A Disintegrin and Metalloproteinase 17 (ADAM17) Mediates Inflammation-induced Shedding of Syndecan-1 and -4 by Lung Epithelial Cells

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Syndecans are cell surface proteoglycans that bind and modulate various proinflammatory mediators and can be proteolytically shed from the cell surface. Within the lung, syndecan-1 and -4 are expressed as transmembrane proteins on epithelial cells and released in the bronchoalveolar fluid during inflammation. We here characterize the mechanism leading to the generation of soluble syndecan-1 and -4 in cultured epithelial cells and murine lung tissue. We show that the bladder carcinoma epithelial cell line ECV304, the lung epithelial cell line A459 and primary alveolar epithelial cells express and constitutively release syndecan-1 and -4. This release involves the activity of the disintegrin-like metalloproteinasen ADAM17 as demonstrated by use of specific inhibitors and lentivirally transduced shRNA. Stimulation of epithelial cells with PMA, thrombin, or proinflammatory cytokines (TNFa/IFNγ) led to the down-regulation of surface-expressed syndecan-1 and -4, which was associated with a significant increase of soluble syndecans and cell-associated cleavage fragments. The enhanced syndecan release was not related to gene induction of syndecans or ADAM17, but rather due to increased ADAM17 activity. Soluble syndecan-1 and -4 were also released into the bronchoalveolar fluid of mice. Treatment with TNFa/IFNγ increased ADAM17 activity and syndecan release in murine lungs. Both constitutive and induced syndecan shedding was prevented by the ADAM17 inhibitor. ADAM17 may therefore be an important regulator of syndecan functions on inflamed lung epithelium.

Syndecans are a family of cell surface proteoglycans that play regulatory roles in wound healing, inflammation, angiogenesis, and neuronal patterning. There are four members of the syndecan family (syndecan-1, -2, -3, and -4) each consisting of an ectodomain carrying heparan sulfate- or chondroitin sulfate-rich glucosaminoglycan chains, a transmembrane domain, and a short cytoplasmic tail (1). Syndecan-1 is predominantly found on endothelial and epithelial cells whereas syndecan-4 is ubiquitously expressed (2). Syndecans are also released as soluble variants that have been found in various body fluids including serum of cancer patients, wound fluid, or bronchoalveolar fluid of inflamed lungs (3–7).

Recent research with syndecan-1−/− and syndecan-4−/− mice has demonstrated that syndecans play an important role in the regulation of inflammation and wound healing (1). Syndecans act as coreceptors modulating binding and signaling of cytokines, chemokines, and adhesion molecules. Syndecan-1 deficiency results in increased acute lung inflammation. Syndecan-1 cleavage by matrix metalloproteinase 7 (MMP7) helps to establish a gradient for the chemokine KC guiding transepithelial migration of neutrophils into the airway (8). These activities can be partially reversed by soluble syndecans competing with transmembrane syndecans for their extracellular ligands (9).

Soluble syndecans are generated by proteolytic shedding at the cell surface (4, 10, 11). A basal shedding activity results in the constitutive release of syndecans by cultured cells. Cell stimulation with PMA, thrombin, or proinflammatory cytokines enhances the shedding (4, 12, 13). Matrix metalloproteinases including MMP7, MMP9 and MT-MMP1 were found to be capable of cleaving syndecans (8, 11, 12, 14). However, it remains unclear whether other members of the metalloproteinase family would contribute to syndecan shedding under physiological and pathophysiological conditions. Especially, a disintegrin and a metalloprotease 10 (ADAM10) and the closely related protease ADAM17 appear to be likely candidates for syndecan shedding because they are coexpressed with syndecans in various cell types including epithelial cells (15) and are responsible for constitutive or inducible shedding of several epithelial surface molecules including TNFa, transmembrane chemokines, E-cadherin, and junctional adhesion molecule A (16–19). Although it has been proposed that ADAM17 could be a physiologically relevant syndecan sheddase, its involvement in the release of soluble syndecan has not been directly studied.

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2 The abbreviations used are: MMP7, matrix metalloproteinase 7; ADAM, A Disintegrin and Metalllopeptiaanse; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; CTF, C-terminal cleavage fragment; TNF, tumor necrosis factor; IFN, interferon; DAPI, 4’,-6-diamidino-2-phenylindole; PMA, phorbol 12-myristate 13-acetate; POD, peroxidase.
Syndecan Shedding by ADAM17

We here characterize the shedding mechanism leading to the generation of soluble syndecan-1 and -4 by epithelial cells in vitro and in vivo. We demonstrate that epithelial cells shed syndecan-1 and -4 in a constitutive and inducible fashion. Pharmacologic and genetic evidence is provided that constitutive and induced shedding of both syndecans is critically mediated by ADAM17. Finally, we demonstrate that soluble syndecan-1 and -4 are released into the bronchoalveolar fluid of murine lungs treated with proinflammatory cytokines and that this release is effectively blocked by inhibition of ADAM17. ADAM17 may therefore be an important regulator of syndecan functions on inflamed lung epithelium.

MATERIALS AND METHODS

Recombinant Proteins, Antibodies, Fluorescent Dyes, and Inhibitors—Mouse monoclonal antibodies to syndecan-1 (DL-101, IgG1) and syndecan-4 (5G9, IgG2a) and goat polyclonal antibody to murine syndecan-4 (N-19) were from from Santa Cruz Biotechnology (Santa Cruz, CA). Human IFN-γ and human TNFα were from Peprotech (Hamburg, Germany). Murine IFNγ and murine TNFα, mouse monoclonal antibodies against human ADAM10 and ADAM17, respectively, mouse IgG2a, and IgG1 isotype controls, recombinant catalytic domain of ADAM17, and normal rabbit IgG were from R&D Systems (Wiesbaden, Germany). PE-conjugated or POD-conjugated secondary antibodies were from Jackson (Newmarket, UK). Mouse monoclonal antibody to β-actin was from Abcam (Cambridge, MA). The ELISA for mouse albumin was from Bethyl Laboratories (San Jose, CA). Human syndecans were detected by incubating conditioned media with mAb 281–2 (10 ng/ml, Sigma) and anti-syndecan-4 5G9 (0.6 μg/ml) or anti-syndecan-4 5G9 (0.6 μg/ml) after another 48 h of incubation. To transduce ECV304 cells, 1 × 10^5 cells were seeded into six wells and after 24 h, 20% of the culture medium was replaced by culture supernatant containing lentivirus. To enhance the efficiency of transduction polybren (8 μg/ml, Sigma) was added. After 48 h, the cells were transduced for a second time.

Syndecan Cleavage Assays—Cells were grown in 6-well dishes to 80–90% confluence in fully supplemented medium for 48 h. Cells were washed once with sterile PBS and received 1000 μl of serum-free medium with GI254023 (10 μM), GW280264 (10 μM) or dimethyl sulfoxide (DMSO). After 1 h, the cells were stimulated with PMA (100 ng/ml), trap-6 (5 μM), or TNFα/IFNγ (both 10 ng/ml), for the indicated periods of time. Digestion of cells with the recombinant ADAM17 catalytic domain (5 ng/ml) was performed in serum-free DMEM supplemented with antibiotics for 3 h at 37 °C. For analysis of shed syndecan, conditioned media were harvested, supplemented with a protease inhibitor mixture (Complete, Roche, Manheim Germany), cleared by high speed centrifugation, and analyzed by dot blotting (see below). For analysis of syndecan surface expression, cells were scraped off in 1 ml of ice-cold PBS and examined by flow cytometry (see below).

Dot Blotting for Syndecan-1 and -4—Conditioned media were diluted in blotting buffer (0.15 m NaCl buffered to pH 4.5 with 50 m sodium acetate, and with 0.1% Triton X-100), and applied to cationic polyvinylidene difluoride-based membrane (Hybond-N^+\textsuperscript{+}, Amersham Biosciences) under vacuum in an immunodot apparatus (Slot blot, Amersham Biosciences, Freiburg, Germany). By acidifying the samples in blotting buffer, only highly anionic molecules in the conditioned medium, such as proteoglycans, are retained by the cationic membrane. The membranes were washed twice with blotting buffer, blocked for 1 h with PBS supplemented with 0.5% bovine serum albumin, 3% nonfat dry milk, and 0.5% Tween 20 (all from Sigma). Human syndecans were detected by incubating membranes overnight at 4 °C with anti-syndecan-1 mAb DL-101 (0.6 μg/ml) or anti-syndecan-4 5G9 (0.6 μg/ml) followed by incubation with POD-coupled goat anti-mouse (diluted 1:20.000 in PBS-T). For murine syndecans, mAb 281–2 to murine syndecan-1 (1 μg/ml) and goat polyclonal antibody against murine syndecan-4 (1 μg/ml), followed by the appro-
appropriate POD-coupled goat anti-rat and rabbit anti-goat antibodies (diluted 1:20,000 in PBS-T), were used. After addition of chemiluminescence substrate (ECL advanced, Amersham Biosciences), signals were recorded and quantified using a luminescence image analyzer LAS3000 and Multi Gauge 3.0 software (Fujifilm, Tokyo, Japan).

**Western Blotting**—Samples were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions using 10% Tris-glycine gels. Proteins were transferred onto polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences) that were probed with normal rabbit IgG (0.5 μg/ml) followed by incubation with POD-coupled goat anti-rabbit IgG (see above). Equal loading and transfer of proteins to the membrane was verified by detection of β-actin using a specific monoclonal antibody.

**Flow Cytometric Analysis**—Cells were analyzed for syndecan expression by staining with anti-syndecan-1 mAb DL-101, anti-syndecan-4 5G9. Isotype controls for mouse IgG1 and mouse IgG2a, respectively, were used in parallel. Lentivirally transduced ECV304 cells were assessed for down-regulation of the respective target protein using mouse monoclonal antibodies against ADAM10 and ADAM17 (1 μg/ml and 5 μg/ml resp.) and a PE-conjugated anti-mouse antibody (1:10,000) (18). The fluorescence signal was then analyzed by flow cytometry (Guava EasyCye Mini, Guava Technologies).

**RT-PCR**—The mRNA expression levels of syndecan-1, syndecan-4, ADAM10, and ADAM17 were quantified by real-time PCR and normalized to the mRNA expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). RT-PCR analysis was performed as described (26). Briefly, RNA was extracted using RNeasy Kit (Qiagen) and quantified by spectrophotometry (NanoDrop, Peqlab, Erlangen, Germany). RNA (1 μg) was reverse transcribed using the RevertAid First Strand cDNA Synthesis kit (Fermentas, St Leon-Rot, Germany) in a 20-μl volume according to the manufacturers’ protocols. PCR reactions were then performed in triplicates of 20-μl volumes containing 1 μl of cDNA template, 10 μl of 2× LightCycler SYBR Green I Master mix (Roche), and 0.5 μM forward and reverse primer. The following primers were used: syndecan-1 forward, ggtgtgtcctgccagaag; syndecan-1 reverse tctgtgtggggagtgaag; syndecan-4 forward, tcgatccgagagactgaggt; syndecan-4 reverse, ccagatctccagacgcac; adam10 forward, tccagcctcgaccagaa; adam10 reverse, gcgtctcatgtgcacccatttg; adam17 forward, gaagtgccaggagcaaatg; adam17 reverse, cgggcactcactgctattacc, GAPDH forward, cagtcagccccagacatttg; GAPDH reverse, gagttcctgtaacctgg. All PCR reactions were run on a LightCycler 480 System (Roche) with 40 cycles of 10 s denaturation at 95 °C, followed by 20 s annealing at 55 °C and 15 s amplification at 72 °C. Standard curves were determined for each gene of interest with serial dilutions of its cDNA inserted in pcDNA3.1. Data were obtained as the threshold cycle CT value and calculated as the ΔCT value as follows: ΔCT = CTgene of interest − CTGAPDH.

**ADAM17 Activity Assay**—Lysates of cultured cell lines (70,000 cells) or lung tissue (3 mg) were prepared and analyzed for ADAM10/17 activity using a fluorogenic peptide-based assay kit from R&D Systems following the manufacturer’s instructions. The enzymatic activity was expressed in relation to a serially diluted standard of recombinant ADAM17 run in parallel.

**FIGURE 1. Release of syndecan-1 and -4 by epithelial cell lines.** A, serum-free medium from cultured ECV304 cells and A549 cells were harvested after the indicated time periods and blotted onto nitrocellulose at pH 4.5. Membranes were then probed with monoclonal antibodies to syndecan-1 or -4, respectively. B, ECV304 and A549 cells were incubated in the presence of the metalloproteinase inhibitors GI254023, GW280264, and TAPI-1 (10 μM each) or DMSO control (0.1%) for 16 h, and subsequently conditioned media were analyzed for the presence of syndecan-1 and -4 by dot blotting. A representative dot blot is shown. For statistical analysis, the signal intensities were quantified by densitometry and calculated as means and S.D. from three independent experiments. Asterisks indicate statistically significant differences (p < 0.05) compared with DMSO control. C, ECV304 and A549 cells were incubated in the absence or presence of GW280264 (10 μM), and subsequently cells were analyzed for surface expression of syndecan-1 and -4 by flow cytometry.
Animals—C57/BL 6 N female mice (20–25 g) with an age of 9–10 weeks were obtained from Janvier (Le Genest-Saint-Isle, France) and used as lung donors.

Intranasal Administration of Inhibitors in Mice and Bronchoalveolar Lavage—Mice were anesthetized with ketamin/xylazin (27 mg and 430 mg/kg body weight). Five minutes after anesthesia, mice were intranasally treated with GW280264, GI254023 (30 μg/kg each), or DMSO control (0.6%) in 50 μl of PBS. After 30 min, the mice were awake again, and no direct effect of the inhibitors on the mice was observed. After 4 h of inhibitor treatment, animals were sacrificed, and lungs were lavaged three times with a total volume of 1 ml of PBS.

Isolated Perfused Lungs—One hour after intranasal application of inhibitors, mice were anesthetized with nembutal (3.5 g of pentobarbital/kg BW). The preparation and perfusion (1 ml/min with non-recirculating RPMI 1640 medium supplemented with hydroxyethyl starch) of mouse lungs through the pulmonary artery were performed as described previously (27). Briefly, anesthetized animals were positioned in an open-tempered (37 °C) chamber and intubated. The abdomen and chest were opened to expose the heart and lungs. The left atrium and pulmonary artery were cannulized, and perfusion was started. Lungs (n = 3 per group) were then intratracheally challenged with TNFα/IFNγ (5 and 20 μg/kg, respectively, in 50 μl of PBS) or vehicle control. The lungs were perfused and ventilated for 4 h under baseline conditions with an end-inspiratory pressure of 8 cm H2O and an end-expiratory pressure of 3 cm H2O, resulting in a tidal volume of 200 μl as measured by numerical integration of airflow velocity. The lungs were then disconnected, the left lung was lavaged with 500 μl of PBS, and the lung tissue and the bronchoalveolar lavage (BAL) fluid were frozen immediately in liquid nitrogen and kept at −80 °C.

Statistics—Data were statistically analyzed using one-way analysis of variance (ANOVA) with post hoc analysis by Tukey’s honestly significant difference test.
variance and subsequent group comparisons. Where required, p values were adjusted by the Bonferroni procedure (GraphPad Prism 5.01, GraphPad Software, San Diego, CA).

RESULTS

Shedding of Syndecan-1 and -4 by Epithelial Cells Involves ADAM17—Syndecan-1 and -4 are both highly expressed on the surface of ECV304 cells (bladder carcinoma epithelial cell line) and A549 cells (alveolar lung cancer epithelial cell line). Dot blot analysis of culture supernatants revealed that both cell lines release increasing amounts of soluble syndecan-1 and -4 over time (Fig. 1A). In the presence of the dual ADAM17 and ADAM10 inhibitor GW280264 (16, 20), the release of both syndecans was profoundly reduced (Fig. 1B). Similar results were obtained with the commercially available metalloproteinase inhibitor TAPI-1. Moreover, the dual ADAM17 and MMP inhibitor TMI-1 as well as the selective ADAM17 inhibitor TMI-2 (21) reduced the production of soluble syndecan-1 and -4 (supplemental Fig. S1). By contrast, the inhibitor GI254023 blocking ADAM10 but not ADAM17 (16, 20) only had a minor effect. Flow cytometry analysis revealed that inhibition of syndecan release by GW280264 was associated with an increased surface expression of syndecan-1 and -4 (Fig. 1C). These findings suggest that ADAM17 could be involved in the release of syndecans by the epithelial cell lines.

To confirm that ADAM17 is capable of mediating syndecan cleavage, we added the recombinant catalytic domain of ADAM17 to ECV304 and A549 cells and found enhanced release of syndecan-1 and -4 (Fig. 2A). To further examine the role of ADAM17 in the generation of soluble syndecan, expression of ADAM10 and ADAM17 was silenced by lentivirally transduced shRNA. Down-regulation of ADAM10 and ADAM17 on the stably transduced ECV304 cells was confirmed by flow cytometry (Fig. 2B). Reduction of ADAM17 expression was associated with suppression of syndecan release, whereas silencing of ADAM10 had no effect (Fig. 2C). These results show that endogenous ADAM17 is critical for the generation of soluble syndecan-1 and -4.

Inducible Shedding of Syndecan-1 and -4 Is Mediated by ADAM17—Cell stimulation with PMA, thrombin, or proinflammatory cytokines has been reported to induce the release of syndecans (4, 12, 13). In line with this, stimulation of epithelial cells with PMA or the thrombin receptor agonist trap6 leads to a rapid release of soluble syndecans (Fig. 3A and supplemental Fig. S2), whereas the combination of TNFα and IFNγ required a longer stimulation period to induce an increase of soluble syndecans in the conditioned medium (Fig. 3A). The stimulation of syndecan release by PMA or TNFα/IFNγ was associated with a reduction of syndecan surface expression (Fig. 3B), suggesting that cell stimulation led to enhanced shedding of syndecan-1 and -4. Again this inducible syndecan shedding was effectively blocked by the metalloproteinase inhibitor TAPI-1. Moreover, specific down-regulation of ADAM17 led to reduced syndecan release, whereas silencing of ADAM10 had no effect (Fig. 5A).
To exclude the possibility that the induced syndecan release would be influenced by increased gene expression, syndecan-1 and syndecan-4 mRNA was quantified by RT-PCR analysis. These studies showed that cytokine stimulation for 24 h did not increase expression of syndecan-1 and -4 (Fig. 5B). Moreover, the cytokine-induced increase in proteolytic shedding was not associated with increased mRNA expression or surface expression of ADAM17 as demonstrated by RT-PCR analysis and flow cytometry (Fig. 5, B and C). An activity assay for ADAM17 using a fluorogenic peptide, however, revealed that cytokine stimulation of the epithelial cell lines led to increased activity of the protease (Fig. 5D). Therefore, enhanced syndecan shedding should be the result of increased ADAM17 activity induced by the proinflammatory cytokines.

Syndecan Shedding Generates a C-terminal Fragment—Syndecan shedding has been found to occur at a single site proximal to the cell membrane (10) and should not only release the N-terminal ectodomain but also generate a C-terminal cleavage fragment (CTF) containing the transmembrane domain. For other shedding substrates including notch, E-cadherin, CX3CL1, CXCL16, and CD44, CTFs have been found to undergo intramembranous cleavage via γ-secretase (23, 28–30). To study the generation and turn over of syndecan CTFs, we fused the C terminus of syndecan-1 and -4 to a 2Z-His tag which is readily detected via its high affinity interaction with the Fc part of IgG (23). Upon transfection of the tagged constructs, soluble syndecan-1 and -4 were released into the conditioned medium. This was increased by PMA and blocked by the inhibitor GW280264, as expected (Fig. 6A). Western blot analysis using rabbit IgG for detection of the tagged proteins revealed the presence of the three different protein bands in HEK293 cells transfected with syndecan-1–2Z-His, and two different protein bands in HEK293 cells transfected with the syndecan-4–2Z-His, but none of the proteins was detected in wt-HEK293 cells transfected with an empty control vector (Fig. 6A). The smallest protein bands of syndecan-1–2Z-His and syndecan-4–2Z-His migrated at ~110 kDa and slightly above. These proteins most likely represented cleavage products, consisting of the 17-kDa tag and syndecan CTFs. The generation of these fragments was considerably enhanced when shedding was induced by PMA, but reduced when shedding was blocked with GW280264 (Fig. 6A).

Treatment with the γ-secretase inhibitor DAPT had no effect on the release of soluble syndecans (Fig. 6B). Interestingly, the inhibitor led to a profound increase of the 25-kDa CTFs without affecting the expression of non-cleaved syndecan. However, when shedding of syndecans was blocked by inhibition of ADAM17 no such accumulation was seen upon treatment with the γ-secretase inhibitor. These findings suggest that ADAM17 is relevant for the release of syndecans and the generation of cellular cleavage fragments that undergo further proteolytic degradation potentially by γ-secretase.

Syndecan Shedding in the Lung—Because soluble variants of syndecan-1 and -4 have been found in the bronchoalveolar fluid (8), we questioned whether the generation of soluble syndecans in the lung would involve a mechanism similar to that found in cell lines. First, we examined primary human alveolar epithelial cells for the presence and shedding of syndecans. Release of syndecan-1 and -4 was blocked by combined inhibition of ADAM17 and ADAM10 but not by inhibition of ADAM10 only (Fig. 7A). As seen for the epithelial cell lines shedding of both syndecans by primary alveolar
epithelial cells was enhanced by PMA or proinflammatory cytokines and blocked by the ADAM10/17 inhibitor. Again inhibition of shedding was associated with increased surface expression whereas stimulated shedding was linked to decreased surface expression of syndecan-1 and -4 (supplemental Fig. S3).

To demonstrate syndecan shedding in vivo, mice were intranasally treated with either GW280264 or GI254023 and analyzed for the presence of soluble murine syndecan-1 and -4. In these experiments, the level of soluble syndecans was markedly reduced by the dual ADAM10/17 inhibitor GW280264 but not by the ADAM10 inhibitor GI254023, suggesting that syndecans in the lung undergo proteolytic shedding via ADAM17 (Fig. 7B). Moreover, when isolated, perfused mouse lungs were challenged by intratracheal administration of TNFα and IFNγ, levels of syndecan-1 and -4 in the bronchoalveolar fluid were profoundly increased. Again, this increase was suppressed by the ADAM10/17 inhibitor (Fig. 7C).

As seen for cultured epithelial cell lines, cytokine treatment of the murine lungs led to a marked increase of ADAM17 activity without affecting mRNA expression of the protease (Fig. 7D).

DISCUSSION

In the present study we have investigated the proteolytic release of soluble syndecan-1 and -4 ectodomains from epithelial cells. By pharmacologic inhibition as well as by transcriptional silencing we have identified ADAM17 as an important sheddase on epithelial cells responsible for the conversion of syndecan-1 and -4 into soluble ectodomains and cell-associated C-terminal trunk molecules. In particular, ADAM17 mediates not only constitutive shedding by epithelial cells but also shedding in response to inflammatory stimuli such as PMA, thrombin, or IFNγ/TNFα. Also in intact lungs consti-
tutive and stimulated release of syndecans was sensitive to ADAM17 inhibition. Collectively, these findings suggest a critical role of ADAM17 in the constitutive and regulated shedding of syndecan-1 and -4 in the alveolar space.

Because the discovery of soluble syndecans metalloproteinases has been implicated in the proteolytic release of soluble ectodomains from transmembrane syndecans. Syndecan shedding is effectively suppressed by the broad spectrum metalloproteinase inhibitor batimastat, which blocks the proteolytic activity of MMPs as well as ADAMs (31). To date, syndecan shedding activity has been mostly reported for members of the MMP family. MMP9 has been found to mediate chemokine-induced shedding of syndecan-1 and -4 by HeLa cells (12). Endogenous MT1-MMP has been implicated in syndecan-1 shedding by mammary fibroblasts (14) and overexpression of this protease enhances syndecan-1 shedding by HEK293T cells (11). MMP7-deficient mice were found to shed less syndecan-1 into the bronchoalveolar fluid of bleomycin-treated mice, and addition of recombinant MMP7 to cultured murine mammary gland cells or mouse myeloma cells increased syndecan-1 shedding (8). These data indicate that different MMPs are capable of shedding syndecans, which may depend on the cell type and stimulatory conditions. Apart from the observation that ADAM12 can interact with syndecans (32) the role of ADAMs as syndecan sheddases has not yet been investigated directly. Interestingly, TIMP-3 but not TIMP-1 was found to suppress syndecan shedding (11, 13). TIMP3 not only blocks a number of MMPs but also ADAM17, whereas TIMP-1 inhibits a number of MMPs and ADAM10 but not ADAM17 (33, 34). From these inhibition studies an involvement of ADAM17 appears possible. In fact, our study demonstrates that ADAM17 is the most relevant sheddase for syndecan-1 and -4 in epithelial cells. Because the inhibitor TMI-2, which blocks ADAM17 but not MMP7, MMP9, and MT1-MMP1 (21), is an effective inhibitor of syndecan shedding in epithelial cells a contribution of MMPs appears unlikely.

Shedding via ADAM17 has been reported for numerous other proteins, all belonging to the class of type 1 transmembrane molecules with a single transmembrane domain (19). ADAM17 mediates their shedding proximal to the cell membrane and in many cases and this requires no specific cleavage site (35). Several ADAM17-mediated cleavage events can be activated by PMA, ionomycin, chemokines, or cytokines (35). Whereas PMA-induced cleavage is a very rapid event, cleavage stimulated by IFN-γ and TNFα requires a longer time period. In a previous study we have reported that TNFα/IFN-γ-induced shedding of the junctional adhesion molecule JAM-A in endothelial cells does not require enhanced biosynthesis or surface expression of ADAM17, but rather involves an altered activity of the protease (18). Our present data indicate that syndecan shedding in epithe-

**FIGURE 6.** Detection of C-terminal cleavage fragments of syndecan-1 and -4. A, HEK293 cells were transfected with syndecan-1–2Z-His, syndecan-4–2Z-His, or transfected with control vector pcDNA3.1 (+) and treated with PMA (100 ng/ml) or DMSO for 2 h in the absence or presence of metalloproteinase inhibitors GI254023 or GW280264. Subsequently, conditioned media were analyzed for the presence of released syndecan ectodomains by dot blotting. The cell lysates were subjected to SDS-PAGE and Western blotting using rabbit IgG for detection of the 2Z-Tag. B, HEK293 cells expressing syndecan-1–2Z-His or syndecan-4–2Z-His were exposed to metalloproteinase inhibitors DMSO, GI254023, GW280264, or DAPT for 16 h. Subsequently, soluble and cell-associated syndecans were analyzed by dot blot analysis of the conditioned medium and Western blotting of the cell lysates, respectively. Asterisks indicate statistically significant differences compared with the control (p < 0.05).
Syndecan Shedding by ADAM17

A primary human alveolar epithelial cells were treated with PMA (100 ng/ml) or DMSO for 2 h or stimulated with IFNγ/TNFα (each 10 ng/ml) or vehicle control for 16 h in the presence or absence of metalloproteinase inhibitors (GI254023 and GW280264, 10 μM), and subsequently conditioned media were investigated for soluble syndecan-1 and -4. Data are shown as dot blot analysis of three independent experiments. B, mice (n = 3 for each group) were intranasally treated with metalloproteinase inhibitor GW280264 or vehicle control for 4 h, sacrificed, and analyzed for the presence of syndecan-1 and -4 in the lung fluid obtained from bronchoalveolar lavage (BAL). C, mice were intranasally treated with metalloproteinase inhibitor GW280264 or vehicle control for 1 h. Afterward, perfused lungs were treated intratracheally with a combination of IFNγ/TNFα (20 and 5 μg/kg, respectively) or vehicle control (n = 3 for each group) and ventilated for 4 h. Subsequently, BAL fluid was analyzed for released syndecan-1 and -4. Data are shown as dot blots and means and S.D. of densitometric measurement. Statistically significant effects induced by IFNγ/TNFα and the inhibitor are indicated by crosses and asterisks, respectively (p < 0.05). D, ex vivo perfused murine lungs (n = 3) were treated intratracheally with a combination of IFNγ/TNFα (20 and 5 μg/kg, respectively) or PBS and subsequently lung lysates were analyzed for ADAM17 activity using a fluorogenic substrate and for ADAM17 mRNA expression by RT-PCR, respectively. Asterisks indicate statistically significant differences (p < 0.05).

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