

# Transmembrane collagen XVII, an epithelial adhesion protein, is shed from the cell surface by ADAMs

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**Collagen XVII, a type II transmembrane protein and epithelial adhesion molecule, can be proteolytically shed from the cell surface to generate a soluble collagen. Here we investigated the release of the ectodomain and identified the enzymes involved. After surface biotinylation of keratinocytes, the ectodomain was detectable in the medium within minutes and remained stable for >48 h. Shedding was enhanced by phorbol esters and inhibited by metalloprotease inhibitors, including hydroxamates and TIMP-3, but not by inhibitors of other protease classes or by TIMP-2. This profile implicated MMPs or ADAMs as candidate sheddases. MMP-2, MMP-9 and MT1-MMP were excluded, but TACE, ADAM-10 and ADAM-9 were shown to be expressed in keratinocytes and to be actively involved. Transfection with cDNAs for the three ADAMs resulted in increased shedding and, vice versa, in TACE-deficient cells shedding was significantly reduced, indicating that transmembrane collagen XVII represents a novel class of substrates for ADAMs. Functionally, release of the ectodomain of collagen XVII from the cell surface was associated with altered keratinocyte motility *in vitro*.**

**Keywords:** basement membrane/hemidesmosome/  
metalloprotease/secretase/skin

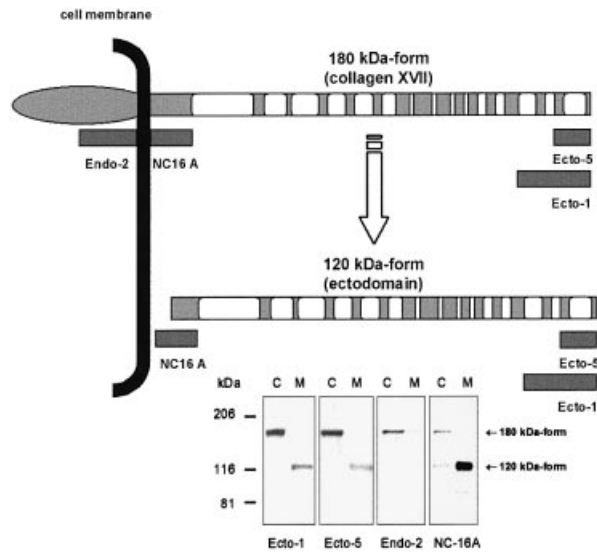
## Introduction

The family of type II transmembrane collagens includes collagens XIII, XVII and ectodysplasin A as adhesion molecules and/or ligand binding receptors on epithelial cell surfaces, and the types I and II macrophage scavenger receptors, and the MARCO protein, which have been proposed to function as receptors in host defense (Krieger and Herz, 1994; Schäcke *et al.*, 1998; Snellman *et al.*, 2000; Elomaa *et al.*, 2001; Sankala *et al.*, 2002). Collagens

XIII, XVII and ectodysplasin A are epidermal proteins with a collagenous ectodomain that colocalizes with integrins on basal keratinocyte surfaces. Collagen XIII and ectodysplasin A are associated with  $\alpha 3\beta 1$  integrin and the actin cytoskeleton in focal contacts (Ezer *et al.*, 1999; Snellman *et al.*, 2000), and collagen XVII with  $\alpha 6\beta 4$  integrin and the keratin intermediate filaments in hemidesmosomes (Borradori and Sonnenberg, 1999). These features imply specific functions in epithelial cell adhesion and suggest putative ligand interactions between the transmembrane collagens and the integrins in the basal keratinocytes.

Collagen XVII is a component of hemidesmosomes which mediate adhesion of keratinocytes and certain other epithelial cells to the underlying basement membrane. Its intracellular domain interacts with the  $\beta 4$ -integrin subunit and is essential for the incorporation of the molecule into the hemidesmosome, and the extracellular juxtamembranous NC16A domain binds to the  $\alpha 6$ -integrin subunit (Borradori and Sonnenberg, 1999). Each collagen XVII molecule is a trimer of three 180 kDa  $\alpha 1$ (XVII) chains, with a globular intracellular N-terminal domain of 466 amino acids, a short transmembrane domain of 23 amino acids and an extracellular C-terminal domain of 1008 amino acids in length. The ectodomain consists of 15 collagenous subdomains characterized by typical collagenous -G-X-Y- repeat sequences, intervened by 16 short noncollagenous sequences. The overall structure is flexible, rod-like and triple helical, with a significant thermal stability (Schäcke *et al.*, 1998; Tasanen *et al.*, 2000a; Areida *et al.*, 2001). The critical role of collagen XVII in maintaining epithelial adhesion is best demonstrated by human skin diseases. Mutations in the collagen XVII gene, *COL17A*, lead to junctional epidermolysis bullosa, a blistering disorder with epidermal detachment from the basement membrane as a consequence of minor friction (McGrath *et al.*, 1995; Tasanen *et al.*, 2000b). In autoimmune diseases of the pemphigoid group, autoantibodies target both full-length collagen XVII and the shed ectodomain and lead to diminished epidermal adhesion and skin blistering (Schumann *et al.*, 2000).

Collagen XVII was the first collagen shown to be constitutively shed from the cell surface, yielding a soluble basement membrane collagen (Hirako *et al.*, 1998; Schäcke *et al.*, 1998). Subsequently, shedding of collagen XIII (Snellman *et al.*, 2000) and ectodysplasin A (Chen *et al.*, 2001; Elomaa *et al.*, 2001) was described. Although furin has been predicted to be involved in the shedding cascade (Schäcke *et al.*, 1998; Snellman *et al.*, 2000; Chen *et al.*, 2001), it has remained unclear whether it cleaves directly the ectodomains of all of these proteins or is perhaps involved in the activation of other, physiological, sheddases. In the present study we demonstrate that three members of the ADAM family act as physiological



**Fig. 1.** Schematic representation of collagen XVII and the epitope regions recognized by antibodies. The full-length collagen XVII is a type II integral transmembrane protein, which is cleaved to yield a soluble ectodomain. White bars, collagenous sequences; light gray bars, non-collagenous sequences; black line, the cell plasma membrane. Dark gray bars, epitope regions of the domain-specific antibodies. Endo-2 recognizes the intracellular domain, NC-16A the juxtamembranous extracellular NC16A-domain, and Ecto-1 (Schäcke *et al.*, 1998) and Ecto-5 the distal C-terminus of the molecule. The lower panel shows immunoblots of keratinocyte cell layer (C) and media (M) with the above antibodies. Ecto-1, Ecto-5, and NC16A recognized both the 180 kDa full-length collagen XVII and the 120 kDa soluble ectodomain. In contrast, Endo-2 recognized only full-length collagen XVII.

sheddases of collagen XVII, and that furin is likely to be involved in the activation of these enzymes.

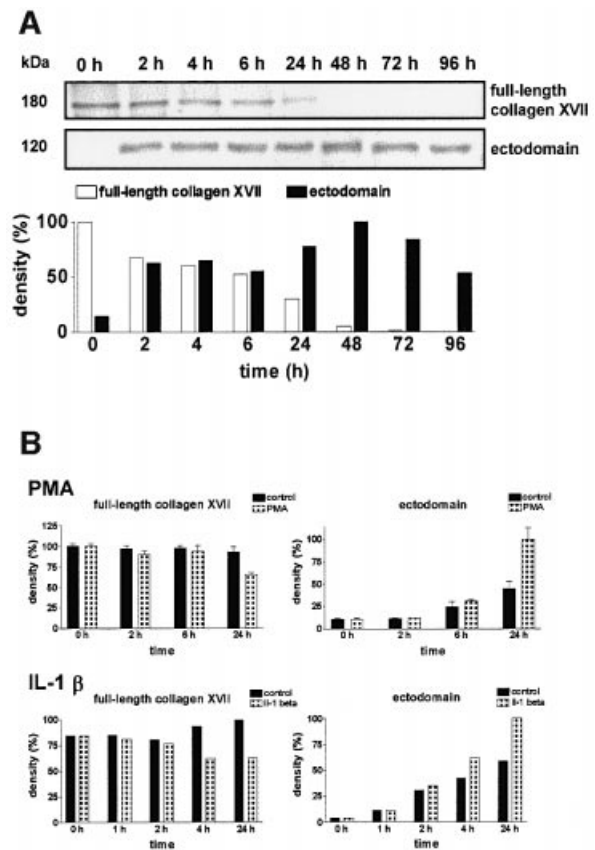
## Results

### Domain-specific antibodies define the shed ectodomain of collagen XVII

For characterization of the two forms of collagen XVII, two novel antibodies, Endo-2, recognizing the intracellular domain, and Ecto-5, against the distal-most 50 amino acids of the extracellular C-terminus of collagen XVII (Figure 1), were produced. The antibody NC16A to the juxtamembranous extracellular domain (Schumann *et al.*, 2000) was also used. Since both NC16A and Ecto-5 recognized full-length collagen XVII and the soluble ectodomain, the authentic shedding product clearly spans almost the entire extracellular domain of collagen XVII. Correspondingly, Endo-2 recognized only the full-length molecule (Figure 1). Thus, the sheddase cleavage site is predicted to localize close to the cell membrane within the extracellular NC16A domain.

### Release of collagen XVII ectodomain from the cell surface

Human keratinocytes were surface biotinylated, washed carefully, and the release of the labeled ectodomain into fresh medium was followed over time. The biotinylated ectodomain could be immunoprecipitated from the medium after 9 min, and after 2 h significant amounts



**Fig. 2.** Shedding of collagen XVII ectodomain. (A) Time-chase experiments with normal human keratinocytes. The cells were surface biotinylated, chased with biotin-free medium and cultured for 96 h. Aliquots of culture medium and cell extract were immunoprecipitated with antibody Ecto-1 or Endo-2 and analyzed by protein blotting with streptavidin-alkaline phosphatase. The lower panel shows the densitometric analysis of the signals. Note that the intact ectodomain was stable in the medium for at least 72 h. (B) Shedding was stimulated by phorbol esters and IL-1 $\beta$ . Keratinocytes were cultured for 24 h in the presence of 100 nM PMA or IL-1 $\beta$  (5 ng/ml). Aliquots were drawn at regular intervals and analyzed by immunoblotting with Endo-2 and NC16A antibodies. The signals were analyzed densitometrically with TotalLab1D (Phoretics) software.

were recovered (Figure 2). Full-length collagen XVII was progressively cleaved, and after 48 h no intact molecules were found in the cell extracts. Interestingly, the intact ectodomain was still recovered from the medium after 72–96 h, indicating a high stability of the soluble form of collagen XVII. The ectodomain was purified from the medium by immunoaffinity chromatography and subjected to N-terminal sequencing. However, despite repeated trials with sufficient amounts of protein the sequence remained elusive, because the newly generated N-terminus was blocked.

### Modulation of shedding

For characterization of the enzymes involved in the shedding of collagen XVII, keratinocytes were cultured in the presence of protease inhibitors (Table I). Release of the ectodomain was completely inhibited by phenanthroline and by the MMP- and sheddase-targeting hydroxamate derivatives BB 3103, BB 3241, and IC-3. About 50% inhibition was observed with 25  $\mu$ M hydroxamate FN-439 and with 500 nM TIMP-3, ~10% with 500 nM TIMP-1,

**Table I.** Effect of proteinase inhibitors on shedding of collagen XVII

Inhibitor	Concentration (μM)	Inhibition ± SD (%)
None		0
1,10- <i>o</i> -phenanthroline	100	91 ± 9
Pepstatin A	100	3 ± 4
E-64	100	8 ± 8
Phosphoramidon	10	4 ± 6
AEBSF	1000	5 ± 11
Aprotinin	25	11 ± 13
Benzamidine	1000	14 ± 9
FN-439	20	20 ± 7
	200	44 ± 10
BB 3103	10	87 ± 16
BB 3241	10	92 ± 9
IC-3 (TAPI)	25	95 ± 4
TIMP-1	0.5	11 ± 4
TIMP-2	1	1 ± 4
TIMP-3	0.15	15 ± 4
	0.5	51 ± 4
Selective gelatinase inhibitor <sup>a</sup>	100	4 ± 3

<sup>a</sup>Cyclic synthetic peptide: CTTHWGFTLC.

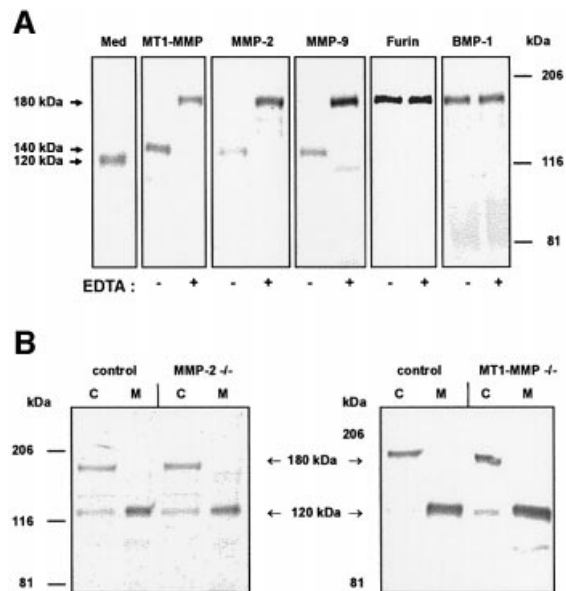
Shedding in the presence or absence of inhibitors was analyzed by immunoblotting followed by semiquantitative densitometry of the signals. The inhibition is expressed as a percentage ± SD ( $n = 3$ ) of controls.

but none with the aspartate protease inhibitor pepstatin A, the cysteine peptidase inhibitor E-64, or the serine protease inhibitors AEBSF, aprotinin, or benzamidine. In addition, no effect was seen with TIMP-2 or with CTTHWGFTLC, a specific cyclic peptide inhibitor of MMP-2 and MMP-9.

Potential stimulators of collagen XVII shedding were also added to keratinocytes. No effect on shedding of collagen XVII was observed with 5 ng/ml TNF- $\alpha$  or 5–10 ng/ml TGF- $\beta$ 2 (not shown). In contrast, 6 h after addition of 5 ng/ml IL-1 $\beta$ , increased ectodomain release, with a concomitant decrease of full-length collagen XVII, was seen (Figure 2B). Addition of 100 nM phorbol 12-myristate 13-acetate (PMA) induced shedding after 24 h (Figure 2B). Both agents are well-known stimulators of shedding and inducers of several ADAMs (Hooper *et al.*, 1997; Flannery *et al.*, 1999).

#### Exclusion of furin, BMP-1, MMP-2, MMP-9 and MT1-MMP as collagen XVII sheddases

The above inhibitor and stimulator profiles indicated that the protease(s) involved in shedding of collagen XVII belong(s) to the metalloproteinases, e.g. MMPs or sheddases of the ADAM family. Enzymes associated with keratinocyte surfaces, such as furin, BMP-1, MMP-2, MMP-9 and MT1-MMP (MMP-14), were tested for their capacity to cleave authentic purified collagen XVII (Figure 3A). No cleavage was observed with recombinant furin or BMP-1. All three MMPs cleaved collagen XVII into a 140 kDa fragment *in vitro*. Immunoblotting with the antibody Endo-2 demonstrated that in addition to the ectodomain, the fragment contained part of the endodomain and, therefore, this cleavage was not physiologically relevant (not shown). The observations were corroborated by studies on MT1-MMP- and MMP-2-deficient cells. After 24 h in culture, the shed ectodomain and full-length collagen XVII were analyzed by immunoblotting



**Fig. 3.** Furin, BMP-1, MMP-2, MMP-9 and MT1-MMP are not directly involved in the processing of collagen XVII. (A) Purified native collagen XVII was digested with recombinant human MT1-MMP, MMP-2, MMP-9, furin and BMP-1. The digests in the absence (–) or in the presence (+) of 5 mM EDTA were immunoblotted with antibody Ecto-1. Furin and BMP-1 did not cleave collagen XVII. MMP-2, MMP-9 and MT1-MMP generated a 140 kDa fragment which was distinct from the authentic 120 kDa fragment in culture medium (Med). (B) Ectodomain shedding in MMP-2-deficient gastric carcinoma cells (left panel) and MT1-MMP-deficient murine keratinocytes (right panel). In both cultures the 120 kDa ectodomain was generated similarly to controls. C, cell extract; M, medium. Immunoblotting was with a mixture of antibodies Endo-2 and NC16A (left panel) or Endo-2 and MO-NC16A (right panel).

(Figure 3B). MMP-2-deficient human gastric carcinoma cells, which had only low MT1-MMP expression, and primary MT1-MMP-deficient murine keratinocytes generated the ectodomain as efficiently as control keratinocytes (Figure 3B), indicating that these MMPs were not involved in the shedding of collagen XVII.

Support for the involvement of TACE, instead of the above MMPs, was obtained from comparisons of the inhibitor profiles of metalloproteinases and collagen XVII shedding. Recombinant human MMP-2, MMP-9 and TACE were used to cleave fluorescein-coupled gelatin or internally quenched fluorescent oligopeptides. Inhibitors of collagen XVII shedding, i.e. IC-3, BB 3241 and BB 3103 strongly inhibited MMP-2, MMP-9 and TACE, whereas the hydroxamate FN-439, a weak inhibitor of shedding, had only little effect on the activity of the MMPs and TACE (Table II). In contrast, the gelatin inhibitor, which did not inhibit collagen XVII shedding, strongly inhibited MMP-2 and MMP-9, but not TACE.

The role of furin in collagen XVII shedding was of interest, since a furin-specific chloromethyl ketone inhibitor prevents collagen XVII processing (Schäcke *et al.*, 1998). The fact that recombinant furin did not cleave purified collagen XVII *in vitro* (Figure 3A) and was not sensitive to hydroxamates (Table II) suggested that furin is indirectly involved in the activation of a convertase. To verify this, TACE expression was analyzed in keratinocytes treated with the furin inhibitor (Figure 4C). Immunoblots of control cell lysates showed two distinct

**Table II.** Effect of proteinase inhibitors on recombinant proteases

Inhibitor	Concentration ( $\mu$ M)	% activity			
		MMP-2	MMP-9	TACE	Furin
BB 3103	25	0 $\pm$ 4	1 $\pm$ 2	0 $\pm$ 4	101 $\pm$ 12
BB 3241	25	0.5 $\pm$ 3	0 $\pm$ 3	0 $\pm$ 1	102 $\pm$ 9
FN-439	25	36 $\pm$ 3	58 $\pm$ 3	45 $\pm$ 7	93 $\pm$ 3
Gelatinase inhibitor <sup>a</sup>	25	19 $\pm$ 11	10 $\pm$ 10	98 $\pm$ 12	98 $\pm$ 9
IC-3 (TAPI)	25	0 $\pm$ 4	0.3 $\pm$ 1	0 $\pm$ 1	102 $\pm$ 9
Furin inhibitor <sup>b</sup>	100	97 $\pm$ 4	88 $\pm$ 3	97 $\pm$ 8	6 $\pm$ 4

<sup>a</sup>Cyclic synthetic peptide: CTTHWGFTLC.

<sup>b</sup>Decanoyl-RVKR-chloromethyl ketone.

The enzymes (5 nM MMP-2; 4 nM MMP-9; 20 nM CAT-DIS-TACE; 10 nM human furin) and inhibitors were preincubated for 30 min at room temperature and the fluorogenic substrate was added as described in Materials and methods. The proteolytic activity is expressed as a percentage  $\pm$  SD ( $n = 3$ ) in relation to controls.

TACE-positive bands, representing the proform and the mature form of the enzyme (Figure 4B and C). Addition of 50  $\mu$ M furin inhibitor to the cultures inhibited the activation of TACE, as shown by nearly complete absence of the mature form (Figure 4C). In contrast, addition of BB 3103 did not block TACE activation, as shown by the presence of the proform and the mature form in both controls and hydroxamate-treated samples (Figure 4C). Importantly, 10 mM 1,10-*o*-phenanthroline was added to the extraction buffers, since it was recently shown that an additional form of TACE lacking the pro- and cytoplasmic domains is detected if cell lysates are prepared without 1,10-*o*-phenanthroline (Schlondorff *et al.*, 2000).

#### Expression of ADAMs in human keratinocytes

Based on the above observations, the ADAM family proteinases were considered as candidate sheddases for collagen XVII. RT-PCR amplification of human keratinocyte mRNA demonstrated that TACE and the related sheddases ADAM-9 and ADAM-10 were expressed in these cells (not shown). Immunofluorescence staining of human skin with antibodies to TACE and ADAM-9 produced a positive signal in the lower epidermis in the vicinity of collagen XVII (Figure 4A), and ADAM-10 was found within the entire epidermis. Immunoblotting of keratinocyte lysates (Figure 4B) demonstrated that the proform and the active form of TACE (Schlondorff *et al.*, 2000) and ADAM-9 (Roghani *et al.*, 1999), and the active form of ADAM-10 (Qi *et al.*, 1999) were present in these cells.

#### TACE and other ADAMs contribute to shedding of collagen XVII

To assess the role of TACE in collagen XVII shedding, HaCaT cells were transiently transfected with full-length murine TACE cDNA. Since TACE is expressed in many cell lines, including HaCaT, different cDNA concentrations (0–20  $\mu$ g) were used. The transfection efficiency was estimated by analysis of TACE protein expression with immunofluorescence staining and determination of the relative number of transfected cells with a strongly positive TACE signal within a defined culture area. A transfection efficiency of ~20% was obtained with 20  $\mu$ g of TACE cDNA (Figure 5A). A dose-dependent increase in shedding, with a concomitant decrease in full-length

collagen XVII, was observed with increasing cDNA concentrations. Transfection with 20  $\mu$ g of TACE cDNA led to a nearly 2-fold increase of shedding (Figure 5B). Enhanced shedding in transfected cells was effectively inhibited with the hydroxamates BB 3103 or BB 3241 (Figure 6).

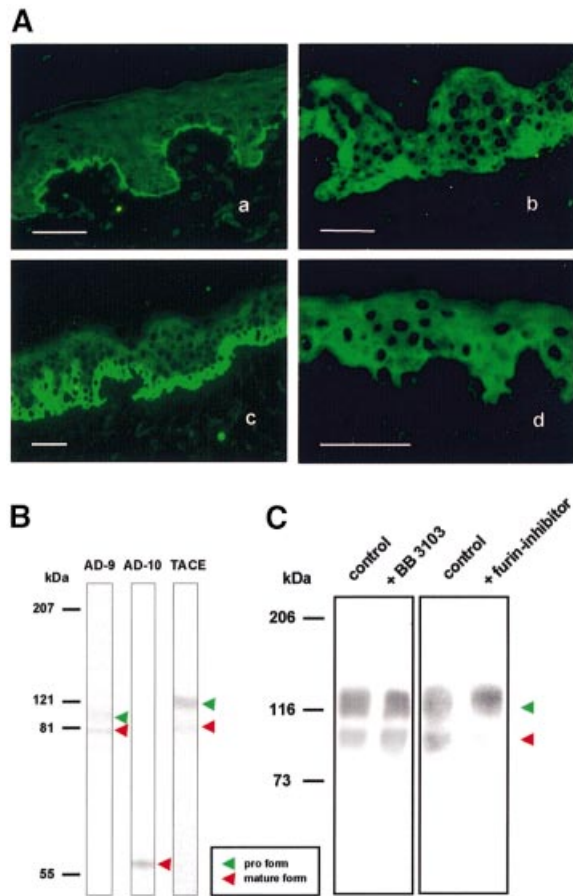
In parallel, HaCaT cells were transfected with full-length cDNA for human ADAM-9 and bovine ADAM-10. The estimated transfection efficiencies with 15  $\mu$ g of cDNA for ADAM-9 and ADAM-10 were 12 and 10%, respectively (Figure 5A). Transfection with 15  $\mu$ g of ADAM-10 cDNA caused an ~1.5-fold increase, and with 15  $\mu$ g of ADAM-9 cDNA a 1.75-fold increase in shedding of the ectodomain (Figure 5B).

The prediction that TACE sheds collagen XVII was confirmed with keratinocytes derived from TACE-deficient mouse embryos. Compared with age-matched normal murine keratinocytes, shedding of collagen XVII was reduced by ~40% in TACE-deficient cells (Figure 7A). In another approach, normal and TACE-deficient murine fibroblasts were transfected with collagen XVII cDNA, and shedding was analyzed. Similarly to TACE-deficient keratinocytes, transfected TACE-deficient fibroblasts shed collagen XVII less efficiently. Also in this case the reduction was ~40% (Figure 7B).

#### Shedding of collagen XVII ectodomain is associated with altered cell motility

Since the release of collagen XVII ectodomain from the keratinocyte surface is likely to influence the interaction of the cell with its environment, we analyzed cell motility after transfection of HaCaT cells with cDNAs for TACE, ADAM-9 and -10. For this purpose, *in vitro* wound closure assays were performed (Cha *et al.*, 1996). In this assay, scrape wounds were generated in confluent serum-free HaCaT cultures and, after extensive washing with PBS, the cells were allowed to migrate into the denuded area for 22 h at 37°C. Cells transfected with an empty vector migrated into the wound and 're-epithelialized' it to ~50% in 11 h and fully in ~22 h (Figure 8A). Cells transfected with one of the ADAMs were less motile, as indicated by a lower number of cells in the denuded area after 11 h and 22 h (Figure 8A).

The inhibitory activity of collagen XVII shedding on cell motility was verified with the addition of ~1 nM

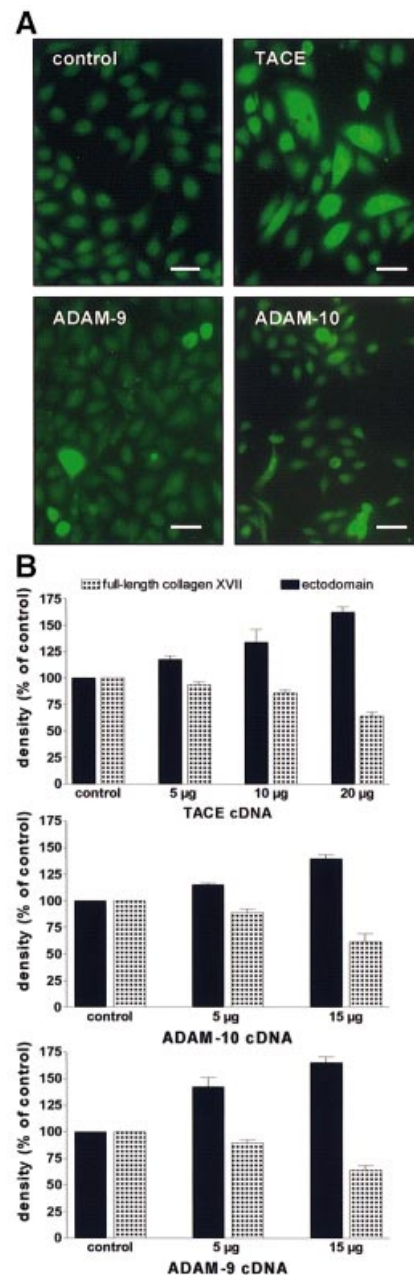


**Fig. 4.** Expression of ADAMs in human keratinocytes. (A) Immunofluorescence staining of collagen XVII and ADAMs in the skin. Collagen XVII antibody NC16A produced a linear signal at the basement membrane zone (a). TACE (b) and ADAM-9 (c) immunoreactivity was stronger in the lower epidermis, in the neighborhood of collagen XVII, whereas ADAM-10 immunoreactivity (d) was distributed throughout the entire epidermis. Scale bar: 250  $\mu$ m. (B) Immunoblot of keratinocyte extracts with antibodies to the ectodomains of TACE and ADAM-9, and the endodomain of ADAM-10. TACE and ADAM-9 antibodies recognized two distinct protein bands of ~120 and 85 kDa, and 110 and 79 kDa, respectively, representing the proform and the active form of the enzymes. ADAM-10 antibody recognized one band of ~60 kDa, corresponding to the active form. (C) Activation of TACE in keratinocytes was inhibited by a furin inhibitor, but not by hydroxamates. Keratinocytes were cultured for 24 h in serum-free medium with or without 50  $\mu$ M furin inhibitor decanoyl-RVKR-chloromethyl ketone or 25  $\mu$ M hydroxamate BB 3103. Keratinocyte lysates were immunoblotted with TACE antibodies. The furin inhibitor, but not BB 3103, prevented the conversion of pro-TACE to active TACE. Molecular weight markers are shown on the left.

purified ectodomain to the culture medium after induction of the scrape wound. Similarly to the experiment described above, untreated control cells readily migrated into the scraped area, covering it to >50% in 16 h. In contrast, keratinocyte migration from the cut edge was decreased when the purified ectodomain was added to the culture medium (Figure 8B), suggesting that the shed ectodomain negatively regulates cell motility.

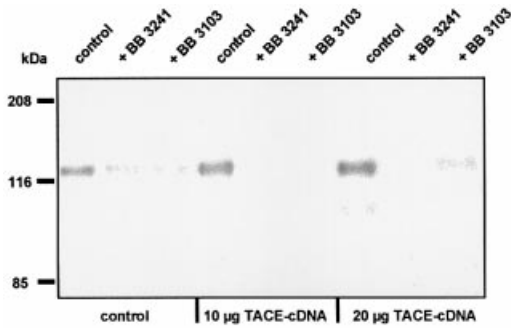
## Discussion

A wide variety of proteins, including cytokines, growth factors and their receptors, and cell adhesion molecules,

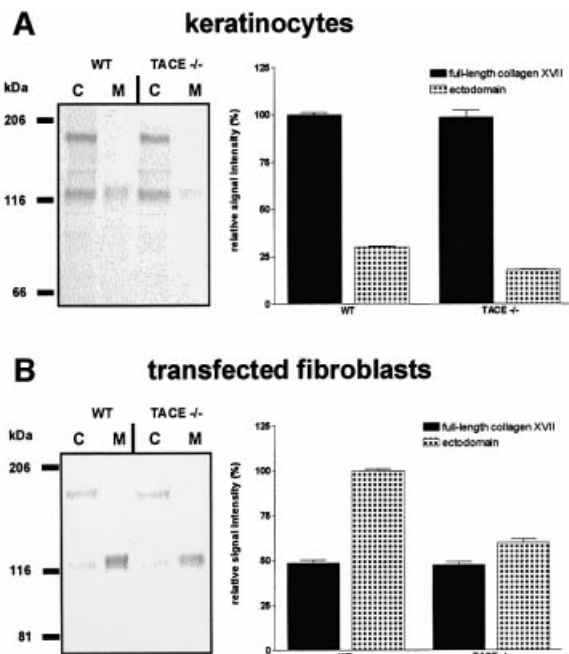


**Fig. 5.** Transient transfections of HaCaT cells with cDNAs for TACE, ADAM-9 and ADAM-10. (A) Immunofluorescence staining of transfected cells. Cells transfected with empty vector (control) remained negative, but ADAM-transfected cells showed strong positive signals. Scale bar: 100  $\mu$ m. (B) HaCaT cells were transfected with different concentrations of cDNA for TACE (upper panel), ADAM-10 (middle panel) and ADAM-9 (lower panel) and shedding of collagen XVII was assessed with immunoblotting with a mixture of antibodies Endo-2, NC16A and Ecto-1. Densitometric analysis of the signals (expressed as a percentage of the control  $\pm$  SD;  $n = 3$ ) showed a dose-dependent increase of ectodomain shedding, with a concomitant decrease of full-length collagen XVII.

are synthesized as integral type I or type II transmembrane proteins that can be released by selective proteolysis from the cell surface (Hooper *et al.*, 1997). The cleavage generally occurs close to the extracellular face of the membrane, generating a physiologically active protein. Release of soluble growth factors and cytokines may change their biological activities, whereas shedding of transmembrane receptors may render cells unresponsive to



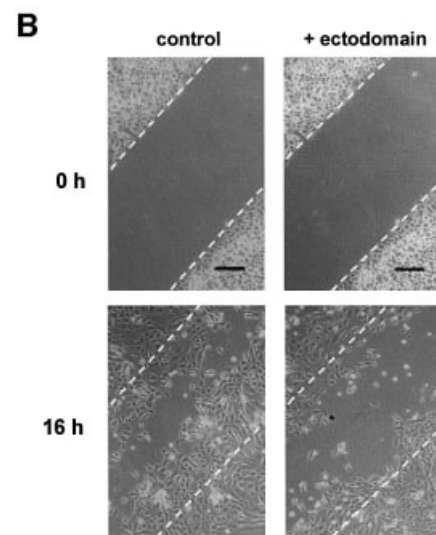
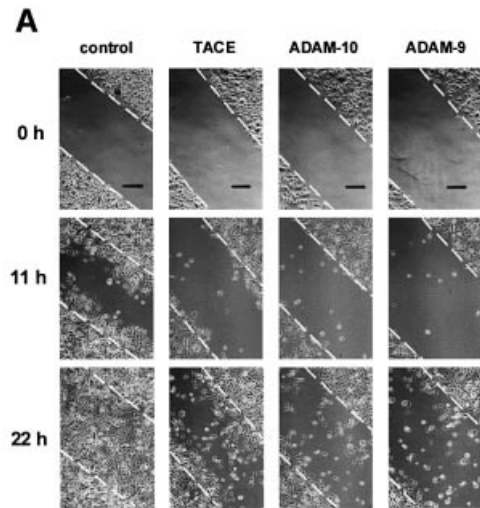
**Fig. 6.** Hydroxamates inhibit increased collagen XVII shedding in TACE-transfected cells. The cells were electroporated with empty vector (control), or with 10 or 20 µg of TACE cDNA. After 24 h the cells were washed, 10 µM BB 3103 or BB 3241 added, and after 6 h the ectodomain in the medium detected with immunoblotting using antibodies NC16A and Ecto-1. Both hydroxamates strongly inhibited shedding in this system.



**Fig. 7.** Decreased collagen XVII shedding in TACE-deficient cells. (A) Murine keratinocytes from wild-type (WT) and TACE  $-/-$  mice were analyzed for collagen XVII shedding *in vitro*. Cells (C) and media (M) were sampled after 6 h and immunoblotted with a mixture of antibodies MO-NC16A and Endo-2 (left panel). The relative intensity of the signals of three individual experiments is expressed as a percentage  $\pm$  SD (right panel). (B) Wild-type (WT) and TACE  $-/-$  fibroblasts were transfected with human collagen XVII cDNA. After 2 days the cells were analyzed for collagen XVII shedding. Cells (C) and media (M) were sampled after 6 h and immunoblotted with a mixture of antibodies MO-NC16A and Endo-2 (left panel). The relative intensity of the signals of two different experiments is expressed as a percentage  $\pm$  SD (right panel).

particular ligands, and the resulting soluble molecules may act to modulate the activity of their ligands (Black and White, 1998). Thus, the process of ectodomain shedding can influence the interaction of cells with their environment in multiple ways.

Many of the recently characterized sheddases are metalloproteinases (Hooper *et al.*, 1997). They are localized at the cell surface, or are themselves integral



**Fig. 8.** Increased collagen XVII shedding correlates with decreased cell motility. In wound closure assays cells migrate from the edges of a 'scrape wound' in the monolayer (interrupted lines) to cover the denuded space (between the lines). Scrape wounds were made in serum-free cultures, and after washing the cells were allowed to re-epithelialize for 22 h at 37°C. Micrographs of non-fixed cell layers at 0, 11, 16 or 22 h after wounding are shown. Scale bars: 200 µm. (A) Transfection of HaCaT cells with ADAM cDNA negatively regulated cell motility. Transfections were with 15 µg of empty vector (control), 15 µg of TACE, 15 µg of ADAM-10, or 15 µg of ADAM-9 cDNA. (B) Addition of purified collagen XVII ectodomain to normal human keratinocytes inhibited cell motility. After wounding, 1 nM collagen XVII ectodomain was added to the culture, and the cells were permitted to migrate into the denuded space for 16 h. Ectodomain-treated cells did not cover the denuded area as efficiently as control cells.

membrane proteins which after activation can act close to the cell membrane in the extracellular space. The prototype sheddase TACE is a member of the ADAM family and was designated as ADAM-17. The substrate spectrum of TACE is broad, including TNF- $\alpha$ , L-selectin, IL-6 receptor and the p75 TNF receptor (Moss *et al.*, 1997; Merlos-Suarez *et al.*, 1998; Peschon *et al.*, 1998). Both TACE and ADAM-10 may also participate in the processing of amyloid precursor protein (Buxbaum *et al.*,



1998; Amour *et al.*, 2000). Another member of the family, ADAM-9, mediates the shedding of heparin-binding epidermal growth factor-like growth factor (Izumi *et al.*, 1998). Kuzbanian, the *Drosophila* homolog of mammalian ADAM-10, releases the Notch ligand Delta from cells (Qi *et al.*, 1999). Thus, the ADAMs constitute a family of sheddases that regulate plasma membrane composition and release soluble signaling molecules and ligand binding receptors from cells. In the present study, we expand the substrate spectrum of ADAM sheddases by showing that collagen XVII, a representative of the family of transmembrane collagens, is a substrate for TACE, ADAM-9 and ADAM-10.

Genetic evidence proves collagen XVII is an essential keratinocyte adhesion molecule. Mutations in the COL17A1 gene lead to severe epidermal dysadhesion and skin blistering in humans (McGrath *et al.*, 1995). The shed ectodomain forms a soluble collagen which remains stable for days in keratinocyte media *in vitro* and which can be isolated from the epidermis and amniotic fluid *in vivo*, suggesting that it functions as a ligand-binding matrix molecule (Zone *et al.*, 1990; Schumann *et al.*, 2000).

The sheddase cleavage site within the ectodomain of collagen XVII remains elusive. Epitope mapping placed the putative site within the 76 amino acid NC16A domain adjacent to the cell membrane, but determination of the cleaved peptide bond was impeded by the fact that the newly generated N-terminus was blocked and not amenable to amino acid sequencing. Since shedding of collagen XVII in keratinocytes was completely inhibited by the synthetic furin inhibitor decanoyl-RVKKR-chloromethyl ketone, and since the NC16A domain contains a conserved furin cleavage motif, RIRR, we first assumed that furin releases the ectodomain from the cell surface (Schäcke *et al.*, 1998). However, recombinant furin did not cleave immunopurified full-length collagen XVII *in vitro* and, more importantly, shedding was completely blocked by hydroxamate inhibitors, such as BB 3103, BB 3241 or IC-3, which do not inhibit furin.

Based on the above data and the fact that the furin inhibitor completely blocked the activation of TACE proform, it is likely that furin activates ADAMs that shed collagen XVII. TACE, ADAM-10 and -9 are expressed as an inactive proform in keratinocytes. They all contain a conserved sequence in the prodomain which is comparable to the cysteine switch sequence of MMPs (Nagase and Wossner, 1999). In addition, in the boundary between the prodomain and the metalloproteinase domain there is a four basic amino acid consensus motif for furin cleavage (RRRR in ADAM-9, RKKR in ADAM-10 and RVKR in ADAM-17), suggesting that the mechanism of activation of these ADAMs includes a cysteine switch and proteolytic removal of the prodomain by furin (Merlos-Suarez *et al.*, 1998; Lammich *et al.*, 1999; Roghani *et al.*, 1999; Schlondorff *et al.*, 2000).

Our results demonstrate that all three ADAMs can shed collagen XVII in keratinocytes, since transient transfections with TACE, ADAM-9 and ADAM-10 lead to increased shedding of the ectodomain, and since TACE-deficient keratinocytes exhibited a remaining shedding activity of ~60%. Three observations argue that TACE may be the major sheddase. First, shedding was sensitive

to TIMP-3, but not to TIMP-1 or TIMP-2. TIMP-3 is known to inhibit shedding of proTNF- $\alpha$  and the adhesion molecules L-selectin, syndecan-1 and -4 (Borland *et al.*, 1999; Fitzgerald *et al.*, 2000). Secondly, expression of TIMP-3 in keratinocytes is downregulated by IL1- $\beta$  (Airola *et al.*, 1998), a stimulator of collagen XVII shedding. Thirdly, TIMP-3, but not TIMP-1 and TIMP-2, inhibited recombinant TACE *in vitro* (Amour *et al.*, 1998). In contrast, the proteolytic activity of ADAM-10 and ADAM-9 is inhibited also by TIMP-1 (Dethlefsen *et al.*, 1998; Amour *et al.*, 2000). Compared with TIMP-3, an equimolar concentration of TIMP-1 inhibited collagen XVII shedding by only ~10%. The synergistic action of the three ADAMs has also been observed in another system: all three enzymes have been postulated to represent potential  $\alpha$ -secretases that can cleave amyloid precursor protein or corresponding peptides *in vitro* (Buxbaum *et al.*, 1998; Schlondorff and Blobel, 1999; Fahrenholz *et al.*, 2000).

The biological context of collagen XVII shedding is predicted to involve keratinocyte adhesion, differentiation and migration. These processes are very complex and involve a number of receptors and ligands depending on the spatial and temporal biological context (Aumailley and Gayraud, 1998; Nguyen *et al.*, 2000), but a scenario could be visualized in which shedding of collagen XVII ectodomain loosens or releases the cell from some of its present binding partners and allows it to embark on other functions as required. We have previously shown that recombinant Col15 domain, the largest collagenous domain of collagen XVII, promotes epithelial cell adhesion in a  $\beta$ 1 integrin-dependent manner, and that denatured Col15 binds cells more efficiently than its native counterpart (Tasanen *et al.*, 2000a). We concluded that the shed ectodomain, which contains Col15, may exert this function, and that shedding may generate new cell binding sites. The hypothesis is supported by the *in vitro* re-epithelialization assays of the present study, which showed that increased shedding of collagen XVII induced by transient transfections with ADAM cDNA, or simulation of shedding through addition of exogenous ectodomain, was associated with decreased keratinocyte motility.

Basal keratinocytes in the skin express  $\alpha$ 2,  $\alpha$ 3,  $\alpha$ 5 and  $\alpha$ V integrin subunits in association with the  $\beta$ 1 subunit and utilize them for cell migration on collagen and laminin e.g. during wound healing.  $\alpha$ 2 $\beta$ 1 is used for adhesion to collagen, but this integrin does not recognize the ectodomain of collagen XVII (Nykqvist *et al.*, 2001).  $\alpha$ 6 $\beta$ 4 and  $\alpha$ 3 $\beta$ 1 are laminin 5-receptors and regulate anchorage and motility of epithelial cells (Ryan *et al.*, 1999). O'Toole *et al.* (1997) observed an inhibitory effect of laminin 5 on keratinocyte motility and showed that this could be reversed by blocking the  $\alpha$ 3 integrin receptor. Ryan *et al.* (1999) demonstrated that basal keratinocytes can utilize integrin  $\alpha$ 3 $\beta$ 1 to interact with a hitherto unknown alternative ligand. Therefore, it is feasible to postulate that the shed ectodomain of collagen XVII is an alternative ligand that can bind to  $\alpha$ 3 $\beta$ 1 integrin and block it for other, migration-enhancing ligands.

Taken together, it is likely that an interplay between stimulatory and inhibitory regulators determines collagen XVII shedding activity in a given cell at a given time. Future studies must show how the enzyme activities,

enzyme–substrate interactions and ligand binding activities are modulated. Finally, the possibility cannot be excluded that hitherto unknown constitutive sheddases, like the as yet uncharacterized shedding activities of TRANCE/OPGL (Schlondorff *et al.*, 2001), also participate in the regulation of keratinocyte adhesion and migration.

## Materials and methods

### Production of bacterial fusion proteins and domain-specific collagen XVII antibodies

For production of domain-specific antibodies to collagen XVII, bacterial fusion proteins were generated as described (Tasanen *et al.*, 2000a,b). The fusion protein GST–Ecto-5 spanned amino acids 1447–1497 and GST–Endo-2 spanned amino acids 367–466 (Figure 1). The corresponding primers for Ecto-5 were: sense primer 5'-GCGCGGATCCGGCACTCCAGGACCCAAAGGT-3' and antisense primer 5'-GCGCGAATTCTCAGGCTTGACAGCAATATC-3'; and for Endo-2, sense primer 5'-CGGGATCCAGCGGGAAGGTCTTACAGCC-3' and antisense primer 5'-GGAATTCCTCCACCAGCTGCAGCA-3'. In parallel, a fusion protein corresponding to the NC16A domain of murine collagen XVII, MO-NC16A, spanning amino acids 499–573, was produced. The corresponding cDNA was amplified by RT-PCR from murine keratinocyte mRNA with sense primer 5'-GCGCGGATCCGAGGAGGTAAGAAAGCTGAAG-3' and antisense primer 5'-GCGCGAATTCATCTGAGATTCCCGTTCTCTG-3'. Rabbit antibodies to the fusion proteins were produced by standard procedures (Eurogentec, Ougrée, Belgium).

### Cell cultures

Normal human keratinocytes were cultured in serum-free keratinocyte growth medium (KGM; Life Technologies, Grand Island, NY) as described (Schäcke *et al.*, 1998). The human keratinocyte cell line HaCaT was a generous gift of Dr N.Fusenig (German Cancer Research Center, Heidelberg, Germany). Prior to experiments, cells were grown in the presence of 50 µg/ml L-ascorbate for 48 h in order to allow for full prolyl- and lysyl hydroxylation of newly synthesized collagens.

TACE-deficient murine keratinocytes were isolated from *tace*<sup>ΔZn/ΔZn</sup> mouse embryos (Dotto, 1999). The mice were homozygous for a targeted mutation in the metalloproteinase domain of TACE which leads to expression of inactive TACE (Peschon *et al.*, 1998). MT1-MMP-deficient murine keratinocytes were isolated from the skin of MT1-MMP knockout mice (Zhou *et al.*, 2000) and cultured as described above for human keratinocytes. The MMP-2-deficient human gastric carcinoma cell line (MKN 45), which had only low MT1-MMP expression, was obtained from the Japanese Cancer Resource Bank (JCRB). Murine fibroblasts and the MKN 45 cells were maintained in DMEM supplemented with L-glutamine, 10% fetal calf serum (FCS) and penicillin/streptomycin (Life Technologies GmbH, Karlsruhe, Germany).

### Surface biotinylation and time chase experiments

For time chase analysis of the release of the ectodomain, semiconfluent keratinocytes were cultured with 50 µg/ml for 48 h, washed extensively and surface biotinylated with *d*-biotinoyl-*ε*-aminocaproic acid *N*-hydroxysuccinimide ester according to the manufacturer's recommendations (Roche Biochemicals, Mannheim, Germany). After washing with phosphate-buffered saline (PBS) five times, the cells were cultured in fresh medium supplemented with ascorbate, and aliquots were collected at regular intervals over 96 h. Culture medium and cell extract were immunoprecipitated with the antibodies Ecto-1 and Endo-2, respectively. The precipitates were immunoblotted with a streptavidin-coupled alkaline phosphatase (Sigma, Germany) to detect biotinylated collagen XVII. The software TotalLabID V1.00 from Phoeix (Biostep GmbH, Jahnsdorf, Germany) was used for semiquantitative analysis of the signals.

### Protein extractions

For protein extractions, the media and cells were processed separately as described (Schäcke *et al.*, 1998). As a modification, 10 mM 1,10-*o*-phenanthroline was added to the extraction buffer. After centrifugation at 13 000 *g* for 5 min at 4°C the supernatant was used for further analysis. The medium was collected on ice, proteinase inhibitors were added immediately and cell debris removed by centrifugation. The supernatant

was precipitated with ethanol and resuspended in a buffer containing 50 mM Tris–HCl, 65 mM NaCl, 5 mM EDTA, 1 mM Pefabloc pH 7.4. Total protein content was determined with a micro-Lowry assay kit (Bio-Rad), and samples containing 50 µg of protein were analyzed by immunoblotting.

### Immunoblotting and immunoprecipitation of collagen XVII

SDS–PAGE and immunoblotting with collagen XVII antibodies Endo-2, Ecto-5, murine or human NC16A (Schumann *et al.*, 2000), or Ecto-1 (Schäcke *et al.*, 1998) were performed using standard techniques. For immunoprecipitation, cell extracts were precleared with protein A–Sepharose (Amersham Pharmacia Biotech) for 30 min and centrifuged at 2000 *g* for 5 min. The supernatants were incubated with antibody Endo-2/protein A–Sepharose complexes at 4°C overnight. After extensive washing, collagen XVII was eluted with 0.1 M glycine pH 2.5 for 5 min and immediately neutralized with 1 M Tris.

### Digestion of collagen XVII with recombinant proteases

Immunoprecipitated collagen XVII was digested with recombinant MMP-14 (MT1-MMP) and pro-MMP-9 (Alexis Deutschland, Grünberg, Germany), recombinant pro-MMP-2, recombinant BMP-1, and recombinant furin (Calbiochem-Novabiochem, Bad Soden, Germany). The proforms of MMP-2 and MMP-9 were activated with 1 mM APMA for 1 h at 37°C. Collagen XVII was incubated with 1–5 nM recombinant enzymes in 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM CaCl<sub>2</sub>, 15 µM ZnCl<sub>2</sub> and 0.3% Brij 35 for up to 4 h at 37°C. The reactions were stopped with 5 mM EDTA and the samples precipitated with methanol/chloroform for immunoblotting with antibodies Ecto-1 and Endo-2.

### Protease inhibitors

The following protease inhibitors were used: AEBF [4-(2-aminoethyl)-benzolsulfonylfluoride hydrochloride; Merck, Darmstadt, Germany], phosphoramidon, 1,10-*o*-phenanthroline, aprotinin, benzamide, E-64 [L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido(4-guanidino)-butane], pepstatin A (Sigma), the MMP-inhibitor 4-Abz-Gly-Pro-D-Leu-D-Ala-NHOH (FN-439; Calbiochem-Novabiochem) and the furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (Calbiochem-Novabiochem). The hydroxamate inhibitors BB 3103, BB 3241 were from British Biotech and IC-3 from Immunex Corp. TIMP-1, -2 and TIMP-3 were from Chemicon International (Hofheim, Germany) and the selective gelatinase inhibitor H-Cys-Thr-Thr-His-Trp-Gly-Phe-Thr-Leu-Cys-OH (Koivunen *et al.*, 1999) from Bachem (Heidelberg, Germany).

### Collagen XVII shedding assay

Subconfluent keratinocytes or HaCaT cells were washed twice with PBS, and fresh medium containing 50 µg/ml ascorbate and inhibitors or stimulators was added for 4–6 h. Media were precipitated with methanol/chloroform and aliquots containing 75 µg of total protein were immunoblotted with antibody NC16A. Semiquantitative densitometry of the signals was performed using the computer program TotalLabID (Biostep GmbH).

### Protease activity assay with fluorescent substrates

Pro-MMPs were activated with 1 mM APMA for 60 min at 37°C, and 5 nM MMP-2 and 4 nM MMP-9 in buffer containing 50 mM Tris–HCl, 200 mM NaCl, 5 mM CaCl<sub>2</sub>, 1 µM ZnCl<sub>2</sub>, 0.05% Brij 35 pH 7.5, were incubated with 15 µg/ml fluorescein-conjugated gelatin (DQ-gelatin from pig skin; Molecular Probes), and the release of soluble fluorescent fragments was followed with a spectrofluorometer with microplate reader set (FluoroMax-2; Jobin Yvon, Munich, Germany;  $\lambda_{ex}$  = 485 nm,  $\lambda_{em}$  = 530 nm). The activities of recombinant furin and CAT-DIS TACE were determined with internally quenched fluorescent substrates in assay buffer. For furin, 5 µM Abz-RVKRGLA-m-nitro-YD-OH (Bachem;  $\lambda_{ex}$  = 320 nm,  $\lambda_{em}$  = 425 nm; Anglikler *et al.*, 1995) and for TACE, 15 µM MCA-PLAQAV-dinitrophenyl-2,3-diaminopropionyl-RSSSR-NH<sub>2</sub> (Sigma;  $\lambda_{ex}$  = 325 nm,  $\lambda_{em}$  = 395 nm) was used.

### Immunofluorescence staining

Immunofluorescence staining of human skin and keratinocytes was performed as described (Schumann *et al.*, 2000). As first antibodies, collagen XVII antibody NC16A, TACE antibodies against the extracellular pro- and metalloproteinase domains (Santa Cruz Biotechnology) and the intracellular C-terminus (Chemicon International, Hofheim, Germany), and antibodies to ADAM-10 endodomain (Chemicon) and ADAM-9 ectodomain (R&D Systems, Wiesbaden, Germany) were used.



The second antibodies were fluorescein isothiocyanate (FITC)-labeled anti-rabbit immunoglobulins (Sigma).

### Transient transfections

One day before transfection with pcDNA3 vectors containing full-length cDNAs for murine TACE, human ADAM-9 cDNA and bovine ADAM-10, HaCaT cells were seeded and grown to 70% confluence. The cells were harvested and counted, washed twice with PBS and resuspended in 1.25% DMSO in KGM. The cDNA constructs were added to  $0.6 \times 10^7$  cells and electroporated with Gene Pulser (Bio-Rad) with 0.25 V and 960  $\mu$ FD capacitance. After electroporation the cells were transferred into pre-warmed medium, washed after 24 h, and shedding assays were performed.

TACE-deficient murine fibroblasts were transfected with pcDNA3 vector encoding the full-length human collagen XVII (kindly provided by L.Borradori, Geneva). One day before transfection the cells were seeded at a density of  $\sim 25$  000 cells/cm<sup>2</sup> and grown in DMEM/10% FCS. The transfection was performed with Effectene. Transfection reagent (Qiagen, Hilden, Germany) was used following the manufacturer's recommendations. Cells were used 24–48 h post-transfection to obtain maximum levels of gene expression.

### Migration assays

Scratch assays were performed with normal human keratinocytes or HaCaT cells in 6-well plates (Cha *et al.*, 1996). The cells were grown to confluence and a 2 mm broad denuded scratch was produced in the culture with a pipette tip. After washing with PBS to remove all floating cells, the cells at the cut edge were allowed to migrate into the empty zone for 11–22 h. Photographs of identical locations within each scratch were taken at different time points. Since the doubling time of keratinocytes under these conditions was  $\sim 30$  h, the assay assessed mostly cell motility, not proliferation (Woodley *et al.*, 1988).

## Acknowledgements

The authors gratefully acknowledge the generous gifts of TACE cDNA, antibodies, IC-3 and TACE-deficient fibroblasts from J.Peschon, Immunex Corp., Seattle. The hydroxamate inhibitors BB 3103 and BB 3241 were kindly provided by H.Mills, British Biotech Pharmaceuticals Ltd, Oxon, UK. The cDNA for ADAM-9 was a generous gift from C.Blobel and G.Westkamp, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, NY. We are grateful to P.Dotto and Y.Aoyama, Cutaneous Biology Research Center, Harvard Medical School, Charlestown, MA, for help with culturing murine keratinocytes, to W.Stetler-Stevenson, NCI, Bethesda, MD, for a gift of recombinant MMP-2, to R.Timpl, Max-Planck-Institute for Biochemistry, Martinsried, Germany, for a gift of recombinant BMP-1, and to N.Koshikawa and V.Quaranta, Scripps Research Institute, La Jolla, CA, for help with the MMP-2-deficient cell line. This work was supported in part by grants from the German Research Council, DFG (Grant Nos. SFB 293-B3 and BR1475/6-1) and from the European Union (Contract No. QLGI-CT-2001-02007) to L.B.-T., and from the Academy of Finland and the Oulu University Hospital to K.T.

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Received January 9, 2002; revised July 24, 2002;  
accepted August 15, 2002