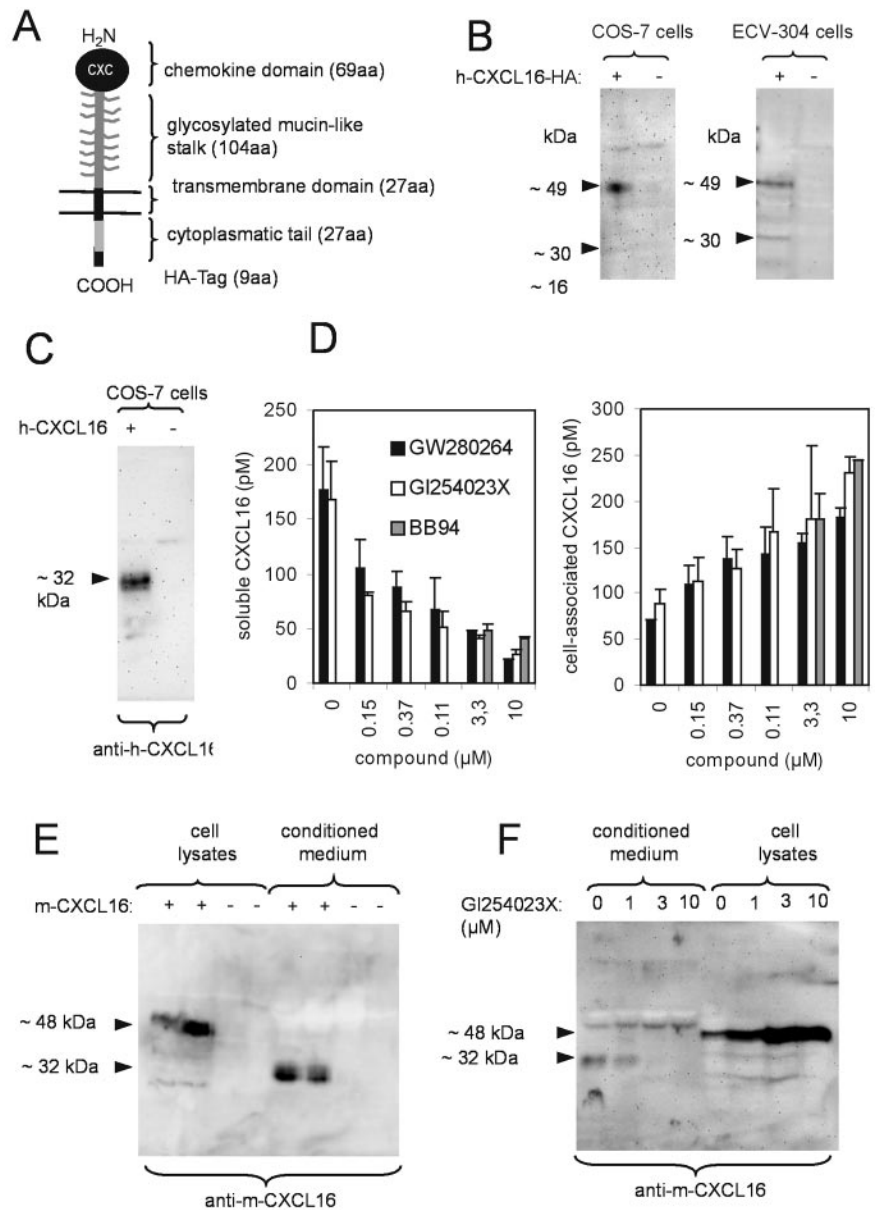
GI254023X that both inhibit ADAM10, but of which only the former blocks ADAM17 (20), showed a very similar concentration-dependent inhibition of CXCL16 release. Therefore, ADAM17 inhibition does not seem to be required for blocking constitutive CXCL16 shedding.

We then thought to determine whether the murine CXCL16 sharing 49% of its amino acid sequence with that of its human homologue would also be subject to metalloproteinase-mediated cleavage. Generation of murine CXCL16 cDNA from spleen and transfection in COS-7 cells resulted in a prominent broad band migrating at 48 kDa as detected by a polyclonal Ab against murine CXCL16 (Fig. 1*E*). This murine 48-kDa protein most likely corresponds to the human 49-kDa form of HA-tagged CXCL16. Moreover, analysis of concentrated media samples from COS-7 cells transfected with murine CXCL-16 revealed a dominant protein band with a very similar molecular size as that found for the soluble form of human CXCL16 (both 32 kDa). As shown in Fig. 1*F*, the release of the soluble 32-kDa variant of murine CXCL16 was completely suppressed by the inhibitor GI254023X, preferentially blocking ADAM10 but not ADAM17, and instead, the 48-

kDa form of CXCL16 was accumulated in the cell lysates (Fig. 1*F*). Taken together, these data strongly suggest that the soluble 32-kDa forms of human as well as murine CXCL16 were generated by metalloproteinase-mediated proteolytic cleavage of cell-associated proteins.

Ectodomain cleavage of surface-expressed CXCL16

We next determined whether CXCL16 cleavage would in fact modulate the surface expression of the transmembrane chemokine. We first examined whether anti-CXCL16 Abs from rabbit or goat could be used to analyze CXCL16 surface expression by flow cytometry. As shown in Fig. 2*A*, the rabbit Ab to CXCL16 reacted with CXCL16-transfected ECV-304 but not with the nontransfected cells. A very similar Ab reactivity was seen with the goat Ab against CXCL16 (data not shown), indicating that both Abs were indeed suitable to detect CXCL16 on the cell surface. Flow cytometry was then used to study the effect of CXCL16 shedding on the surface expression of the chemokine on CXCL16-ECV-304 cells. Inhibition of CXCL16 cleavage by the ADAM10 inhibitor
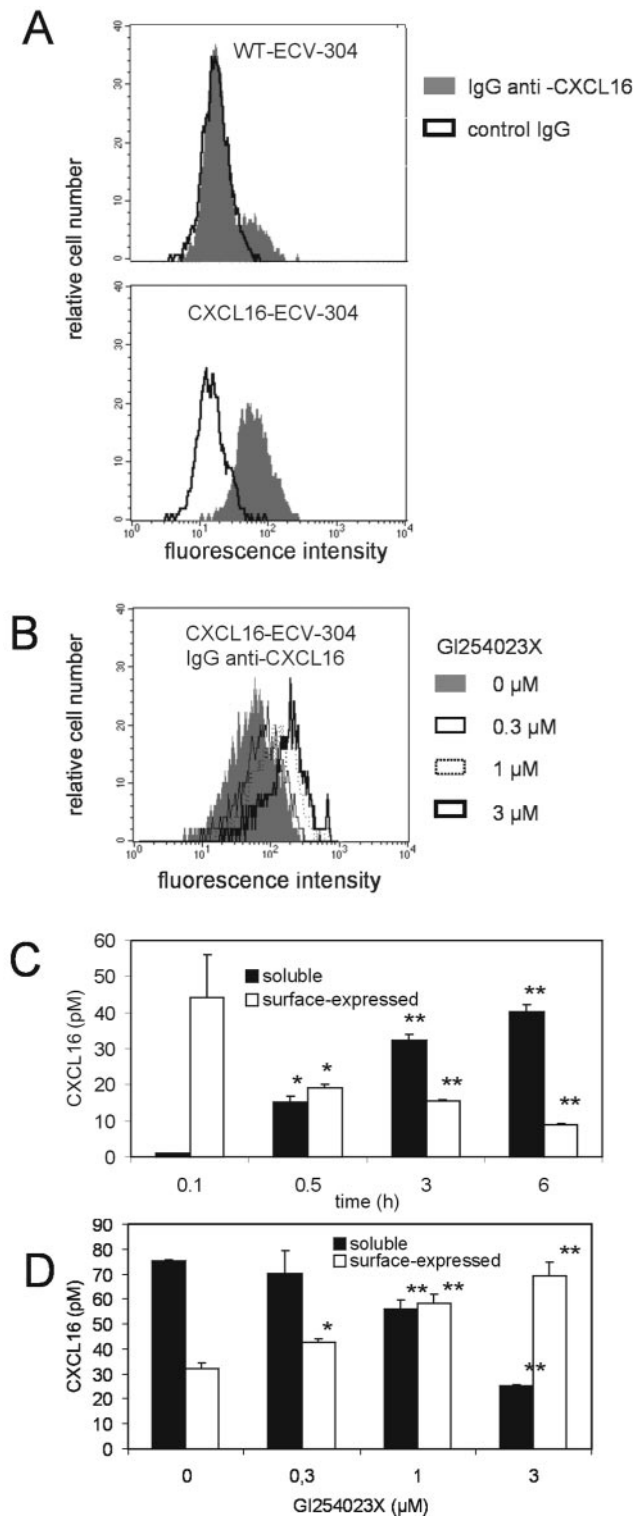
GI254023X dose dependently increased CXCL16 surface expression (Fig. 2B), which was associated with a reduced release of soluble CXCL16 (data not shown). To analyze whether surface-expressed CXCL16 is subject to proteolytic shedding, CXCL16-ECV cells were labeled with a biotinylated Ab to CXCL16. Subsequently, cells were washed and incubated for different periods of time at 37°C. The complex of biotinylated Ab and shed CXCL16 in the conditioned medium was captured by an anti-CXCL16 Ab immobilized to a microtiter plate. A similar procedure was followed with the cell lysates containing the cell-associated CXCL16. Subsequently, the amount of the bound chemokine Ab complex was determined by quantification of its biotin conjugate. As shown in Fig. 2C, Ab-labeled CXCL16 was released into the media in a time-dependent fashion that was associated with a reduction of surface-expressed CXCL16. In the presence of the ADAM10 inhibitor GI254023X, however, the majority of labeled CXCL16 remained on the cell surface (Fig. 2D). These data indicate that a metalloproteinase activity is responsible for the down-regulation of surface-expressed CXCL16.

Constitutive cleavage of CXCL16 involves the activity of ADAM10

To further investigate the role of ADAM10 in the CXCL16 cleavage, we used MEFs that were generated from ADAM10-deficient mice (17). As shown by Western blotting with an antiserum against murine ADAM10 (Fig. 3A, *left panel*) these cells completely lack ADAM10 because neither the 85-kDa proform nor the 64-kDa mature form, which are both present in wild-type MEFs of the same genetic background, can be detected. To restore ADAM10 expression, ADAM10$^{-/-}$ MEFs were stably transfected with bovine HA-tagged ADAM10. As seen with an anti-HA-tag Ab, both the processed and immature forms of bovine ADAM10-HA were present in the retransfected cells (Fig. 3A, *right panel*). Following transfection with human CXCL16, MEFs were cultured for 4 h in fresh serum-free medium and subsequently, the shedding of CXCL16 was analyzed by quantification of the chemokine in the media and in the cell lysates using ELISA. As shown in Table I, the ADAM10$^{-/-}$ MEFs released reduced levels of CXCL16 but contained increased amounts of the chemokine in their cell lysates compared with the ADAM$^{+/+}$ MEFs. The amount of released CXCL16 expressed as percentage of the total chemokine content was reduced from 51% in the ADAM10$^{+/+}$ cells to 13% in the ADAM10$^{-/-}$ cells. To exclude that differences between the two cell lines independent from ADAM10 may account for their different shedding activity, CXCL16 shedding was also quantified in the same ADAM10$^{-/-}$ cell line that was retransfected with ADAM10. As expected, the shedding activity was completely restored upon retransfection of ADAM10 (Fig. 3B).

To investigate the shedding of CXCL16 under more physiological conditions endogenous expression of CXCL16 was induced in MEFs by cytokine stimulation. Costimulation with IFN-γ and TNF-α led to a profound time-dependent up-regulation of cell-associated CXCL16 and the release of soluble protein as detected

FIGURE 2. Flow cytometry analysis of human CXCL16 surface expression and shedding of surface-labeled CXCL16 from stably transfected ECV-304 cells. *A*, WT-ECV-304 and CXCL16-ECV-304 cells were incubated with a rabbit anti-human CXCL16 Ab or an IgG control on ice. Subsequently, cell-bound Abs were detected by a fluorescein-conjugated secondary Ab and fluorescence was recorded by flow cytometry. *B*, CXCL16-ECV-304 cells were incubated for 4 h in serum-free medium in the presence of various concentrations of the ADAM10 inhibitor GI254023X. Subsequently, CXCL16 expression was analyzed by flow cytometry. Inhibition of CXCL16 release by the inhibitor was controlled in parallel by quantification of released CXCL16 in the conditioned media. Histograms shown in *A* and *B* were representative of three separate experiments. *C* and *D*, CXCL16-ECV-304 cells were labeled with biotinylated anti-CXCL16 Ab and repeatedly washed to remove free Ab. Cells were either incubated for various periods of time (*C*) or incubated for 4 h in the presence of increasing dosages of the ADAM10 inhibitor GI254023X (*D*). Subsequently, the labeled chemokine was quantified in the lysates and conditioned media (both 0.75 ml per dish) by capturing and detecting the biotinylated Ab/CXCL16 complex with a CXCL16-specific ELISA. Data in *C* and *D* are given as mean and SD of triplicates of one representative of three separate experiments and statistically significant differences in CXCL16 shedding caused by the inhibitor are indicated by asterisks (∗, $p < 0.05$; ∗∗, $p < 0.01$).
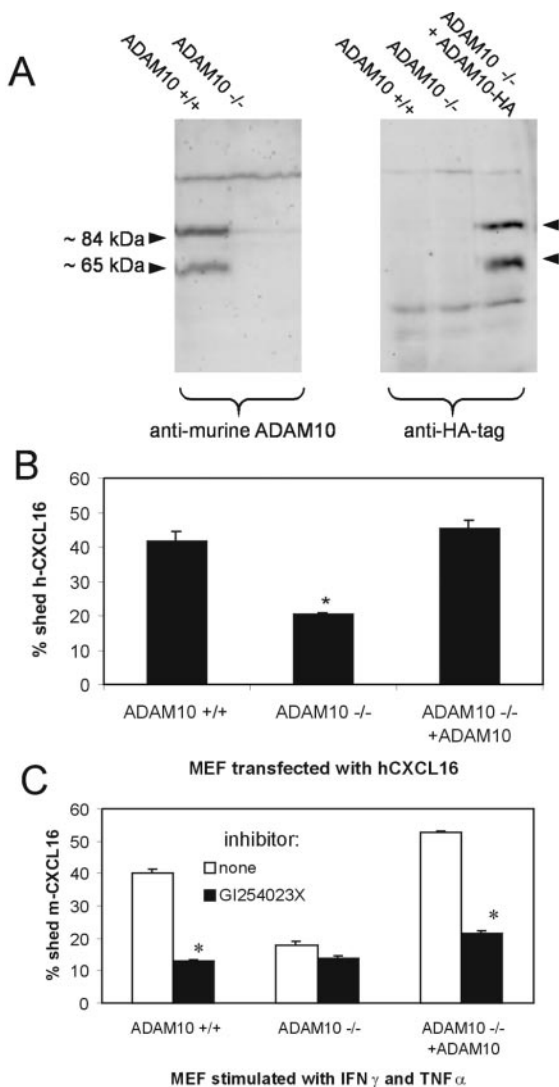
FIGURE 3. CXCL16 shedding in ADAM10-deficient MEFs. *A*, SDS-lysates of ADAM10$^{+/+}$ MEFs, ADAM10$^{-/-}$ MEFs, and ADAM10$^{-/-}$ MEFs retransfected with HA-tagged bovine ADAM10 were analyzed for the presence and absence of ADAM10 by Western blotting using Abs against the murine ADAM10 (*left*) or the HA tag, respectively (*right*). *B*, Human CXCL16 was transfected in ADAM10$^{+/+}$, ADAM10$^{-/-}$, and ADAM10-retransfected ADAM10$^{-/-}$ MEFs. Shedding of CXCL16 was analyzed by incubation of cells for 4 h in serum-free medium and subsequent quantification of soluble and cell-associated CXCL16 by ELISA. The amount of shed soluble human CXCL16 is expressed as the percentage of the total CXCL16 content including soluble and cell-associated molecules. The statistically significant difference in CXCL16 shedding between the ADAM10$^{-/-}$ MEFs and the ADAM10$^{+/+}$ or ADAM10-retransfected ADAM10$^{-/-}$ cells is indicated by an asterisk ($p < 0.05$). *C*, MEFs were costimulated with IFN-γ and TNF-α (both 20 ng/ml) to express endogenous murine CXCL16. The cells were incubated in serum-free medium for 4 h in the absence or presence of the preferential ADAM10 inhibitor GI254023X and shedding of murine CXCL16 was analyzed by an ELISA specific for murine CXCL16 and calculated as described for *B*. Data in *B* and *C* are given as mean and SD of one representative experiment performed in triplicates. Statistically significant differences in CXCL16 shedding caused by deletion of ADAM10 (*B*) or by the inhibitor (*C*) are indicated by asterisks ($p < 0.05$). Results are representative for at least three independent experiments.

by the ELISA specific for murine CXCL16 (data not shown). The level of total induced CXCL16 protein was not influenced by the absence or presence of ADAM10 (data not shown). Quantification

of shed CXCL16 in relation to the total protein revealed that the ADAM10$^{-/-}$ cells released less CXCL16 (18%) compared with the wild-type cells (40%, Fig. 3*C*). Moreover, the CXCL16 shedding was restored in the cells retransfected with ADAM10 (52% shed CXCL16). As expected, the ADAM10 inhibitor GI254023X (3 μM) markedly reduced CXCL16 cleavage in the wild type and the ADAM10 retransfected cells, but not in the ADAM10$^{-/-}$ cells (Fig. 3*C*), confirming that the inhibition of CXCL16 shedding in wild-type fibroblasts by the inhibitor is indeed due to inhibition of ADAM10. Notably, in the presence of the broad spectrum inhibitor batimastat (BB94) or GW280264X (both 3 μM), the residual CXCL16 release from ADAM10$^{-/-}$ cells was almost completely reduced (data not shown) suggesting that other metalloproteinases contribute to the residual CXCL16 cleavage in ADAM10$^{-/-}$ cells. To investigate the possibility that stimulation with IFN-γ and TNF-α might have altered the expression or the activity of endogenous ADAM10 in murine fibroblasts, the cells were transfected with human CXCL16 and subsequently stimulated with a combination of the two cytokines (both 20 ng/ml) for 24 h. As expected, stimulation with IFN-γ and TNF-α increased the expression of murine CXCL16 in the conditioned medium as well as in the cell lysate and did not interfere with the expression of transfected human CXCL16 (Table II). Because the percentage of shed human CXCL16 was not modulated by the two cytokines (Table II) ADAM10 does not appear to be regulated by IFN-γ and TNF-α.

Enhancement of CXCL16 release by PMA does not involve ADAM10

As described for other substrates of disintegrin-like metalloproteinases including TNF-α, L-selectin, IL-6R, and CX3CL1, shedding can be rapidly enhanced by cell stimulation with PMA (16, 21, 25). To investigate whether PMA would have a similar effect on CXCL16 shedding, CXCL16-transfected COS-7 cells were stimulated with PMA (200 ng/ml) and subsequently assayed for the release of soluble CXCL16. One-hour treatment with PMA was sufficient to increase the CXCL16 release. This PMA-induced increase was maximal after 2 h and not further enhanced during longer treatment (Fig. 4*A*). Because this PMA response is very rapid it does not seem to be due to enhanced biosynthesis of CXCL16 or metalloproteinases. Indeed, the increased release of CXCL16 during 2 h of treatment with PMA was associated with an equivalent reduction of the protein concentration in the cell lysate (Fig. 4*B*), showing that PMA did not alter the amount of totally expressed CXCL16 (shed plus cell-associated molecules). Western blot analysis of the 50-fold concentrated conditioned media revealed that stimulation of CXCL16-transfected COS-7 cells with PMA for 2 h enhanced the release of 32-kDa CXCL16 protein and did not generate additional CXCL16 variants (Fig. 4*C*). To investigate the role of metalloproteinases in the PMA-induced increase in CXCL16 release, CXCL16-transfected COS-7 cells were pretreated with different metalloproteinase inhibitors and subsequently stimulated with PMA for 2 h (Fig. 4*D*). The broad spectrum inhibitor batimastat (BB94) and the combined ADAM17/ADAM10 inhibitor (GW280264X) both blocked both the constitutive and the PMA-induced shedding. The preferential ADAM10 inhibitor (GI254023X) also reduced the constitutive CXCL16 release; however, the PMA-induced increase of CXCL16 shedding in the inhibitor-treated cells remained similar to that in the untreated cells. The latter finding may indicate that ADAM10 does not contribute to the PMA-inducible shedding. Indeed, when CXCL16 transfected ADAM10$^{-/-}$ MEFs were assayed for their response to PMA, CXCL16 shedding was increased upon PMA stimulation and this increase was even more pronounced than that found in ADAM10$^{-/-}$ MEFs retransfected with ADAM10 (Fig.

Table I. *Expression and release of human CXCL16 in ADAM10$^{-/-}$ and ADAM10$^{+/+}$ MEFs*

	Soluble CXCL16 (pM)		Cell-Associated CXCL16 (pM)		Total CXCL16 (fmol/dish)[a]		Shed CXCL16 (% of total)[b]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ADAM10$^{+/+c}$	310[d]	11	271	12	581	19	51.2	1.0
ADAM10$^{-/-}$	140[e]	21	928[e]	85	1068[e]	102	13.5[e]	0.6

[a] The amount of total CXCL16 molecules per dish was calculated as the soluble plus cell-associated CXCL16.
[b] Shed CXCL16 was expressed as percent soluble of the total CXCL16.
[c] ADAM10$^{+/+}$ and ADAM10$^{-/-}$ MEFs (150,000 cells per dish) were transfected with human CXCL16 for 48 h, followed by 4 h incubation in serum-free medium. Subsequently, the concentration of soluble and cell-associated CXCL16 was determined in the media and cell lysates (both 0.75 ml per dish) by ELISA.
[d] Data are given as mean and SD of one representative of three experiments each performed in triplicates.
[e] Statistically significant differences in CXCL16 shedding in ADAM10$^{+/+}$ vs ADAM10$^{-/-}$ cells ($p < 0.05$).

4E). In summary, our data provide several lines of evidence that CXCL16 shedding predominantly involves ADAM10. By contrast, ADAM10 does not seem to be responsible for the increase in CXCL16 shedding seen in response to PMA.

CXCL16 is expressed and shed by cytokine-stimulated vascular cells

Because the other transmembrane chemokine CX3CL1 is profoundly up-regulated in cultured ECs and vascular SMCs by costimulation with the proinflammatory cytokines IFN-γ and TNF-α (8, 26), we investigated these cells for the expression of CXCL16 mRNA by quantitative TaqMan RT-PCR. To study possible upregulation of the chemokine, cells were stimulated with IFN-γ or TNF-α or a combination of both. In nonstimulated ECs and SMCs, the level of CXCL16 mRNA was very low and stimulation for 24 h with either TNF-α or IFN-γ alone led to a slight up-regulation (Fig. 5, *A* and *B*). However, CXCL16 mRNA was profoundly increased in cells costimulated with IFN-γ and TNF-α. By ELISA, only minute amounts of CXCL16 protein could be detected in the cell lysates and conditioned media of nonstimulated ECs and SMCs. However, after 24 h of stimulation with IFN-γ and TNF-α, CXCL16 protein was profoundly up-regulated (Fig. 5*C*). In ECs, even more CXCL16 was released than remained associated with the cells. Because IL-4 is a possible negative regulator for IFN-γ- or TNF-α-induced responses in ECs such as the induction of CX3CL1 (26), we next stimulated ECs with IFN-γ/TNF-α (both 10 ng/ml) for 24 h in the presence or absence of IL-4 (20 ng/ml). As described for CX3CL1, the generation of soluble and cell-associated CXCLl6 in IFN-γ/TNF-α-stimulated cells was markedly suppressed by IL-4 (Fig. 5*D*).

We then addressed the question of whether ADAM10 protein would be present in ECs and could therefore be a candidate en-

zyme for CXCL16 cleavage. Indeed, as seen by Western blotting with an Ab against human ADAM10, two immunoreactive proteins of 85 and 64 kDa are expressed in unstimulated as well as in IFN-γ/TNF-α-stimulated ECs matching the size of the immature and the processed form of ADAM10 (Fig. 6*A*). Inhibition experiments were then performed with the mixed ADAM10/ADAM17 inhibitor (GW280264X) and the preferential ADAM10 inhibitor (GI254023X). As shown in Fig. 6*B*, both inhibitors profoundly reduced the release of CXCL16 from IFN-γ/TNF-α-stimulated ECs. The inhibition was concentration dependent and very similar for both inhibitors suggesting that they are both equally capable of blocking a protease activity that is involved in the constitutive cleavage of CXCL16 in IFN-γ/TNF-α-stimulated ECs and very likely represents ADAM10 but not ADAM17. We then studied the up-regulation of CXCL16 on the surface of IFN-γ/TNF-α-stimulated ECs by flow cytometry. As expected, the inhibition of constitutive CXCL16 shedding by GI254023X or GW280264X further increased the surface expression of the transmembrane chemokine (Fig. 6, *C* and *D*). In addition, the ADAM10/ADAM17 inhibitor GW280264X fully prevented the down-regulation of CXCL16 surface expression in response to PMA (Fig. 6*D*), whereas the ADAM10 inhibitor GI254023X had no effect (data not shown), suggesting that the metalloproteinase involved in the PMA-inducible shedding is not ADAM10 and may represent ADAM17.

Discussion

The recently discovered CXC-chemokine CXCL16 is one of the two known chemokines that are linked to the plasma membrane via a mucin-like stalk and a transmembrane domain. It is found as a membrane-bound and soluble form with completely different biological functions. Although soluble CXCL16 functions as a

Table II. *Effect of IFN-γ and TNF-α on the shedding of transfected human CXCL16 in MEFs*

	Transfected Human CXCL16[a]						Endogenous Murine CXCL16[b]					
	Soluble (pM)		Cell-associated (pM)		Shed (% of total)[c]		Soluble (pM)		Cell-associated (pMI)		Shed (% of total)[c]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
None	203[c]	21.6	152.4	7.6	57.0[d]	1.4	2.9	0.1	3.3	0.1	46.4	1.3
IFN-γ/TNF-α	186.2	11.9	139.2	1.9	57.1[d]	1.5	11.5	1.3	11.6	1	49.9	1.9

[a] ADAM10$^{+/+}$ MEFs (150,000 cells per dish) were transfected with human CXCL16 for 24 h, followed by 24 h incubation in the presence or absence of IFN-γ and TNF-α (both 20 ng/ml), thereafter, cells were incubated for 4 h in serum-free medium and the concentration of soluble and cell-associated human CXCL16 was determined as explained in Table I.
[b] As a control, the up-regulation of endogenous murine CXCL16 by IFN-γ and TNF-α was determined in parallel.
[c] The amount of total CXCL16 molecules per dish was calculated as the soluble plus cell-associated CXCL16 and shed CXCL16 was expressed as percent soluble of the total CXCL16.
[d] The shedding of human CXCL16 in untreated and IFN-γ/TNF-α-stimulated MEFs was not significantly different ($p < 0.01$).

classical chemoattractant, the transmembrane molecule promotes binding and adhesion of lymphocytes, oxidized LDL particles, or bacteria (5, 6, 9, 27). It is therefore of immediate interest to understand how the two forms are generated and whether their expression is differentially regulated. In this report, we present in vivo and in vitro evidence for the expression of CXCL16 in vascular cells. We demonstrate that IFN-γ and TNF-α synergize to induce CXCL16 expression in vascular cells and that these cells spontaneously release considerable amounts of CXCL16. We provide evidence that soluble CXCL16 is generated by cleavage of the transmembrane molecule via metalloproteinase activity. By transfection of exogenous CXCL16 in different cell lines as well as by up-regulation of endogenous CXCL16 in murine fibroblasts with IFN-γ and TNF-α, we show that this cleavage regulates the cell surface expression of the transmembrane molecule and that the majority of the cleavage is mediated by the disintegrin-like metalloproteinase ADAM10. This report demonstrates that CXCL16 is a novel substrate for ADAM10-mediated ectodomain shedding and therefore implies a role of ADAM10 in the regulation of this chemokine's biological activity.

In the present study, we provide the first piece of direct evidence that the soluble 32-kDa variant of murine CXCL16 is generated from a 48-kDa transmembrane molecule by a metalloproteinase-mediated cleavage event. We demonstrate that the release of soluble 32-kDa CXCL16 is markedly reduced in the presence of metalloproteinase inhibitors leading to profound accumulation of the 48-kDa molecule in the cell lysates. The size of released murine CXCL16 estimated in our study matches quite well with the data reported by Matloubian et al. (5) (34 kDa), considering that the latter authors fused the murine CXCL16 with an additional N-terminal flag tag. The molecular mass is almost twice the size of the predicted protein backbone (19 kDa) which is due to heavy glycosylation of the soluble molecule as demonstrated by Wilbanks et al. (9). Notably, besides a 48-kDa protein we have found additional CXCL16 molecules of reduced molecular mass in the cell lysates. Similar observations were made by Matloubian et al. (5) and Shimaoka et al. (6), reporting the identification of cell-associated CXCL16 variants of 34 kDa (murine CXCL16) or 30 and 27 kDa (human CXCL16), respectively, which could represent the complete CXCL16 protein, but bearing different glycosylation. Different cell types and protocols used for expression could account for such a differential glycosylation. In fact, tissue-specific variations in the molecular size of CXCL16 proteins expressed in spleen and lymph node have been observed (5). However, the existence of a CXCL16-specific 16 kDa-protein band in the cell lysates, as seen in our present study, cannot be explained by the differential glycosylation. This band could rather represent the
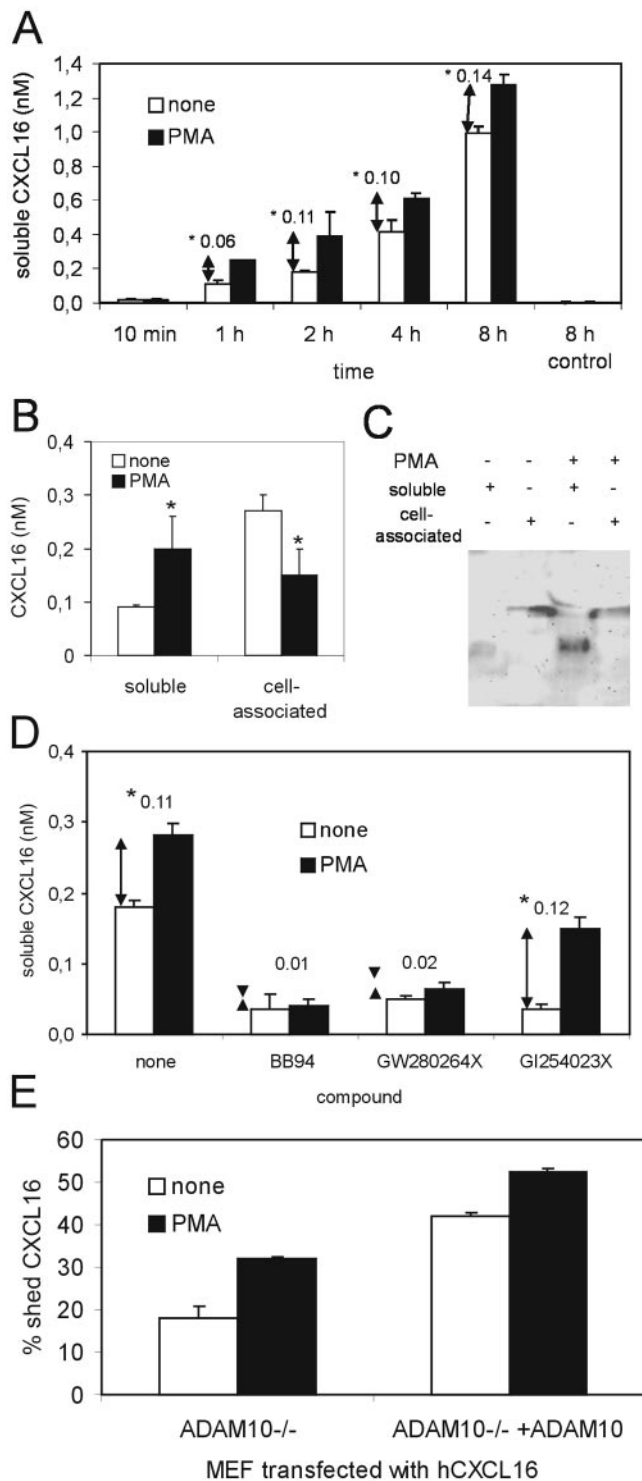
FIGURE 4. Effect of PMA on CXCL16 shedding. *A*, COS-7 cells were transiently transfected with human CXCL16 and subsequently incubated in serum-free medium in the absence or presence of PMA (200 ng/ml) for various periods of time and shed CXCL16 was quantified by ELISA. As a control, mock-transfected COS-7 cells were stimulated with PMA for 8 h which did not lead to the expression of endogenous CXCL16. In *A* and *D*, the increase of soluble CXCL16 in response to PMA is indicated by arrows and given as concentration value in nanomoles per liter. *B*, CXCL16-expressing COS-7 cells were stimulated with PMA for 2 h or left nonstimulated. Subsequently, cell-associated CXCL16 in the cell lysates and released CXCL16 in the conditioned media (both 0.75 ml per dish) were quantified by ELISA. *C*, Cell-associated and released CXCL16 from CXCL16-expressing COS-7 cells that were left unstimulated or treated with PMA for 2 h were analyzed by Western blotting with an anti-CXCL16 Ab. *D*,

CXCL16 expressing COS-7 cells were treated with the metalloproteinase inhibitors batimastat (BB94), GW280264X, and GI254023X and subsequently stimulated with PMA for 2 h or left nonstimulated. Released CXCL16 in the conditioned media was quantified by ELISA. ADAM10$^{+/+}$, ADAM10$^{-/-}$, and ADAM10-retransfected ADAM10$^{-/-}$ MEFs were transiently transfected with human CXCL16 and subsequently incubated in serum-free medium in the presence or absence of PMA (200 ng/ml) for 4 h. To normalize variations in the transfection efficiency for each single transfection of the different MEF cell lines, CXCL16 shedding was expressed as the percentage of soluble CXCL16 in the media in relation to the total CXCL16 including soluble and cell-associated molecules. Data shown in *A*, *B*, *D*, and *E*, represent means and SD of one experiment performed in triplicates. All results were reproduced at least three times. Statistically significant differences in CXCL16 shedding between untreated and PMA-treated cells are indicated by asterisks ($p < 0.05$).

FIGURE 5. Induction of CXCL16 mRNA and protein in vascular cells. Isolated human ECs from umbilical vein (*A*) and serum-starved human SMCs from aorta (*B*) were stimulated for 24 h with either increasing concentrations of IFN-γ or TNF-α alone or a combination of 10 ng/ml IFN-γ and increasing concentrations of TNF-α. CXCL16 mRNA expression was quantified by TaqMan RT-PCR and expressed as the percentage of that determined for GAPDH. *C*, ECs and serum-starved SMCs were stimulated with a combination of IFN-γ and TNF-α (each 10 ng/ml) for 24 h or left unstimulated. CXCL16 protein expression in the conditioned media and cell lysates was determined by the ELISA. Statistically significant differences in CXCL16 protein expression between untreated and cytokine-treated cells are indicated by asterisks ($p < 0.05$). *D*, ECs were left unstimulated or stimulated with a combination of IFN-γ and TNF-α (both 10 ng/ml) for 24 h in the presence or absence of IL-4 (20 ng/ml). Subsequently, soluble and cell-associated CXCL16 was quantified by ELISA. Statistically significant differences in CXCL16 protein expression induced by IL-4 are indicated by asterisks ($p < 0.05$).
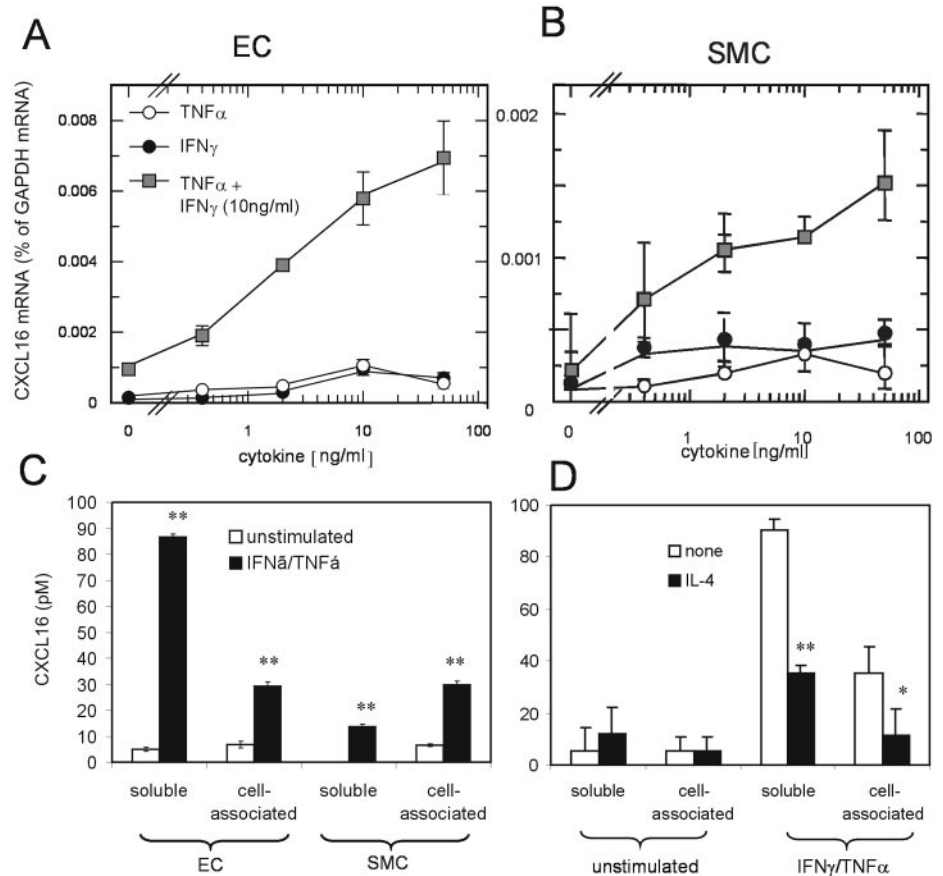
trunk of the cleaved protein residing in the cell membrane. In fact, its molecular mass exactly matches the difference in size between the major forms of soluble and cell-expressed CXCL16 and, moreover, it appears to be N-terminally truncated and C-terminally intact as it was detectable by its N-terminal HA tag but not via its C-terminal chemokine domain. However, compared with the full-size molecule this trunk was represented by only a faint protein band, suggesting that it has been subject to further degradation as it is reported for other substrates of ADAM10 such as the Notch or its ligand Delta1 (28) or the β-amyloid precursor protein (29).

Our findings indicate that ADAM10 mediates the major proportion of CXCL16 shedding under physiological conditions. The role of ADAM10 in CXCL16 cleavage was consistently demonstrated by different experimental approaches, by the use of specific inhibitors, with fibroblasts generated from mice with targeted disruption of the ADAM10 gene, and by retransfection of ADAM10. Inhibition studies were conducted with hydroxamate metalloproteinase inhibitors that had been characterized and validated for the inhibition of ADAM10 and ADAM17 (20). The release of CXCL16 was profoundly blocked by inhibition of ADAM10 and additional inhibition of ADAM17 did not modulate the effect. The ability of the compounds to block CXCL16 cleavage was the same in human ECs, murine fibroblasts, and ECV-304 and COS-7 cells, suggesting that the underlying mechanism is very similar. Nevertheless, our studies do not fully exclude any effects of the inhibitors on CXCL16 cleavage that are unrelated to ADAM10. Strong evidence for the involvement of ADAM10 in the cleavage of CXCL16 was provided by our finding that ADAM10-deficient MEFs display a considerably reduced potential to shed CXCL16 and that the loss in shedding activity was completely rescued upon retransfection of ADAM10. Even more importantly, shedding of endogenous CXCL16 in cyto-

kine-stimulated MEFs was profoundly reduced in the absence of endogenous ADAM10 and completely reconstituted by retransfection of ADAM10 cDNA providing strong evidence that ADAM10 is a physiological sheddase for CXCL16.

ADAM10 has been implicated in a number of shedding events that occur spontaneously in unstimulated cells. ADAM10 was found to cleave the amyloid precursor protein β-amyloid precursor protein (17), the cellular prion protein PrP (23), the Notch ligand Delta1 (28), the IL-6R (24), and the L1 adhesion molecule (30) under normal cell culture conditions. We have recently demonstrated that ADAM10 is also involved in the spontaneous cleavage of CX3CL1 which is so far the only other known transmembrane chemokine besides CXCL16 (20). Notably, in our recent study and in our present work, deletion of ADAM10 in MEFs did not completely abrogate CX3CL1 or CXCL16 shedding. As shown here for CXCL16, the residual shedding in ADAM10$^{-/-}$ MEFs was sensitive to broad spectrum inhibitors of metalloproteinases but not to the preferential ADAM10 inhibitor, although all compounds were potent inhibitors of the constitutive cleavage in ADAM10$^{+/+}$ MEFs, COS-7 cells, and human ECs. This suggests that in the ADAM10-deficient MEFs, other as yet undefined metalloproteinases could partially compensate for the cleavage of CXCL16 and possibly also for that of other shedding substrates such as CX3CL1. For CX3CL1, others have demonstrated that PMA enhances the shedding and that this up-regulation is mediated to a large extent by the activity of ADAM17 (21, 22). Our observation that the combined ADAM17/ADAM10 inhibitor was capable of blocking the PMA-induced shedding in COS-7 cells may suggest that ADAM17 is also involved in the PMA-inducible cleavage of CXCL16. However, we did not observe enhanced CXCL16 shedding in PMA-stimulated ECV-304 cells (A. Ludwig, unpublished data) although these cells expressed ADAM17 and exhibited a
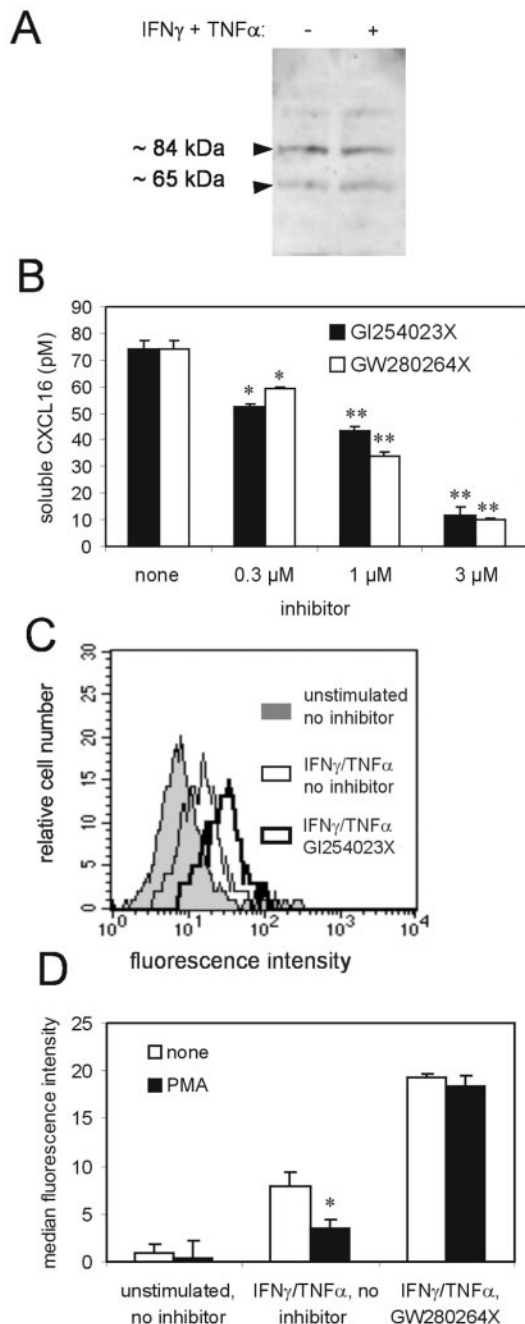
FIGURE 6. CXCL16 shedding by ECs. ECs were costimulated with IFN-γ and TNF-α (both 10 ng/ml) for 24 h or left unstimulated. *A*, SDS cell lysates were subjected to Western blot analysis with a rabbit anti-human ADAM10 Ab. *B*, Cytokine-stimulated ECs were incubated for 4 h in fresh medium in the absence or presence of increasing dosages of the metalloproteinase inhibitors GW280264X and GI254023X. The inhibition of the CXCL16 release by the inhibitors was quantified by ELISA. Statistically significant differences in CXCL16 shedding in the inhibitor-treated vs the untreated cells are indicated by asterisks (∗, $p < 0.05$; ∗∗, $p < 0.01$). *C* and *D*, Unstimulated or IFN-γ/TNF-α-stimulated ECs were treated with the metalloproteinase inhibitors GI254023X (*C*) or GW280264X (*D*) and incubated in the presence or absence of PMA for 4 h (*D*). Subsequently, CXCL16 surface expression was analyzed by flow cytometry using the rabbit anti-human CXCL16 Ab. Results are given as histograms (*C*) or calculated as MFI from which unspecific fluorescence (MFI = 7.1 ± 0.5, determined in parallel by labeling with an IgG control Ab) was subtracted (*D*). Statistically significant differences in CXCL16 shedding in response to PMA are indicated by asterisks ($p < 0.05$). Data in *A*, *B*, and *D* are given as mean and SD of one experiment performed in triplicates. All results shown are representative of three independent experiments.

clearly enhanced shedding activity for CX3CL1 (20). Therefore, despite major similarities in the cleavage mechanism of the two chemokines with respect to the involvement of ADAM10, the background of cell type-specific differences in the PMA-inducible cleavage of CXCL16 and CX3CL1 still remains to be resolved.

Not much is known about the functional role of CXCL16 under inflammatory conditions. To date, CXCL16 protein expression has been consistently demonstrated for APCs only (5, 6, 9, 31). In this study, we provide first evidence that the two proinflammatory cytokines TNF-α and IFN-γ synergistically induce CXCL16 in ECs, SMCs, and fibroblasts. Conversely, IL-4 down-regulates CXCL16 expression in EC. This pattern of induction is similar to that previously described for CX3CL1 in ECs (26) and in SMCs (8) which may indicate that both CX3CL1 and CXCL16 are induced in TH-1-dominated inflammatory responses controlled by master cytokines such as IFN-γ and TNF-α (26). Our data demonstrate that a considerable proportion of all CXCL16 expressed in ECs, SMCs, and fibroblasts is spontaneously released from these cells under normal culture conditions by a metalloproteinase activity. In fibroblasts, the most relevant metalloproteinase for CXCL16 shedding was identified as ADAM10. In ECs, ADAM10 could fulfil a similar function as suggested by the similar inhibition profile of the preferential ADAM10 inhibitor on CXCL16 shedding in fibroblasts and ECs. The shedding of CXCL16 is likely to have an impact on the chemokine's biological functions in the vascular tissue. During an inflammatory reaction in the vasculature the following situation can be envisaged: upon induction of CXCL16, the protein will first appear locally on the surface of vascular cells stimulated by inflammatory insult. Transmembrane CXCL16 would promote various adhesive events that have been described by a number of in vitro experiments such as binding of CXCR6-expressing leukocytes (13), scavenging ox-LDL (6) and mediating phagocytosis of bacteria (27). For the recruitment of leukocytes, CXCL16 expression would first be relevant in ECs where the molecule could capture CXCR6-expressing leukocytes in the blood flow. Due to continuous shedding of the chemokine, CXCL16 would not accumulate on the endothelium over time. Moreover, shedding of the molecule could facilitate detachment of cells bound via CXCL16, leading to more transient interaction similar to that we have previously reported for CX3CL1 (20). However, besides the endothelium, the underlying tissue consisting of SMCs, fibroblasts, and possibly macrophages would also express CXCL16 under inflammatory conditions as suggested by our present data and by previous work of others (6, 31). Underneath the endothelium, continuous shedding of the chemokine as well as enhanced shedding via yet undefined stimuli that act similar to PMA would lead to the accumulation of the soluble chemokine and the generation of a chemotactic gradient. Such a gradient could further direct the transmigrating leukocytes from the endothelium into the blood vessel wall and the underlying tissue. Thus, during leukocyte recruitment CXCL16 may initially function as an adhesion molecule on the endothelium and in a later phase rather as a T cell chemoattractant in the vascular tissue due to its shedding by ADAM10. Further studies are required to prove the importance of the role of CXCL16 in the vessel wall in health and disease.

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