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# Shedding of Collagen XVII/BP180 in Skin Depends on Both ADAM10 and ADAM9*[S]

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**Collagen XVII is a transmembrane collagen and the major autoantigen of the autoimmune skin blistering disease bullous pemphigoid. Collagen XVII is proteolytically released from the membrane, and the pathogenic epitope harbors the cleavage site for its ectodomain shedding, suggesting that proteolysis has an important role in regulating the function of collagen XVII in skin homeostasis. Previous studies identified ADAMs 9, 10, and 17 as candidate collagen XVII sheddases and suggested that ADAM17 is a major sheddase. Here we show that ADAM17 only indirectly affects collagen XVII shedding and that ADAMs 9 and 10 are the most prominent collagen XVII sheddases in primary keratinocytes because (*a*) collagen XVII shedding was not stimulated by phorbol esters, known activators of ADAM17, (*b*) constitutive and calcium influx-stimulated shedding was sensitive to the ADAM10-selective inhibitor GI254023X and was strongly reduced in *Adam10*$^{-/-}$ cells, (*c*) there was a 55% decrease in constitutive collagen XVII ectodomain shedding from *Adam9*$^{-/-}$ keratinocytes, and (*d*) $H_2O_2$ enhanced ADAM9 expression and stimulated collagen XVII shedding in skin and keratinocytes of wild type mice but not of *Adam9*$^{-/-}$ mice. We conclude that ADAM9 and ADAM10 can both contribute to collagen XVII shedding in skin with an enhanced relative contribution of ADAM9 in the presence of reactive oxygen species. These results provide critical new insights into the identity and regulation of the major sheddases for collagen XVII in keratinocytes and skin and have implications for the treatment of blistering diseases of the skin.**

Collagen XVII (also called BP180 or BPAG2) is a hemidesmosomal adhesion component in the skin and mucosa and belongs to the emerging group of collagenous transmembrane proteins (1). This type II oriented transmembrane protein is involved in the molecular pathology of human skin diseases. Mutations in the *COL17A1* gene are associated with junctional epidermolysis bullosa, a genetic skin blistering disease (2). Patients with bullous pemphigoid and related autoimmune bullous dermatoses have tissue-bound and circulating autoantibodies targeting collagen XVII (3). Structural and functional changes of collagen XVII play an important role in these diseases, although the molecular pathology is not yet fully understood. The collagen XVII consists of three 180-kDa $\alpha$1 (XVII) chains, each with an intracellular N-terminal domain, a short transmembrane stretch, and a flexible extracellular C-terminal ectodomain with collagenous (Col)[2] subdomains that are interrupted by short non-collagenous (NC) sequences. The human and murine collagen XVII molecules differ in size and in the number of the Col and NC domains. Human collagen XVII consists of 1497 amino acid residues with 15 Col and 16 NC domains, whereas the murine form, which is 86% identical (4), consists of 1433 amino acid residues with 13 Col and 14 NC domains. In humans the extracellular linker domain NC16A between the plasma membrane and the Col15 domain is functionally important because it is believed to play a role in both ectodomain shedding and in the proper folding of the triple helical structure of collagen XVII (5–7).

Our previous studies revealed two forms of collagen XVII, the 180-kDa membrane-anchored form and the soluble 120-kDa form. The latter represents the extracellular collagenous ectodomain, which is released by cleavage by membrane-anchored metalloproteinases of the a disintegrin and metalloproteinase (ADAM) family (8). The shed ectodomain of collagen XVII is very stable *in vivo* and *in vitro*. In wound scratch assays, both addition of the purified soluble ectodomain or overexpression of ADAMs suppressed cell motility (8), indicating that the ectodomain has a role in regulating keratinocyte-matrix interactions. In the context of the known functions of collagen XVII as an adhesion molecule, its shedding could therefore regulate its functions in keratinocyte migration, differentiation, and proliferation.

ADAMs are also involved in the release of several other type I or type II transmembrane proteins and are considered to be critical regulators of epidermal growth factor receptor signaling, tumor necrosis factor $\alpha$ release, and Notch signaling to name a few examples (9, 10). Previously ADAM9, ADAM10, and ADAM17 had been identified as potential sheddases for

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[2] The abbreviations used are: Col, collagenous; NC, non-collagenous; ADAM, a disintegrin and metalloproteinase; AP, alkaline phosphatase; IM, ionomycin; mEF, mouse embryonic fibroblast; E, embryonic day; GI, GI254023X; PMA, phorbol 12-myristate 13-acetate.

collagen XVII in keratinocytes by overexpression in cell-based assays (8). Moreover *Adam17*$^{-/-}$ keratinocytes had 50% diminished collagen XVII shedding, which was interpreted to suggest that ADAM17 represents an important, if not the major, physiological collagen XVII sheddase (8). The major goal of the current study was to further explore the contribution of ADAM17 and other candidate sheddases to the release of collagen XVII from primary keratinocytes and mouse skin. The identification of the major collagen XVII sheddases and their regulation is critical for understanding the role of collagen XVII shedding in the pathogenesis of skin diseases.

## EXPERIMENTAL PROCEDURES

*Animals—Adam8*$^{-/-}$ (11), *Adam9*$^{-/-}$ (12), *Adam15*$^{-/-}$ (13), *Adam17*$^{-/-}$ mice (14), and wild type littermate controls were maintained in an accredited animal facility at the Hospital for Special Surgery according to the guidelines of the American Veterinary Association, and all experiments were approved by the Hospital for Special Surgery Institutional Animal Care and Use Committee.

*Cell Lines and Reagents*—The *Adam17*$^{-/-}$ fibroblast cell line (E2 cells) and the *Adam10*$^{-/-}$ fibroblast cell line were derived from E13.5 and E9.5 embryos, respectively (15, 16). Immortalized fibroblasts derived from *Adam9*$^{-/-}$, *Adam12*$^{-/-}$, and wild type E13.5 embryos were generated by transfecting primary mouse embryonic fibroblast (mEF) cells from the appropriate mouse lines (17) with a vector carrying the SV40 large T antigen. Wild type mEFs express ADAMs 9, 10, 12, 15, 17, and 19 (17). All cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and antibiotics. The ionophores monensin, valinomycin, and nystatin were purchased from Sigma-Aldrich. Chloride ionophore 1 was purchased from Fluka, and ionomycin was obtained from Calbiochem. Batimastat (BB94) was kindly provided by Dr. D. Becherer (GlaxoSmithKline, Research Triangle Park, NC), and marimastat was provided by Dr. Ouathek Ouerfelli (Memorial Sloan-Kettering Cancer Center). The hydroxamate inhibitors GI254023X (GI) and GW280264X are described elsewhere (18) and were also provided by Dr. Becherer. The following protease inhibitors were used: 4-(2-aminoethyl)benzolsulfonylfluoride hydrochloride (Roche Applied Science) and 1,10-*ortho*-phenanthroline (Sigma-Aldrich). All other reagents were obtained from Sigma-Aldrich unless otherwise indicated.

*Expression Vectors*—The expression vector for alkaline phosphatase (AP)-tagged Kit ligand 2 has been described previously (19). The full-length cDNA for human collagen XVII was generated as described previously (20) and cloned into the NotI site of pcDNA3 (Invitrogen). To produce the C-terminal AP-tagged murine truncated collagen XVII construct, we generated a PCR fragment spanning nucleotides 294–2777 (amino acids 1–828) of murine collagen XVII, including the largest collagenous subdomain Col13 (GenBank™ accession number NM007732) and cloned it into pAPtag5 (Genhunter, Nashville, TN; see Fig. 1*B* for a diagram of the AP-tagged collagen XVII fragment). For amplification of the fragment we used the forward primer 3′-cgcgggctagccaccatggatgtgaccaagaaaagc-5′ and reverse primer 3′-cgcggaagcttctccgggcacagtgattgttga-5′ with *Pfu*Turbo DNA polymerase (Stratagene) and the Integrated Molecular Analysis of Genomes and their Expression (IMAGE) Consortium mouse cDNA clone 40086691 (Open Biosystems) as template. The proper insertion and the sequence of murine AP-collagen XVII construct (Col13-AP) was verified by DNA sequencing.

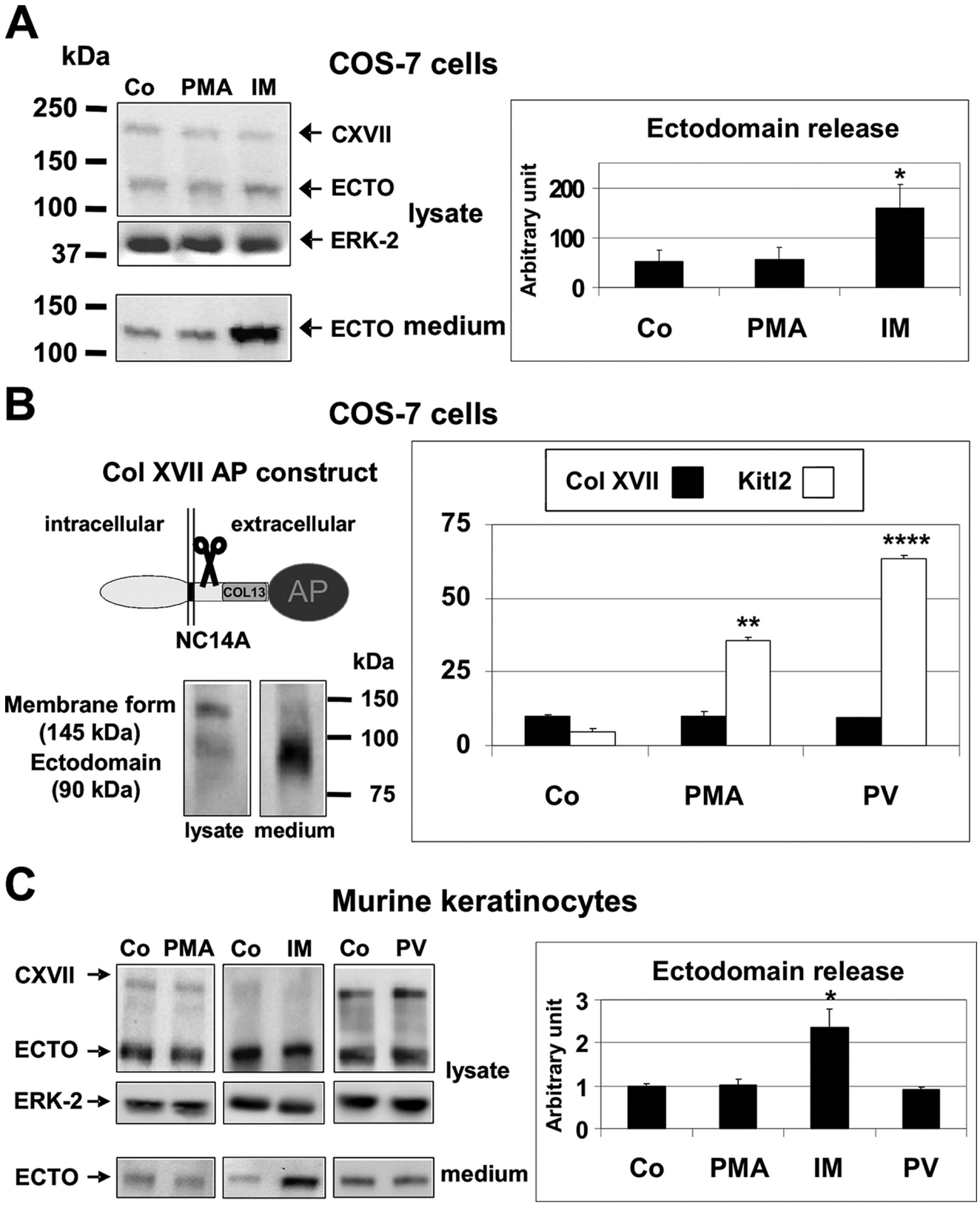
*mRNA Expression Analysis*—For real time PCR, total RNA from primary wild type and *Adam17*$^{-/-}$ keratinocyte cultures was extracted using an RNeasy Mini kit (Qiagen), and 1 $\mu$g of total RNA was reverse transcribed using a First Strand cDNA Synthesis kit (Fermentas). Relative quantification of gene expression was performed by real time PCR using iQ SYBR Green Supermix on the iCycler iQ thermal cycler (Bio-Rad) following the manufacturer's protocols. Primer sequences were as follows: mouse glyceraldehyde-3-phosphate dehydrogenase: sense primer, 5′-tggagaaacctgccaagtatg-3′; antisense primer, 5′-gttgaagtcgcaggagacaac-3′; mouse ADAM9: sense primer, 5′-tgaccatcccaacgtacaga-3′; antisense primer, 5′-ttccaaaactggcattctcc-3′; mouse ADAM10: sense primer, 5′-tctccggaatccgtaacatc-3′; antisense primer, 5′-tccaggaacttctccacacc-3′; mouse ADAM17: sense primer, 5′-cagcagcactccataaggaaa-3′; antisense primer, 5′-tttgtaaaagcgttcggta-3′; and mouse collagen XVII: sense primer, 5′-ctggattaggcaaggctgag-3′; antisense primer, 5′-cttgactccccatgtcacct-3′. Relative expression was normalized for levels of glyceraldehyde-3-phosphate dehydrogenase. The generation of amplification products of the correct size was confirmed using agarose gel electrophoresis.

*Cell Culture, Transfection, and AP Shedding Assay*—Immortalized fibroblast cell lines and COS-7 cells were seeded on 12-well plates at 75% confluency and transfected with 1.5 $\mu$g of DNA/well with the indicated plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations, and 50 $\mu$g/ml ascorbic acid was added to the media to allow for hydroxylation of collagen and proper triple helix formation. Fresh Opti-MEM (Invitrogen) medium with or without the indicated reagents was added the next day after transfection and incubated for the designated time period. The AP activity in 100 $\mu$l of supernatant and 10 $\mu$l of cell lysate was measured by colorimetry as described previously (17, 21, 22). The normalized percentage of shedding was calculated as ratio of supernatant AP activity divided by total AP activity (supernatant AP activity + cell lysate AP activity). All experiments were repeated at least three times with similar results.

For verification of the expression of the collagen XVII-AP fusion protein and its shedding in murine fibroblasts, the media and cell lysates were processed separately after the indicated times as described earlier (23) and analyzed by Western blot with murine collagen XVII NC14A rabbit antiserum (MO-NC14A (8)).

*Keratinocyte and Epidermis Sheet Preparation and Cultivation*—Keratinocytes were isolated from the skin of ADAM-deficient mice and their wild type littermates essentially as described previously (24, 25). Briefly the skin was washed with 70% ethanol and phosphate-buffered saline, and epidermis and dermis were detached by overnight digestion with 5 units/ml dispase II (Roche Applied Science) at 4 °C. The epidermis was mechanically separated from the dermis and incubated with 0.25% trypsin (w/v) and 2 mM EDTA for 30 min at 37 °C with vigorous shaking. After stopping the reaction with phosphate-buffered saline containing 10% fetal calf serum, the

keratinocyte suspension was passed through a 70-$\mu$m sieve, and $10^5$ cells/cm$^2$ were plated in defined serum-free keratinocyte medium supplemented with 100 pM cholera toxin, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, and 0.25 $\mu$g/ml amphotericin B (all from Invitrogen) on 6-well plates coated with gelatin (2.5 mg/ml; Sigma-Aldrich). The cells were maintained in defined serum-free keratinocyte medium at 37 °C, 5% $CO_2$, and 95% humidity, and medium was replaced every 48 h. Subcon-



fluent cells derived from passages 2–5 were used for the experiments, and all cells were cultured with medium containing 50 μg/ml ascorbate for 48 h to allow full prolyl and lysyl hydroxylation of newly synthesized collagens.

To isolate epidermis sheets from ADAM9-deficient mice and their wild type littermates, the skin was removed from euthanized 2–3-day-old pups, flattened on a Petri dish, and cut into epidermis sheets of equal size using an 8-mm biopsy punch. After 1-h digestion with dispase II at 37 °C, the epidermis was detached from the dermis with tweezers and transferred to a 12-well plate with serum-free keratinocyte growth medium. After a maximum of 2-h incubation at 37 °C, the epidermis sheets were used for the experiments outlined under "Results."

For ectodomain shedding analysis by immunoblotting, cell lysates and media were processed separately as described previously (23). Briefly the cells were washed twice with phosphate-buffered saline and lysed on ice for 30 min in lysis buffer (1% Nonidet P-40, 0.1 M NaCl, and 25 mM Tris-HCl, pH 7.4) containing 1 mM Pefabloc (Merck), 2 mM EDTA, 10 mM 1,10-*ortho*-phenanthroline, and 10 μl/ml protease inhibitor mixture set III (Calbiochem). Then the cell lysate was collected with a rubber cell scraper, centrifuged for 30 min at 13,000 × *g* at 4 °C, and then used immediately or stored at −80 °C. Total protein content was determined using the microtiter BCA™ Protein Assay kit (Pierce), and 30 μg of total protein/sample was used for SDS-PAGE. The medium was collected on ice, 1 mM Pefabloc and 2 mM EDTA were added immediately, and cell debris were then removed by centrifugation. Proteins were precipitated with chloroform-methanol and centrifuged, and the pellets were dissolved in Laemmli sample loading buffer containing 5 mM dithiothreitol and heated at 95 °C for 5 min.

*Preparation of Mouse Tissue Lysates*—Mouse tissues were dissected immediately after euthanasia (performed according to the guidelines of the American Veterinary Association; all animal experiments were approved by the Hospital for Special Surgery Internal Animal Care and Use Committee). We homogenized 0.5 g of each tissue (lung, liver, skeletal muscle, and skin) in 600 μl of lysis buffer consisting of 0.1 M Tris-HCl, pH 6.8, 1 M urea, 1% Nonidet P-40, 10 mM EDTA, 1 mM 4-(2-aminoethyl)benzolsulfonylfluoride hydrochloride, and proteinase inhibitor mixture (26) using a Polytron homogenizer for 3 min on ice (Kinematica, Littau, Switzerland). All lysates were centrifuged at 15,000 × *g* for 30 min to remove debris, and the supernatants were used for microtiter detergent-compatible colorimetric protein detection using the BCA Protein Assay kit (Pierce). Samples of equal protein content (30 μg) were mixed with 5-fold concentrated Laemmli buffer containing 50 mM dithiothreitol, heated at 95 °C for 5 min, and then analyzed by Western blotting (see below).

*Immunofluorescence Microscopy and Western Blot Analysis*—For immunofluorescence microscopy, cryosections of mouse skin were fixed in ice-cold acetone for 10 min, washed in Tris-buffered saline, and blocked with 10% normal goat serum in Tris-buffered saline for 30 min at room temperature. The polyclonal goat anti-ADAM9 antiserum (R&D Systems) was used as primary antibody, and fluorescein isothiocyanate-labeled AffiniPure donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as secondary antibody. Mounting medium supplemented with 4′,6-diamidino-2-phenylindole was purchased from Vector Laboratories (Burlingame, CA).

For immunoblotting, the proteins were separated by electrophoresis on 7 or 10% SDS-polyacrylamide gels as indicated. Immunoblotting was performed with rabbit polyclonal antiserum against the human collagen XVII NC16A domain (NC16A (27) and human collagen XVII cytodomain (Endo-2 (8)), murine collagen XVII NC14A domain (MO-NC14A (8)), murine ADAM17 cytoplasmic domain (28), and murine ADAM9 cytoplasmic domain (29). Anti-ADAM10 cytoplasmic domain antibodies were from R&D Systems. Immunoblot signals of collagen XVII ectodomain in the media of at least three independent experiments were analyzed and quantified with Quantity One software (Bio-Rad). All values are expressed as means ± S.D. Two populations of data were statistically analyzed using the unpaired two-tailed *t* test and considered significantly different at *p* values smaller than 0.05.

## RESULTS

*Collagen XVII Shedding Is Stimulated by Short Term Treatment with Ionomycin but Not PMA or Pervanadate*—ADAM10 and ADAM17 have different responses to treatment of cells with phorbol esters or calcium ionophores in cell-based assays. Low concentrations of PMA (20 ng/ml) predominantly activate ADAM17, whereas the calcium ionophore ionomycin stimulates both ADAMs 10 and 17 (30, 31). Because we had shown previously that ADAMs 9, 10, and 17 have the potential to shed collagen XVII and that these ADAMs were constitutively

FIGURE 1. **Collagen XVII shedding is stimulated by ionomycin but not by PMA or pervanadate.** *A*, COS-7 cells transfected with full-length human collagen XVII were treated with DMSO as vehicle control (*Co*) or with 20 ng/ml PMA or 2.5 μM IM for 25 min. Representative immunoblots of the 180-kDa full-length human collagen XVII (*CXVII*) and the 120-kDa shed ectodomain (*ECTO*) from lysates and media probed with the polyclonal rabbit anti-human NC16A antibody are shown. ERK-2 was used as lysate loading control. Collagen XVII shedding was significantly stimulated by ionomycin but not PMA. The graph summarizes the densitometric analysis of collagen XVII ectodomain shedding in three independent experiments (mean ± S.D.; *, $p < 0.05$). *B*, COS-7 cells were either transfected with a cDNA vector coding for an AP-tagged murine collagen (*Col*) XVII construct or an AP-tagged construct for Kit ligand 2 (*Kitl2*; Ref. 19), which was used as a representative substrate of ADAM17. The C-terminally truncated collagen XVII construct consists of its cytoplasmic domain, transmembrane domain, and the extracellular domains NC14A, Col13, and NC13, which were fused in-frame with a C-terminal AP tag. A Western blot probed with the MO-NC14A monoclonal antibodies on the *left* shows the 145-kDa membrane-bound form in the cell lysate and the shed 90-kDa form in the supernatant. Twenty-four hours after transfection the cells were washed, and fresh medium with DMSO vehicle control (*Co*), 20 ng/ml PMA, or 100 μM pervanadate (*PV*) was added. After 60 min, 100 μl of culture media and 10 μl of cell lysate were used for the AP assay. Treatment with either PMA or pervanadate failed to stimulate collagen XVII shedding in contrast to the significant stimulation of shedding of the ADAM17 substrate Kit ligand 2. Data are represented as mean ± S.D. ($n = 3$; **, $p < 0.01$; ****, $p < 0.0001$). *C*, murine keratinocytes were treated with DMSO vehicle as control (*Co*), 20 ng/ml PMA, or 100 μM pervanadate (*PV*) for 60 min or with 1 μM IM for 20 min, and the cell lysates and the concentrated supernatants were subjected to Western blot analysis with the polyclonal rabbit anti-mouse MO-NC14A antibody with ERK-2 levels serving as loading control. The graph summarizes the analysis of immunoblots of three independent experiments (one representative blot is shown) as the ratio of released ectodomain from treated cells *versus* control cells (mean ± S.D.). As in transfected COS-7 cells, shedding of endogenously expressed collagen XVII from murine keratinocytes was stimulated by ionomycin (*, $p < 0.05$) but not by PMA or pervanadate. *Error bars* indicate S.D.

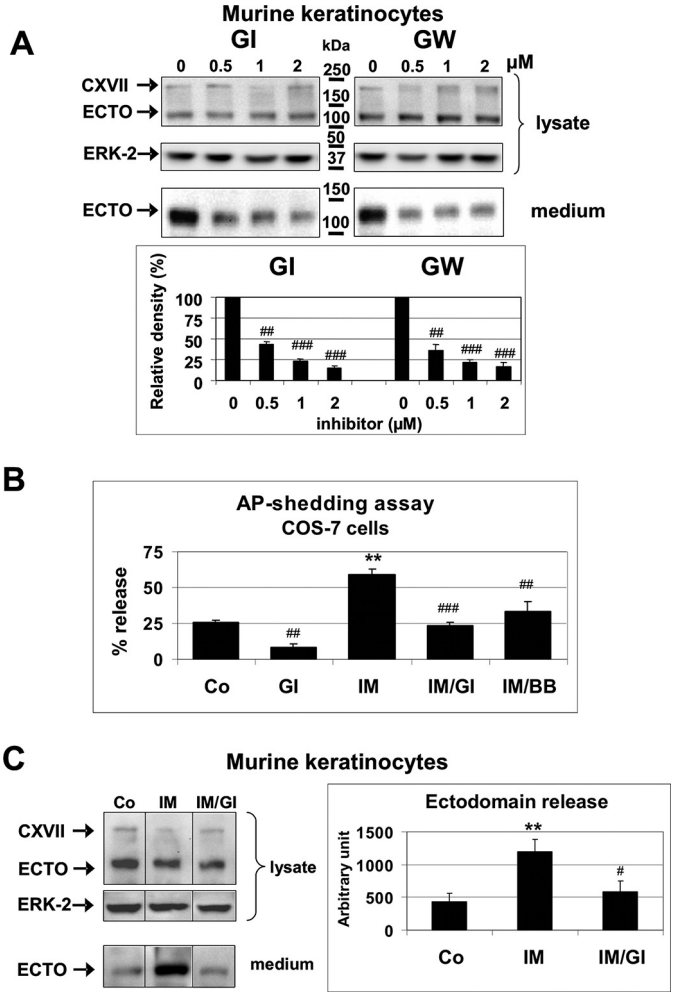



FIGURE 2. **Constitutive and ionomycin-stimulated collagen XVII shedding is strongly inhibited by the ADAM10-selective GI254023X.** *A*, inhibition of constitutive collagen XVII shedding from murine primary keratinocytes by increasing concentrations of the hydroxamate inhibitors GI and GW280264X (*GW*). Confluent murine keratinocytes were incubated with DMSO vehicle control or with 0.5, 1, and 2 $\mu$M inhibitor for 3 h in culture medium. Western blots of the cell lysates were probed for full-length collagen XVII (*CXVII*) and the ectodomain (*ECTO*) with a mixture of MO-NC14A and Endo-2 antibodies with ERK-2 serving as loading control. The released ectodomain (*ECTO*) in cell supernatants was visualized using the MO-NC14A antibody. Each immunoblot is representative of three independent experiments, the results of which are summarized as mean ± S.D. in the graph in the *lower panel* (##, $p < 0.01$; ###, $p < 0.001$). Collagen XVII shedding is strongly inhibited by 1 $\mu$M GI, a concentration that is selective for ADAM10 over ADAM17 (34, 35). *B*, the constitutive and stimulated shedding of murine AP-tagged collagen XVII in transfected COS-7 cells was analyzed by AP assay. The cells were incubated for 25 min without (*Co*) or with 2.5 $\mu$M IM and/or 1 $\mu$M GI or the non-selective hydroxamate BB94 (*BB*). Data represent the mean ± S.D. ($n = 3$). Both constitutive and stimulated shedding (**, $p < 0.01$) was efficiently inhibited by 1 $\mu$M GI (##, $p < 0.01$; ###, $p < 0.001$). *C*, inhibition of ionomycin-stimulated shedding of collagen XVII from murine keratinocytes with GI. Western blot analysis of cell lysates probed with both MO-NC14A and the Endo-2 antibody with ERK2 used as loading control and Western blots of concentrated supernatants probed with MO-NC14A after treatment with 1 $\mu$M ionomycin for 20 min revealed strong inhibition of shedding in the presence of 1 $\mu$M GI. The graph in the panel on the *right* summarizes the densitometric analysis of immunoblots of the released collagen XVII ectodomain from three different experiments (mean ± S.D.; **, $p < 0.01$; #, $p < 0.05$).

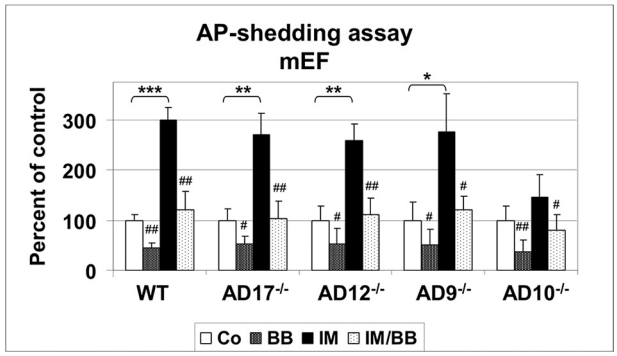
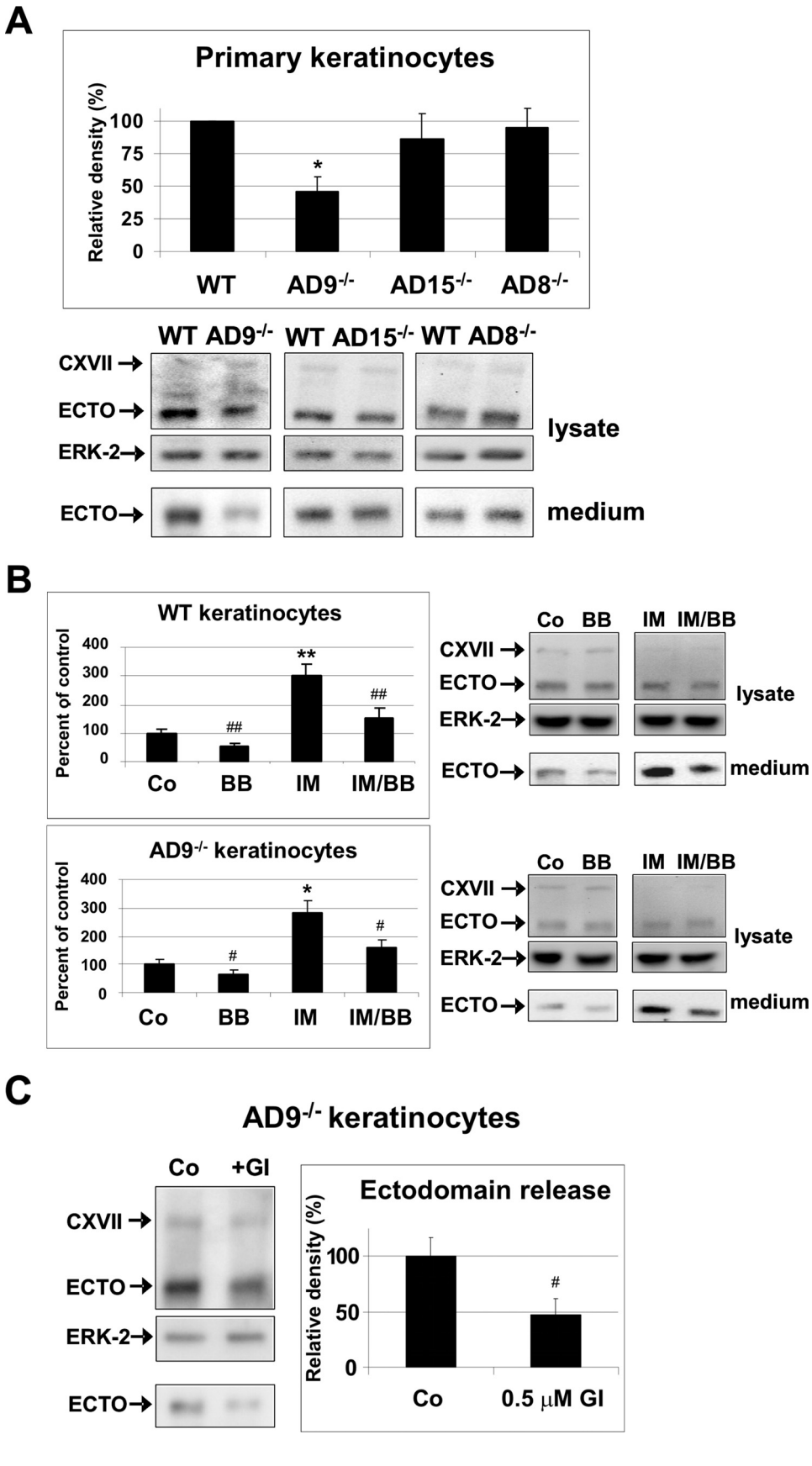



FIGURE 3. **Loss-of-function experiments demonstrate that ADAM10 is required for ionomycin-stimulated shedding of collagen XVII in mouse embryonic fibroblasts.** Immortalized wild type (*WT*) and ADAM (*AD*)-deficient mouse embryonic fibroblasts were transfected with a cDNA vector coding for AP-tagged murine collagen XVII. Twenty-four hours after transfection the cells were washed, and fresh medium with DMSO vehicle as control (*Co*), 1 $\mu$M batimastat (*BB*), 2.5 $\mu$M IM, or 2.5 $\mu$M ionomycin plus 1 $\mu$M batimastat (*IM/BB*) was added. After 30 min, 100 $\mu$l of culture media and 10 $\mu$l of cell lysate were used for the AP assay. The constitutive shedding activity of each cell line is set to 100% to be able to compare the relative increases in shedding upon stimulation with IM. Collagen XVII shedding was stimulated by ionomycin in *Adam9*$^{-/-}$, *-12*$^{-/-}$, *-17*$^{-/-}$, and wild type murine immortalized embryonal fibroblasts but not in *Adam10*$^{-/-}$ cells. Constitutive and stimulated shedding was strongly sensitive to batimastat. Data represent the mean ± S.D. ($n = 4$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; #, $p < 0.05$; ##, $p < 0.01$).

expressed in COS-7 cells (32), we analyzed the shedding of human full-length collagen XVII in response to PMA, pervanadate, and ionomycin in transiently transfected COS-7 cells. We observed no stimulation of shedding by 20 ng/ml PMA (Fig. 1*A*) or 100 $\mu$M pervanadate for 1 h (not shown), whereas treatment with 2.5 $\mu$M ionomycin strongly increased the release of the soluble collagen XVII ectodomain into the culture supernatant (Fig. 1*A*). For further analysis, a C-terminally truncated murine collagen XVII-alkaline phosphatase fusion protein (Col13-AP) was generated, and its expression and shedding were validated in transfected COS-7 cells. When shedding of the collagen XVII ectodomain-AP fusion protein was assessed by Western blot analysis, the 145-kDa transmembrane form was detected in the cell lysates, and the 90-kDa soluble form was detected in the culture supernatant (Fig. 1*B*). Stimulation with 20 ng/ml PMA or 100 $\mu$M pervanadate for 1 h did not increase the AP activity released into the supernatant, although both stimuli strongly enhanced shedding of the ADAM17 substrate Kit ligand 2 (19), which was used in parallel as a positive control in transiently transfected COS-7 cells to show that ADAM17 was activated under these conditions (Fig. 1*B*). Moreover shedding of endogenous collagen XVII from murine keratinocytes was also not stimulated by 20 ng/ml PMA or 100 $\mu$M pervanadate for 1 h (Fig. 1*C*). However, treatment of murine keratinocytes with 1 $\mu$M ionomycin for 20 min (Fig. 1*C*) resulted in strong stimulation of collagen XVII shedding as did stimulation with a 25 $\mu$M concentration of the calmodulin inhibitor trifluoroperazine (data not shown). Furthermore collagen XVII shedding from murine keratinocytes was only stimulated by incubation with ionomycin but not with ionophores for sodium (monensin; 20 $\mu$M), potassium (valinomycin; 1 $\mu$M), monovalent cations (nystatin; 20 $\mu$M), or chloride ions (chloride ionophore 1; 1 $\mu$M) (data not shown). The lack of stimulation by 1-h treatment with PMA or pervanadate in COS-7 cells and murine keratinocytes (see above) as well as in HaCaT keratinocytes and immortalized mEFs (data not shown) was not consistent with the hypothesis that ADAM17 is a major sheddase for collagen XVII as stimulation of shedding by these agents is a characteristic feature of ADAM17 (30, 31).

*Constitutive and Ionomycin-stimulated Shedding of Collagen XVII Is Strongly Inhibited by the ADAM10-selective Hydroxamate GI254023X*—Because ADAM10 is activated by the calcium ionophore ionomycin (IM) or the calmodulin inhibitor trifluoroperazine but not by PMA (30, 31, 33, 34) we further corroborated the involvement of ADAM10 in collagen XVII shedding by testing the inhibition of constitutive collagen XVII shedding in murine keratinocytes by GI, a hydroxamate inhibitor with selectivity for ADAM10 *versus* ADAM17 at 1 $\mu$M (34, 35). Moreover we used GW280264X, which has nearly identical inhibitory activity for ADAM17 and ADAM10 (18). As shown in Fig. 2*A*, both inhibitors blocked constitutive shedding of collagen XVII at 1 $\mu$M by about 80%. In addition, ionomycin-stimulated shedding of collagen XVII was also inhibited by 1 $\mu$M GI or the non-selective hydroxamate batimastat (BB94) in COS-7 cells transfected with murine collagen XVII-AP (Col13-AP) (Fig. 2*B*) and by 1 $\mu$M GI in primary murine keratinocytes (Fig. 2*C*) consistent with a major contribution of ADAM10 to constitutive and IM-stimulated shedding of collagen XVII.

*Loss of Function Experiments with ADAM-deficient Fibroblasts Corroborate That ADAM10 Is Required for Ionomycin-stimulated Shedding of Collagen XVII*—To evaluate the contribution of various ADAM proteases to collagen XVII shedding in cell-based assays, we transiently expressed murine collagen XVII-AP (Col13-AP) in immortalized mEFs from wild type mice or mice lacking one of the three potential collagen XVII sheddases (ADAM9, ADAM10, or ADAM17). As controls, we also included *Adam12*$^{-/-}$ mEFs in the analysis because we had seen higher collagen XVII shedding in COS-7 and HaCaT cells transfected with ADAM12 (data not shown) and because ADAM12 was recently reported to play a potentially inhibitory role in healing of chronic wounds (36). Analysis of the AP activity released into the superna-



tant of ADAM-deficient cells expressing collagen XVII showed a comparable ionomycin-stimulated shedding in wild type cells and in *Adam9*$^{-/-}$, *-12*$^{-/-}$, or *-17*$^{-/-}$cells, whereas *Adam10*$^{-/-}$ cells showed no significant ionomycin-stimulated shedding (Fig. 3*A*).

*Collagen XVII Shedding Is Strongly Diminished in Adam9*$^{-/-}$ *Keratinocytes*—The experiments using mouse embryonic fibroblasts described above allowed a side by side comparison of the response to activators of shedding in cells isolated from *Adam10*$^{-/-}$ mice and mice lacking other ADAMs. Because *Adam10*$^{-/-}$ mice die at E9.5 and thus too early to allow preparation of primary keratinocytes, the use of mEF cells from *Adam10*$^{-/-}$ mice thus represented the only currently feasible approach to perform loss-of-function experiments for ADAM10 in cell-based assays. However, it is important to note that it is difficult to compare levels of constitutive shedding in immortalized mEF cells because immortalization with SV40 large T antigen is likely to affect the activation state of these cell lines compared with primary mEF cells.

To analyze the effects of deletion of ADAM9 on constitutive shedding of endogenous collagen XVII, we prepared primary keratinocytes from *Adam8*$^{-/-}$, *-9*$^{-/-}$, or *-15*$^{-/-}$ mice and their wild type littermate controls. To avoid interindividual variation, at least four different individuals of each ADAM-deficient mouse strain and their wild type littermates were included in the analysis as described under "Experimental Procedures." Primary keratinocytes at passages 2–5 were cultured in defined serum-free keratinocyte medium until confluent, then washed twice with phosphate-buffered saline, and incubated for 5 h in fresh medium. The media and the cell lysates were analyzed by immunoblotting for full-length collagen XVII or the ectodomain, and shedding was quantified by densitometry. These experiments confirmed a strong reduction in collagen XVII shedding in *Adam9*$^{-/-}$ cells (46.0 ± 11.3%; $n = 5$) compared with wild type controls, whereas no significant change in shedding was observed in keratinocytes from *Adam8*$^{-/-}$ or *Adam15*$^{-/-}$ mice (Fig. 4*A*). However, similar to our results in *Adam9*$^{-/-}$ fibroblasts, the ionomycin-stimulated shedding of collagen XVII was not affected in *Adam9*$^{-/-}$ keratinocytes compared with controls, arguing against a role for ADAM9 in ionomycin-stimulated shedding (Fig. 4*B*).

*ADAM9 and ADAM10 Cooperate in the Constitutive Shedding of Collagen XVII*—To determine whether ADAM9 and ADAM10 cooperate in the constitutive shedding of collagen XVII, we incubated *Adam9*$^{-/-}$ keratinocytes with the ADAM10-selective inhibitor GI254023X to assess the contribution of ADAM10 to constitutive shedding in keratinocytes in the absence of ADAM9. We detected significant inhibition of collagen XVII shedding by 0.5 $\mu$M GI254023X in *Adam9*$^{-/-}$ keratinocytes, suggesting that ADAMs 9 and 10 cooperate in the constitutive shedding of collagen XVII (Fig. 4*C*). In agreement with this finding, constitutive shedding of collagen XVII from *Adam10*$^{-/-}$ mEF cells can also be further reduced by batimastat (see Fig. 3*A*), demonstrating that part of the remaining constitutive activity is also due to a metalloproteinase, presumably ADAM9.

*Knock-out of ADAM17 in Keratinocytes Does Not Alter Expression or Maturation of ADAMs 9 and 10*—To clarify the reason for the 50% diminished collagen XVII shedding in *Adam17*$^{-/-}$ keratinocytes, we analyzed the expression of ADAMs 9 and 10 at the mRNA and protein levels in *Adam17*$^{-/-}$ keratinocytes. Quantitative real time reverse transcription-PCR analysis of keratinocyte mRNA did not uncover significant differences in the expression of ADAM9, ADAM10, or collagen XVII (supplemental Fig. 1A).

Immunoblot analysis of ADAM9 and ADAM10 also did not uncover differences in their expression or maturation in *Adam17*$^{-/-}$ keratinocytes as determined by the appearance of the faster migrating mature form of either ADAM (supplemental Fig. 1B). Thus the cause for the reduced collagen XVII shedding in *Adam17*$^{-/-}$ keratinocytes remains to be determined. This result is in agreement with previous studies that failed to detect differences in the expression of ADAMs 9 and 10 in primary embryonic *Adam17*$^{-/-}$ fibroblasts (17).

*Collagen XVII Shedding Is Stimulated by Hydrogen Peroxide in Wild Type Cells but Not in Adam9*$^{-/-}$ *Keratinocytes*—Recent investigations have shown that oxidative stress induces ADAM9 expression in several human prostate cancer cell lines (37, 38). Therefore we transfected wild type mEFs with collagen XVII-AP and analyzed its shedding in semiconfluent (about 70%) cultures after 5.5 h of stimulation with increasing concentrations of $H_2O_2$ (100–500 $\mu$M). We observed a dose-dependent increase of collagen XVII ectodomain shedding with a 1.8-fold induction at 500 $\mu$M $H_2O_2$ (Fig. 5*A*). When collagen XVII-AP shedding was analyzed in *Adam9*$^{-/-}$ mEFs under identical conditions, the addition of $H_2O_2$ did not stimulate collagen XVII shedding. To assess the involvement of ADAM10 in hydrogen peroxide-stimulated shedding of collagen XVII, we also treated *Adam10*$^{-/-}$ mEFs with increasing concentrations

FIGURE 4. **Constitutive collagen XVII shedding is strongly diminished in *Adam9*$^{-/-}$ keratinocytes and can be further reduced by the ADAM10-selective GI254023X, whereas ionomycin-stimulated shedding is not affected in the absence of ADAM9.** *A*, shedding in primary murine keratinocytes was analyzed as described under "Experimental Procedures." Keratinocytes derived from at least four different mice for each *Adam*$^{-/-}$ deficient strain and their wild type littermate controls were used for each experiment. All experiments were performed in duplicate, and cell lysates and concentrated media were probed for full-length collagen XVII (*CXVII*) and/or the ectodomain (*ECTO*) by Western blot. The percentage of ectodomain release was calculated by densitometric quantification. Shedding of collagen XVII was strongly diminished in *Adam9*$^{-/-}$ keratinocytes (46.0 ± 11.3%; $n = 5$; *, $p < 0.05$) but not in keratinocytes from *Adam8*$^{-/-}$ (95.5 ± 14.9%; $n = 4$) or *Adam15*$^{-/-}$ mice (86.5 ± 19.1%; $n = 4$). Representative immunoblots of membrane-bound collagen XVII in the lysate and the ectodomain in the medium are shown. *B*, analysis of the ionomycin stimulation (1 $\mu$M; 20 min) of *Adam9*$^{-/-}$ keratinocytes (*AD9*$^{-/-}$) revealed comparable responses to IM and sensitivity to batimastat (*BB*; #, $p < 0.05$; ##, $p < 0.01$) as in wild type keratinocytes (*WT*). The graph represents the densitometric analysis of collagen XVII ectodomain immunoblot signals from three experiments (mean ± S.D.; *, $p < 0.05$; **, $p < 0.01$). The DMSO vehicle control activities (*Co*) are normalized to 100% to be able to compare activated shedding between different experiments. *C*, confluent cultures of *Adam9*$^{-/-}$ keratinocytes were washed, and fresh medium with DMSO vehicle as control (*Co*) or 0.5 $\mu$M GI was added and incubated for 3 h. The cell lysates and concentrated media were probed for full-length collagen XVII and/or the ectodomain by Western blot, and the inhibition of shedding was determined by densitometric comparison of the blots of supernatants from control- or GI-treated keratinocytes from three experiments, each with keratinocytes from different mice (mean ± S.D.; #, $p < 0.05$). Collagen XVII shedding in *Adam9*$^{-/-}$ keratinocytes was further reduced by the GI, which is selective for ADAM10 over ADAM17 at 0.5 $\mu$M, indicating that ADAM9 and ADAM10 are both involved in the constitutive shedding of collagen XVII.

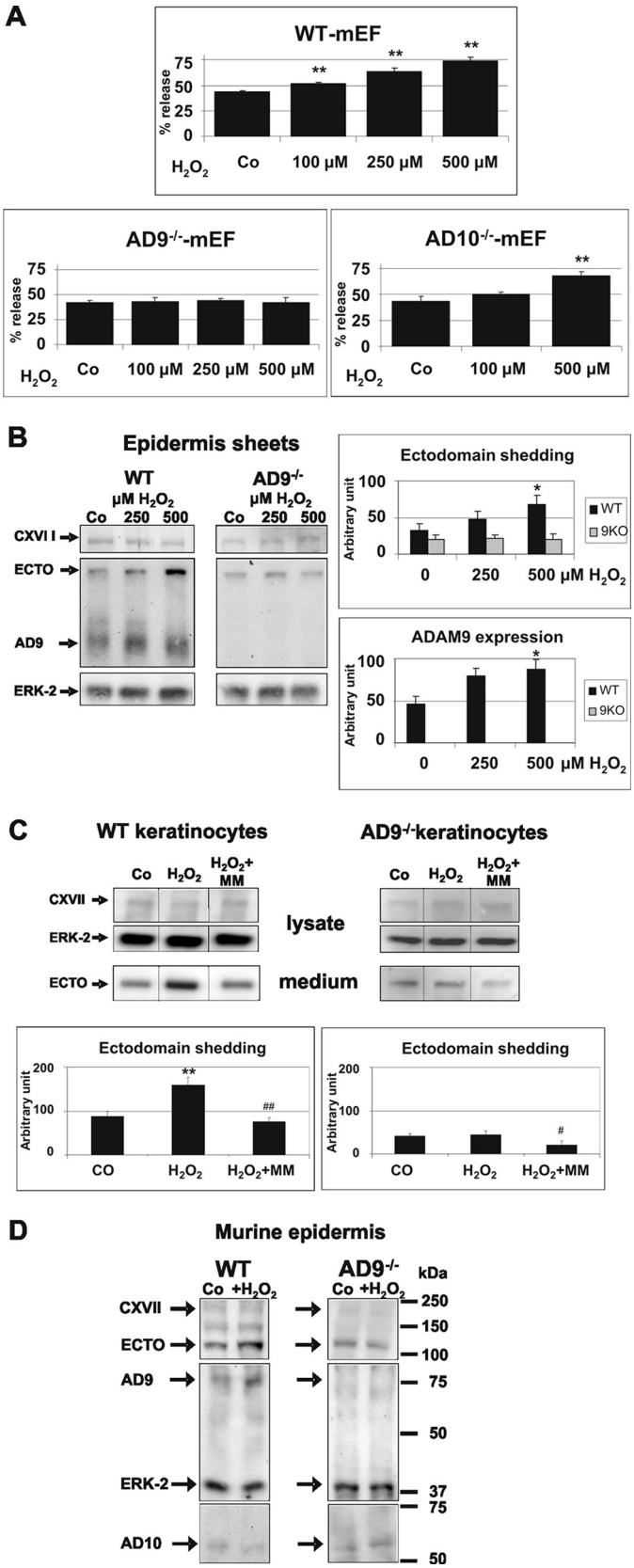



FIGURE 5. **Collagen XVII shedding is stimulated by $H_2O_2$ in cells and skin from wild type but not *Adam9*$^{-/-}$ mice.** *A*, *Adam9*$^{-/-}$, *Adam10*$^{-/-}$, and wild type (*WT*) mEFs were transfected with murine AP-tagged collagen XVII cDNA and treated with increasing concentrations of $H_2O_2$ for 5.5 h. AP assays revealed a dose-dependent increase in collagen XVII shedding in *Adam10*$^{-/-}$ and wild type but not in *Adam9*$^{-/-}$ cells. Data are shown as mean ± S.D. ($n = 3$; **, $p < 0.01$). *B*, epidermis sheets were prepared from 3-day-old wild type or *Adam9*$^{-/-}$ pups as described under "Experimental Procedures" and incubated with 0, 250, and 500 µM $H_2O_2$ for 5.5 h. Lysates of epidermis sheets from three different experiments, each using tissue from a different animal, were analyzed by Western blotting for the presence of full-length collagen XVII (*CXVII*) and the collagen XVII ectodomain (*ECTO*), ADAM9 (*AD9*), and ERK-2 as loading control. Densitometric analysis of immunoblots for the collagen XVII ectodomain and for ADAM9 content revealed a dose-dependent increase of the collagen XVII ectodomain concomitant with increased ADAM9 expression in epidermis sheets from wild type but not from *Adam9*$^{-/-}$ mice (data represent the mean ± S.D.; $n = 3$; *, $p < 0.05$). *C*, wild type and *Adam9*$^{-/-}$ keratinocytes were treated with 300 µM $H_2O_2$ with or without 3 µM marimastat (*MM*) for 8.5 h. Cell lysates and methanol-chloroform-precipitated media of three different experiments were analyzed by Western blotting for the presence of full-length collagen XVII (*CXVII*; in lysates) or ectodomain (*ECTO*; in medium) and ERK-2 as lysate loading control. Densitometric analysis of immunoblots for collagen XVII ectodomain revealed a nearly 2-fold increase in $H_2O_2$-treated wild type but not *Adam9*$^{-/-}$ keratinocytes, and the $H_2O_2$-stimulated shedding was sensitive to marimastat (data represent the mean ± S.D.; $n = 3$; **, $p < 0.01$; #, $p < 0.05$; ##, $p < 0.01$). *D*, *in vivo* application of $H_2O_2$ on shaved skin on the back of wild type mice (details under "Experimental Procedures") increased the level of the collagen XVII ectodomain as well as of ADAM9 (*AD9*) in skin lysates. These effects were not seen in skin lysates of *Adam9*$^{-/-}$ animals. Representative immunoblots of three independent experiments are shown. *Co*, control; *9KO*, ADAM9 knock-out.

of $H_2O_2$ (100–500 µM). These cells had a dose-dependent increase of collagen XVII shedding similar to that in wild type mEFs (Fig. 5*A*).

To test the relevance of these findings in the context of *ex vivo* skin explant cultures, we treated epidermis sheets derived from *Adam9*$^{-/-}$ mice and their wild type littermates with 100–500 µM $H_2O_2$ for 5.5 h at 37 °C. Western blot analysis with the polyclonal rabbit anti-collagen XVII antiserum MO-NC14A, anti-ADAM9 cytotail antibodies, or polyclonal rabbit antibodies against ERK-2 used as a loading control showed a dose-dependent increase in the ectodomain of collagen XVII in wild type skin with a concomitant increase in ADAM9 expression. However, no increase in collagen XVII shedding was observed in epidermis sheets from *Adam9*$^{-/-}$ mice (Fig. 5*B*).

To corroborate that $H_2O_2$ also stimulated collagen XVII shedding in primary keratinocytes, we treated these cells with 300 µM $H_2O_2$ for 8.5 h. The viability of the keratinocytes treated under these conditions was verified by trypan blue staining and microscopy, which revealed no differences compared with untreated controls (data not shown). $H_2O_2$ stimulation resulted in an ~2-fold increase in collagen XVII ectodomain release in wild type keratinocytes but not in keratinocytes lacking ADAM9 (Fig. 5*C*).

To assess whether hydrogen peroxide also affects the expression of ADAM9 *in vivo*, we treated the shaved back skin of *Adam9*$^{-/-}$ mice and their wild type littermates with two doses of 100 µl of 30% $H_2O_2$ within 24 h. Six hours after the second application, the animals were sacrificed, and a biopsy of the treated skin and a control area in untreated back skin of the same animal were extracted and analyzed by immunoblotting for both collagen XVII forms, ADAM9, and ERK-2 as loading control. Skin samples from all three $H_2O_2$-treated wild type mice showed up-regulated expression of ADAM9 protein and a concomitant increase in collagen XVII shedding (a representative blot is shown in Fig. 5*D*). In contrast, no differences in the shed forms of collagen XVII were seen in *Adam9*$^{-/-}$ mice ($n = 3$; Fig. 5*D*). Finally we found no up-regulation of ADAM10 expression after $H_2O_2$ treatment (Fig. 5*D*).

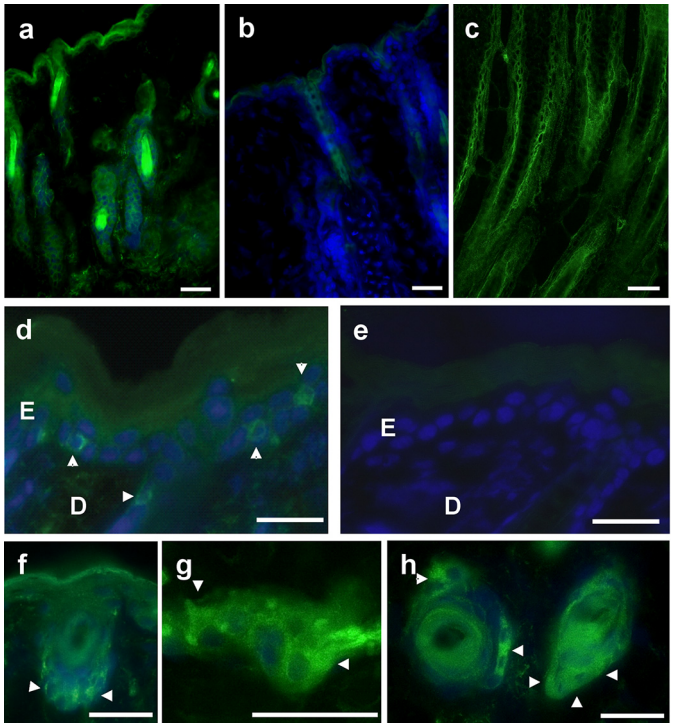



FIGURE 6. **ADAM9 is strongly expressed in the epidermis and in the outer root sheaths of hair follicles.** Immunofluorescence staining of wild type (*a*, *c*, *d*, and *f–h*) and *Adam9*$^{-/-}$ skin sections (*b* and *e*) with an anti-ADAM9 ectodomain antibody is shown. *Green*, FITC staining of ADAM9; *blue*, 4′,6-diamidino-2-phenylindole cell nucleus counterstaining. ADAM9 expression is localized to the epidermal keratinocytes and interfollicular keratinocytes of hair follicles (*a*). This staining pattern for ADAM9 was not seen in *Adam9*$^{-/-}$ skin where only minor unspecific staining in the surface stratum corneum and in hair was observed (*b*). Immunostaining of longitudinal skin sections of anagen stage hair follicles showed ADAM9 staining in keratinocytes of the outer root sheath (*c*). ADAM9 immunoreactivity in wild type epidermis (*d* and *f–h*) and murine *Adam9*$^{-/-}$ epidermis (*e*) is shown. Wild type epidermis (*d* and *g*) and hair follicles (*f* and *h*) are shown at higher magnification. *E*, epidermis; *D*, dermis. ADAM9 reactivity is distributed all over the epidermis and the outer root sheath of the hair follicle. Both contain solitary cells with high ADAM9 expression that are most likely basal keratinocytes near the basement membrane (*d* and *f–h*; indicated by *white arrowheads*). *Scale bar*, 100 $\mu$m.

*ADAM9 Expression in Mouse Skin*—The results presented above show that both ADAM9 and ADAM10 can participate in collagen XVII shedding. The ionomycin-stimulated shedding of collagen XVII depends on ADAM10, whereas increased shedding following stimulation by $H_2O_2$ depends on ADAM9. To analyze the expression pattern of ADAM9 in the skin, we used a polyclonal anti-ADAM9 ectodomain antibody for staining of wild type mouse skin. ADAM9-positive immunostaining was detected in the keratinocytes in all layers of the interfollicular epidermis and in the hair follicles (Fig. 6*a*). Some solitary cells preferentially localized near the basal membrane exhibited very high ADAM9 expression (Fig. 6, *d* and *f–h*; indicated by *white arrowheads*). There was only weak, if any, positive immunostaining of dermal cells (Fig. 6, *a* and *d*). Immunostaining of longitudinal back sections of anagen stage hair follicles of 9-day-old mice revealed ADAM9 staining in the outer root sheath (Fig. 6, *c*). No ADAM9 signal was seen in *Adam9*$^{-/-}$ skin, confirming the specificity of the antibody (Fig. 6, *b* and *e*).

## DISCUSSION

Collagen XVII is a hemidesmosomal membrane protein, which is important for the anchorage of the epidermis to the underlying basement membrane (1, 39–41). Our previous studies revealed two forms of collagen XVII, the 180-kDa membrane-anchored form and the soluble 120-kDa form, which represents almost the entire extracellular collagenous ectodomain. Here we used ADAM-deficient embryonic fibroblasts and primary keratinocytes as well as mouse skin samples to identify the major physiological collagen XVII sheddases.

Our results demonstrate that both ADAMs 9 and 10 are prominent collagen XVII sheddases that both contribute to its constitutive shedding. The evidence pointing toward ADAM10 as a major collagen XVII sheddase is based on several findings. First, collagen XVII shedding is not stimulated by short term treatment with phorbol esters, arguing against the involvement of ADAM17, a sheddase for many cell surface molecules whose activity in cell-based assays can be strongly stimulated by phorbol esters (30). It should be noted that the previously published PMA induction of collagen XVII shedding resulted from longer stimulations (6–24 h) at 5-fold higher PMA concentrations (8). Under these conditions, various cellular processes, including biosynthesis and transport through the secretory pathway, are strongly induced, and it has been shown that the expression of several ADAMs is up-regulated (42). Second, the strong simulation of collagen XVII shedding by calcium influx is consistent with a contribution of ADAM10 as this ADAM is known to respond to ionomycin treatment in cell-based assays (30, 33) and keratinocytes (43, 44). Third, constitutive and calcium influx-stimulated shedding of collagen XVII is highly sensitive to low concentrations of the ADAM10-selective inhibitor GI254023X (18, 34, 35). Fourth, calcium influx-stimulated shedding of collagen XVII was normal in *Adam9*$^{-/-}$, *Adam12*$^{-/-}$, or *Adam17*$^{-/-}$ mEFs but was abrogated in *Adam10*$^{-/-}$ mEFs.

The mechanism underlying the calcium influx-dependent stimulation of ADAM10 activity remains to be determined. Although Nagano *et al.* (33) have reported that ionomycin induces ADAM10 maturation and its accumulation at the cell surface, Horiuchi *et al.* (30) have reported that stimulation of ADAM10 is independent of prodomain removal or relocalization. Recently treatment of human keratinocytes with proinflammatory cytokines, especially IL1-$\beta$, has been reported to increase ADAM10 activity due to its maturation by prodomain removal (44). The previously reported finding that IL1-$\beta$ treatment leads to pronounced increases in collagen XVII shedding in human keratinocytes (8) therefore most likely reflected activation of ADAM10. Up-regulation of IL1-$\beta$ in keratinocytes is involved in UV-induced skin blistering and acute wound closure (45, 46) and is known to contribute to the pathogenesis of inflammatory epithelial skin diseases such as psoriasis (47). Moreover significant up-regulation of ADAM10 expression has been observed in human oral squamous cell carcinoma and prostatic adenocarcinomas (48, 49).

In addition to identifying ADAM10 as a major collagen XVII sheddase, we also found evidence for a significant role of ADAM9 in constitutive shedding of collagen XVII. In *Adam9*$^{-/-}$ skin and keratinocytes *in vitro*, shedding of collagen XVII was strongly decreased. This decrease was not caused by indirect effects on ADAM10 because neither expression of ADAM10 nor calcium influx-stimulated shedding of collagen

XVII were detectably affected in *Adam9*$^{-/-}$ keratinocytes. The involvement of ADAM9 was further corroborated by its strong expression in normal mouse epidermis and hair follicles. Consequently because overexpressed ADAM9 can process collagen XVII in cell-based assays, we hypothesize that the high levels of ADAM9 in keratinocytes and in skin result in a significant contribution to the constitutive but not calcium influx-stimulated shedding of collagen XVII in these cells and tissues.

A recent study demonstrated that oxidative stress induces ADAM9 expression in human prostate cancer cell lines (37, 38). When we used $H_2O_2$ stimulation as a tool to induce ADAM9 expression in cultured cells as well as in isolated epidermis sheets and in mouse skin *in vivo*, we observed a dose-dependent stimulation of ADAM9 expression and collagen XVII shedding. Importantly no $H_2O_2$-dependent increase in collagen XVII shedding was seen using *Adam9*$^{-/-}$ cells, epidermis, or skin. In contrast, $H_2O_2$ stimulation of collagen XVII shedding was intact in *Adam10*$^{-/-}$ mEFs, and no up-regulation of ADAM10 expression was observed in $H_2O_2$-treated skin, further corroborating that this effect does not involve ADAM10.

In a previous study, we observed a 50% reduction in shedding of collagen XVII in *Adam17*$^{-/-}$ keratinocytes (8). However, this effect is likely to be indirect because the properties of the main collagen sheddases do not match those of ADAM17 with respect to short term PMA stimulation or the response to an ADAM10-selective inhibitor, which does not inhibit ADAM17 at the concentration used here. Reverse transcription-PCR and immunoblotting ruled out differences in expression level of collagen XVII as well as expression and activation of ADAMs 9 and 10 in wild type *versus* *Adam17*$^{-/-}$ keratinocytes (see supplemental Fig. 1), so the mechanism responsible for the reduced collagen XVII shedding in *Adam17*$^{-/-}$ keratinocytes remains to be determined.

Taken together, our results suggest that both ADAMs 9 and 10 contribute to constitutive shedding of collagen XVII in keratinocytes and in adult skin. Moreover the activity of ADAM10 can be up-regulated by calcium influx and potentially also other stimuli, whereas ADAM9-dependent collagen XVII shedding is increased in the presence of reactive oxygen species through transcriptional up-regulation of ADAM9 expression (50). Because reactive oxygen species are generated through insults to the skin, such as by infiltrating activated leukocytes during inflammation, by exposure to UV irradiation, or in cutaneous neoplasias, we propose that such insults affect the relative contribution of ADAMs 9 and 10 to collagen XVII shedding in healthy and diseased skin (51). Any increase in shedding of collagen XVII is predicted to alter keratinocyte motility, proliferation, and differentiation. However, it should be noted that the overall consequences of increased expression of ADAMs 9 and 10 in skin will likely be determined by the sum of all processing events of the substrates cleaved by these enzymes. In addition, the non-catalytic domains of these ADAMs, such as their disintegrin and cysteine-rich domain, might also affect cell-cell or cell-matrix interactions in skin independently of their catalytic properties. Future studies will be aimed at analyzing collagen XVII shedding in the skin of conditional ADAM10 knock-out mice as mice lacking ADAM10 die too early during embryogenesis to study skin development (16). Moreover it will be of interest to test how inactivation of the cleavage site of collagen XVII in mice affects its function in skin.

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